# ASSOCIATIONS BETWEEN FUNCTIONAL VARIATIONS IN FCγRIIIA AND TLR-9 AND SUSCEPTIBILITY TO SEVERE MALARIA ANEMIA IN CHILDREN BELOW 3 YEARS IN WESTERN KENYA

**By**

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**A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Medical Biotechnology**

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# ABSTRACT

*Plasmodium falciparum* malaria in holoendemic transmission areas is a major cause of childhood morbidity and mortality. The greatest burden of this morbidity and mortality is as a consequence of severe malaria anemia (SMA) in children aged below 5 years. Understanding the immunogenetic basis of naturally acquired immunity to *P. falciparum* infection and SMA would aid in designing a rationally based malaria vaccine. Variants within the FcγR mediate immunity through engagement of immunoglobulin (Ig) G and other immune mediators such as interferon gamma (IFN-γ), resulting into erythrophagocytosis and production of inflammatory cytokines. Toll like receptors (TLRs) trigger transcription of pro-inflammatory cytokines and induction of adaptive immune responses. The two receptors collectively through both innate and adaptive immune responses may determine malaria disease pathogenesis. The role of these single nucleotide polymorphisms (SNPs) in conditioning susceptibility to SMA has however not been investigated in *P. falciparum* holoendemic transmission areas such as in western Kenya. In this cross-sectional study, the association between FcγRIIIA -176F/V, TLR-9 (-1237T/C) genotypes/SNPs combinations, SMA (Hb<6.0g/dL) and circulating levels of IFN-γ were investigated in children (n=301) with *falciparum* malaria from Siaya in western Kenya. Haematological and parasitological parameters were determined in all study participants. FcγRIIIA -176F/V and TLR-9 (-1237T/C) genotypes were determined using TaqMan 5' allelic discrimination assay. Circulating levels of IFN-γ were determined using Cytokine 25-plex Ab Bead Kit. All data analysis was performed using SPSS (Version 19.0). Multivariate logistic regression analysis (controlling for confounding factors) revealed that children with FcγRIIIA -176V/ TLR-9 -1237C SNP combinations had a 64% reduced odds of developing SMA (OR=0.36, 95%CI 0.20-0.64; *p*=0.001) while carriers of FcγRIIIA -176V/ TLR-9 -1237T SNP combination were more susceptible to SMA (OR=2.04, 95%CI, 1.19-3.50; *p*=0.009). Children with SMA had higher circulating IFN-γ levels compared to non-SMA (*p*=0.008). Consistently, the FcγRIIIA -176V/TLR-9 -1237T (VT) carriers had higher levels of circulating IFN-γ (*p*=0.011) relative to non-carriers supporting the observation that higher IFN-γ levels are associated with SMA. These results demonstrate that FcγRIIIA-176F/V and TLR-9 (-1237T/C) variants condition susceptibility to SMA and functional changes in circulating IFN-γ levels. This study therefore provides critical information that can be utilized in the design of an effective malaria vaccine since the intermediate step is to understand the natural immune response in a population before designing a long-lasting effective vaccine against the disease.



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# CHAPTER ONE

## 1.0. 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 estimated 655,000 deaths per year, predominantly in children under the age of 5 years living in Sub-Saharan Africa (WHO, 2011). *P. 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) (Marsh *et al*., 1995; Giha *et al*., 2005). Of these pathologies, severe malaria anemia (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 infant mortality associated with malaria (Ekvall, 2003).

The mortality rate in children with SMA is about 8.6% compared with 3.6% in children with severe anemia due to other causes (Obonyo *et al*., 2007). Severe anemia may develop rapidly in the course of a malaria illness, especially in the presence of high parasite densities and co-pathogens such as bacteria and HIV-1 (Newton *et al*., 1997; Otieno *et al*., 2006; Were *et al*., 2011). Studies have shown that plasma levels of the pro-inflammatory cytokines, for instance tumor necrosis factor (TNF-α) and 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



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pathogenesis of SMA. High level of parasitemia mainly in non-immune individuals can result into massive lysis and clearance of erythrocytes leading to profound anemia (Phillips *et al*., 1986). Despite efforts aimed at ameliorating the anemia burden, SMA remains an important childhood health burden in sub-Saharan Africa (Brabin *et al*., 2001). 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).

