ASSOCIATION BETWEEN CD14 (CD14 C-159T) PROMOTER POLYMORPHISM AND SUSCEPTIBILITY TO SEVERE MALARIA ANEMIA IN CHILDREN, BELOW 3 YEARS RESIDENT IN SIAYA DISTRICT, WESTERN KENYA

BY

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DEPARTMENT OF ZOOLOGY

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DECLARATION

I hereby declare that this thesis is the result of my original work and its findings have not been presented for an award of a degree certificate in any other institution.

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To my children, Elly Kingsley, Basil Wesley, Sister Janeal and baby Raychelle, for always making me see a reason to forge ahead. To my late parents, Dad, Elias Owade Odera, and Mum, Angeline Awino, for instilling hard work and focus in me as a young girl.

ABSTRACT

Among the infectious diseases worldwide, malaria is one of the leading causes of child morbidity and mortality. Severe malaria anemia (SMA) is the most common clinical manifestation of acute *Plasmodium falciparum* malaria in western Kenya, a holoendemic transmission area. It affects children aged between 3 and 36 months mostly, leading to morbidity and mortality in this region. Previous studies have associated variations in the promoter region of CD14, a gene which expresses as membrane-bound receptor (mCD14) and as soluble receptor (sCD14), with outcome of severe malaria in adult populations in some malaria endemic regions. However, the association between the CD14 C-159T and susceptibility to SMA in children resident in western Kenya is unknown. In addition, the distribution of allele frequencies in SMA and non-SMA children populations is unclear. As such, this cross-sectional study investigated the associations between polymorphisms in the CD14 C-159T and susceptibility to SMA in children (n=240; aged 3-36 months), resident in *P. falciparum* holoendemic region of Siaya District, western Kenya. The allele frequencies in SMA versus non-SMA were also determined. The sample was drawn from a target population of 720 children who present to the hospital with varying disease conditions in a year. The CD14 C-159T genotyping was carried out using a high-throughput TaqMan® single nucleotide polymorphism (SNP) genotyping assay technique. The association between CD14 C-159T genotypes and SMA was determined by multivariate logistic regression, controlling for the confounding effects of gender, age, G-6-PD deficiency, sickle cell trait, HIV-1 status, and bacteremia. Comparison of alleles and genotype distribution among the clinical groups was performed using chi-square (χ^2) test. Multivariate logistic regression analysis revealed that relative to the wild type (CC genotype), there was no association between polymorphism in CD14 -C159T and susceptibility to SMA (CT, OR =0.75, CI =0.38-1.47, P=0.398, and TT, OR =1.23, CI =0.63-2.36 P=0.544). Further analysis revealed that the genotypic and allelic frequencies were comparable between SMA and the non-SMA (P=0.422). There was deviation from the Hardy Weinberg Equilibrium by the variant allele. The results from this study show that CD14 C-159T promoter polymorphisms are not associated with susceptibility to SMA in children from Siaya District, with allele and genotype frequencies being comparable in the SMA and non-SMA groups. This study, contributes to the wealth of knowledge on association studies that try to reveal the pathogenesis of malaria. Further research on CD14 C-159T and its functionally related immune genes should be conducted, to help decipher its exact role in severe malaria anemia. This would help in revealing the challenges associated with pathogenicity of *P.falciparum* malaria in pediatric populations resident in *P. falciparum* holoendemic transmission regions.

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CHAPTER ONE

INTRODUCTION

1.1. Background information

Among the infectious diseases worldwide, malaria is one of the most severe public health problems. It is a leading cause of death and disease in many developing countries, where young children and pregnant women are the most affected (WHO, 2006). In the year 2010, there were approximately 3.3 billion people at risk of infection and 216 million episodes of malaria, worldwide (WHO, 2011). This led to 655,000 malaria deaths globally, of which 91% were in the African region, with 86% being children under 5 years of age (WHO, 2011).

Majority of malaria-related morbidity and mortality in African is due to infection with *Plasmodium falciparum*. Severe *P. falciparum* infection is generally characterised by unarousable coma, renal impairment, pulmonary oedema, severe monocytic anemia, hypoglycaemia, hyperlactatemia, as well as hyperparasitaemia (Wenisch *et al.*, 1996). Of all these manifestations, severe malaria anemia (SMA) is the most common clinical outcome in western Kenya accounting for the greatest proportion of malaria-associated morbidity and mortality among infants and young children in *P. falciparum* holoendemic transmission areas of the world (Breman *et al.*, 2001). Other presentations such as cerebral malaria (CM), hyperparasitemia, hypoglycaemia, and renal insufficiency occur rarely (Ong'echa *et al.*, 2006).

Polymorphisms in certain genes have been associated with susceptibility to SMA. SNPs in Interleukin-1 beta gene (IL-1β) promoter at loci IL-1β -31C/T and -511A/G IL-1 have been found to condition susceptibility to SMA, whereby carriage of the TA haplotype has been demonstrated to produce relatively higher IL-1β and associated with elevated hemoglobin (Hb) concentration, while low IL-1β producing CA haplotype

individuals have been reported to be at an increased risk of developing SMA (Ouma *et al.*, 2008a). Another gene that has been implicated in severe malaria in other populations is CD14 gene, which expresses membrane-bound receptor (mCD14) and soluble receptor (sCD14), majorly for lipopolysaccharides (Wenisch *et al.*, 1996). The mCD14 is found on the surfaces of monocytes, macrophages and granulocytes, while sCD14 is found in the serum and urine (Buckova *et al.*, 2003). Five major genetic variants have been identified in the promoter of the CD14 gene (C–159T, A–1619G, G–1359T, A–1145C, and A–809C) and are associated with atopic diseases and IgE levels in different ethnic groups. Of the variants, C-159T has been associated with complicated *P. falciparum* malaria in humans. The CD14-159TT homozygote has been associated with high levels of sCD14 (LeVan *et al.*, 2001). A study in adult population in Thailand indicated that elevated levels of sCD14 in serum were associated with complicated *P. falciparum* malaria in humans, manifesting as cerebral malaria (CM) and renal failure (Wenisch *et al.*, 1996). Severe malaria in western Kenya, however, manifests itself as SMA which causes morbidity and mortality mostly among the children aged 3 to 36 months. It is unclear whether the genetic variants within CD14C−159T are associated with susceptibility to SMA (Hb≤6.0 g/dL, any density parasitaemia) in pediatric populations resident in *P. falciparum* holoendemic transmission region of western Kenya. As such, the current study investigated the associations between variants within the CD14C−159T and susceptibility to SMA (Hb≤6.0 g/dL, any density parasitaemia) in pediatric populations resident in *P. falciparum* holoendemic transmission region of western Kenya.

Allele frequencies have been found to be influenced by historical isolation, environmental positive selections as well as ethnic differences in humans (LeVan *et al.*, 2001). The CD14C−159T SNP has been reported to show significant allele frequency variations in different ethnic populations (LeVan *et al.*, 2001; Kedda *et al.*, 2005; Wang

et al., 2005). For example, a number of studies have reported the T allele as a risk for total or specific IgE, atopy, wheezing or asthma phenotypes (LeVan *et al.*, 2001; Kedda *et al.*, 2005; Wang *et al.*, 2005), while others reported it to be protective against atopy in population sub-groups (Baldini *et al.*, 1999; Sharma *et al.*, 2004). The T allele has also been consistently suggested to be associated with higher levels of soluble CD14 (sCD14) when compared to the C allele (LeVan *et al.*, 2001). As such, it is hypothesized that the presence of T allele in a population would be associated with a positive selection leading to relatively higher levels of sCD14, which may alter susceptibility to severe malaria. Currently, it is unclear whether there are differences in distribution patterns of alleles and genotypes in SMA versus non-SMA in pediatric populations resident in *P. falciparum* holoendemic regions such as in Siaya District in western Kenya. As such, the current study determined the genotype and allelic frequencies of CD14 C-159T in SMA and non-SMA children presenting with *P. falciparum* malaria in a holoendemic region of Siaya district, western Kenya.

