POLYMORPHISMS OF TOLL LIKE RECEPTOR 4 (-8984C/G AND 299ASP/GLY) AND INTERFERON GAMMA LEVELS IN CHILDREN WITH SEVERE *PLASMODIUM FALCIPARUM* MALARIA ANAEMIA IN SIAYA COUNTY, WESTERN KENYA

BY

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DEPARTMENT OF BIOMEDICAL SCIENCES AND TECHNOLOGY

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DECLARATION

I declare that this is my original work and has not been presented to any other University or institution for a degree or any other award.

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I am indebted to my family, my parents and siblings for their encouragement and emotional support in every way possible to make this work a success.

Finally and most importantly, I thank the Lord for granting me life and the capability to carry out and successfully finish this work.

DEDICATION

This thesis is sincerely dedicated to my dear wife Frida Achieng and children Barbara, Wendy, Candy and Joshua in appreciation of their support, encouragement and sacrifice that was highly felt during the study.

May God bless you.

ABSTRACT

Plasmodium falciparum (*P. falciparum*) malaria is a major cause of childhood morbidity and mortality in Sub-Saharan Africa despite the integrated approaches put in place to control the disease. Most of the mortality in holoendemic transmission areas occurs due to severe *P. falciparum* disease complication of severe malarial anaemia (SMA), a condition which largely presents in children below five years. Molecular determinants have been implicated in the pathogenesis of SMA (Hb<6.0 g/dl with any density parasitemia). Toll-like receptors (TLRs) induce production of pro-inflammatory cytokines such as interferon gamma (IFN-γ) and possesses promoter polymorphisms that could contribute to the development of SMA by influencing the circulating levels of IFN-γ, a major cytokine that activates phagocytes in malaria infection.TLR 4 is a well-established receptor for the toxigenic glycosylphosphatidylinositol (GPI) of *P. falciparum*. However, the contribution of these receptor polymorphisms in the pathogenesis of SMA and circulating levels of IFN-γ remains largely unknown in peadriatic populations resident in *P. falciparum* holoendemic areas of western Kenya. Identifying genes that naturally condition susceptibility to SMA in children population is important in designing a long lasting malaria vaccine. The purpose of the current study was to assess the associations between TLR-4 polymorphisms and IFN-γ levels in children (aged 3-36 months) that presented with clinical symptoms of falciparum malaria in Siaya County Referral Hospital (SCRH), western Kenya. Specifically, it determined the associations between TLR-4 (-8984C/G and 299Asp/Gly) polymorphic variants and susceptibility to SMA and functional differences in IFN-γ production. In addition, the study determined the functional differences in IFN-γ levels between the clinical groups. In this case-control study, 414 children with SMA and their age- and sex-matched with non-SMA (Hb ≥6.0g/dl with any density parasitemia) controls were targeted. Parasite genomic DNA was extracted from stored blood spot samples and genotyped for TLR-4 (-8984C/G and 299Asp/Gly) polymorphisms using TaqMan real-time polymerase chain reaction technique. Circulating IFN-γ levels was quantified from 50µl of stored plasma using Human Cytokine 25-plex Antibody Bead Assay. Haematological and parasitological parameters were determined in all study participants prior to administration of medication. Multivariate logistic regression analysis (controlling for confounders), demonstrated that none of the genotypes TLR-4 -8984C/G (GG vs. GC, OR, 0.78, 95% CI, 0.55-1.67, P=0.89 and GG vs. CC, OR, 0.62, 95% CI, 0.20-1.89,P=0.740) and TLR-4 +299Asp/Gly (Asp/Asp vs. Gly/Asp, OR, 1.67, 95% CI, 0.24-11.62, P =0.60 and Asp/Asp vs. Gly/Gly, OR, 2.06, 95% CI, 0.330-12.99, P=0.44) or haplotypes TLR-4 -8984G and +299Gly(OR, 0.89; 95% CI, 0.46-1.47; P= 0.51), TLR-4 -8984G and +299Asp (OR, 0.79; 95% CI, 0.35-1.76; P= 0.56) and TLR-4 -8984C and +299Asp (OR, 0.84; 95% CI, 0.52-1.36; P=0.486) showed any significant associations with susceptibility to SMA. Further analyses revealed that there were no differences between individual genotypes and circulating IFN-γ levels. However, carriers of C/Asp (-8984C and +299Asp) haplotype showed significantly lower levels of circulating IFN-γ (P=0.041) relative to non-carriers. The circulating levels of IFN-γ were similar between the clinical groups (P=0.224).These results demonstrate thatTLR-4 (-8984C/G and 299Asp/Gly) variants are not associated with SMA in this population, but co-inheritance of functional variations in TLR-4 condition changes in circulating IFN-γ levels. The findings are important in further enhancing knowledge on the immune genes that may alter pathogenesis of malaria, thus making more informed decisions in designing rational novel interventions in the control against malaria. Therefore, the pathological role of lower IFN-γ should be investigated as it may act peadriatric as a basis of vaccine development against malaria.

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ABBREVIATIONS AND ACRONYMS

ADCC	-	Antibody-Dependent Cell-mediated Cytotoxicity
CM	-	Cerebral Malaria
DNA	-	Deoxyribonucleic Acid
EDTA	-	Ethylene Diatetra Acetic Acid
ELISA	-	Enzyme Linked Immunosorbent Assay
FTA	-	Free To Air
G-6-PD	-	Glucose 6 Phosphate Dehydrogenase
GPI	-	Glycosylphosphatidylinositol
Hb	-	Haemoglobin
HDP	-	High Density Parasitemia
HIV-1	-	Human Immunodeficiency Virus subtype 1
IFN-γ	-	Interferon Gamma
IL	-	Interleukin
μL	-	Microliter
NADP	-	Nicotinamide Adenine Dinucleotide Phosphate
PAMPs	-	Pathogen Associated Molecular Patterns
PCR	-	Polymerase Chain Reaction
pDCs	-	Plasmacytoid Dendritic Cell
RANTES	-	Regulated upon Activation, Normal T- cell Expressed and Secreted
PRRs	-	Pattern Recognition Receptors
RBC	-	Red Blood Cell
RDW	-	Red Blood Cell Distribution Width
RSP-2	-	Ring Surface Protein-2
SCRH	-	Siaya County Referral Hospital
SMA	-	Severe Malarial Anaemia
SNPs	-	Single Nucleotide Polymorphisms

SPSS	-	Statistical Package for the Social Sciences
TBE	**-**	Tris- Boric-Ethylene Diatetra Acetic Acid
TLRs	**-**	Toll-Like Receptors
TNF	**-**	Tumour Necrosis Factor
UV	-	Ultra-Violet
WHO	-	World Health Organization

OPERATIONAL TERMS

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.

Severe malaria Life-threatening form of malaria that is characterized by symptoms including kidney failure, hyper parasitemia, mental confusion (cerebral malaria), severe Anaemia, pulmonary edema, acute respiratory distress syndrome (ARDS) and hemorrhage.

Allele An alternative form of a gene (one member of a pair) that is allocated at a specific position on a specific chromosome.

Genotype A set of alleles that determines the expression of a particular characteristic or trait.

Haplotype Combination of alleles at adjacent locations (loci) on the chromosome that are transmitted together.

SNP Single Nucleotide Polymorphism is a genetic variation in a DNA sequence that occurs when a single nucleotide- A, T, C or G in a genome is altered.

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CHAPTER ONE

INTRODUCTION

1.1 Background Information

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium* and is transmitted by the bites of infective female *Anopheline* mosquitoes. Of the five species of human malaria parasites, *Plasmodium falciparum* (*P. falciparum*) is the most virulent and accounts for an estimated 300,000 deaths per year, predominantly in children under the age of 5 years living in sub-Saharan Africa (WHO, 2016). *Plasmodium falciparum* malaria is a complex clinical syndrome comprising a number of life-threatening conditions including severe malarial anaemia (SMA), cerebral malaria (CM), metabolic acidosis, respiratory distress, hypoglycaemia and other, less frequent complications such as hypotension (systolic blood pressure <50 mmHg in children aged <5 years) (Giha *et al*., 2005; Marsh *et al*., 1995). Of these pathologies, severe malaria anaemia (Hb<6.0 g/dL) immunopathogenesis remains the least understood, although it is a major health problem in endemic areas for children and pregnant women and a main cause of the infant mortality associated with malaria (Ekvall, 2003). It accounts for the greatest degree of global malaria-related morbidity and mortality (WHO, 2016).

The mortality rate in children with SMA is about 8.6% compared with 3.6% in children with severe anaemia due to other causes (Newton *et al*., 2007). The majority of this mortality occurs in children under 5 years of age due to their lack of naturally acquired immunity against malaria (Breman *et al*., 2004). Despite efforts aimed at ameliorating the anaemia burden, SMA remains an important childhood health burden in sub-Saharan Africa (Brabin *et al.,* 2001). Severe anaemia may develop rapidly in the course of malaria illness, especially in

the presence of high parasite densities and co-pathogens such as bacteria and HIV-1(Newton *et al*., 2007; Otieno *et al*., 2006; Were *et al*., 2011). Higher levels of *Plasmodium falciparum* parasitemia mainly in non-immune individuals can result into massive lysis and clearance of erythrocytes leading to profound anaemia (Phillips *et al*., 2000). Additional studies illustrate that SMA risk peaks at 1 year of age in holoendemic transmission regions and at approximately 2 years of age in areas with moderate and low transmission intensities, such that the overall risk of SMA decreases with increasing age (Reyburn *et al*., 2005).

Severe malarial anaemia susceptibility is also associated with polymorphic variability in genes that condition functional changes in levels of circulating inflammatory and hematopoietic mediators such as IFN-γ, IL-10, IL-1β and stem cell growth factors (SCGF) (Ouma *et al*., 2008; Were, *et al*., 2008; Ouma *et al*., 2010). Toll like receptors (TLRs) are cell surface receptors whose activation leads to induction of anti-microbial genes and inflammatory cytokines that link innate and adaptive immunity (Akira *et al*., 2003). Variations in Toll-like receptors have been associated with susceptibility to infectious disease in several studies (Agnese *et al*., 2002; Lorenz*et al*., 2002; Ouma *et al*., 2011; Ouma *et al*., 2006). Toll like receptor 4 is a well-established receptor for the glycosylphosphatidylinositol (GPI) of *P. falciparum* (Coban *et al*., 2007; Ferwerda *et al*., 2007; Lofgren,*et al*., 2010). Asp299Gly rs4986790 was selected based on amino acid change from aspartate to glycine that affects receptor binding which could eventually affect the efficiency of the immune response. Toll like receptor 4 Asp299Gly has been shown in northern Ghana to increase the risk of severe peadriatic malaria by1.5-fold but had no impact on the risk of infection (Mockenhaupt *et al*., 2006b). No data is available regarding association of TLR-4 rs2770150 (-8984C/G) SNP with different diseases. However, the (-8984C/G) SNP was selected because it occur in the promoter region and thus regulate TLR-4 gene expression by influencing the

binding of transcription factors (Esposito *et al*., 2012).However, none of these studies investigated the association between these TLR 4 polymorphic variants and susceptibility to SMA in peadriatic populations residing in *P. falciparum* holoendemic transmission regions of western Kenya. The present study investigated the association between TLR-4 (-8984C/G and 299Asp/Gly) genotypes/haplotypes and susceptibility to SMA in peadriatic populations less than 3 years living in *P. falciparum* malaria holoendemic transmission area of Siaya County, western Kenya.