The susceptibility to SMA is also associated with polymorphic variability in genes that condition functional changes in levels of circulating inflammatory and hematopoietic mediators such as IL-10, IL-1β and stem cell growth factors (SCGF) (Ouma *et al*., 2008a; Ouma *et al*., 2008b; Ouma *et al*., 2010). Variations in Toll-like receptors and FcγRs have been associated with susceptibility to infectious disease in several studies (Agnese *et al*., 2002; Lorenz *et al*., 2002; Ouma *et al*., 2006; Ouma *et al*., 2011). However, none of these studies have investigated the association between these variants with susceptibility to SMA in pediatric populations residing in *P. falciparum* holoendemic transmission regions such as in Siaya in western Kenya. The present study therefore investigated the associations between, FcγRIIIA -176F/V, TLR-9 (-1237T/C) genotype/Single Nucleotide Polymorphism (SNP) combinations and SMA in pediatric populations less than 3 years living in *P. falciparum* malaria holoendemic transmission area of Siaya District, western Kenya. In addition, this study determined how these genetic variants affect levels of circulating IFN-γ in children naturally exposed to *P. falciparum* infection.

**1.2. Problem statement for study**

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 pediatric

population residing in *P. falciparum* holoendemic transmission area such as Siaya District in western Kenya (McElroy *et al.*, 1999; Obonyo *et al.*, 2007). However, the host-related genetic factors that modulate such pathogenesis and complication associated with *P. falciparum* malaria remains unclear. A better understanding of these factors is essential for the identification of populations at-risk and for the development of more effective prophylactic and therapeutic measures.

FcγRs may contribute to the pathogenesis of SMA by mediating phagocytosis of IgG-coated erythrocytes. Erythrocytes of children with SMA have been shown to have increased surface IgG (Waitumbi *et al.*, 2000), a condition that could trigger FcγR-mediated erythrophagocytosis. Moreover, the clearance half-times of IgG-sensitized red blood cells has been shown to be dissimilar and to correlate directly with haematocrit in patients with acute *P. falciparum* malaria (Lee *et al.*, 1989; Huang *et al.*, 2011) indicating that FcγR-mediated clearance of uninfected erythrocytes may be important in the development of severe anemia in *P. falciparum* malaria. Human FcγRIIIA exhibits the genetic polymorphisms, FcγRIIIA-176F/V that influences capacities for IgG binding and phagocytosis of IgG-opsonized targets (Omi *et al.*, 2002) and may therefore determine an individuals' susceptibility to SMA. However, little is known about the contribution of these receptor polymorphisms to the pathogenesis of SMA in children below 3 years of age.

Previous studies have indicated the role of toll-like receptors (TLRs) in immunity against inflammatory and infectious diseases (Hemmi *et al.*, 2000; Akira, 2003). The TLR-9 which is a receptor for CpG-DNA and hemozoin (Coban *et al.*, 2005; Ng *et al.*, 2010) have been shown to harbour promoter polymorphisms that influence circulating levels of pro-inflammatory cytokines such as IFN-γ in malaria infection (Sam-Agudu *et al.*, 2010). Production of IFN-γ as triggered by different sets of stimuli has also been shown to be

involved in malaria pathogenesis (Ong'echa *et al.*, 2003). However, there is little evidence to support the role of TLR-9 and its variants in influencing the production of IFN-γ during SMA pathogenesis. The current study therefore explored the associations between FcγRIIIA-176F/V and TLR-9 (-1237T/C) polymorphisms and susceptibility to SMA in children below 3 years residing in a holoendemic *P. falciparum* region of Siaya District in western Kenya. In addition, polymorphism-mediated differences in the levels of circulating IFN-γ in children were also determined.