1.2. Statement of the problem

Severe malarial anemia (SMA; Hb≤6.0 g/dL, any density parasitaemia) is one of the most serious manifestations of *P. falciparum* malaria in holoendemic region of western Kenya. It affects children aged 3 to 36 months mostly, leading to mortality and morbidity. Previous studies have associated variations in the promoter region of CD14C−159T with the outcome of severe malaria in a Thailand population, and allele T with high levels of sCD14 which have been found to exacerbate severe malaria. It is unclear whether the genetic variants within CD14C−159T are associated with susceptibility to SMA (Hb≤6.0 g/dL, any density parasitaemia) in pediatric populations resident in *P. falciparum* holoendemic transmission region of western Kenya. In

addition, it is unclear whether there are differences in distribution patterns of alleles and genotypes in SMA versus non-SMA in pediatric populations resident in *P. falciparum* holoendemic regions such as in Siaya District in western Kenya. It is hoped that children below the age of 5 years will be the first beneficiary of a malaria vaccine once it becomes available since this is the most vulnerable populations. As such, carrying out a study in such pediatric populations would advice on the most relevant components that can benefit that particular population. As such, the current study was designed to determine the association between the CD14 C–159T promoter polymorphism and severe malaria anemia (SMA) in children aged 3 to 36 months, resident in *P. falciparum* holoendemic region of Siaya District, western Kenya. The allele and genotype distribution pattern between the SMA and non-SMA pediatric populations was also determined.

1.3. Justification

Single nucleotide polymorphism (SNP) in the promoter region of CD14 gene (CD14 C-159T), TT homozygote had been associated with elevated levels of soluble CD14 in severe *P. falciparum* malaria expressing as renal failure (Wenisch *et al.*, 1996). Severe malaria in western Kenya manifests mainly as SMA among children aged 3-36 months. Some children are more prone to develop SMA than others even though the conditions surrounding them may be similar. Ascertaining genetic conditions that predispose such children to SMA would be paramount in an attempt to identify at risk children populations. In addition, genes associated with naturally-acquired immunity can be manipulated in the design of a long-lasting effective vaccine against malaria. As such, information generated from this study through identification of at risk population for intervention and genes associated with protection will eventually lead to a reduction in

morbidity and mortality in holoendemic *P. falciparum* transmission regions, such as in Siaya District in western Kenya.

1.4. Objectives

1.4.1. General objective

To determine the association between the CD14 C–159T promoter polymorphism and severe malaria anemia (SMA; (Hb≤6.0 g/dL, any density parasitaemia) in children aged 3 to 36 months, resident in *P. falciparum* holoendemic region of Siaya District, western Kenya.

1.4.2. Specific objectives

a) To determine the association between CD14 C-159T genotypes and susceptibility to SMA (Hb≤6.0 g/dL, any density parasitaemia) in children presenting with *P. falciparum* malaria in a holoendemic region of Siaya District, western Kenya.

b) To determine the genotype and allelic frequencies of CD14 C-159T in SMA (Hb≤6.0 g/dL, any density parasitaemia) and non-SMA children presenting with *P. falciparum* malaria in a holoendemic region of Siaya District, western Kenya.

1.5. Study hypotheses

a) There is no association between CD14 C-159T genotypes and susceptibility to SMA in children presenting with *P. falciparum* malaria in a holoendemic region of western Kenya.

b) There are no differences in genotype and allelic frequencies of CD14 C-159T in SMA and non-SMA children presenting with P. *falciparum* malaria in a holoendemic region in a holoendemic region of Siaya District, western Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1. *Plasmodium falciparum* and severe malaria

Malaria in humans is transmitted by female *Anopheles* mosquitoes. It is a protozoan disease caused by one of the four members of genus *Plasmodium*: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale,* and *Plasmodium malariae* (Sisowath *et al.*, 2007). *Plasmodium falciparum* is the most virulent of the parasites and kills more people annually, mostly in sub-Saharan Africa (Sisowath *et al.*, 2007). In the year 2010, World Health Organization (WHO) estimated the occurrence of 216 million malaria episodes resulting in approximately 655,000 deaths, of which 91% occurred in the African region (WHO, 2011). The greatest disease burden was born by children under five years of age and pregnant women, with a global malaria related mortality of 86% (WHO, 2011). *P. falciparum* malaria ranges from asymptomatic infections to symptomatic cases presenting with fever, chills, sweating, headache and muscle aches. A sub-population of the symptomatic cases express severe, life-threatening complications (severe malaria) which manifest by a variety of clinical syndromes dependent on properties of both the host and the parasite (Wenisch *et al.*, 1996).

Severe *P. falciparum* infection is generally characterized by unarousable coma, renal impairment, pulmonary oedema, severe monocytic anemia, hypoglycemia, hyperlactatemia, as well as hyperparasitemia (Wenisch *et al.*, 1996). Of all these manifestations, severe malaria anemia (SMA) accounts for the greatest proportion of malaria-associated morbidity and mortality among infants and young children in *P. falciparum*-holoendemic transmission areas of the world (Breman *et al.*, 2001). In western Kenya, SMA (Hb<6.0g/dL, any density parasitemia), is the most common clinical outcome while the other presentations such as cerebral malaria (CM), hyperparasitemia, hypoglycemia, and renal insufficiency occur, but in

rare situations (Ong'echa *et al.*, 2006). Defined according to World Health Organization (WHO), severe malaria anemia refers to Hb concentrations <5.0 g/dL (or a hematocrit <15.0%) in the presence of any density parasitemia (WHO, 2000). However, this definition leaves out many severe anemic individuals hence in this region, a modified definition of SMA based on age and geographically-matched Hb concentrations (i.e., Hb<6.0g/dL, with any density parasitemia) is used (McElroy *et al.*, 1999). In sub-Saharan Africa, it remains an important childhood health burden and a major public health problem because of the very large numbers of children affected, which are likely to increase as drug resistance spreads (Brabin *et al.*, 2001; Taylor *et al.*, 2006). Manifestation of SMA has been attributed to lysis of infected and uninfected erythrocytes (Dondorp *et al.*, 1999; Price *et al.*, 2001), splenic sequestration of erythrocytes, dyserythropoiesis and bone marrow suppression (Phillips *et al.*, 1986; Abdalla, 1990). Severity of malaria anemia is enhanced in the presence of co-infections such as bacteremia, HIV-1, and hookworm (Berkley *et al.*, 2005; Otieno *et al.*, 2006; Were *et al.*, 2011). It has been found to correlate with parasitemia and schizontemia (Looareesuwan *et al.*, 1983), as well as with high levels of bilirubin and creatinine in serum, showing the ill effects of malaria on the liver (Goljan *et al.*, 2006).

Once the infection occurs, the pathophysiology of malaria is mediated by host and parasite interactions which depend on factors such as endemicity patterns, natural acquisition of malaria immunity, parasite virulence, multiplication rate, age, antigenic variation and polymorphic variability in both the host and parasite (Trape *et al.*, 1994; Akanmori *et al.*, 1995; Abdalla, 2004).

2.2. Human genes and susceptibility to SMA

Most previous studies have tried to explain the genetic aspects of *Plasmodium falciparum* and development of resistance to different anti-malarial drugs at a relatively fast

rate and alternative treatments that have been suggested from time to time (Greenwood *et al.*, 1991; Sidhu *et al.*, 2006; Sisowath *et al.*, 2007). Children in sub-Saharan Africa are repeatedly infected with *P. falciparum,* but life-threatening anemia develops in only a fraction of the infected (Greenwood *et al.*, 1991) . In an attempt to investigate the detailed role of human genetics in conditioning susceptibility to malaria disease, studies to find the extent to which human genes predispose children to severe anemia, have been ongoing with others implicating certain genes while others do not (Greenwood *et al.*, 1991; Sisowath *et al.*, 2007).

Successful type 1 response to malaria requires a well-timed and proportional release of interleukin (IL)-12, interferon (IFN)-γ, and tumor necrosis factor-alpha (TNF-α) to minimize parasitemia and preserve erythropoiesis (Crutcher *et al.*, 1995; Stevenson *et al.*, 1995). The pro-inflammatory phase is followed by an equally timely response by type 2 cytokines such as IL-10, transforming growth factor (TGF)-β, and IL-4, to avoid inflammatory host damage (Clark *et al.*, 2006). Toll like receptors (Shio *et al.*, 2011), Interleukin-6 (IL-6) and CD14 (LeVan *et al.*, 2001) among others have been involved in microbial recognition as well as many non-infectious stimuli to acute inflammatory responses. Polymorphisms in the regulatory regions of these genes have been associated with altered cytokine responses and outcome in a variety of experimental and clinical conditions (Vercelli *et al.*, 2001; Liu *et al.*, 2012). A study of malaria in West African children showed that a human leukocytes class I antigen (HLA-Bw53) and an HLA class II haplotype (DRB1*1302-DQB1*0501), common in West Africans but rare in other racial groups, are independently associated with protection from severe malaria (LeVan *et al.*, 2001). A low interleukin-10:TNF-α ratio was associated with malaria anemia in children residing in a *P. falciparum* holoendemic malaria region in western Kenya (LeVan *et al.*, 2001). At the same time, polymorphic variability in the interleukin (IL)-1beta promoter has

been found to condition susceptibility to severe malarial anemia and functional changes in IL-1beta production in the same region (Ouma *et al.*, 2008a). It is unclear whether polymorphisms within the CD14 will alter susceptibility to SMA in pediatric populations resident in Siaya District in western Kenya.