Studies have shown that plasma levels of the pro-inflammatory cytokines, for instance tumor necrosis factor (TNF-α), Interferon gamma (IFN-γ) and Interleukin -10 (IL-10) are significantly higher in children with severe malaria than uncomplicated malaria (Perkins*et al*., 2000), implying that the balance between these inflammatory mediators may play a significant role in the pathogenesis of SMA. The production of these cytokine depends on the interaction between pathogen associated molecular patterns (PAMPs) and cell surface receptors hence linking the innate and adaptive immune systems (Janeway *et al*., 2002). Severe malarial anaemia susceptibility is also associated with polymorphic variability in genes that condition functional changes in levels of circulating inflammatory and hematopoietic mediators such as IFN-γ, IL-10, IL-1β and stem cell growth factors (SCGF) (Ouma, *et al*., 2008;; Ouma *et al*., 2010). Interferon gamma (IFN-γ) is the only form of type II IFN. It regulates several components of the immune system such as antigen presentation, antimicrobial mechanism, leukocyte development and immune cells trafficking (Gil *et al*., 2001). It is the most widely studied interferon in malaria infection since it is primarily involved in host defense against intracellular pathogens. Its protective role as an immune mediator emerges as early as at the liver stage (Miller *et al*., 2014; Perlaza *et al*., 2011). Recent longitudinal study in children population of western Kenya has shown that high levels

of circulating IFN-γ are associated with enhanced SMA severity (Ouma *et al*., 2011). However, none of these studies have investigated the association between TLR 4 polymorphic variants and circulating IFN-γ levels in children populations residing in *P. falciparum* holoendemic transmission regions of western Kenya. As such the present study investigated the association between TLR-4 (-8984C/G and 299Asp/Gly) genotypes/haplotypes and circulating IFN-γ levels in peadriatic populations less than 3 years living in *P. falciparum* malaria holoendemic transmission area of Siaya County, western Kenya. In addition, differences in circulating IFN-γ plasma levels between SMA and non-SMA patients were also examined in children naturally exposed to *P. falciparum* infection.

1.2 Statement of the Problem

Severe malarial anaemia (SMA) is one of the most severe complications of *P. falciparum* malaria and an important cause of childhood mortality and morbidity in children population residing in *P. falciparum* holoendemic transmission areas such as western Kenya. However, the host-related genetic factors that modulate such pathogenesis and complication associated with *P. falciparum* malaria remains unclear. A better understanding of the factors involved in the pathogenesis of SMA is essential for the identification of candidate genes to be manipulated in the design of a vaccine against this disease sequeleae.

Previous studies have indicated the role of toll-like receptors (TLRs) in immunity against inflammatory and infectious diseases. The TLR4 Asp299Gly polymorphism has been shown in northern Ghana to increase the risk of severe peadriatic malaria but had no impact on the risk of infection. The variant also has been shown to predispose children to cerebral malaria (CM) and increases production of IFN-γ in severe *P. falciparum* infection. Production of IFN-γ as triggered by different sets of stimuli has also been shown to be involved in malaria

pathogenesis. However, it is not clear what influence polymorphic variants on TLR-4 have on the production of IFN-γ during SMA pathogenesis in children below 3 years of age. Therefore the variants within the promoter of TLR 4 rs2770150 (-8984C/G) and exon rs4986790 (299Asp/Gly) (HapMap, 2003) were targeted in the current study to establish whether these variants alter functional susceptibility to SMA (Hb<6.0 g/dl with any density parasitemia) through differential production of IFN-γ. These polymorphisms were selected based on allelic frequencies of the mutant allele >10% in the African reference population. The study was conducted using samples obtained from patients aged below 3 years (since majority in this age group suffer more from malaria related morbidity and mortality) and presenting with clinical symptoms of malaria at Siaya County Referral Hospital (SCRH), western Kenya.

1.3 Objectives

1.3.1. General objective

To assess association between TLR 4 polymorphisms and susceptibility to SMA and functional changes in IFN-γ levels in children (<3years) with severe *Plasmodium falciparum* malaria at Siaya County Referral Hospital (SCRH), western Kenya.

1.3.2. Specific objectives

1. To determine associations between TLR-4 (-8984C/G and 299Asp/Gly) genotypes/haplotype and SMA in children presenting at SCRH in western Kenya.
2. To establish differences between TLR-4 (-8984C/G and 299Asp/Gly) genotype/haplotypes and IFN-γ production in children presenting at SCRH in western Kenya.

3. To compare differences in circulating IFN-γ levels between children with SMA and non-SMA presenting at SCRH in western Kenya.

1.3.3. Null hypotheses

1. There are no associations between TLR-4(-8984C/G and 299Asp/Gly) genotypes/haplotypes and SMA in children presenting at SCRH in western Kenya.
2. There are no differences between TLR-4(-8984C/G and 299Asp/Gly) genotypes/haplotypes and IFN-γ level production in children presenting at SCRH in western Kenya.
3. There are no differences in interferon gamma (IFN-γ) levels between children with SMA and non-SMA presenting at SCRH in western Kenya.

1.4 Significance of the Study

The results of this study have provided important information that TLR-4 (-8984C/G and 299Asp/Gly) variants are not associated with SMA in this population, but co-inheritance of these variants condition functional changes in circulating IFN-γ levels. Since the role of these receptors is to interact with pathogen associated molecular patterns and immune complexes, the findings of this study are important in further enhancing knowledge on the immune genes that may alter pathogenesis of malaria, thus making more informed decisions in designing rational novel interventions in the control against malaria in children living in *P. falciparum* holoendemic transmission areas, such as western Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1 Mechanisms of Severe *P. falciparum* Malaria

The primary complications of *P. falciparum* malaria include cerebral malaria (CM), pulmonary oedema, acute renal failure, severe anaemia, and/or bleeding, which rapidly progress to death within hours or days (WHO, 2016). Clinical outcome of SMA in children living in holoendemic *P. falciparum* transmission region is characterized by a younger age, monocytosis, thrombocytopenia, reticulocytosis, dyserythropoiesis, elevated pigment-containing monocytes (PCM), respiratory distress, conjunctival and palmar pallor, splenomegaly, signs of malnutrition, and protracted fever and emesis (Novelli *et al*., 2010). Some of the mechanisms that have been implicated in the pathogenesis of severe anaemia in malaria include reduced erythropoietin activity, pro-inflammatory cytokines and increased erythrocyte destruction mediated by parasites, eythrophagocytosis, antibody and complement lysis (el Hassan *et al*., 2001; Stoute *et al*., 2003) and repeated cycles of invasion, replication, and bursting of erythrocytes due to infection by *P. falciparum* parasites (Wickramasinghe *et al*., 2000).

Studies in mice models suggest that anti-erythrocyte auto-antibodies may be involved in the destruction of uninfected erythrocytes (Helegbe *et al*., 2009). In addition, it has been documented that released *P. falciparum* glycosylphosphatidylinositol (GPI) can insert into unparasitized erythrocyte membranes and cause its recognition by circulating anti-GPI antibodies leading to subsequent elimination (Brattig *et al*., 2008).Induction of phagocytosis and complement activation by ring surface protein 2 (RSP-2) tagged to normal erythrocytes and erythroid precursor cells in the bone marrow of anemic malaria patients has also been identified as a mechanism of destruction of normal erythrocytes and dyserythropoiesis (Layez

et al., 2005). Reduced regulated upon activation normal T-cell expressed and secreted (RANTES) levels as a result of naturally-acquired hemozoin by monocytes may also contribute, in part, to suppression of erythropoiesis in children with malarial anaemia (Were *et al*., 2006). Hemozoin acquisition by phagocytes has been shown to suppress cellular immunity and enhance malaria disease severity (Casals-Pascual *et al*., 2006; Lyke *et al*., 2003). Moreover, SMA has been characterized by a shortened life span of circulating erythrocytes (Looareesuwan *et al*., 2000) which is enhanced in the presence of other co-pathogens, such as HIV-1, hookworm and bacteria (Bassat *et al*., 2009; Davenport *et al*., 2010; Otieno *et al*., 2006; Were *et al*., 2011).

2.2 Role of Toll-like Receptors in Severe Malaria Pathogenesis

Toll-like receptors (TLRs) are type 1 transmembrane proteins differentially expressed among immune cells (Akira, 2003; Janeway *et al*., 2002). They bind to pathogen-associated molecular patterns (PAMPs) and trigger the activation of signal transduction pathways that in turn induce dendritic cell maturation and cytokine production (Akira *et al*., 2006).Toll-like receptor 4, a major pathogen recognition receptor (PRR) expressed on membrane surface of innate immune cells is genetically encoded by the *TLR4* gene (Gene ID = 7099; 9q33.1), which spans a genomic region of ~13.3 kb with three exons (Takeda *et al*., 2003).

Several single-nucleotide polymorphisms (SNPs) that alter susceptibility to infectious and inflammatory diseases have been identified in TLRs (Papadopoulos *et al*., 2010; Ricci *et al*., 2010; Schroder *et al*., 2005). *In vitro* and animal model have shown that two most-studied non-synonymous SNPs of *TLR4*: an A to G transition (SNP ID = rs4986790), resulting in Aspartate-Glycine substitution at position 299 (TLR4 Asp299Gly) and C to T transition (SNP ID = rs4986791), resulting in threonine-isoleucine substitution at position 399

(TLR4Thr399Ile). These changes altered GPI binding, 50 % reduction in TLR4 expression on the membrane surface of innate immune cells, induce Lipopolysaccharides (LPS) hyporesponsiveness and excessive production of pro-inflammatory cytokines such as IFN-γ(Ferwerda *et al*., 2007; Prohinar*et al*., 2010). Subsequent case–control studies then established associations of TLR4 Asp299Gly or Thr399Ile polymorphism with death from septic shock and susceptibility to typhoid fever, tuberculosis, meningitis, chagas disease and respiratory syncytial virus infection in infected infants, children below 5 years and adults (Lofgren *et al*., 2010; Prohinar *et al*., 2010).

Toll like receptor 4 Asp299Gly has also been shown in northern Ghana to increase the risk of severe peadriatic malaria by 1.5- fold but had no impact on the risk of infection (Mockenhaupt *et al*., 2006b). Taken together, these studies demonstrate that TLRs have the capacity to mount acute inflammatory responses against invading pathogens through induction of antimicrobial genes and inflammatory cytokines (Takeda *et al*., 2003). In addition there is no data available regarding functional relationships between TLR-4 rs2770150 (-8984C/G) SNP with different diseases including SMA. However, the (-8984C/G) SNP was selected because it occurs in the promoter region and thus regulate TLR-4 gene expression by influencing the binding of transcription factors. However, the functional associations between the TLR-4 (-8984 C/G and 299A/G) polymorphisms and susceptibility to SMA in peadriatic populations from western Kenya remains unknown. As such, the current study investigated the association of the functional polymorphisms between TLR-4 (-8984 C/G and 299Asp/Gly) and susceptibility to SMA in peadriatic populations resident in western Kenya.

2.3 Interferon gamma (IFN-γ): Pathology and Protection in Malaria

Interferon gamma is multifunctional cytokine produced by T lymphocytes, macrophages, dendritic cells and natural killer cells (NKs) (Hensmann *et al*., 2001; Miller *et al*., 2009). It is the most widely studied interferon in malaria infection since it is primarily involved in host defense against intracellular pathogens. Its protective role as an immune mediator emerges as early as in the liver stage (Miller *et al*., 2014; Perlaza *et al*., 2011). It plays an important role in inflammatory responses and is often associated with the development of overt Th1-like cell-mediated responses (Gajewski *et al*., 2002), and hence forms an important part of the immune system (Chehimi *et al*., 2004). The production of IFN-γ depends on the interaction between pathogen associated molecular patterns (PAMPs) and cell surface receptors (TLRs) hence linking the innate and adaptive immune systems (Janeway *et al*., 2002). Toll Like receptor 4 Asp299Gly polymorphism has been shown in northern Ghana to increase the production of IFN-γ in severe *P. falciparum* infection (Mockenhaupt *et al*., 2006b). However, there is no data to show what influence TLR-4 -8984 C/G has on circulating IFN-γ levels. A study in Papua New Guinean children indicated that early production of malaria-specific IFN-γ leads to immunity against clinical malaria (D'Ombrain *et al*., 2007). Moreover, elevated levels of IFN-γ at the acute phase of uncomplicated *P. falciparum* malaria has been shown to limit progression to clinical malaria (Torre *et al*., 2002). Rapid IFN-γ production inhibits intra-erythrocytic replication of malaria parasite preventing the onset of clinical malaria (Horowitz *et al*., 2010) further demonstrating the critical role of IFN-γ in malaria pathogenesis. A study in Thai adults demonstrated elevated IFN-γ levels in patients with complicated malaria at the initial stage of the disease than uncomplicated malaria (Tangteerawatana *et al*., 2007). Recent longitudinal study in peadriatic population in western Kenya has shown that high levels of circulating IFN-γ are associated with enhanced SMA