### 1.3.0. Objectives

#### 1.3.1. General objective

To investigate the functional associations between FcγRIIIA-176F/V, TLR-9 (-1237T/C) genotypes/SNP combinations and susceptibility to SMA in children below 3 years of age residing in a holoendemic *P. falciparum* transmission region in Siaya District of western Kenya.

#### 1.3.2. Specific objectives

1. To determine the association between FcγRIIIA-176F/V, TLR-9 (-1237T/C) genotypes/SNP combinations and SMA in children below 3 years presenting with acute malaria in Siaya District, western Kenya.
2. To determine the associations between FcγRIIIA-176F/V and TLR-9 (-1237T/C) genotypes/SNPs combinations and interferon gamma (IFN-γ) production in children below 3 years presenting with acute malaria in Siaya District of western Kenya.
3. To determine functional roles of polymorphism-mediated interferon gamma (IFN-γ) in children with SMA and non-SMA presenting with acute malaria in Siaya District, western Kenya.

### 1.4. Research questions

1. What are the associations between FcγRIIIA-176F/V, TLR-9 (-1237T/C) genotypes/SNPs combination and severe malaria anemia in children below 3 years presenting with acute malaria in Siaya District, western Kenya?
2. What are the associations between FcγRIIIA-176F/V and TLR-9 (-1237T/C) genotypes/SNPs combinations and interferon gamma (IFN-γ) production in children below 3 years presenting with acute malaria in Siaya District, western Kenya?
3. What are the functional roles of polymorphism-mediated interferon gamma (IFN-γ) in children with SMA and non-SMA presenting with acute malaria in Siaya District, western Kenya?

### 1.5. Justification of the study

The results of this study have provided an important insight on the potential role of FcγRIIIA-176F/V and TLR-9 (-1237T/C) polymorphisms in the pathogenesis of severe anaemia in *P. falciparum* malaria. Since the major role of these receptors is to interact with pathogen associated molecular patterns and immune complexes, the finding that these polymorphisms are able to impact on severe malaria outcome can be applied in designing vaccines that target these polymorphisms. The study has further added information on the possible underlying molecular basis required for the development of protective immunity against severe malaria anemia (SMA). Following the finding that the carriers of FcγRIIIA - 176V/ TLR-9 -1237T SNPs combinations are more susceptible to severe malaria anemia, they are therefore a group at risk of SMA development which should be identified for early intervention before the disease develops to anemia to help reduce child mortality due to SMA.



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# CHAPTER TWO

## 2.0 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 anemia, and/or bleeding, which rapidly progress to death within hours or days (WHO, 2000). 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 anemia in malaria include reduced erythropoietin activity, pro-inflammatory cytokines and increased erythrocyte destruction mediated by parasites, erythrophagocytosis, antibody and complement-mediated lysis (el Hassan *et al.*, 1997; Stoute *et al.*, 2003), repeated cycles of invasion, replication, and bursting of erythrocytes due to infection by *P. falciparum* parasites (Wickramasinghe and Abdalla, 2000).

Recent 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 unparasitised erythrocytes and dyserythropoiesis (Layez *et al.*, 2005).

Reduced regulated upon activation, normal T-cell expressed, and secreted (RANTES) levels (Were *et al.*, 2006) as a result of naturally-acquired hemozoin by monocytes may also contribute, in part, to suppression of erythropoiesis in children with malarial anemia (Were *et al.*, 2009). Hemozoin acquisition by phagocytes has been shown to suppress cellular immunity and enhance malaria disease severity (Lyke *et al.*, 2003; Casals-Pascual *et al.*, 2006). Moreover, SMA has been characterized by a shortened life span of circulating erythrocytes (Looareesuwan *et al.*, 1987) and enhanced in the presence of other co-pathogens, such as HIV-1, hookworm and bacteria (Otieno *et al.*, 2006; Bassat *et al.*, 2009; Davenport *et al.*, 2010; Were *et al.*, 2011).