2.3. The cluster of differentiation 14 gene (CD14) and *P. falciparum* malaria

The gene, cluster of differentiation 14 gene, has a standard name, CD14. It is found on chromosome 5q31-q33, on which other genes as IL3, IL4, IL5, IL9, IL10, and IL 13 among others are found. It consists of 193 nucleotides encoding a pattern recognition receptor (PRR) comprising the mannose receptor and scavenger receptors (Pugin *et al.*, 1994; Medzhitov and Janeway, 1998). The CD14 exists as a single–copy gene although the protein it encodes is found in two distinct forms (Haziot *et al.*, 1988; Buckova *et al.*, 2003). These are, a 50- to 55-kDa glycosylphosphatidylinositol-anchored membrane molecule, membrane CD14, (mCD14) (Janeway, 1992) expressed on the surface of monocytes, macrophages, granulocytes and β- lymphocytes (Haziot *et al.*, 1988; Buckova *et al.*, 2003), and a 48-kDa soluble form lacking the glycosylphosphatidylinositol anchor (Bazil, 1986).

It is suggested that the soluble CD14 (sCD14) is derived from monocytes (Bazil, 1991) as well as the liver (Su *et al.*, 1999), and is found in the serum and urine (Buckova *et al.*, 2003). Membrane-bound CD14 (mCD14) recognizes bacterial compounds like lipopolysaccharides (LPS), lipoteichoic acid (LTA), or peptidoglycan (PGN), while sCD14 mediates the response of endothelial and epithelial cells, which do not express CD14, to microbes (Kitchens *et al.*, 2001; Sonja von Aulock, 2005). Recognition and signaling through CD14 involves a Lipopolysaccharide (LPS) binding-protein acting on bacterial LPS and then presenting the LPS monomers to binding sites on the CD14 (Ulevitch and Tobias, 1999). The CD14 then binds to the LPS resulting in cellular activation and production of

pro-inflammatory cytokines (e.g. TNF-α, IL-6), and chemokines as well as activation of inducible nitric oxide synthase (iNOS) (Schroeder *et al.*, 1997; Ulevitch and Tobias, 1999). Toll-like receptor 4 (TLR4) initiates LPS signal transduction within macrophages, making the TLR4 molecule a critical transmembrane component of the CD14 signaling pathway for LPS (Poltorak *et al.*, 2000).

Five major genetic variants have been found to occur in the promoter of the CD14 gene identified as C–159T, A–1619G, G–1359T, A–1145C and A–809C (Baldini *et al.*, 1999). Of the five SNPs, the most studied and linked to different atopic conditions is the C-159T. Studies from populations in Brazil (de Faria *et al.*, 2008), Tunisia (Lachheb *et al.*, 2008), Poland (Kowal *et al.*, 2008), and India (Sharma *et al.*, 2004) have linked C-159T to atopic asthma. In contrary, studies in populations from the Czech Republic (Buckova *et al.*, 2003), Korea (Hong *et al.*, 2007), China (Liang *et al.*, 2006), Australia (Kedda *et al.*, 2005), Germany (Heinzmann *et al.*, 2003), and the United States (Woo *et al.*, 2003) show that C–159T does not play a role in the development of atopic asthma. A recent meta-analysis, however, suggested that C-159T could be a protective factor for atopic asthma in Asian population children (Zhang *et al.*, 2011). Still in Asian population, C-159T was associated with tuberculosis whereby the TT homozygote carriers were reported to have an increased risk of TB compared with those individuals with the TC and CC homozygote (Zhang *et al.*, 2011). A meta-analysis on susceptibility to TB confirmed susceptibility by TT homozygote and determined C/T heterozygote as a risk factor for TB in Asians, but not in Latinos (Zhang *et al.*, 2011). The C–159T TT homozygote is reported to be associated with lower serum IgE levels in some populations, while the CC genotype is associated with higher levels of total serum IgE and a higher number of positive atopic skin test results (Zhang *et al.*, 2011). Furthermore, the C allele has been associated with atopy, specifically to molds, in a Chinese population (Zhang *et al.*, 2011). Such studies indicate that the CD14 C–159T polymorphism

impacts differently on different diseases and on populations with different ethnic backgrounds.

Studies that link C-159T to *P. falciparum* malaria have indicated that high levels of sCD14 are associated with complicated *P. falciparum* malaria with the highest levels detected in patients with renal failure (a severe *P. falciparum* malaria case) in a Thai population (Wenisch *et al.*, 1996). Furthermore, the high levels of sCD14 have been associated with C-159T, in which homozygous carriers of the T allele were found to have significant increase in serum levels of sCD14 among the Hispanic and non-Hispanic white populations (Baldini *et al.*, 1999). Despite these available literatures, it is unclear whether the genetic variants within CD14C−159T are associated with susceptibility to SMA (Hb≤6.0 g/dL, any density parasitaemia) in pediatric populations resident in *P. falciparum* holoendemic transmission region of western Kenya. As such, the current study investigated the associations between variants within the CD14C−159T and susceptibility to SMA (Hb≤6.0 g/dL, any density parasitaemia) in pediatric populations resident in *P. falciparum* holoendemic transmission region of western Kenya.

2.4. The Allele frequencies

Allele frequency refers to proportion of individuals in a population that carry a given allele. It is usually expressed as a proportion or a percentage and shows the genetic diversity of a species population or the richness of its gene pool. The Hardy-Weinberg model describes and predicts genotype and allele frequencies in a non-evolving population. The model has five basic assumptions that; the population is large (i.e., there is no genetic drift); there is no gene flow between populations, from migration or transfer of gametes; mutations are negligible; individuals are mating randomly; and that natural selection is not operating on the population (Haziot *et al.*, 1988). Alleles are supposed to be distributed randomly in a

population, that is, obey Hardy–Weinberg rule (Janis *et al.*, 2005). However, certain forces do lead to changes in the distribution and frequencies of alleles hence leading to a condition that the alleles are not randomly distributed. The forces are selection, genetic drift, mutation, migration random mating and meiotic drive, and they finally result in evolution. Whenever such forces are not acting on the genes, the population is said to be in Hardy–Weinberg Equilibrium. From distribution genotype frequencies also referred to as Hardy Weinberg Proportion (HWP), allele frequencies get determined. Genotype frequencies represent the abundance of each genotype within a population as a fraction of the population size (Haziot *et al.*, 1988).