severity in peadriatic populations (Ouma *et al*., 2011) indicating the role of this cytokine in malaria pathogenesis. However, the functional associations between the TLR-4 (-8984 C/G and 299A/G) polymorphisms and IFN-γ production in peadriatic populations from western Kenya remains unknown. As such, the current study investigated the relationship between circulating IFN-γ levels and TLR-4 (-8984 C/G and 299A/G) polymorphisms in children populations resident in western Kenya.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was carried out in Siaya County Referral Hospital (SCRH), western Kenya, and the surrounding community (Figure 1). This is a major government hospital for the population living in the *P. falciparum* holoendemic transmission region of Siaya County in western Kenya (Ong'echa *et al*., 2006). It is bordered by Busia, Vihiga, Kakamega and Kisumu Counties. It is approximately 2530 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. Severe malaria anaemia and hyperparasitemia are the most common clinical outcome of severe malaria and primary cause of peadriatic morbidity and mortality (Bloland *et al*., 1999; Ong'echa *et al*., 2006). The annual population growth rate is 1% and the mortality rate for infants is 11.3%, while under five mortality rates is 10.2% (Kenya National Bureau of Statistics, 2010). The mosquito vectors in this area are commonly *Anopheles gambiaes.s. Anopheles arabiensis*, and *Anopheles funestus* (Beach *et al*., 1993). The average temperature lies between 15^oC and 30^oC.The County population is 500,000 (Kenya census, 2009). The intensity of malaria transmission in this holoendemic is experienced during the seasonal rainfalls in April to August and November to January (Beier *et al*., 1994). Residents are predominantly of the Luo ethnic group (>99%) thus the study area had a homogenous population that is suitable for genetic based studies (Bloland *et al*., 1999; Ong'echa *et al*., 2006). The main economic activities in the County are subsistence farming, fishing and small scale business (MOFaP, 2002-2008).

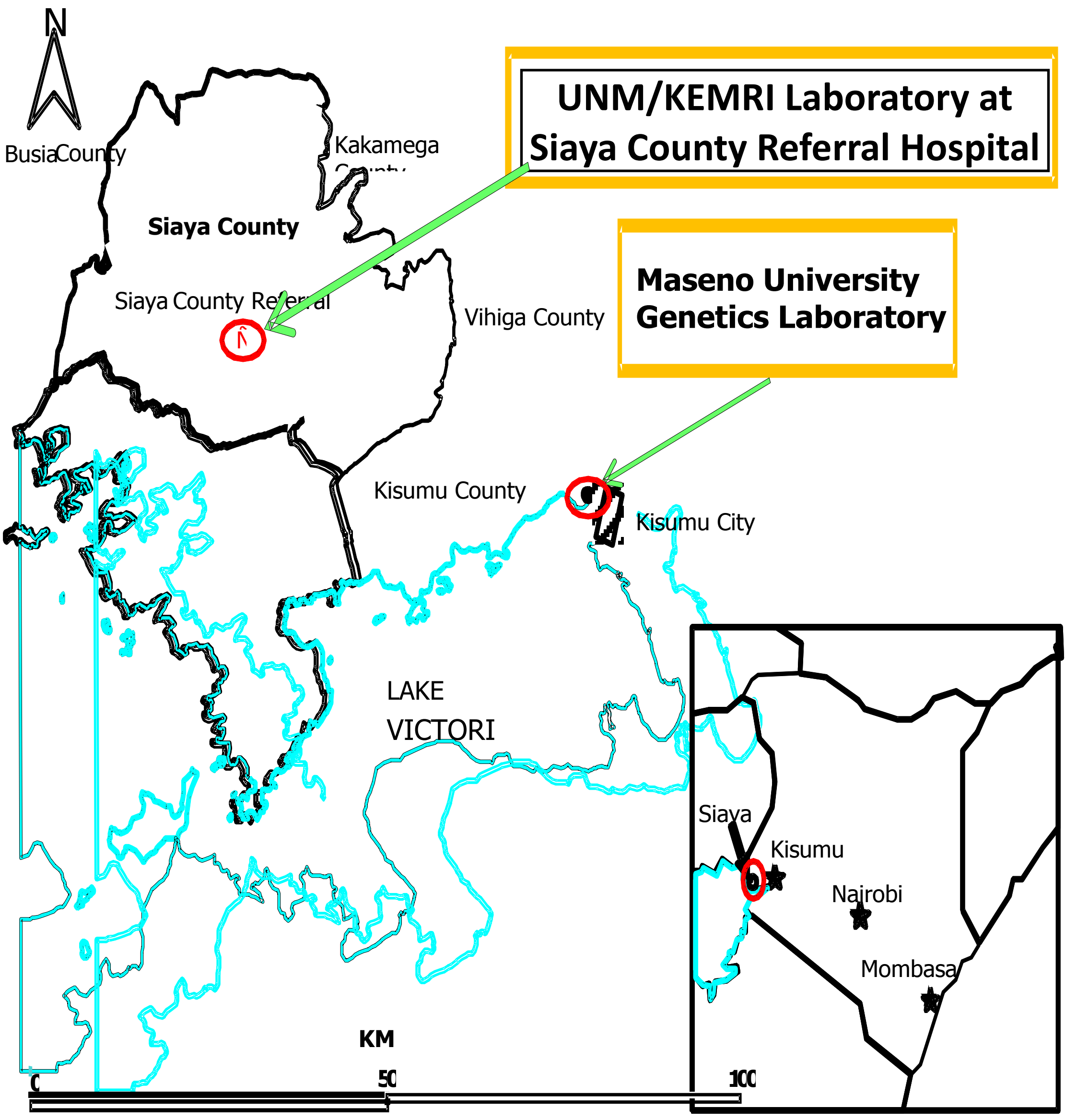

Figure 1. Map of study area and relative position of Siaya County Referral Hospital and Maseno University Genetics Laboratory in western Kenya, adopted from (Ong'echa *et al*., 2006).

3.2 Study Design and Patient Population

3.2.1. Recruitment of study participants

This was a case control study of children with severe malarial anaemia (positive smear for asexual *P. falciparum,* parasitemia and haemoglobin <6.0 g/dl). Controls were non-severe anaemia children of similar age and same gender with *P. falciparum,* parasitemia (of any density) and Hb≥6.0 g/dL. Children of both sexes were recruited in SCRH in western Kenya during their initial hospitalization for treatment of malaria using a questionnaire and existing medical records. Enrolment was confined to children less than 3 years.

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. It was made clear that enrolment decision was made after initial HIV-1 screening of the child and obtaining informed consent. The questionnaires were used to collect relevant demographic and clinical information.

Venous blood samples (<3.0 mL) was collected in ethylene diatetra acetic acid (EDTA)-containing tubes at the time of enrollment, prior to provision of treatment or any supportive care. Blood samples were used for malaria diagnosis, haematological measurements, HIV testing, and bacterial culture. Based on the HIV-1 test results, malaria parasitemia, and haemoglobin (Hb) status, children that satisfied all inclusion criteria were enrolled into the study.

3.3 Eligibility Criteria

Upon enrolment into the study, HIV-1 status, parasitemia and haematological measurements of the child were determined. Based on a previous longitudinal study examining the

distribution of >10,000 Hb measurements in an age- and geographically-matched reference population in western Kenya, SMA was defined as Hb<6.0 g/dL with any density parasitemia (McElroy *et al*., 1999). This definition of SMA is appropriately defined by Hb distributions according to age, gender, and geographic context. Since previous studies demonstrated that HIV-1 and bacterial co-infection impact on the development and severity of malarial anaemia (Otieno *et al*., 2006), and all children were tested for these co-pathogens. Children with acute malaria were stratified into two categories: Non-severe malarial anaemia (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 anaemia (SMA): Children with a positive smear for asexual *P. falciparum,* parasitemia (of any density) and Hb< 6.0 g/dL (McElroy *et al*., 1999).

3.3.1. Inclusion criteria

i. Age between ≥3 months and≤36 months of both sexes.
ii. Malaria parasitemia (any density) and Hb<11.0g/dL.
iii. Parent/guardian was willing and able to sign consent form.
iv. Parent/guardian able to keep schedule and study appointments.
v. Distance to the hospital ≤15km.
vi. Parent/guardian able to provide two contacts familiar with the child's where about during the study period.

3.3.2. Exclusion criteria

i. Children with cerebral malaria.
ii. Children with malaria from non-*falciparum* species.
iii. History of any HIV-1related symptoms such as oral thrush.
iv. Clinical evidence of acute respiratory infection.
v. Prior hospitalization or had reported antimalarial use within the previous two weeks.

vi. Intent to relocate during the study period.

vii. Unwillingness to enroll the child in the study.

3.3.3. Sample size determination

The study was carried out in a total of 414 malaria naive infected patients (aged 3-36 months) who presented with clinical symptoms of *P. falciparum* malaria at SCRH. This sample size was based on the actual number of children below 3 years that presented with clinical symptoms of malaria at SCRH within the 24 months of the study (average 16 patients per month).

According to Dupont *et al*., (1990), the Bonferroni correction for 2 different loci required a per-comparison alpha of 0.004 for sample size calculations. However, given the increased power provided by the procedure, it is estimated that sample sizes based on a $\alpha \leq 0.05$ provided a balance between Type I and Type II errors. Based on this rationale, to achieve a power of 0.8 with a Type I error rate of $\alpha \leq 0.05$, allele frequency difference of 0.25(HapMap, 2003) between cases (SMA) and controls (Non-SMA) was required. The following formula was used to determine sample size (Whitley & Ball, 2002).

$$n = \left(\frac{r+1}{r}\right)\frac{(\overline{p})(1-\overline{p})(Z_{\beta}+Z_{\alpha/2})^2}{(p_1-p_2)^2}$$

Where;

n is the sample size of the case-control group.

r is the ratio of controls to cases (assumed to be 1 when using equal proportions)

p is a measure of variability (similar to the standard deviation)

(p_1-p_2) is the effect size (difference in proportion)

Z_β represents the desired power (typically 0.84 for 80% power)

Z_α represents confidence interval (typically 1.96 for 95% CI)

Based on previous studies (Ouma, Davenport, Were, *et al*., 2008), to detect an odds ratio (OR) of 2, equal number of cases and controls were used. Therefore r=1. The proportion ($\overline{p}$) of children with malaria and had the genotypes understudy was determined, OR:

$$p = \frac{ORp}{p\ (OR-1)+1}$$

$$p = \frac{2.0(0.25)}{(0.25)(2.0-1)+1} = \frac{0.5}{1.25} = 0.4$$

The average proportion of children exposed to *P. falciparum* malaria and who possess the genotypes was given by (0.4+0.25)/2=0.375. Therefore $\overline{p}$ =0.375

$$n = 2\frac{(0.375)(1-0.375)(0.84+1.96)^2}{(0.4-0.25)^2} = 163$$

Therefore the minimum number of cases or controls that was needed to achieve an 80% power was 163. However in this current study, 193 SMA (cases) and 221 non-SMA (controls) children were recruited over a two year in the mother study.

3.4 Laboratory Procedures

3.4.1. Malaria diagnosis

Thick and thin blood films were prepared from finger prick blood and stained with10% Giemsa for 15 minutes. To determine parasitemia, 10% Giemsa-stained thick blood smears were prepared and examined under a microscope at high-power magnification. The number of *P. falciparum* parasites per 300 white blood cells (WBC) was determined, and number of parasites per microliter of blood estimated using the following formula; parasite density/ µL= WBC count/ µL x trophozoites/300. Following microscopic confirmation of the diagnosis of

P. falciparum parasitaemia, venous blood was collected into EDTA vacutainers (Becton Dickinson, USA) and blood spots made on free to air (FTA) Classic® cards (Whatman Inc., Clifton, NJ, USA), air dried and stored with desiccants at room temperature until use.

3.4.2. Haematological, sickle cell trait (HbAS), G6PD, HIV and bacteremia determinations

Haemoglobin levels and complete blood counts were determined using the Beckman Coulter ACT diff2™ (Beckman-Counter Corporation, Miami, FL, USA). The presence of sickle cell trait (HbAS), bacteremia, HIV-1 and G6PD deficiency were determined and positive results excluded from the data analyses since these confounding factors have previously been demonstrated to individually alter susceptibility to SMA (Aidoo *et al.*, 2002; Otieno *et al.*, 2006; Were *et al.*, 2011).