**2.2. Fcγ receptors (FcγRs) in pathology and protection in malaria**

FcγR are cell surface proteins expressed on the surface of antigen presenting cells (APCs) which function to help in recognition and elimination of invading pathogens (Marsh *et al.*, 1989; Cabrera *et al.*, 2004). FcγRs bind to the Fc portion of the immunoglobulin G (IgG) and thereby link antigen recognition by antibodies with cell-based effector mechanisms (Unkeless *et al.*, 1988; Hulett and Hogarth, 1994). Human FcγR genes form a clustered gene family, which are mapped to chromosome 1q and are located at q21-q23 (Ravetch and Kinet, 1991; Indik *et al.*, 1995; Daeron, 1997). FcγRs are involved in the clearance of immune complexes, phagocytosis of antibody-coated pathogens, enhancement of antigen presentation, secretion of reactive oxygen intermediates (ROI), antibody-dependent cellular cytotoxicity (ADCC), and cytotoxicity of antibody-coated tumor cells (Fanger *et al.*, 1989; Morganelli *et al.*, 1992). The structural heterogeneity of FcγR isoforms contributes to differences in

preferential binding affinity, distinct signal transduction pathways, and cell type-specific distributions (Salmon and Pricop, 2001).

FcγR polymorphisms exist on the extracellular domain where binding sites are located (Takai, 2002). Studies in a Kenyan pediatric population have shown that FcγR -131R/R alleles protect against high-density parasitemia (HDP; ≥10,000 parasites/μL) (Ouma *et al.*, 2006). FcγRIIIA plays an important role in the clearance of circulating immune complexes (Clarkson *et al.*, 1986). The dimorphism in the amino acid position 176(F/V) of the FcγRIIIA, has been shown to influence the binding of IgG subtypes in that FcγRIIIA -176V had a higher affinity compared to FcγRIIIA -176F for $IgG_1$ and $IgG_3$ (Omi *et al.*, 2002). In *P. falciparum* infections, FcγRIIIA-176V/V is associated with low parasitemia and low risk of clinical malaria (Aribot *et al.*, 1996). As such, defining the association between FcγRIIIA-176F/V genotypes and SMA in pediatric populations residing in holoendemic *P. falciparum* transmission areas is important since this clinical phenotype accounts for the largest degree of malaria-induced morbidity and mortality (Obonyo *et al.*, 2007). Since previous studies have identified associations between FcγR polymorphisms and SMA, the current study further explored whether FcγRIIIA-176F/V genotypes are associated with susceptibility to SMA and levels of circulating interferon gamma (IFN-γ) in children less than 3 years resident in Siaya District, western Kenya.

**2.3. Role of Toll-like receptors in severe malaria pathogenesis**

Toll-like receptors (TLRs) are type 1 transmembrane proteins differentially expressed among immune cells (Janeway and Medzhitov, 2002; Akira, 2003). They are receptors 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). TLR-9 occupies 5kb on chromosome 3p21.3 and consists of two exons

and encodes 1028 amino acids (Du *et al.*, 2000). It is inter-localized, to allow them to interact with pathogens that have previously been phagocytozed and thus minimize the chances of the recognition of self-antigen (Hemmi *et al.*, 2000). The only human dendritic cell subset that express TLR-9 are plasmacytoid dendritic cells (pDCs) (Hochrein *et al.*, 2002).

Hemozoin, a heme metabolite during malaria infection, activates the innate immune system via a TLR-9-mediated MyD88-dependent pathway resulting into signals that upregulate TNF-α, IL-12p40, monocyte chemo-attractant protein 1 (MCP-1), and IL-6 production by dendritic cells (Coban *et al.*, 2005). It is also a carrier that facilitates entry of plasmodial-DNA into the host cell, where the latter can bind to, and stimulate TLR-9 (Pichyangkul *et al.*, 2004). Previous studies have demonstrated that unmethylated CpG-DNA is a ligand for TLR-9 (Hemmi *et al.*, 2000; Bauer *et al.*, 2001; Hoene *et al.*, 2006; Knuefermann *et al.*, 2007). TLR-9 discriminates between microbial and self CpG-DNA motifs because the microbial motifs consist of unmethylated CpG dinucleotides flanked by two 5′-purines and two 3`-pyrimidines (Krieg *et al.*, 1995). Both *in vivo* and *in vitro* studies have shown that bacterial CpG-DNA elicits production of IFN-γ by NK and CD4+ T cells (Klinman *et al.*, 1996). TLR-9 has been shown to induce production of IFN-γ, IFN-β, IL-6 and IL-12 as a result of bacterial and viral invasion (Kang *et al.*, 2006).