The CD14 C-159T alleles have been expressed in different population with either T or C impacting on outcome of different conditions (Baldini *et al.*, 1999; Buckova *et al.*, 2003; Tan *et al.*, 2006; Wang *et al.*, 2013). Some studies have associated CD14-159 T allele with exposure to atopic disease, elevated levels of soluble CD14 and with the total serum IgE level in some populations (Baldini *et al.*, 1999; Buckova *et al.*, 2003; Tan *et al.*, 2006; Wang *et al.*, 2013). Others have associated the allele T with lower IgE levels and/or a reduced risk for atopy (Koppelman *et al.*, 2001; Buckova *et al.*, 2003; Leynaert *et al.*, 2006). Some studies, however, found no association between these variants and any disease outcome (Baldini *et al.*, 1999). These reports indicate that the CD14C-156T alleles may differ in populations with differing backgrounds leading to differential susceptibility to infectious diseases. Currently, it is unclear whether there are differences in distribution patterns of alleles and genotypes in SMA versus non-SMA in pediatric populations resident in *P. falciparum* holoendemic regions such as in Siaya District in western Kenya. As such, the current study determined the genotype and allelic frequencies of CD14 C-159T in SMA and non-SMA children presenting with *P. falciparum* malaria in a holoendemic region of Siaya district, western Kenya.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study site

The study was conducted in Siaya District Hospital (SDH), in Siaya district, western Kenya (Figure 1). Siaya District Hospital is the major government hospital for the population living in the *P. falciparum* holoendemic transmission region of Siaya District in western Kenya (Ong'echa *et al.*, 2006). The annual population growth rate was 0.9 1% in the year 2002. Infant mortality rate in the district stood at 102/1000 and under five mortality was 113/1000 in the same year (McElroy *et al.*, 2000). The intensity of malaria transmission in this holoendemic region is experienced during the seasonal rainfalls in April to August and November to January (Beier *et al.*, 1994). Inhabitants of the study area are predominantly of the Luo ethnic tribe (>96%), with the population being culturally homogeneous hence suitable for genetic study (Bloland *et al.*, 1999; Ong'echa *et al.*, 2006). It is bordered by Busia, Vihiga, Butere-Mumias, Bondo and Kisumu districts. It is approximately 1520 sq km in size and lies between Latitude 0° 26' to 0° 18' North and Longitude 33° 58' East and 34° 33' West. It has 3 main geo-morphological areas; dissected uplands, moderate lowlands and the Yala swamp.

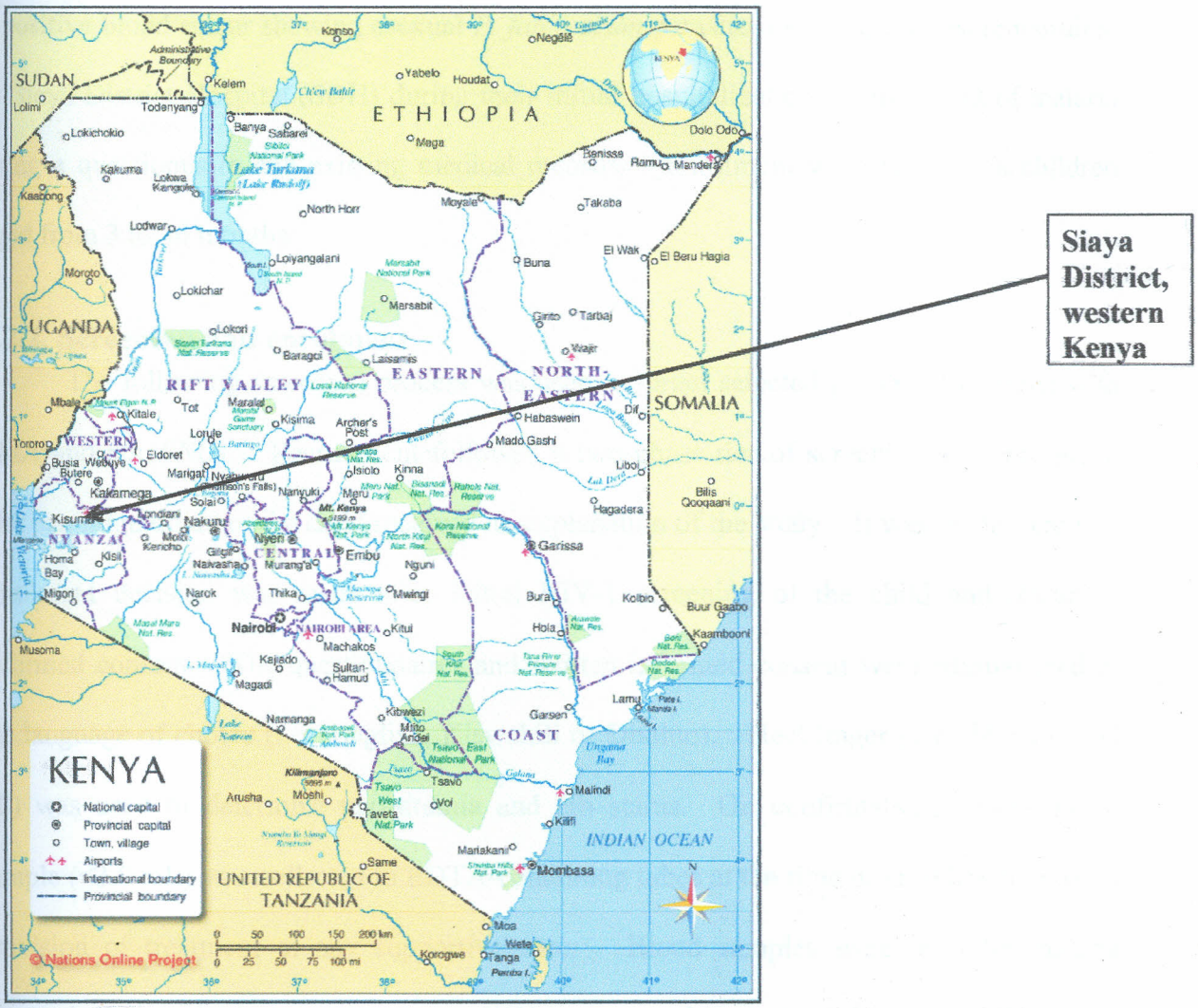

FIGURE 1: Siaya District Map by Administrative Units- 2007. (Courtesy of Siaya District Development Office - Source: Siaya District Development Plan 2008 - 2012).

3.2. Study design and patient population

3.2.1. Recruitment of study participants

This was a case-control study of which the cases were children with severe malarial anemia (SMA; positive smear for asexual *P. falciparum*, parasitemia and hemoglobin <6.0 g/dL). Controls were children of similar age and same gender with non-severe malaria

anemia (Hb≥6.0g/dL, any density parasitaemia) from *P. falciparum* malaria accompanied by a positive blood smear showing asexual *P. falciparum* parasitemia. Children were recruited at Siaya District Hospital (SDH) during their initial hospitalization for treatment of malaria using a questionnaire and existing medical records. Enrolment was confined to children aged from 3 to 36 months.

3.2.2. Screening and enrolment

The following screening process was used to target selected groups of children with SMA and non-SMA. Recruitment followed a two-phase tier of screening and enrolment. The parent/guardian of the child received an explanation of the study. It was made clear that enrolment decision was made after initial HIV-1 screening of the child and obtaining informed consent. The questionnaires and written informed consent were administered in the language of choice (i.e. English, Kiswahili or Dholuo). Heel/finger-prick blood (<100 μL) was used to determine parasitemia and Hb status. On confirmation, venous blood sample (<3.0 mL) was collected in EDTA-containing tubes at the time of enrollment, prior to provision of treatment or any supportive care. Blood samples were used for malaria diagnosis, hematological measurements, HIV testing, and bacterial culture. Children that satisfied all inclusion criteria were enrolled into the study. All participants promptly received appropriate antimalarial treatment and required supportive therapy as per the Kenya Ministry of Health (MoH) guidelines.

3.3. Eligibility criteria

3.3.1. Inclusion criteria

Any patient aged between 3 to 36 months, whether male or female, visiting SDH for the first time and presenting with symptoms of malaria was included into the study. Study

subjects were selected based on the period they had resided in the study site to validate the immune responses on children naturally exposed to *P. falciparum* infection.

3.3.2. Exclusion criteria

Any participant who had been hospitalized at any time in their life before the time of enrolment and any subject who had received anti-malarial therapy within 2 weeks before the time of recruitment was not taken in for the study. Patients with other forms of malaria parasite such as *P. malariae* and *P. ovale* were also excluded from the study. Upon enrolment into the study, HIV-1 status, parasitemia and haematological measurements of the child were determined. The children with acute malaria were stratified into two categories: Non-Severe malarial anemia (non-SMA) group- Children with a positive smear for asexual *P. falciparum,* parasitemia (of any density) and Hb≥6.0 g/dL, and Severe malarial anemia (SMA) - Children with a positive smear for asexual *P. falciparum,* parasitemia (of any density) and Hb < 6.0 g/dL (McElroy *et al.*, 1999).