The presence of the HbAS was determined by cellulose acetate electrophoresis with Titan III plates according to the manufacturer's protocols (Helena Biosciences, Oxford, United Kingdom). Briefly, heamolysates prepared from blood samples and Hemo AFSC controls were dispensed onto 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 Haemoglobin types scored using the Hemo AFSC control.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency was determined by a fluorescent spot test (Trinity Biotech PIc., Bray, Ireland). Briefly, blood was heamolyized and spotted onto filter paper. Assay solutions containing glucose-6-phosphate and oxidized NADP ($NADP^+$) was added and samples excited with Ultraviolet (UV) light at 340nm. Based on the presence or absence of fluorescence emissions, the samples were scored as normal (high emission), intermediate (moderate emission), or deficient (no emission). Human

immunodeficiency virus (HIV) status was determined using two serological methods; Unigold (Trinity Biotech PIc., Bray, Ireland) and determine (Abbot Laboratories, Tokyo, Japan), and positive serological tests were confirmed to previously published methods (Otieno *et al*., 2006). Bacteremia was determined using the Wampole Isostat Peadriatric 1.5 system (Wampole Laboratories), and blood was processed according to the manufacturer's instructions. API biochemical galleries (BioMérieux Inc.) and/or serology were used for identification of blood-borne bacterial isolates.

3.4.3. Deoxyribonucleic acid (DNA) extraction

Genomic DNA was extracted from FTA Classic cards using the Gentra System DNA extraction protocol (Gentra System Inc., Minneapolis, MN, USA) according to the manufacturer's recommendation. In brief, three millimeters of the disc was punched from sample collection filter paper (FTA) and placed into 0.2mL tube. To the tube, 50µL of DNA purification solution 1 was added and incubated at room temperature (25°C) for 15 minutes. The solution wasp petted up and down twice then discarded. DNA solution 1 was added again and incubated at room temperature for 15 minutes. The solution was then pipetted up and down. For elution, 70µL of DNA elution solution 2 was added and incubated in a preheated thermal cycler at 99°C for 15 minutes to release DNA and then cooled at room temperature. The eluted DNA was transferred into a clean 1.5mL eppendorf tube and then stored at -20°C until use.

To confirm the presence of DNA, a house-keeping gene, the human 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 1μLof the test sample. The thermocycling conditions were as follows: Initial denaturation 94º C for 2minutes: Denaturation 94ºC for 45 seconds: Annealing 62ºC for 45 seconds: Extension 72ºC for 2minutes: These were repeated for 40 cycles: Final extension 72ºC for 7minutes.Once amplification was completed; gel electrophoresis was performed for visualization of the amplified gene. A 2% agarose gel was prepared by heating 2 grams of agarose powder in 100mL of 1xTBE buffer, with 5μL of Ethidium bromide for staining of double stranded DNA in the gel. Once the gel had polymerized, the samples were loaded into the wells alongside DNA molecular weight marker. This was run for 2 hours at 80 volts, and results viewed under an ultraviolet (UV) source, and stored on a flash drive or printed out for documentation.

3.4.4. Genotyping of TLR-4 (-8984C/G and 299A/G) variants

Briefly, the TLR-4 variants-8984C/G (rs2770150, assay ID no.C_1844486_20) and299A/G (rs4986790, assay ID no.C_11722238_20) were genotyped using allele-specific PCR amplification. This was performed using predesigned custom made high-throughput TaqMan®SNP genotyping assays technology which accurately discriminated alleles using allele-specific fluorochrome labeled probes based on the manufacturer's instructions (Applied Biosystems, Foster City, CA). For genotyping of TLR-4 variant-8984C/G, a master mix containing 5.0μL of TaqMan® genotyping mix, 0.5μL of SNP assay mix, 3.5μL of PCR grade water and 1.0μL of DNA were added into each micro-well and genotyped using the following cycling parameters; pre-PCR hold stage was done at 60ºC for 30 seconds, hold stage at 90ºC for 10 minutes, cycling stage at 95ºC for 15 seconds and annealing at 60ºC for 1 minute. The hold stage to the annealing stage was repeated 45 times. For genotyping of TLR-4 299Asp/Gly, the same reaction volumes and cycling parameters was used. The results were then stored for further analyses.

3.4.5. Quantification of circulating IFN-γ levels

Plasma samples obtained from venous blood and stored at -80°Cwere used. Circulating IFN-γ concentrations were determined using the Human Cytokine 25-plex Antibody (Ab) Bead kit (BioSource International) according to the manufacturer's instructions. Briefly, the wells were pre-wetted by adding 200 µL of working wash solution into designated wells then incubated for 30 seconds at room temperature. The working wash solution was aspirated from the wells using the vacuum manifold. The bead solution was vortexed for 30 seconds, then sonicated again for 30 seconds. About 25µL of the bead solution was added into each well and the plate immediately protected from light to prevent photo-bleaching.

To the wells, 200µL working wash solution was added and beads allowed to soak for 20 seconds. The wells were washed by aspirating the working wash solution from the wells with the vacuum manifold. The washing step was repeated once and the bottoms of the filter plate blotted on clean paper towels to remove any residual liquid. To each of the wells 50 µL of incubation buffer was added. To the wells designated for standard curves 100µL of each standard dilution was added while to the wells designated for sample, 50µL of assay diluent was added followed by 50µL plasma sample. The filter plates were covered with aluminum foil and incubated for 2 hours at room temperature on orbital shaker at 500 revolutions per minute (rpm). After incubation, the plate was washed twice using 200µL of wash solution. To the washed plates, 100µL of biotinylated antibody was added to each well and then incubated for 1 hour at room temperature on an orbital shaker at 500 rpm to keep the beads suspended during incubation. After incubation 200µL of wash solution was added to each well to soak the beads then aspirated using vacuum manifold. To each of the wells, 100µL of streptavidin-rpe was added and plate incubated for 30 minutes at room temperature on an orbital shaker 500 rpm. The liquid in the wells were removed by vacuum aspiration manifold and then

200μL working wash solution added to the wells, beads allowed to soak for 10 seconds, and then aspirated using the vacuum manifold. Washing step was repeated twice. Plates were read on the Luminex 100™ system (Luminex Corporation) and analyzed using the Bio-plex Manager software (Bio-Rad Laboratories).The detection limit for IFN-γ was2.0 pg/ml. Batch analysis was performed to re-strict experimental variability between assays.

3.5 Data Management and Analysis

Data was managed using Statistical Package for Social Sciences (SPSS) (Version 19.0). Chi-square analyses were used to examine differences between proportions of gender, high parasite density (≥10000 parasites/μl) and genotypes/haplotypes distributions in the clinical groups. A quantitative comparison involving unpaired data such as age, auxiliary temperature and hematological parameters was made using Mann–Whitney U test. Circulating IFN-γ levels were log-transformed to normal distribution. Student t-test was used to determine differences between TLR-4 (-8984C/G and 299A/G) genotypes/haplotypes and means of circulating IFN-γ levels. Across-group comparisons between three independent groups was determined using one way Analysis of variance (ANOVA). TLR-4 (-8984C/G and 299A/G) haplotypes were constructed using HPlus software program (Version 2.5). The associations between genotypes/haplotypes and SMA were determined by multivariate logistic regression, controlling for the confounding effects of age, gender, HIV-1 status (including HIV-1 exposed and definitively HIV-1positive results), sickle cell trait, G6PD and bacteremia. Critical significance levels were set at $p \leq 0.05$.

3.6 Ethical Considerations

Study approval was initially provided by the School of Graduate Studies of Maseno University (SGS) (Appendix I). Ethical approval was obtained from the Kenya Medical

Research Institute National Ethical Review (Appendix II).The study used stored blood samples that were collected after written informed consent was obtained from parents or guardians of study participants. Written informed consent was administered in the language of choice (i.e. English, Kiswahili or Dholuo). Blood collection was carried out by trained and qualified phlebotomists, to reduce risks of bleeding incidents. Sterile disposable lancets were used and all sharps stored in biohazard sharps' containers before disposal at the Kenya Medical Research Institute (KEMRI) incinerator in Kisian, Kisumu. Study participation was voluntary; participants were allowed to withdraw at any time during the study. To ensure confidentiality, all the samples collected were coded for identification using the study identification number and investigators controlled access to the data. No samples were labelled with the participant's name. All participants promptly received appropriate antimalarial treatment and required supportive therapy as per the Kenya Ministry of Health (MoH) guidelines, which included the use of an artemether and Lumefantrin combination drug (Coartem) for non-severe malaria, intravenous quinine for severe malaria, and broad spectrum antibiotics for bacterial infections.

CHAPTER FOUR

RESULTS

4.1 Demographic, Clinical, and Laboratory Characteristics of Study Participants

To determine the role of TLR-4 (-8984C/G and +299A/G) promoter variants in conditioning susceptibility to SMA children (N=414, aged 3 to 36 months) presenting with acute *P. falciparum* malaria were first categorized into two groups according to haemoglobin levels based on a previous age- and geographically defined reference population from western Kenya (McElroy *et al*., 1999), i.e., non-severe malaria (non-SMA) (Hb ≥6.0g/dl; N=221) and severe malaria anaemia (SMA) (Hb<6.0 g/dl; N=193) with any density of parasitemia. The demographic, clinical and laboratory characteristics of the study participants are summarized in Table 1. Proportions of male vs. females (*P*=0.653) and those with high density parasitemia (HDP≥10000 parasites/µl) (*P*=0.213) were similar between the clinical groups. In addition the median parasite density (*P*=0.125) and axillary temperature (*P*=0.161) were similar between the SMA and non-SMA. Haematological parameters, among them haemoglobin (Hb), red blood cells (RBC) count, red cell distribution width (RDW), mean corpuscular haemoglobin concentration (MCHC) which are indicators for erythropoiesis were all significantly different between the clinical groups (*P*<0.001). In addition, participants in non-SMA group were significantly older than those in SMA (*P* <0.001), Table 1.

Table 1: Demographic, Clinical and Laboratory Characteristics of Study Participants

Characteristics	non SMA (Hb≥6.0 g/dl)n=221	SMA (Hb<6.0 g/dl)n=193	*P*-value
Gender, n (%)			
Male	121 (54.8)	98 (50.8)	0.653[a]
Female	100 (45.2)	95 (49.2)	
Age in Months	11 (7.0)	8.0 (5.0)	**<0.001**[b]
Parasites/µl	21360(6074.3)	17622(4498.9)	0.125[b]
HPD (≥10000 parasites/µl no. %)	137/205(66.8)	90/149(60.4)	**0.213**[a]
Haemoglobin g/dL	8.0 (7.0)	5.0(4.2)	**<0.001**[b]
RBCs, $\times 10^{12}$/µL	3.7 (3.2)	2.2 (1.8)	**<0.001**[b]
RDW, %	20.6 (18.4)	23.2 (21.1)	**<0.001**[b]
MCHC g/dL	32.3 (31.1)	31.4 (29.9)	**<0.001**[b]
Axillary temperature,^{o}C	37.5(36.8)	37.4(36.8)	**0.161**[b]

Data are shown as the median (with interquartile range [IQR] in parentheses) unless otherwise noted. Children with parasitemia (*n*=414) were stratified according to a modified definition of SMA based on age- and geographically matched Hb concentrations in children in western Kenya (i.e., Hb<6.0 g/dl with any density of parasitemia) ((McElroy *et al*., 1999)) into non-SMA (*n*=221) and SMA (*n*=193). [a]Statistical significance determined by Chi-square tests. [b]Statistical significance determined by the Mann-Whitney U test. Abbreviations RBCs=Red blood cells: RDW=Red cell distribution width: MCHC=Mean corpuscular haemoglobin concentration.