Several single-nucleotide polymorphisms (SNPs) that alter susceptibility to infectious and inflammatory diseases have been identified in TLRs (Schroder *et al.*, 2005; Papadopoulos *et al.*, 2010; Ricci *et al.*, 2010). For example, recent studies in Ugandan children reported that elevated IFN-γ levels contribute to the pathogenesis of human cerebral malaria (CM), and that children with the C allele at TLR-9 −1237 or the G allele at TLR-9 1174 were more likely to develop CM because these alleles are associated with increased production of IFN-γ in severe *P. falciparum* infection (Sam-Agudu *et al.*, 2010). Studies in

pregnant Ghanaian women with *P. falciparum* showed that variants in the TLR-9 T-1486C increased the risk of maternal malaria (Mockenhaupt *et al.*, 2006). The TLR-9 (-1237T/C) polymorphism has been identified as a risk factor for the development of *H. pylori*-induced premalignant gastric changes (Ng *et al.*, 2010). 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. This study therefore investigated whether TLR-9 (-1237T/C) polymorphisms are associated with susceptibility to SMA and changes in the levels of circulating interferon gamma (IFN-γ) in children less than 3 years resident in Siaya District, western Kenya.

**2.4. Interferon gamma (IFN-γ) in pathology and protection in malaria**

IFN-γ is multifunctional cytokine produced primarily by T lymphocytes and natural killer cells (NKs) (Hensmann and Kwiatkowski, 2001; Miller *et al.*, 2009). It plays an important role in inflammatory responses and is often associated with the development of overt $T_H1$-like cell-mediated responses (Gajewski *et al.*, 1989), and hence forms an important part of the immune system (Chehimi and Trinchieri, 1994). A study involving children 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 longitudinal study in pediatric population in western Kenya has shown that high levels of circulating IFN-γ are associated with enhanced SMA severity in pediatric population (Ouma *et al.*, 2011).

Moreover, a study in Thai adults has also demonstrated IFN-γ levels are elevated in patients with complicated malaria at the initial stage of the disease than uncomplicated malaria (Tangteerawatana *et al.*, 2007) indicating the role of this cytokine in malaria pathogenesis. The study in addition, determined the functional relationship between interferon gamma (IFN-γ) levels with SMA and non-SMA in children presenting with acute malaria in Siaya District, western Kenya.

# CHAPTER THREE

## 3.0. METHODOLOGY

### 3.1. Study site

The study was conducted in Siaya District Hospital, in Siaya District, western Kenya (Appendix 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 is 1% and the mortality rate for infants is 11.3%, while under five mortality rates is 10.2% (KNBS, 2010). 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 (>99%), with the population being culturally homogeneous hence suitable for genetic based studies (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.

### 3.2.0. Study design and patient population

### 3.2.1. Recruitment of study participants

This was a case-control study of children presenting with acute malaria. Acute malaria in children is characterised by oral/axillary temperature > 37.5 °C or two of the following: nausea/vomiting, diarrhoea, headache, myalgias, poor feeding accompanied by a positive blood smear showing asexual *P. falciparum* parasitaemia. Cases were children with

severe malarial anaemia (SMA) (Hb <6.0 g/dl, any density parasitemia). Controls were children of similar age, same gender and from the same geographical area with non-severe malaria anemia (non-SMA) (Hb≥6.0g/dl, any density parasitemia). Children of both sexes were recruited at Siaya District Hospital as per previously established protocols (Ong'echa *et al.*, 2006). Questionnaires and existing medical records were used for recruitment. Enrolment into this study was confined to children who were less than 3 years of age.