3.4. Sample size determination

The current study targeted 240 malaria naïve patients. Children (aged 3-36 months) presenting with clinical symptoms of malaria in Siaya District Hospital were targeted. The sample size was determined using the following formula; $n = N\,x/\,((N\text{-}1)\,E2 + x)$, ($x = Z(c/100)\,2r\,(100\text{-}r)$, $E = \mathrm{Sqrt}[(N - n)x/n(N\text{-}1)]$). Where: *n*- sample size of the case/control group, *N*= is the population size, E=margin of error; X=confidence level (Dupont and Plummer, 1990). Furthermore, the total number to target for this study was also based on the practical number of children aged 3 to 36 months that presented with clinical symptoms of malaria at SDH within the past 2 years (averagely 20 patients per month). The primary objectives of this genetic study was to determine association between CD14, C-159T polymorphisms and SMA, and to identify CD14, C-159T polymorphisms that have

significant allele frequency differences between case (SMA) and control (non-SMA) populations. Based on previous observations in the same study population (Ouma *et al.*, 2008a; Ouma *et al.*, 2008b), the magnitude of significant allele frequency differences ranged from 0.10 to 0.13 between cases and controls. As such, an allele frequency difference of 0.1 [e.g. 0.15 in SMA cases vs. 0.25 in non-SMA (controls)] was selected as the smallest difference to distinguish from chance variation between the groups in the current study. Given the increased power provided by the procedure (Dupont and Plummer, 1990), a minimum number of cases or controls that was needed to achieve an 80% power with a Type I error rate of $\alpha \leq 0.01$ was 113. Based on this rationale, a sample size of 120 SMA cases and 120 parasitemic children without SMA (Non SMA/controls) further increased the power of study.

3.5. Collection and processing of blood samples

3.5.1 Collection of infected blood samples

Thick and thin blood films was prepared from pinprick blood and stained with Giemsa. Following microscopic confirmation of *P. falciparum* infection, venous blood was collected into heparinized vacutainers (Becton Dickinson, USA). Blood spots were made on FTA Classic® cards (Whatman Inc., Clifton, NJ, USA), air dried, and stored at room temperature until use.

3.5.2. Hematological measurements

Hemoglobin (Hb) levels and complete blood counts were determined using the Beckman Coulter ACT diff2™ (Beckman-Counter Corporation, Miami, FL, USA). Bacterial co-infections were investigated by incubation of blood by paediatric isolator microbial tubes, and further sub-cultures. Bacteria were identified by gram staining procedures, colonial characteristics, appearances and biochemical and serological tests

according to the methods used by (Were *et al.*, 2011). Sickle-cell status was determined by alkaline cellulose acetate electrophoresis while G6PD deficiency was determined by a fluorescent spot test using glucose-6-phosphate and oxidized NADP (NADP+). The patient's blood sample, depending on the amount of G6PD present would react to produce 6-phosphogluconane and NADPH, which fluoresce under ultraviolet (UV) light. Depending on the level of emission, the samples were scored as normal (high emission), intermediate (moderate emission), or deficient (no emission). HIV-1 status was determined using two rapid tests, Determine and Unigold. A positive or discordant result was then confirmed using nested PCR. These tests were done due to their confounding effects on the acquisition of severe malarial anemia.

3.5.3. DNA extraction

DNA was extracted from the FTA Classic card and filter papers using the Gentra Systems DNA extraction protocol (Gentra Systems, Minneapollis, MN, USA). Discs, 3 mm in diameter, were punched and put into 1.5mL eppendorf tubes. DNA purification solution (150µL) was added to the discs, making sure they were completely immersed into the solution. This was incubated at room temperature for 15 minutes. The discs were then washed by pipetting the solution up and down several times then discarding it. This wash was repeated twice, making a total of three washes with the DNA purification solution. 150µL of DNA elution solution was then added onto the discs. This was also incubated at room temperature for 15 minutes then washed. Another 150µL of the elution solution was added to the discs, this time incubating it on a heating block that had been pre-set to heat to 99°C for 15 minutes. This was meant to elute the DNA from the discs. After this, the solution containing the DNA was pipetted out and transferred into a clean 1.5mL eppendorf tube. To confirm the presence of DNA, a house keeping gene, the human glucose-3-

phosphate dehydrogenase (hG3PDH), was amplified using the PTC-100 programmable thermal controller (MJ Research Inc). The master mix per 20μL reaction contained 200μM of dNTPs, 10X buffer, 50mM MgCl, 100μM of each primer (forward and reverse), 1U/μL of Taq polymerase and 1μL of the test sample. The thermocycling conditions were as follows: Initial denaturation - 94°C for 2 minutes; Denaturation - 94°C for 45 seconds; Annealing - 62°C for 45 seconds; Extension - 72°C for 2 minutes; these were repeated for 40 cycles and a final extension - 72°C for 7 minutes.

3.5.4. Genotyping of CD14 C-159T

After DNA extraction, the samples were then genotyped using the Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Applied Biosystems SNP-specific probes (TaqMan® SNP Genotyping Assays, assay ID; 2569190) was used to identify the CD14 C-159T SNPs (Ref. Appendix 2, for primer and probe sequences). Each 10μL reaction mix per well contained 5μL of 2X TaqMan Universal PCR master mix, 0.5μL of 20X SNP assay mix, 3.25μL of PCR water, and 1.25μL of the DNA sample (~50ng of extracted DNA sample). The thermo-cycling conditions were as follows: Pre-PCR temperature - 60°C for 30 seconds; Initial denaturation temperature - 95°C for 10 minutes; Denaturation temperature - 95°C for 15 seconds; Annealing/extension temperature- 62°C for 1 minute; These were repeated for 45 cycles; Post-PCR hold - 60°C for 30 seconds. Samples were run alongside pure water as a negative control. The results were then stored for further analyses and manipulation (Genotyping graph, Ref. Appendix 3).

3.6. Statistical analyses

Statistical analyses were performed using SPSS (Version 20.0). Chi-square analysis was used to examine differences between proportions. The association between CD14 C-

159T genotypes and susceptibility to SMA was determined by multivariate logistic regression, controlling for the confounding effects of sickle cell trait age, gender, HIV-1 status [including HIV-1 exposed and definitively HIV-1(+) results], and bacteremia. Critical significance level was set at $p \leq 0.05$

3.7. Ethical considerations

Approval to carry out this study was initially provided by the School of Graduate Studies (SGS) of Maseno University. Ethical approval was obtained from the Kenya Medical Research Institute (KEMRI) Ethical Review Committee (Appendix 4).

CHAPTER FOUR

RESULTS

4.1. Demographic, clinical, and laboratory characteristics of study participants

To determine the association between the CD14 C-159T promoter polymorphisms and severity of malaria anemia in children, a total of 240 children aged 3-36 months with any density *P. falciparum* parasitemia were included in the study. The children were stratified into two groups; SMA (Hb<6 g/dL), n=120 and non-SMA (Hb≥6 g/dL) n=120. The demographic, clinical, and laboratory characteristics of the study participants are summarized in Table 1 below.

The proportion of female versus male participants was comparable between the SMA and non-SMA groups (P=0.121). The parasite density and initial temperature of the participants was comparable between the two clinical categories (P=0.118, and P=0.113 respectively). However, participants in the SMA group were younger than those in non-SMA (P=0.004). Both haemoglobin level and erythrocyte counts were also low in SMA group compared to non-SMA (P<0.001).

Table 1. Clinical, demographic and laboratory characteristics of the study participants

Characteristics	SMA (Hb<6.0g/dL)	Non-SMA (Hb≥6.0g/dL)	*P*- Value
No. Of participants (n=240)	120	120	
Gender, n (%)			
Female	63 (52.5)	51 (42.5)	0.121[a]
Male	57 (47.5)	69 (57.5)	
Age, months*	8.0 (5.0)	10.0 (10.0)	**0.004**[b]
Haemoglobin level, g/dL*	4.7 (1.2)	9.0 (2.2)	**<0.001**[b]
Parasite density, parasite/μL*	18356.3 (39015.4)	25178.1 (48467.2)	0.118[b]
Erythrocyte counts, $X10^{12}$/μL*	2.0(0.7)	4.2 (0.9)	**<0.001**[b]
Temperature, °C*	37.5 (2.0)	37.6 (2.0)	0.113[b]

*Data are the median (interquartile range; IQR) unless otherwise noted. Children with parasitemia (n=240) were stratified according to a modified definition of SMA based on age- and geographically-matched Hb concentrations (i.e., Hb<6.0g/dL, with any density parasitemia) (McElroy *et al.,* 1999) into non-SMA (n=120) and SMA (n=120). [a] Statistical significance determined by the χ^2 analysis. [b] Statistical significance determined by the Mann-Whitney U test.