4.2 Association between Individual TLR-4 (-8984C/G and +299Asp/Gly) Genotypes and SMA

To determine the role of individual genotypes of TLR-4 (-8984C/G and +299Asp/Gly) (independent variables) promoters in conditioning susceptibility to SMA (dependent variables), their distributions were compared between the clinical groups. The distribution of -8984C/G variant in the non-SMA (n = 221) and SMA (n = 193) and the genotype prevalences within the clinical groups were GG (non-SMA, n = 173, 54.4%; SMA, n = 145, 45.6%), GC (non-SMA, n =37, 48.1%; SMA, n = 40, 51.9%) and CC (non-SMA, n = 11, 57.9% ; SMA, n = 8, 42.1%). These distributions were similar in the SMA and non-SMA groups (P = 0.558). The distribution of +299Asp/Gly variant were Asp/Asp (non-SMA, n = 196, 54.0%; SMA, n = 157, 46.0%), Asp/Gly (non-SMA, n =22, 48.9%; SMA, n = 23, 51.1%) and Gly/Gly (non-SMA, n = 3, 50.0%; SMA, n = 3, 50.0%). These distributions were similar between the SMA and non-SMA groups (P = 0.800), Table 2.

Multivariate logistic regression analyses was carried out while controlling for the confounding effects of age, gender, HIV-1 status, sickle cell trait (HbAS), bacteremia, and G6PD deficiency since these factors have previously been demonstrated to individually alter susceptibility to SMA (Aidoo *et al*., 2002; Otieno *et al*., 2006; Were *et al*., 2011). As shown in Table 2, the results demonstrated no significant statistical associations between variations at individual promoter loci of TLR-4 (-8984C/G and +299Asp/Gly) and susceptibility to SMA.

Table 2: Association between TLR-4 (-8984C/G and +299Asp/Gly) genotypes and SMA

	Non-SMA (Hb≥6.0 g/dL)	SMA (Hb<6.0 g/dL)		Association with SMA (Hb<6.0 g/dl)		
Genotypes	**n, (%)**	**n, (%)**	***P*- value**[a]	**OR**	**95% CI**	***P*-value**[b]
TLR-4 (-8984C/G)						
GG	173 (54.4)	145 (45.6)		1.00 reference		
GC	37 (48.1)	40 (51.9)	0.558	0.78	0.46-1.31	0.37
CC	11 (57.9)	8 (42.1)		0.62	0.42-3.20	0.78
TLR-4 (+299Asp/Gly)						
Asp/Asp	196 (54.0)	157 (46.0)		1.00 reference		
Asp/Gly	22 (48.9)	23 (51.1)	0.800	1.67	0.24-11.62	0.60
Gly/Gly	3 (50.0)	3 (50.0)		2.06	0.33-12.99	0.44

Children with acute malaria (*n*=414) were stratified according to the modified definition of severe malarial anaemia (SMA) based on age- and geographically matched Hb concentration (i.e., Hb<6.0g/dl with any density of parasitemia) ((McElroy *et al*., 1999). Data are presented as [n, (%)] for proportions of TLR 4 promoter variants -8984 C > T and (+299 Asp >Gly within the SMA (n = 193) and non-SMA (n = 221) groups [a]Statistical significance determined by Chi-square tests. [b]Statistical significance, odds ratios (OR) and 95% confidence intervals (CI) determined using multivariate logistic regression, controlling for age, gender, HIV-1 infection, sickle cell trait (HbAS), bacteremia, and G6PD deficiency. The reference groups in the multivariate logistic regression analyses were the homozygous wild-type genotypes.

4.3 Association between TLR-4 (-8984C/G and +299Asp/Gly) Haplotypes and SMA

Construction of haplotypes was done using HPlus software (Version 2.5). Haplotypes were constructed based on combinations of individual alleles of the two genotypes (e.g.-8984/G and +299/Gly forms a G/Gly haplotype). Prior to determining the associations between the haplotypes and SMA, the overall distributions of the different haplotypes were determined. The distribution of the haplotype carriers in the clinical groups were as follows; TLR-4 (-8984G and +299Gly) (non-SMA, n = 25/221, 49.0%; SMA, n = 26/193, 51.0%; *P*=0.505); TLR-4 (-8984G and +299Asp) (non-SMA, n = 204/221, 53.0%; SMA, n = 181/193, 47.0%; *P*=0.558) and TLR-4 (-8984C and +299Asp), (non-SMA, n = 48/221, 50.0%; SMA, n = 48/193, 50.0%; *P*=0.449). These distributions were not significantly different in the SMA and non-SMA groups (Table 3).

Multivariate logistic regression model, controlling for potential confounders was used to determine the association between TLR-4 (-8984C/G and +299Asp/Gly) haplotypes (independent variables) and SMA (dependent variables). The analyses (Table 3) revealed that none of the haplotypes TLR-4 -8984G and +299Gly (OR, 0.89; 95% CI, 0.46-1.47; *P*=0.51), TLR-4 -8984G and +299Asp (OR, 0.79; 95% CI, 0.35-1.76; *P*=0.58) and TLR-4 -8984C and +299Asp (OR, 0.84; 95% CI, 0.52-1.36; *P*=0.49) showed significant association with SMA. The CG haplotype, which was constructed from the minor alleles for both SNPs, was excluded from these analyses due to its low frequency<0.005 in this population.

Table 3: Association between TLR-4 (-8984C/G and +299Asp/Gly) haplotypes and SMA

	Non-SMA (Hb≥6.0 g/dL)	SMA (Hb<6.0 g/dL)		Association with SMA (Hb< 6.0 g/dl)		
Haplotype	**n, (%)**	**n, (%)**	***P*-value[a]**	**OR**	**95% CI**	***P*-value[b]**
TLR-4 (-8984G and +299Gly)	25 (49.0)	26 (51.0)		0.89	0.46-1.47	0.51
Non- TLR-4 (-8984G and +299Gly)	196 (54.0)	157 (46.0)	0.505			
TLR-4 (-8984G and +299Asp)	204 (53.0)	181 (47.0)		0.79	0.35-1.76	0.56
Non-TLR-4 (-8984G and +299Asp)	17 (58.6)	12 (41.4)	0.558			
TLR-4 (-8984C and +299Asp)	48 (50.0)	48 (50.0)		0.84	0.52-1.36	0.49
Non-TLR-4 (-8984C and +299Asp)	173 (54.4)	145 (45.6)	0.449			

Children with acute malaria (*n*=414) were stratified according to the modified definition of severe malarial anaemia (SMA) based on age- and geographically matched Hb concentration (i.e., Hb< 6.0g/dl with any density of parasitemia) ((McElroy *et al*., 1999)).Data are presented as [n, (%)] for proportions of TLR 4 haplotype carriers and non-carriers within the SMA (n = 193) and non-SMA (n = 221) groups [a]Statistical significance determined by Chi-square tests. [b]Statistical significance, 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. The reference groups in this multivariate logistic regression analysis were those without the respective haplotypes.

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4.4 Differences between individual TLR-4 (-8984C/G and +299Asp/Gly) genotypes and circulating IFN-γ levels

To determine whether these genotypes were associated with functional changes in concentrations of IFN-γ levels, plasma levels of IFN-γ were compared across the genotypic groups. As presented in Fig.2A and B, there were no significant differences in the concentrations of plasma IFN-γ across genotypes for both TLR-4 -8984C/G (GG; CG and CC; *P*=0.154) and TLR-4 +299Asp/Gly (Asp/Asp and Asp/Gly; *P*=0.453). These results show that the individual polymorphisms in TLR-4 (-8984C/G and +299Asp/Gly) are not associated with the production of IFN-γ in this malaria holoendemic population.

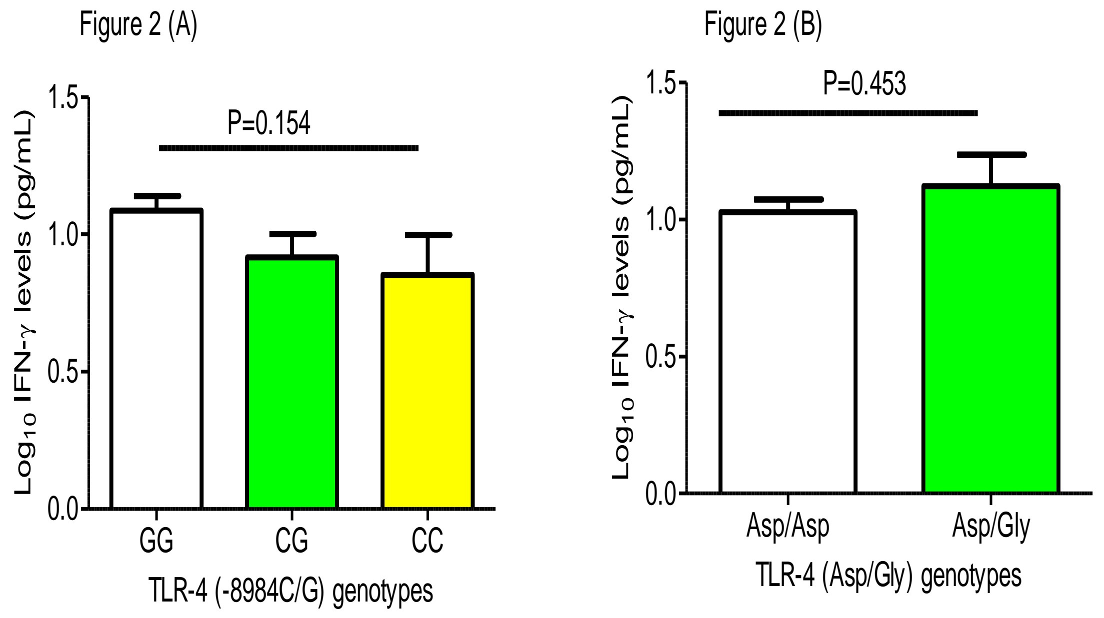
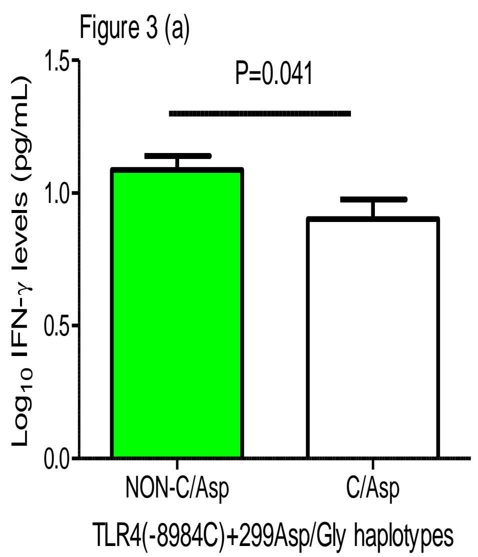
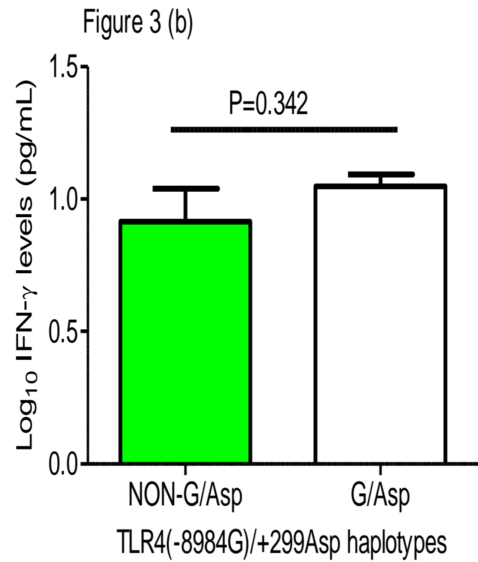


Figure 2. Differences between TLR-4 (-8984C/G and +299A/G) genotypes and circulating IFN-γ levels. Data are represented in column bar chart TLR-4 -8984C/G (GG; CG and CC; *P*=0.154) and TLR-4 +299Asp/Gly (Asp/Asp and Asp/Gly; *P*=0.453). Across-group comparisons were determined using one way ANOVA and Student t- test for figures 2a and 2b respectively. The levels of IFN-γ were log-transformed to normal distribution.