### 3.2.2. Screening and enrolment

The following screening process was used to target 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. Enrolment decision was made after initial HIV-1 screening of children and obtaining informed consent. The questionnaires and written informed consent were administered in the language of choice (i.e. English, Kiswahili or Dholuo). Venous blood samples (<3.0 mL) were 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, plasma isolation for IFN-γ, dry blood spot preparation for DNA extraction and bacteremia determination. Based on the HIV-1 test results, malaria parasitemia, and haemoglobin (Hb) status, children that satisfied all inclusion criteria and did not meet the exclusion criteria were enrolled into the study. Children with malaria and bacteremia were treated according to Kenyan 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 infection.

**3.3.0. Eligibility criteria**

Upon enrolment into the study, HIV-1 status, parasitemia and haematological measurements of the children 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).

**3.3.1. Inclusion criteria**

Malaria parasitemia (any density) and Hb<11.0g/dL; age ≥3 months and ≤36 months; parent/guardian willing and able to sign consent form; able to keep schedule and study appointments; distance to the hospital ≤15km; and able to provide two contacts familiar with the child's whereabouts during the study period.

**3.3.2. Exclusion criteria**

Children with cerebral malaria (rare in this holoendemic area); clinical evidence of acute respiratory infection; prior hospitalization; intent to relocate during the study period; unwillingness to enroll child in the study.

**3.3.3 Sample size determination**

The study was carried out in a total of 301 malaria naïve patients (aged 3-36 months) who presented with clinical symptoms of *P. falciparum* malaria at Siaya District Hospital (SDH). The sample size (n=301) was based on actual number of children below 3 years that presented with clinical symptoms of malaria at Siaya District Hospital within the 24 months of the study (averagely 12 patients per month). The primary objective of the current genetic study was to identify genotypes/SNPs combinations that have significant functional allele frequency differences between case (SMA) and control (non-SMA) populations. According

to (Dupont and Plummer, 1990), the Bonferroni correction for 2 different loci would require a per-comparison alpha of 0.004 for sample size calculations. However, given the increased power provided by the procedure (Dupont and Plummer, 1990), it is estimated that sample sizes based on an $\alpha \leq 0.05$ provides 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 0.2 between cases and controls was required. The following formula was used to determine sample size.

$$n = (\frac{r+1}{r})\frac{(\overline{p})(1-\overline{p})(Z_\beta + Z_{\alpha/2})^2}{(p_1 - p_2)^2}$$ (Whitley and Ball, 2002).

Where;

**n** is the sample size of the case group.
**r** is the ratio of controls to cases.
**p** is a measure of variability (similar to the standard deviation)
**($p_1$-$p_2$)** is the effect size (difference in proportion)
**$Z_\beta$** represents the desired power (typically 0.84 for 80% power)
**$Z_\alpha$** represents confidence interval (typically 1.96 for 95% CI)

Based on previous studies (Ouma *et al.*, 2008b), to detect an odds ratio of 2, equal number of cases and controls should be used. Therefore r=1. To determine proportion ($\overline{p}$) of children with malaria and have the genotypes understudy,