4.2. Association between CD 14 C-159T genotypes and susceptibility to severe malaria anemia

To determine the association between the CD14 -C159T genotypes and susceptibility to SMA, multivariate logistic regression analysis was used while controlling for the confounding effects of age, gender, bacteremia, HIV-1 status, G6PD deficiency and sickle-cell trait (Berkley *et al.*, 1999; Aidoo *et al.*, 2002; Otieno *et al.*, 2006). Using CC as the reference group in the analyses, the results demonstrated no association between the CD14 C-159T genotypes and susceptibility to SMA (CC vs. CT, OR =0.75, CI =0.38-1.47, *P*= 0.398, and CC vs. TT, OR =1.23, CI = 0.63-2.36 *P* = 0.544; Table 2). Since the TC was the most prevalent in the population, additional analyses were carried out in which it was used as the reference group (Table 2). Likewise, there were no significant associations between CC (*P*=0.131) and TT (*P*=0.544) and susceptibility to SMA (Table 2).

Table 2. Association between CD14 C-159T genotypes and SMA

	SMA (Hb level <6.0g/dL)		
Genotypes	**Odds Ratio (OR)**	**95% Confidence Interval**	***P*-value**
CD14-C159T			
CC	1.00 (reference)		
CT	0.75	0.38-1.47	0.398
TT	1.23	0.63-2.36	0.544
TC	1.00 (reference)		
CC	0.61	0.32-1.16	0.131
TT	0.82	0.42-1.57	0.544

Children with acute malaria (n=240) were stratified according to the modified definition of SMA based on age and geographically matched Hb concentration (i.e., Hb<6.0g/dL, with any density parasitemia), (McElroy *et al.*, 1999). Odds ratios (OR) and 95% confidence intervals (CI) were determined using multivariate logistic regression controlling for age, gender, HIV-1 infection, sickle cell trait (HbAS), bacteremia, and G6PD deficiency (Berkley *et al.*, 1999; Aidoo *et al.*, 2002; Otieno *et al.*, 2006). The reference group in the multivariate logistic regression analysis was the homozygous wild-type genotype (CC) and TC (the most prevalent) in the population.

4.3. Distribution of the CD14-C159T genotypes and alleles in study participants

Genotypic distributions of individual CD14 C-159T promoter variant in the SMA (n=120) and non-SMA (n=120) groups, and the combined cohort, are presented in Table 3. The distribution of the individual genotypes in the whole cohort was 33.3% (80/240) CC; 37.1% (89/240) CT; and 29.6% (71/240) TT. In the SMA group, the distribution was 30.0% (36/120) CC; 40.8% (49/120) CT; and 29.2% (35/120) TT, whereas the distribution of the genotypes in the non-SMA group was 36.7% (44/120) CC; 33.3% (40/120) CT; and 30.0% (36/120) TT. The genotype distribution was comparable between SMA and the non-SMA (P=0.422) groups. In the combined cohort, there was departure from the Hardy-Weinberg Equilibrium (HWE) for the CD14 C-159T variant ($\chi 2$ =15.9, P<0.001) while the variant allele (T) frequency was 48%. Additionally, deviation from HWE was also observed in the SMA and non-SMA groups ($\chi 2$=4.0, P=0.045 and $\chi 2$=13.1, P<0.001, respectively). The variant allele (T) in SMA was 50% while in non-SMA it was 47%.

Table 3. Distribution of the CD14-C159T genotypes and alleles in the study participants

	Genotype	SMA (Hb level <6.0g/dL)	Non-SMA (Hb level ≥6.0g/dL)	Total	*P*-value
No of participants		n=120	n=120	240	
CD14 C-159T	CC, n (%)	36(30.0)	44(36.7)	80(33.3)	0.422[a]
	CT, n (%)	49(40.8)	40(33.3)	89(37.1)	
	TT, n (%)	35(29.2)	36(30.0)	71(29.6)	
	Allele				
	C	121(50)	128(53)	249(52)	
	T (variant)	119(50) ($\chi2$=4.0, *P*=0.045)	112 (47) ($\chi2$=13.1, *P*<0.001)	231(48) ($\chi2$=15.9, *P*<0.001)	

Data are presented as proportions (n, %). Abbreviations: SMA, severe malarial anemia (Hb<6.0 g/dL, with any density parasitemia) (McElroy *et al.*, 1999) ; Non-SMA, non-severe malarial anemia (Hb≥6.0 g/dL, with any density parasitemia); *P* (T), frequency of variant/ minor allele in the population. [a] Statistical significance determined by $\chi2$ analysis.

CHAPTER FIVE

DISCUSSION

In holoendemic areas of *P. falciparum* transmission, such as in western Kenya, SMA is the primary clinical manifestation of severe malaria in infants and young children. Although a majority of these infected children present with only mild forms of malaria, others experience severe life-threatening complications that predominantly manifest as SMA (Baldini *et al.*, 1999). Comparing the genetic backgrounds of *P. falciparum*-infected children who develop SMA with those of children who do not develop SMA would therefore help in identifying gene variants associated with susceptibility to SMA. The effect of CD14C-159T promoter polymorphisms on susceptibility to SMA was investigated by examining variants in *P. falciparum*-infected children without SMA, and children with SMA. Analyses showed no significant association between the CD14C-159T promoter polymorphisms and susceptibility to SMA. In addition, the genotypic frequencies were also not significantly different in the SMA and non-SMA groups.

Most previous studies have reported the CD14C-159T polymorphism with atopic diseases, levels of sCD14 and IgE levels among different ethnicities. Some of the studies have confirmed the association of TT genotype with significantly higher levels of sCD14, lower levels of total serum IgE and exposure to atopic diseases (Baldini *et al.*, 1999), while others have reported contrary results (Koppelman *et al.*, 2001; Buckova *et al.*, 2003; Leynaert *et al.*, 2006). It is important to note that the association between the CD14C-159T polymorphism and susceptibility to severe malaria disease in sub-Saharan Africa has not been reported extensively. This point stands despite the fact that the associations between gene variants and susceptibility to SMA remain unclear in *P. falciparum* holoendemic regions such as in Siaya District in western Kenya. The TT homozygote of the CD14C-159T has been associated with elevated levels of sCD14 (Baldini *et al.*, 1999; Kabesch *et al.*,

2004). Elevated levels of sCD14 have further been reported to be associated with acute *P. falciparum* malaria manifesting as renal failure among a Thai population (Wenisch *et al.*, 1996). The current study in an attempt to relate the CD14C-159T genotypes to susceptibility to SMA found no association (CC vs. CT, OR =0.75, CI =0.38-1.47, P= 0.398, and CC vs. TT, OR =1.23, CI = 0.63-2.36 P = 0.544) between any of the variants and susceptibility to SMA. Since the TC was the most prevalent in the population, additional analyses using it as the reference group, still revealed no associations between any of the variants, CC (P=0.131) and TT (P=0.544), and susceptibility to SMA. These results demonstrate that the CD14C-159T polymorphisms may not independently alter susceptibility to SMA in this pediatric population. These observations differ from findings in the Thai population (Wenisch *et al.*, 1996). Such differences may be attributed to differences in disease manifestations in different populations with differing genetic backgrounds. For example, in the Thai population, severe disease manifests as a mixed clinical phenotype (i.e. SMA and cerebral malaria, renal failure) in adults populations while in western Kenya, SMA is the most severe clinical manifestation in pediatric population. Additional differences in malaria disease transmission patterns may also select for particular genes relative to others to confer positive selection. For example, in Thai, the disease transmission pattern is hyper-endemic while in western Kenya, it is holoendemic. Additional studies exploring additional promoter genes are warranted to help decipher variants that could potentially alter susceptibility to SMA in this pediatric population resident in western Kenya.