4.5 Differences between TLR-4 (-8984C/G and +299Asp/Gly) Haplotypes and IFN-γ levels

To determine whether co-inheritance of these receptor polymorphisms were associated with changes in concentrations of IFN-γ levels, circulating concentrations of IFN-γ were compared with SNP combinations' in TLR-4 -8984C/G and +299Asp/Gly. As shown in Figure 3A, haplotype carriers of C/Asp (N=51; P=0.041) was associated significantly with lower levels of IFN-γ than the non-carriers. However, haplotype carriers of TLR-4 (-8984G and +299Asp Figure 3B) (N=174; P=0.342), and TLR-4 (-8984G and +299Gly Figure 3C) (N=20; P=0.43) demonstrated similar levels of IFN-γ with the non-carriers. Haplotype TLR-4 (-8984G and +299Gly) was not present in this population.





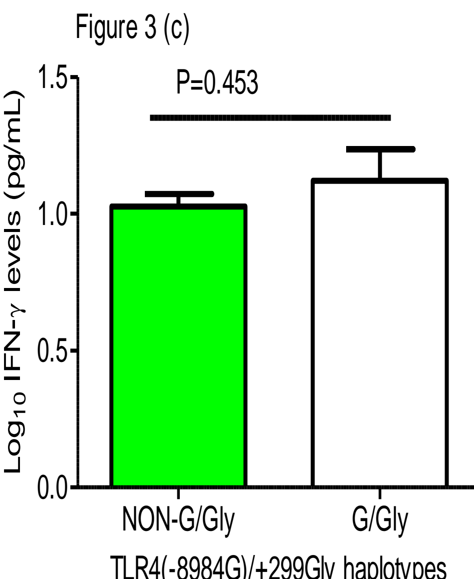


Figure 3. Difference between TLR-4 (-8984C/G and +299Asp/Gly) haplotypes and circulating IFN-γ levels: Data are represented in column bar chart for TLR-4 (-8984C and +299Asp) (C/Asp, 51), TLR-4 (-8984G and +299Asp) (G/Asp, 174) and TLR-4 (-8984G and +299Gly) (G/Gly, 20). Pairwise comparisons in those with and without the combination/haplotype were done using the Student t-test. The levels of IFN-γ were log-transformed to normal distribution.

4.6 Differences in Circulating IFN-γ levels between Children with SMA and Non-SMA

To determine whether the changes in the circulating levels of IFN-γ are associated with severity of acute malaria, the circulating levels were compared between the SMA (*n*=96) and non-SMA (*n*=83) groups. It is critical to note that after the first screening (to determine Hb levels), it was not possible to collect additional blood samples to carryout measurements of IFN-γ levels in some study participants due to the fact that the children either were too anemic or were too sick to ethically allow collection of additional blood samples, hence the reduction in numbers in this analysis. As presented in Figure 4, the results demonstrated that SMA and non-SMA children had similar levels of circulating plasma IFN-γ levels (*P*=0.224).

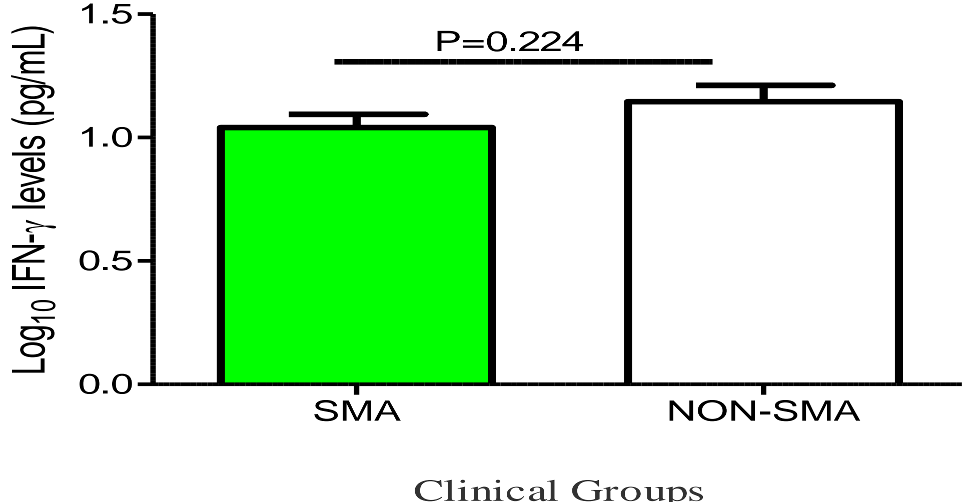


Figure 4. Differences in circulating IFN-γ levels between children with SMA and non-SMA. Data are represented in Column bar chart for the non-SMA (Mean ± SEM 1.145 ± 0.06679:*n*=83) and SMA(Mean ± SEM 1.145 ± 0.06679: *n*=96) groups. There were no significant differences in circulating IFN-γ levels between SMA and non-SMA children (*P*=0.224, Student T-test).

CHAPTER FIVE

DISCUSSION

This is so far the first study that has been conducted to determine the association between these TLR-4 (-8984C/G and +299Asp/Gly) promoter polymorphisms and susceptibility to severe malaria disease outcome (SMA) and functional changes in IFN-γ levels in a peadriatic population resident in a *P. falciparum* holoendemic transmission area of western Kenya. Results obtained from this study of parasitemic children (n=414) aged 3-36months, demonstrated that none of the individual genotypes or haplotypes showed any association with SMA (Hb<6.0g/dL). However, carriage of TLR-4 (-8984C and +299Asp) (C/Asp) haplotype was significantly associated with lower IFN-γ levels. In addition, there were no significant differences in circulating IFN-γ levels between SMA and non-SMA children in this population.

5.1 Association between TLR-4 -8984C/G and 299Asp/Gly genotypes/haplotype and SMA in children presenting at SCRH in western Kenya

Results obtained from this study of parasitemic children (n=414) aged 3-36 months, demonstrated that none of the individual genotypes or haplotypes TLR-4 (-8984C/G and +299Asp/Gly) showed any association with SMA (Hb<6.0g/dL). Data on TLR4 Asp299Gly are quite different from those reported by other authors who, on the other hand, obtained results largely conflicting with each other. Mockenhaupt *et al.* reported that this polymorphism was associated with an increased risk of severe peadriatic malaria in northern Ghana but had no impact on the risk of infection (Mockenhaupt *et al.*, 2006b). On the contrary, Basu *et al.* demonstrated that TLR 4 Asp299Gly was more common in patients with low parasitaemia and concluded that TLR 4 could have a genetic role in controlling the

blood infection level in mild malaria and could indirectly suggest a protective effect of TLR4 Asp299Gly against severe disease (Basu *et al*., 2010). Consistent with the current study, two separate studies, carried out in Brazil and Iran, have recently revealed no impact of individual TLR-4 +299Asp/Gly promoter polymorphism on susceptibility to mild malaria in their respective populations (Leoratti *et al*., 2008; Zakeri *et al*., 2011). Investigations with Burundian children less than 10 years old characterized by mixed clinical phenotypes (cerebral and/or severe malaria anaemia) did not show any association between the TLR-4 (Asp299Gly) polymorphisms and severe malaria (Esposito *et al*., 2012). In general, findings from case–control studies in malaria endemic countries regarding association of TLR-4 Asp299Gly polymorphisms with susceptibility to clinical and severe malaria have been contradictory (Basu *et al*., 2010; Agudu *et al*., 2010; Zakeri *et al*., 2011; Apinjoh *et al*., 2013). These discrepancies may be attributed to clinical definitions of malaria, different genetic backgrounds from ethnic diversity and overall sample (population) size in previous studies.

Since susceptibility to infectious disease occurs through multifactorial, complex, and even contradictory selective pressures (Balaresque *et al*., 2007), haplotypes between TLR-4 (-8984C/G and +299Asp/Gly) were constructed, in an attempt to determine whether co-inheritance of these receptor-SNP combinations could influence susceptibility to SMA. Haplotypes are important markers for identifying associations with disease outcome that are unidentifiable with SNPs (Ouma *et al*., 2008). In the current study, none of the haplotypes was associated with susceptibility to SMA. It might be possible that the association of TLR 4 polymorphisms with the ability to control parasitaemia and the lack of association with disease severity actually reflect differences in the molecular mechanisms underlying protective/ pathological immune responses at different stages of infection.

5.2 Differences between TLR-4 (-8984C/G and 299Asp/Gly) genotype/haplotypes and IFN-γ production in children presenting at SCRH in western Kenya

The present study demonstrated that none of the individual TLR-4 (-8984C/G and +299Asp/Gly) genotypes conditioned changes in IFN-γ levels in this population. However, a study of Ugandan children (aged 4 to 12 years) showed that the TLR-4 (Asp299Gly) genotype was associated with elevated levels of plasma IFN-γ and enhanced cerebral malaria (Sam-Agudu *et al*., 2010), emphasizing the fact that these variants may individually be associated with CM rather than SMA. Moreover, other studies have revealed that individuals infected by malaria have down regulated TLR4 and decreased IFN-γ and that mice with TLR-4 gene knockout produce low IFN-γ levels in response to *Plasmodium chabaudi* AS (Franklin *et al*., 2009). The discrepancies observed between the current study and others may in part be explained by the difference in clinical phenotypes since in the current study population, the main clinical manifestation is SMA in peadriatic populations, while the earlier studies focused on heterogeneous populations in which the most severe clinical manifestation was CM. In addition, pathways of TLR-4 signaling involve polymorphisms in the downstream molecules (for instance, NF-*κ* B and MyD88) that were not investigated in the present study. Haplotypes between TLR-4 (-8984C/G and +299Asp/Gly) were constructed, in an attempt to determine whether co-inheritance of these receptor-SNP combinations could influence IFN-γ levels in this population. The results revealed that carriage of TLR-4 (-8984C and +299Asp) (C/Asp) haplotype was significantly associated with lower IFN-γ levels (although the impact of these haplotypes on promoter binding elements is currently unknown), demonstrating that variation in promoter sequences, such as the TLR-4 promoter, likely alter specific transcription factor recognition sites and consequently affecting cytokine production. For example, presence of the

C/Asp haplotype may create sites for enhanced binding of repressors that favor reduced IFN-γ levels production.

5.3 Differences in IFN-γ levels between children with SMA and non-SMA presenting at SCRH, western Kenya

Despite continued investigations, the exact role of IFN-γ in the pathogenesis of SMA continues to be baffling. For example, high early IFN-γ production has been shown to confer protection against symptomatic malaria episodes in children aged 5 to 14 years from a region of malaria endemicity of Papua New Guinea (D'Ombrain *et al*., 2007). An additional study in an area of holoendemic perennial *P. falciparum* malaria transmission in southern Ghana reported that malaria-specific production of IFN-γ was associated with reduced clinical malaria and fever (Dodoo *et al*., 2002). Collectively, these studies implicate increased IFN-γ production in clinical malaria. However, certain studies have reported an association between higher levels of IFN-γ and severe malaria. For instance, a previous report on a population of children resident in western Kenya demonstrated that IFN-γ was a positive predictor of SMA (Ong'echa, *et al*., 2011). The present study, demonstrated that children with SMA and non SMA had similar IFN-γ concentrations, a finding not consistent with a previous study of the same population (Ouma *et al*., 2011). The results presented here versus those from the previous study (Ong'echa *et al*., 2011) likely differ due to differences in the stratification of the cohort groups. In the present study, the population was stratified into the SMA group (Hb< 6.0 g/dl with any density of parasitemia) and non-SMA groups (Hb≥6.0g/dl with any density of parasitemia), while the previous study (Ong'echa *et al*., 2011) further stratified the overall non-SMA group (Hb≥ 6.0 g/dl with any density of parasitemia) into uncomplicated malaria (UM) (Hb levels of >11.0 g/dl *n*=31) and non-SMA (Hb levels of 6.0 to 10.9 g/dl;

n=37) for the least-angle regression (LAR) analyses. In addition, potential underlying genetic variations that may potentially contribute to differences in functional changes (e.g., IFN-γ) during disease in the population were never controlled for as a variable in the LAR analyses. This continues to highlight the complexity associated with the determination of host immune responses against severe malaria. It has been demonstrated that IFN-γ is involved in alternative activation of macrophages which are specialized for defense against extracellular pathogens (Martinez *et al.,* 2009). However, *P. falciparum* is an obligatory intracellular pathogen (Breman *et al*., 2004) thus the mechanism provided by alternative activation of macrophages may not aid in the defense against it. Furthermore, differences could be due to geographical and demographic differences which may concomitantly be accompanied by various selective pressures. For example, the current pediatric population is naturally exposed to additional conditions such as other parasitic infections, which may independently alter IFN-γ production.