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

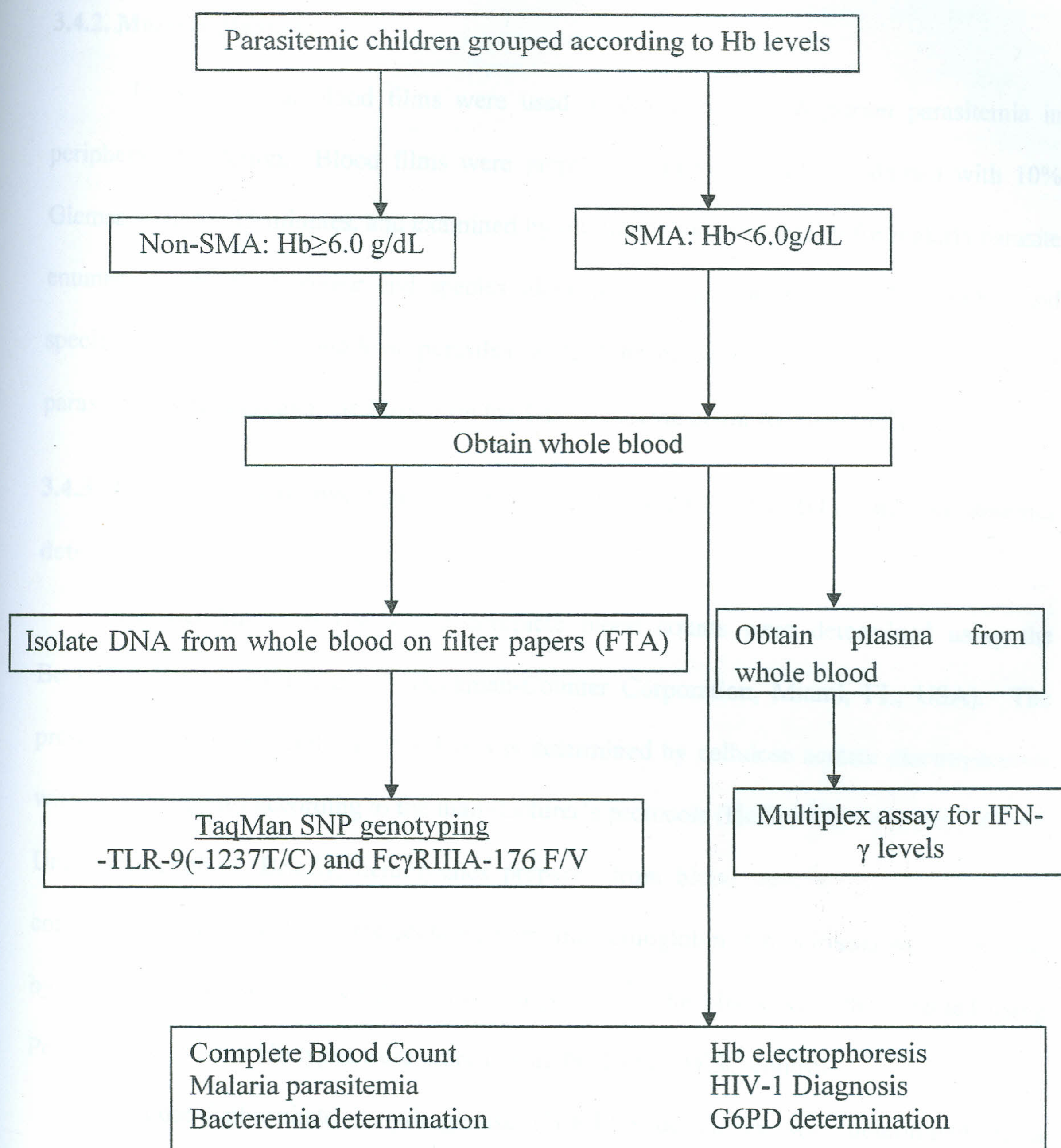
$$p = \frac{2.0(0.2)}{(0.2)(2.0-1)+1} = \frac{0.4}{1.2} = 0.30$$

The average proportion of children exposed to *P.faslciparum* malaria and possess the genotypes is given by (0.3+0.2)/2=0.25. Therefore $\overline{p}$=0.25.

$$n = 2\frac{(0.25)(1-0.25)(0.84+1.96)^2}{(0.3-0.2)^2} = 294$$

The study therefore used a sample size of n=301 (138 SMA and 163 non-SMA children).

### 3.4.0. Experimental design




Flow chart showing the experimental design of the study

### 3.4.1. Laboratory procedures

### 3.4.2. Malaria diagnosis

Thick and thin blood films were used to determine *P. falciparum* parasitemia in peripheral circulation. Blood films were prepared from venous blood, stained with 10% Giemsa stain for 15 minutes, and examined by oil immersion microscopy for malaria parasite enumeration by thick smear and species identification by thin smear. The number and species of asexual *Plasmodium* parasites were determined per 300 leukocytes, and the parasite density was calculated based on the total leukocyte count for each individual.