The distribution of the genotypes was comparable between SMA and the non-SMA (P=0.422) indicating no preference of any of the three genotypes (TT, CT and CC) in either SMA or non-SMA groups. The frequency of the variant allele (T), however deviated from the Hardy-Weinberg equilibrium (HWE) in the SMA, non-SMA and even in the combined

group [(50%, $\chi 2$=4.0, P=0.045), (47%, $\chi 2$=13.1, P<0.001) and (48%, $\chi 2$=15.9, P<0.001) respectively]. It is important to note that the combined group frequency of the T variant allele (48%) was lower than that observed among the Tunisians (asthmatic 57%, controls 68%) but was higher than that Yoruba of Nigeria (30%) (Lachheb *et al.,* 2008), indicating diverse distribution of alleles in different regions, and even among same races. The observed frequency in the current population, however, did not from Hardy-Weinberg Equilibrium (HWE) (P=0.422). As indicated above, the variants within the CD14C-159T may not be under selection by malaria disease in this pediatric population from western Kenya. Identification of genes under selection is a necessary first step in the design of an effective vaccine in a population since it gives directions on genes that can potentially be targeted. Since all of the CD14C-159T variants were not likely to be selected in this population, they are unlikely to be the genes under selection by malaria in this population. It would be critical to map additional genes around the CD14 and beyond, to help unravel genetic variants that alter disease susceptibility in this population. Furthermore, identification of genes may be affected by relatively small sample sizes in genetic studies. Additional analyses in large populations are warranted to further delineate whether these variants could alter susceptibility in SMA. Since the current study also did not follow up the children over time, it would be important to design a longitudinal study in which disease outcome can be monitored over time to establish the associations between these variants and longitudinal outcomes in disease. Finally, it has previously been demonstrated that haplotypes in a gene can be more informative on how genes moderate or amplify their effects in order to give a particular disease outcome (Ouma *et al.,* 2008a; Ouma *et al.,* 2008b). As such, additional studies should explore the possibilities of including haplotypes within the CD14 gene to further reveal possible associations in the context of haplotypes.

Finally, since CD14 acts only in recognition and induction of immune response, it could be argued that genes involved in immune regulation such as IL-10, transforming growth factor (TGF)-β, and IL-4 among others should also be studied together with it, since susceptibility to infectious diseases occurs through complex, multifactorial, and often contradictory selective forces (Balaresque *et al.*, 2007). In summary, the insignificant association of CD14 C-159T genotypes with SMA and allelic and genotypic frequencies observed could be variable across different populations, possibly due to differential exertion of selective pressures on human genome especially host immune response genes that mediate susceptibility and clinical outcomes of diseases such as malaria (Kwiatkowski and Luoni, 2006).

CHAPTER SIX

SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1. Summary of Findings

The current study investigated the association between CD14 C-159T polymorphism and susceptibility to SMA in pediatric population in Siaya District in western Kenya, a *P. falciparum* malaria holoendemic transmission region reported lack of association between CD14C-159T genotypes and susceptibility to SMA. The genotype frequencies were also comparable between the SMA and non-SMA groups. However, the frequency of the variant allele T deviated from the HWE with the allele showing an increase in frequency in the population.

6.2. Conclusions

a) There is no association between the CD14 C-159T promoter polymorphisms and susceptibility to SMA in children presenting with *P. falciparum* malaria in a holoendemic region of Siaya District, western Kenya.

b) The genotype frequencies of the CD14 C-159T are comparable between SMA and the non-SMA in children presenting with *P. falciparum* malaria in a holoendemic region of Siaya District, western Kenya. However, the frequency of variant allele shows a departure from the Hardy-Weinberg Equilibrium (HWE), indicating existence of some selective pressure on this locus.

6.3. Recommendations and future research areas

a) CD14 promoter C-159T polymorphism does not affect the susceptibility to SMA solely, but there is a possibility that it can affect the outcome in association with other same promoter loci SNPs as well as with other related genes. Therefore,

further studies should be done based on association of CD14 promoter C-159T polymorphism with other identified promoter SNPs in the same gene, as well as on association of this gene with other genes such as IL-13, FcγRIIIA, TLR9, with the aim of revealing how such associations affect the outcome of severe *P. falciparum* malaria and other related diseases.

b) There are selective pressures acting on the CD14 gene. Research should be done to identify these pressures as well as other related host immune response genes that mediate susceptibility and clinical outcomes of diseases such as malaria in an attempt to identify at risk populations for effective intervention.

c) There is a need for a similar study that includes measurement of levels of sCD14 to reveal a clear link of how CD14 C-159T genotypes and sCD14 influence outcome of severe malaria in one population.

d) Additional longitudinal studies, investigating the associations of CD14 haplotypes across time can be informative how a combination of these variants moderate disease outcome over time.

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APPENDICES

APPENDIX 1: Operational Terms

Term	Definition
Acute malaria:	Any of various forms of malaria that may be intermittent or remittent, consisting of a chill accompanied by fever with its attendant general symptoms and terminating in a sweating stage.
Allele:	Alternative form of a gene located at a specific location on a specific chromosome.
Genotype:	A set of alleles that determines the expression of a particular characteristic or trait.
Haplotype:	A combination of alleles at adjacent locations (loci) on the chromosome that are inherited together in blocks.
Heterozygous:	Having two different alleles at a specific gene locus.
Homozygous:	Having two identical alleles at a specific gene locus.
Odds Ratio:	Is a measure of association between an exposure and an outcome.
Severe malaria:	Life-threatening form of malaria that is characterized by symptoms including kidney failure, hyper parasitemia, mental confusion (cerebral malaria) , severe anemia, pulmonary edema, acute respiratory distress syndrome (ARDS) and haemorrhage.
SNP:	A genetic variation in a DNA sequence that occurs when a single nucleotide - A, T, C or G - in a genome differs in members of a species.

APPENDIX 2: Primers and probes used

	Primers 5' to 3'	Probes 5' to 3'
Promoter C-159T	**Forward-** CTAGATGCCCTGCAGAATCCTT **Reverse-** CCCTTCCTTTCCTGGAAATATTGCA	Wild -VIC-CTGTTACGGCCCCCCT-MGB Mutant -FAM-CTGTTACGGTCCCCCT-MGB

APPENDIX 3: Amplification plot

Allelic discrimination plot of CD14 (C-159T). Genotyping was done by use of TaqMan® 5' allelic discrimination assay technique (Applied Biosystems, Foster City, CA, USA).

APPENDIX 4: Research Approval

15 FEB 2012

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 **February 13, 2012**

TO: **PROF. COLLINS OUMA (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. JOHN VULULE,**
THE DIRECTOR, CGHR,
KISUMU

FORWARDED
DIRECTOR

RE: **SSC PROTOCOL No. 1733 – (*RE-SUBMISSION – REQUEST FOR STUDY RENEWAL*): IMPACTS OF SURFACE RECEPTORS [TOLL LIKE RECEPTOR (TLR)] AND Fc GAMMA RECEPTOR (FcγR) ON SUSCEPTIBILITY TO PAEDIATRIC SEVERE MALARIAL ANAEMIA**

Reference is made to your letter dated February 7, 2012. We acknowledge receipt of the following documents on February 9, 2012:

(a) ASTMH Abstract # 848 – Kiplagat S *et al*
(b) ASTMH Abstract # 1208 – Ouma C *et al*
(c) ASTMH Abstract # 1292 – Ouma C *et al*
(d) Functional haplotypes of Fc gamma (Fcγ) receptor (FcγRIIA and FcγRIIIB) predict risk to repeated episodes of severe malarial anemia and mortality in Kenyan children. *Hum Genet*

This is to inform you that the Committee determines that the issues raised at the initial review are adequately addressed. Consequently, the study is granted approval for implementation effective this **13th day of February 2012** for a period of one year.

Please note that authorization to conduct this study will automatically expire on **February 11, 2013**. If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to the ERC Secretariat by **January 4, 2013**.

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

Sincerely,

CHRISTINE WASUNNA,
FOR: SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

APPENDIX 5: Consent forms

Screening Form: Written consent for parent/guardian for screening of child for malarial anemia hospital-based prospective study

You are being asked to participate in screening for a research study. The purpose of this document is to provide you with information to consider in deciding whether to participate in the screening procedure for this research study. Your consent should be made based on your understanding of the nature and risks of the procedures. Please ask questions if there is anything you do not understand. Your participation is voluntary and will have no effect on the quality of your child's medical care if you choose not to participate.

Is my participation in this screening procedure voluntary?
Having the screening done is up to you. If your child is eligible for the research study, based on the screening results, you may choose to have your child enrolled in the research study. If you do not want your child screened or to be in the research study, your child will still get the best possible medical care at hospital. You can change your mind at any time. There will not be any loss of care or benefits if you refuse for your child to participate or if you want to withdraw your child at any time. If you decide you want your child to in the research study, you must discuss the research study with a member of the research team and sign the consent form for enrollment (participation).