5.4 Study limitations

The current study had limitation in that the population does not have clinical manifestations of different *P. falciparum* malaria (asymptomatic malaria, mild and severe) to perform an intergroup comparison between the levels of cytokines and genotypes/haplotypes studied.

CHAPTER SIX

SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of Findings

In summary, the results of this study showed that none of the individual promoter loci (genotype) and haplotypes of TLR-4 -8984C/G and TLR-4 +299Asp/Gly had significant association with susceptibility to SMA. Similarly, there was no significant difference between individual TLR-4 -8984C/G genotypes and circulating IFN-γ levels. However, haplotype carriers of TLR-4 (-8984G and +299Asp), and TLR-4 (-8984G and +299Gly) demonstrated similar levels of IFN-γ with the non-carriers. Further analysis revealed that C/Asp haplotype carriers were significantly associated with lower levels of circulating IFN-γ than the non-carriers of C/Asp. In addition, there were no significant differences in circulating IFN-γ levels between SMA and non-SMA groups.

6.2 Conclusions

1. The results demonstrate that SMA in this paediatric population of western Kenya is not associated with individual promoter variants (genotype) and co-inheritance of functional variations in TLR-4 (-8984C/G and +299Asp/Gly) polymorphisms.
2. In this peadriatic population of western Kenya, co-inheritance of variations in TLR-4 (-8984C/G and +299Asp/Gly) polymorphisms conditioned changes in levels of circulating IFN-γ.
3. In this peadriatic population of western Kenya, circulating IFN-γ levels were similar between the clinical (SMA and non SMA) groups.

6.3 Recommendations from Current Study

1. In this population more studies on additional TLR-4 variants are necessary to identify the genotypic/haplotypic characteristics that predispose children to SMA.
2. The pathological role of lower IFN-γ as a result of co-inheritance of functional variations in TLR-4 (-8984C/G and +299Asp/Gly) polymorphisms should be investigated as it may act in peadriatric population as a basis of vaccine development against malaria.
3. More studies with larger sample sizes are necessary to confirm discrepancies a rising from the current and previous studies regarding the levels of IFN-γ in SMA and non-SMA in this population of western Kenya.

6.4 Recommendations for Future Studies

1. Immunogenetics study on basis of SMA development should involve inclusive panel of receptor polymorphisms that influence innate immune response and disease outcome are required as this may provide an immune-genetic basis for the development of vaccines that modulate receptor functions.
2. To further understand the molecular basis of SMA in children, cytokine milieu including IFN-γ should be investigated to enable determination of the role of cytokine balance in susceptibility to SMA.

REFERENCES

Agnese, D. M., Calvano, J. E., Hahm, S. J., Coyle, S. M., Corbett, S. A., Calvano, S. E., & Lowry, S. F. (2002). Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. *J Infect Dis, 186*(10), 1522-1525.

Aidoo, M., Terlouw, D. J., Kolczak, M. S., McElroy, P. D., ter Kuile, F. O., Kariuki, S., Udhayakumar, V. (2002). Protective effects of the sickle cell gene against malaria morbidity and mortality. *Lancet, 359*(9314), 1311-1312.

Akira, S. (2003). Mammalian Toll-like receptors. *Curr Opin Immunol, 15*(1), 5-11.

Akira, S., Uematsu, S., & Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell, 124*(4), 783-801.

Balaresque, P. L., Ballereau, S. J., & Jobling, M. A. (2007). Challenges in human genetic diversity: demographic history and adaptation. *Hum Mol Genet, 16 Spec No. 2*, R134-139.

Bassat, Q., Guinovart, C., Sigauque, B., Mandomando, I., Aide, P., Sacarlal, J., Alonso, P. L. (2009). Severe malaria and concomitant bacteraemia in children admitted to a rural Mozambican hospital. *Trop Med Int Health, 14*(9), 1011-1019.

Basu, M., Maji, A. K., Chakraborty, A., Banerjee, R., Mullick, S., Saha, P., Sengupta, S. (2010). Genetic association of Toll-like-receptor 4 and tumor necrosis factor-alpha polymorphisms with Plasmodium falciparum blood infection levels. *Infect Genet Evol, 10*(5), 686-696.

Beach, R. F., Ruebush, T. K., 2nd, Sexton, J. D., Bright, P. L., Hightower, A. W., Breman, J. G., Oloo, A. J. (1993). Effectiveness of permethrin-impregnated bed nets and curtains for malaria control in a holoendemic area of western Kenya. *Am J Trop Med Hyg, 49*(3), 290-300.

Beier, J. C., Oster, C. N., Onyango, F. K., Bales, J. D., Sherwood, J. A., Perkins, P. V., *et al*. (1994). *Plasmodium falciparum* incidence relative to entomologic inoculation rates at a site proposed for testing malaria vaccines in western Kenya. *Am J Trop Med Hyg, 50*(5), 529-536.

Bloland, P. B., Boriga, D. A., Ruebush, T. K., McCormick, J. B., Roberts, J. M., Oloo, A. J., Campbell, C. C. (1999). Longitudinal cohort study of the epidemiology of malaria infections in an area of intense malaria transmission II. Descriptive epidemiology of malaria infection and disease among children. *Am J Trop Med Hyg, 60*(4), 641-648.

Brabin, B. J., Premji, Z., & Verhoeff, F. (2001). An analysis of Anaemia and child mortality. *J Nutr, 131*(2S-2), 636S-645S;

Brattig, N. W., Kowalsky, K., Liu, X., Burchard, G. D., Kamena, F., & Seeberger, P. H. (2008). *Plasmodium falciparum* glycosylphosphatidylinositol toxin interacts with the

membrane of non-parasitized red blood cells: a putative mechanism contributing to malaria Anaemia. *Microbes Infect, 10*(8), 885-891.

Breman, J. G., Alilio, M. S., & Mills, A. (2004). Conquering the intolerable burden of malaria: what's new, what's needed: a summary. *Am J Trop Med Hyg, 71*(2 Suppl), 1-15.

Casals-Pascual, C., Kai, O., Cheung, J. O., Williams, S., Lowe, B., Nyanoti, M., Roberts, D. J. (2006). Suppression of erythropoiesis in malarial Anaemia is associated with hemozoin in vitro and in vivo. *Blood, 108*(8), 2569-2577.

Chehimi, J., & Trinchieri, G. (2004). Interleukin-12: a bridge between innate resistance and adaptive immunity with a role in infection and acquired immunodeficiency. *J Clin Immunol, 14*(3), 149-161.

Coban, C., Ishii, K. J., Uematsu, S., Arisue, N., Sato, S., Yamamoto, M., Akira, S. (2007). Pathological role of Toll-like receptor signaling in cerebral malaria. *Int Immunol, 19*(1), 67-79
.
D'Ombrain, M. C., Hansen, D. S., Simpson, K. M., & Schofield, L. (2007). Gammadelta-T cells expressing NK receptors predominate over NK cells and conventional T cells in the innate IFN-gamma response to *Plasmodium falciparum* malaria. *Eur J Immunol, 37*(7), 1864-1873.

Davenport, G. C., Ouma, C., Hittner, J. B., Were, T., Ouma, Y., Ong'echa, J. M., & Perkins, D. J. (2010). Hematological predictors of increased severe Anaemia in Kenyan children coinfected with Plasmodium falciparum and HIV-1. *Am J Hematol, 85*(4), 227-233.

Dodoo, D., Omer, F. M., Todd, J., Akanmori, B. D., Koram, K. A., & Riley, E. M. (2002). Absolute levels and ratios of proinflammatory and anti-inflammatory cytokine production in vitro predict clinical immunity to Plasmodium falciparum malaria. *J Infect Dis, 185*(7), 971-979.

Dupont, W. D., & Plummer, W. D., Jr. (1990). Power and sample size calculations. A review and computer program. *Control Clin Trials, 11*(2), 116-128.

Ekvall, H. (2003). Malaria and Anaemia. *Curr Opin Hematol, 10*(2), 108-114. Retrieved from el Hassan, A. M., Saeed, A. M., Fandrey, J., & Jelkmann, W. (2001). Decreased erythropoietin response in *Plasmodium falciparum* malaria-associated anaemia. *Eur J Haematol, 59*(5), 299-304
.
Esposito, S., Molteni, C. G., Zampiero, A., Baggi, E., Lavizzari, A., Semino, M., Principi, N. (2012). Role of polymorphisms of toll-like receptor (TLR) 4, TLR9, toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) and FCGR2A genes in malaria susceptibility and severity in Burundian children. *Malar J, 11*, 196.

Ferwerda, B., McCall, M. B., Alonso, S., Giamarellos-Bourboulis, E. J., Mouktaroudi, M., Izagirre, N., . . . Netea, M. G. (2007). TLR4 polymorphisms, infectious diseases, and

evolutionary pressure during migration of modern humans. *Proc Natl Acad Sci U S A, 104*(42), 16645-16650.

Franklin, B. S., Parroche, P., Ataide, M. A., Lauw, F., Ropert, C., de Oliveira, R. B., Gazzinelli, R. T. (2009). Malaria primes the innate immune response due to interferon-gamma induced enhancement of toll-like receptor expression and function. *Proc Natl Acad Sci U S A, 106*(14), 5789-5794.

Gajewski, T. F., Joyce, J., & Fitch, F. W. (2002). Antiproliferative effect of IFN-gamma in immune regulation. III. Differential selection of TH1 and TH2 murine helper T lymphocyte clones using recombinant IL-2 and recombinant IFN-gamma. *J Immunol, 143*(1), 15-22.

Gatlin, M. R., Black, C. L., Mwinzi, P. N., Secor, W. E., Karanja, D. M., & Colley, D. G. (2009). Association of the gene polymorphisms IFN-gamma +874, IL-13 -1055 and IL-4 -590 with patterns of reinfection with Schistosoma mansoni. *PLoS Negl Trop Dis, 3*(2), e375.

Giha, H. A., Elghazali, G., TM, A. E., IE, A. E., Eltahir, E. M., Baraka, O. Z., Elbashir, M. I. (2005). Clinical pattern of severe *Plasmodium falciparum* malaria in Sudan in an area characterized by seasonal and unstable malaria transmission. *Trans R Soc Trop Med Hyg, 99*(4), 243-251.

Gil, M. P., Bohn, E., O'Guin, A. K., Ramana, C. V., Levine, B., Stark, G. R., Schreiber, R. D. (2001). Biologic consequences of Stat1-independent IFN signaling. *Proc Natl Acad Sci U S A, 98*(12), 6680-6685.

Helegbe, G. K., Huy, N. T., Yanagi, T., Shuaibu, M. N., Yamazaki, A., Kikuchi, M., Hirayama, K. (2009). Rate of red blood cell destruction varies in different strains of mice infected with *Plasmodium berghei-ANKA* after chronic exposure. *Malar J, 8*, 91.

Hensmann, M., & Kwiatkowski, D. (2001). Cellular basis of early cytokine response to *Plasmodium falciparum*. *Infect Immun, 69*(4), 2364-2371.

Horowitz, A., Newman, K. C., Evans, J. H., Korbel, D. S., Davis, D. M., & Riley, E. M. (2010). Cross-talk between T cells and NK cells generates rapid effector responses to *Plasmodium falciparum*-infected erythrocytes. *J Immunol, 184*(11), 6043-6052.

Janeway, C. A., Jr., & Medzhitov, R. (2002). Innate immune recognition. *Annu Rev Immunol, 20*, 197-216.

Layez, C., Nogueira, P., Combes, V., Costa, F. T., Juhan-Vague, I., da Silva, L. H., & Gysin, J. (2005). *Plasmodium falciparum* rhoptry protein RSP-2 triggers destruction of the erythroid lineage. *Blood, 106*(10), 3632-3638.

Leoratti, F. M., Farias, L., Alves, F. P., Suarez-Mutis, M. C., Coura, J. R., Kalil, J., Ramasawmy, R. (2008). Variants in the toll-like receptor signaling pathway and clinical outcomes of malaria. *J Infect Dis, 198*(5), 772-780.

Lofgren, J., Marttila, R., Renko, M., Ramet, M., & Hallman, M. (2010). Toll-like receptor 4 Asp299Gly polymorphism in respiratory syncytial virus epidemics. *Pediatr Pulmonol, 45*(7), 687-692.