Who will be conducting the screening procedure and research study?
The Kenya Medical Research Institute (KEMRI) in collaboration with the University of New Mexico is doing a research study at the Siaya District Hospital and Ombeyi Health Centre to see how children fight malaria. The principal investigator, Dr. Collins Ouma, of the The Kenya Medical Research Institute (KEMRI) would like your child to be screened to see if your child can be in the research study since you live in an area where nearly all children get malaria.

The title of the study is "Impacts of surface receptors [Toll like rececptor (TLR) and Fc gamma receptor (FcγR) on susceptibility to pediatric severe malarial anemia". If you have questions about the study or feel you have been harmed, you can contact Dr. Collins Ouma (KEMRI/University of New Mexico, P.O. Box 1578, Kisumu, Kenya, telephone: 0722-381214).
You can also contact a co-investigator on the study, Dr. John Michael Ong'echa (KEMRI/University of New Mexico, P.O. Box 1578, Kisumu, Kenya, telephone: 0733-447920).
The physician at Siaya District hospital in charge of treatment and care is Dr. Benjamin Esiaba. You can contact Dr. Benjamin Esiaba (telephone 321055/321554) at Siaya District Hospital, Nyanza Province, P.O. Box 144, Siaya. The physician at Ombeyi Health Centre in charge of treatment and care is Dr. Hezron MacKombewa. You can contact Dr. Hezron MacKombewa at Ombeyi Health Centre, Nyanza Province, P.O. Box 3050-40100, Kisumu or through phone (mobile 0722-866943). For questions or problems about your rights as a research subject, please call or write: The National/KEMRI Ethical Review Committee, P.O. Box 54840, Nairobi, telephone: 02-20722541.

Who is sponsoring the research study?

The research study is funded by the National Institutes of Health (NIH) in the United States.

Where will the screening procedure and research study be conducted?
The screening and research study will be conducted at the Siaya District Hospital and in Ombeyi Health Centre, Ombeyi, Muhoroni.

What is the purpose of the research?
The purpose of the research study is to better understand the causes of severe malaria in children from this region. We want to learn how children fight malaria during the first three years of life. We will enroll children who are 3 to 18 months of age and follow them for 3 years from the date of enrollment. Through this, we will learn ways to help improve treatment or prevention of malaria. Before joining the research study, your child must have several tests to see if he or she is in the groups of children that the research study will be done in.

Who is being asked to participate in this screening?
Your child is being asked to participate because your child has the signs and symptoms of malaria.

What procedures will be performed for screening purposes?
Since the research study will only include children with malaria and anemia (low blood), we will screen your child to see if he or she has malaria and low blood. If you choose for your child to be screened, we will ask you about your child's health and check for malaria and low blood. To check for malaria and low blood, we will get blood (several drops) by sticking your child's heel or finger with a small needle.

Also, we know that HIV/AIDS is a problem in this area. HIV/AIDS affects the way the body fights infections. There are blood tests that can be done to see if your child has the virus that causes AIDS. This virus is called HIV. We would like to test your child's blood for HIV exposure. The research study will enroll children with HIV exposure and those without HIV exposure.

It is important for the research study to know who has HIV exposure since it affects the way your child fights infections. If your child has HIV exposure, this means that he or she may or may not have HIV. To see if your child has HIV exposure, we must do a rapid blood test from the several drops of blood we will get by sticking your child's heel or finger with a small needle. You will receive results of the rapid test (for HIV exposure) the same day. If your child has a positive rapid test for HIV, we will do an additional test. You will receive the results of the additional test at Siaya District Hospital (for those whose tests are carried out in Siaya) or Ombeyi Health Centre (for those in Ombeyi) within two weeks. We will give you a schedule telling you when to return. If the results of the test are positive, you will need to bring your child back for another test to confirm the results two weeks from now. When you return in two weeks, we will need to get blood (several drops) by sticking your child's heel or finger with a small needle for the additional test. This second test should confirm if your child has HIV in his or her body.

If you want your child to be tested for HIV, you will have to talk to one of our HIV counselors.

The counselor will give you facts about HIV. These facts will be about the HIV blood tests and how you can keep your child from getting HIV. Whether the blood test is negative or positive, you will be the only person to get the result of your child's HIV test. The results of the HIV test will be kept private to the extent allowed by law.

What are the possible risks, side effects, and discomforts of the screening?
Receiving your child's HIV test results can be stressful. If your child tests positive, this may upset you. However, knowing the tests results will benefit your child, because you will be able to act to protect your child's health. If your child's blood tests are positive for HIV, we would like you to talk with a doctor. However, if you choose, only you will get the HIV test results of your child. You do not have to speak with a doctor. We will not tell any other person the result of the test unless you ask us to do so. If others find out about the HIV results, it could cause some social stigma (problems in the community). Testing for HIV and talking to HIV/AIDS counselors will be free of charge. If your child has a positive HIV-1 test result, this means that you may have HIV-1. As such, if you want, we kindly ask that you seek HIV-1 testing and counseling at the Adult Comprehensive Care Clinic at SDH or Ombeyi Health Centre.

If you want, we will refer your child to the Patient Support Center (PSC) at SDH (for those in Siaya) and Ombeyi Health Centre (for those in Ombeyi) so that your child can get medicine that can help fight the HIV virus. HIV can also cause low blood in your child. Counselors will give you facts about problems of low blood. They will also talk with you about why it is important to go to follow-up visits and take the vitamins and drugs for malaria and HIV.

Pricking your child's heel or finger may cause slight discomfort and bruising. Only a person trained to get blood will take blood from your child. If your child has no problems with bleeding, taking blood should not cause harm. There may be a small bruise or short time of discomfort when we do the finger- or heel-stick. Additional risks include the unintentional disclosure (release) of health information that could cause problems for you and your child in the community.

What are the possible benefits from taking part in this research study?
There may be no direct benefits for participation in the research study.

What treatments or procedures are available if I decide that my child will not participate in this screening procedure?
The procedure for the screening that includes, testing for malaria, HIV, and low blood can be performed by the Ministry of Health if you do not want to participate in the screening.

Will I be told of any new information or new risks that may be found during the course of this study?
You will be told of any significant new developments (findings) that may cause you to change your mind about your child participating in the screening or research study.

Will my insurance provider or I be charged for any costs of any procedures performed as part of this screening procedure?
Neither you nor your insurance provider will be charged for the costs of any of the procedures performed for the purpose of screening.

Who will pay if my child is injured during the screening?
In the unlikely event that your child becomes ill or injured as a direct result of participating in the screening, you will receive medical care that will be provided free of charge from the Ministry of Health. The cost of the health care will be paid for by the project.

Will I be paid for participating in this screening procedure?
You will not be paid for your child's participation in the screening.

Who will know about my child's participation in this screening?
Information related to you and your child will be treated in strict confidence to the extent provided by law. Your child's identity will be coded and will not be associated with any published results. Your child's code number and identity will be kept in a locked file of the Principal Investigator. In order to monitor this research study, representatives from the federal agencies such as NIH (National Institutes of Health) and OHRP (Office of Human Research
Protection) may inspect the research records which may reveal your identity.

Parent/guardian's name: _____ Child's name:_____
(Please Print) (Please Print)

Date: _____ Study #:_____

Parent/guardian's statement:
□ The above screening process has been explained to me. The screening consent form has been read to me or I have read the screening consent form. My questions have been answered to my satisfaction. I have received a copy of this form. I was told that being in the research study is my choice. I was told that for my child to be in the research study that I must discuss the research study with a member of the study team. I was told that to be in the research study, I must sign the consent form for the research study that is separate from this form. I agree for my child to be screened for taking part in the research study. By signing this form, I give my consent for my child to having screening for the research study.

Signatures:
Parent/guardian's signature: _____ Date: _____

Witness Signature: _____ Date: _____

Parent/guardian' thumbprint: _____

HIV Testing:
□ The above screening process has been explained to me. The consent form for screening has been read to me or I have read the screening consent form. My questions have been answered to
my satisfaction. I have been told that HIV counseling is available to me before I decide if my child will have HIV testing. I agree that my child's blood sample can be tested for HIV.

Parent/guardian's signature:_____ Date :_____

Parent/guardian's thumbprint: _____

****
***

STUDY EMPLOYEE CERTIFICATION

I certify that the nature and purpose, the potential benefits and possible risks associated with participation in this research study have been explained to the above individual and that any questions about this information have been answered.

Study employee's signature: _____ **Date:** _____