Looareesuwan, S., Merry, A. H., Phillips, R. E., Pleehachinda, R., Wattanagoon, Y., Ho, M., Weatherall, D. J. (2000). Reduced erythrocyte survival following clearance of malarial parasitaemia in Thai patients. *Br J Haematol, 67*(4), 473-478.

Lorenz, E., Mira, J. P., Frees, K. L., & Schwartz, D. A. (2002). Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. *Arch Intern Med, 162*(9), 1028-1032.

Lyke, K. E., Diallo, D. A., Dicko, A., Kone, A., Coulibaly, D., Guindo, A., Plowe, C. V. (2003). Association of intraleukocytic Plasmodium falciparum malaria pigment with disease severity, clinical manifestations, and prognosis in severe malaria. *Am J Trop Med Hyg, 69*(3), 253-259
.

Marsh, K., Forster, D., Waruiru, C., Mwangi, I., Winstanley, M., Marsh, V., *et al*. (1995). Indicators of life-threatening malaria in African children. *N Engl J Med, 332*(21), 1399-1404.

Martinez, F. O., Helming, L., & Gordon, S. (2009). Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol, 27*, 451-483.

McElroy, P. D., Lal, A. A., Hawley, W. A., Bloland, P. B., Kuile, F. O., Oloo, A. J., Nahlen, B. L. (1999). Analysis of repeated haemoglobin measures in full-term, normal birth weight Kenyan children between birth and four years of age. III. The Asemobo Bay Cohort Project. *Am J Trop Med Hyg, 61*(6), 932-940.

Miller, C. H., Maher, S. G., & Young, H. A. (2009). Clinical Use of Interferon-gamma. *Ann N Y Acad Sci, 1182*, 69-79.

Miller, J. L., Sack, B. K., Baldwin, M., Vaughan, A. M., & Kappe, S. H. (2014). Interferon-mediated innate immune responses against malaria parasite liver stages. *Cell Rep, 7*(2), 436-447.

Mockenhaupt, F. P., Cramer, J. P., Hamann, L., Stegemann, M. S., Eckert, J., Oh, N. R., Schumann, R. R. (2006b). Toll-like receptor (TLR) polymorphisms in African children: common TLR-4 variants predispose to severe malaria. *J Commun Dis, 38*(3), 230-245.

Mockenhaupt, F. P., Hamann, L., von Gaertner, C., Bedu-Addo, G., von Kleinsorgen, C., Schumann, R. R., & Bienzle, U. (2006a). Common polymorphisms of toll-like receptors 4 and 9 are associated with the clinical manifestation of malaria during pregnancy. *J Infect Dis, 194*(2), 184-188.

Newton, C. R., Warn, P. A., Winstanley, P. A., Peshu, N., Snow, R. W., Pasvol, G., & Marsh, K. (2007). Severe anaemia in children living in a malaria endemic area of Kenya. *Trop Med Int Health, 2*(2), 165-178.

Novelli, E. M., Hittner, J. B., Davenport, G. C., Ouma, C., Were, T., Obaro, S., Perkins, D. J. (2010). Clinical predictors of severe malarial anaemia in a holoendemic *Plasmodium falciparum* transmission area. *Br J Haematol, 149*(5), 711-721.

Ong'echa, J. M., Davenport, G. C., Vulule, J. M., Hittner, J. B., & Perkins, D. J. (2011). Identification of inflammatory biomarkers for pediatric malarial Anaemia severity using novel statistical methods. *Infect Immun, 79*(11), 4674-4680.

Ong'echa, J. M., Keller, C. C., Were, T., Ouma, C., Otieno, R. O., Landis-Lewis, Z., Perkins, D. J. (2006). Parasitemia, Anaemia, and malarial Anaemia in infants and young children in a rural holoendemic *Plasmodium falciparum* transmission area. *Am J Trop Med Hyg, 74*(3), 376-385.

Otieno, R. O., Ouma, C., Ong'echa, J. M., Keller, C. C., Were, T., Waindi, E. N., Perkins, D. J. (2006). Increased severe Anaemia in HIV-1-exposed and HIV-1-positive infants and children during acute malaria. *AIDS, 20*(2), 275-280.

Ouma, C., Davenport, G. C., Awandare, G. A., Keller, C. C., Were, T., Otieno, M. F., . . . Perkins, D. J. (2008). Polymorphic variability in the interleukin (IL)-1beta promoter conditions susceptibility to severe malarial Anaemia and functional changes in IL-1beta production. *J Infect Dis, 198*(8), 1219-1226.

Ouma, C., Davenport, G. C., Garcia, S., Kempaiah, P., Chaudhary, A., Were, T., . . . Perkins, D. J. (2011). Functional haplotypes of Fc gamma (Fcgamma) receptor (FcgammaRIIA and FcgammaRIIIB) predict risk to repeated episodes of severe malarial Anaemia and mortality in Kenyan children. *Hum Genet*.

Ouma, C., Davenport, G. C., Were, T., Otieno, M. F., Hittner, J. B., Vulule, J. M., . . . Perkins, D. J. (2008). Haplotypes of IL-10 promoter variants are associated with susceptibility to severe malarial Anaemia and functional changes in IL-10 production. *Hum Genet, 124*(5), 515-524.

Ouma, C., Keller, C. C., Davenport, G. C., Were, T., Konah, S., Otieno, M. F., . . . Perkins, D. J. (2010). A novel functional variant in the stem cell growth factor promoter protects against severe malarial Anaemia. *Infect Immun, 78*(1), 453-460.

Ouma, C., Keller, C. C., Opondo, D. A., Were, T., Otieno, R. O., Otieno, M. F., . . . Perkins, D. J. (2006). Association of FCgamma receptor IIA (CD32) polymorphism with malarial Anaemia and high-density parasitemia in infants and young children. *Am J Trop Med Hyg, 74*(4), 573-577.

Papadopoulos, A. I., Ferwerda, B., Antoniadou, A., Sakka, V., Galani, L., Kavatha, D., . . . Netea, M. G. (2010). Association of toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms with increased infection risk in patients with advanced HIV-1 infection. *Clin Infect Dis, 51*(2), 242-247.

Perkins, D. J., Weinberg, J. B., & Kremsner, P. G. (2000). Reduced interleukin-12 and transforming growth factor-beta1 in severe childhood malaria: relationship of cytokine balance with disease severity. *J Infect Dis, 182*(3), 988-992.

Perlaza, B. L., Sauzet, J. P., Brahimi, K., BenMohamed, L., & Druilhe, P. (2011). Interferon-gamma, a valuable surrogate marker of Plasmodium falciparum pre-erythrocytic stages protective immunity. *Malar J, 10*, 27.

Phillips, R. E., Looareesuwan, S., Warrell, D. A., Lee, S. H., Karbwang, J., Warrell, M. J., Weatherall, D. J. (2000). The importance of anaemia in cerebral and uncomplicated falciparum malaria: role of complications, dyserythropoiesis and iron sequestration. *Q J Med, 58*(227), 305-323.

Prohinar, P., Rallabhandi, P., Weiss, J. P., & Gioannini, T. L. (2010). Expression of functional D299G.T399I polymorphic variant of TLR4 depends more on coexpression of MD-2 than does wild-type TLR4. *J Immunol, 184*(8), 4362-4367.

Reyburn, H., Mbatia, R., Drakeley, C., Bruce, J., Carneiro, I., Olomi, R., Riley, E. M. (2005). Association of transmission intensity and age with clinical manifestations and case fatality of severe Plasmodium falciparum malaria. *JAMA, 293*(12), 1461-1470.

Ricci, E., Malacrida, S., Zanchetta, M., Mosconi, I., Montagna, M., Giaquinto, C., & De Rossi, A. (2010). Toll-like receptor 9 polymorphisms influence mother-to-child transmission of human immunodeficiency virus type 1. *J Transl Med, 8*, 49.

Sam-Agudu, N. A., Greene, J. A., Opoka, R. O., Kazura, J. W., Boivin, M. J., Zimmerman, P. A., John, C. C. (2010). TLR9 polymorphisms are associated with altered IFN-gamma levels in children with cerebral malaria. *Am J Trop Med Hyg, 82*(4), 548-555.

Schroder, N. W., Meister, D., Wolff, V., Christan, C., Kaner, D., Haban, V., Schumann, R. R. (2005). Chronic periodontal disease is associated with single-nucleotide polymorphisms of the human TLR-4 gene. *Genes Immun, 6*(5), 448-451.

Stoute, J. A., Odindo, A. O., Owuor, B. O., Mibei, E. K., Opollo, M. O., & Waitumbi, J. N. (2003). Loss of red blood cell-complement regulatory proteins and increased levels of circulating immune complexes are associated with severe malarial Anaemia. *J Infect Dis, 187*(3), 522-525.

Takeda, K., Kaisho, T., & Akira, S. (2003). Toll-like receptors. *Annu Rev Immunol, 21*, 335-376. doi:10.1146/annurev.immunol.21.120601.141126120601.141126

Tangteerawatana, P., Pichyangkul, S., Hayano, M., Kalambaheti, T., Looareesuwan, S., Troye-Blomberg, M., & Khusmith, S. (2007). Relative levels of IL4 and IFN-gamma in complicated malaria: association with IL4 polymorphism and peripheral parasitemia. *Acta Trop, 101*(3), 258-265.

Torre, D., Speranza, F., Giola, M., Matteelli, A., Tambini, R., & Biondi, G. (2002). Role of Th1 and Th2 cytokines in immune response to uncomplicated *Plasmodium falciparum* malaria. *Clin Diagn Lab Immunol, 9*(2), 348-351.

Were, T., Davenport, G. C., Hittner, J. B., Ouma, C., Vulule, J. M., Ong'echa, J. M., & Perkins, D. J. (2011). Bacteremia in Kenyan children presenting with malaria. *J Clin Microbiol, 49*(2), 671-676.

Were, T., Hittner, J. B., Ouma, C., Otieno, R. O., Orago, A. S., Ong'echa, J. M., Perkins, D. J. (2006). Suppression of RANTES in children with *Plasmodium falciparum* malaria. *Haematologica, 91*(10), 1396-1399.

Whitley, E., & Ball, J. (2002). Statistics review 4: Sample size calculations. *Critical Care, 6*(4), 335-341.

WHO. (2000). Severe *falciparum* malaria. World Health Organization, Communicable Diseases Cluster. *Trans R Soc Trop Med Hyg, 94 Suppl 1*, S1-90.

WHO. (2007). WHO, Fact Sheet No. 94, May 2007.

WHO. (2010). WORLD MALARIA REPORT. Geneva Switzertland: WHO Press.

WHO. (2016). WHO, World malaria report, 2016. (WHO Press).

Wickramasinghe, S. N., & Abdalla, S. H. (2000). Blood and bone marrow changes in malaria. *Baillieres Best Pract Res Clin Haematol, 13*(2), 277-299.

Zakeri, S., Pirahmadi, S., Mehrizi, A. A., & Djadid, N. D. (2011). Genetic variation of TLR-4, TLR-9 and TIRAP genes in Iranian malaria patients. *Malar J, 10*, 77.

APPENDICES

AppendixI: School of Graduate Studies (SGS) Research Approval letter.

MASENO UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: MSC/PH/00096/2013

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

Date: 31st August, 2017

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR PHILIP OTIENO ONYONA— MSC/PH/00096/2013

The above named is registered in the Master of Science in Programme of the School of Public Health and Community Development, Maseno University. This is to confirm that his research proposal titled "Association between Toll Like Receptor-4(-8984C/G and 299C/G) Polymorphisms and Susceptibility to Pediatric Severe Plasmodium Falciparum Malaria Anemia in Western Kenya" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

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

DEAN SCHOOL OF GRADUATE STUDIES MASENO UNIVERSITY 26 SEP 2017 P.O. Box PRIVATE BAG, MASENO

Maseno University *ISO 9001:2008 Certified*

AppendixII: KEMRI ERC Research approval letter.

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

15 FEB 2012

FORWARDED

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

TO: PROF. COLLINS OUMA (PRINCIPAL INVESTIGATOR)

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

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

In Search of Better Health