### 3.4.3. Haematological investigations, Sickle cell trait, G-6-PD, HIV and bacteraemia determination

Hemoglobin (Hb) levels and complete blood counts were determined using the Beckman Coulter ACT diff2™ (Beckman-Counter Corporation, Miami, FL, USA). The presence of the sickle cell trait (HbAS) was determined by cellulose acetate electrophoresis with Titan III plates according to the manufacturer's protocols (Helena Bio-Sciences, Oxford, United Kingdom). Briefly, hemolysates prepared from blood samples and Hemo AFSC controls were dispensed onto the acetate paper, and hemoglobin (Hb) variants were separated by electrophoresis with an alkaline buffer at pH 8.6. The plates were then stained using Ponceau S stain, and Hb types were scored using the Hemo AFSC control.

Glucose-6-phosphate dehydrogenase (G-6-PD) deficiency was determined by a fluorescent spot test (Trinity Biotech Plc., Bray, Ireland). Briefly, blood was hemolyzed and spotted onto a filter paper. Assay solution containing glucose-6-phosphate and oxidized NADP ($NADP^+$) was added, and samples were excited with UV light at 340 nm. Based on the presence or absence of fluorescence emissions, the samples were scored as normal (high emission), intermediate (moderate emission), or deficient (no emission). HIV-1 status was

determined using two serological methods (Unigold [Trinity Biotech Plc., Bray, Ireland] and Determine [Abbott Laboratories, Tokyo, Japan]), and positive serological tests were confirmed according to previously published methods (Otieno *et al.*, 2006). Bacteremia was determined using the Wampole Isostat Pediatric 1.5 system (Wampole Laboratories), and blood was processed according to the manufacturer's instructions.

### 3.4.4. Genotyping of TLR-9 (-1237T/C, rs5743836) and FcγRIIIA-176F/V, (rs396991) variants.

DNA was extracted using the Gentra System (Gentra System Inc., Minneapolis, MN, USA) according to the manufacturer's recommendation. Briefly thermal cycler was preheated at 99°C. 3mm of disck 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 was then pipetted up and down twice then discarded. The washing step was repeated twice. DNA solution 1 was again added and incubated again at room temperature for 15 minutes. The solution was then pipetted up and down. For elution, 70μL of DNA elusion solution 2 was added and incubated at 99°C in the thermal cycler for 15 minutes to release DNA and then cooled at room temperature. The eluted DNA was then transferred into a clean tube and then stored at -20°C until use.

The TLR-9 variants (-1237T/C) and FcγRIIIA-176F/V were genotyped using allele-specific PCR amplification. This was performed using a high-throughput TaqMan® SNP genotyping assays technology. The FcγRIIIA-176F/V (rs396991, assay ID C__25815666_10) and TLR-9-1237C/T (rs5743836, assay ID C__32645383_10) promoter polymorphism were genotyped using the high-throughput TaqMan® 5' allelic discrimination

Assay-By-Design method which accurately discriminates alleles using allele-specific fluorochrome labeled probes (Applied Biosystems, Foster City, CA, USA). For genotyping of FcγRIIIA-176F/V (rs396991), a master mix containing 5.0μL of TaqMan® genotyping mix, 0.5μL of rs396991 SNP assay mix, 3.5μL of PCR grade water and 1.0μL of DNA was added into each micro-well and genotyped using the following cycling parameters; pre-PCR hold stage was done at 60°C for 30 seconds, hold stage at 90°C for 10 minutes, cycling stage at 95°C for 15 seconds and annealing at 60°C for 1 minute. The hold stage to the annealing stage was repeated 45 times. For genotyping of TLR-9(-1237T/C, rs5743836), the same reaction volumes and cycling parameters were used. The graphical plots for both TLR-9(-1237T/C and FcγRIIIA-176F/V are shown in appendix 3 (a) and 3 (b), respectively.

### 3.4.5. IFN-γ measurements

Plasma samples were obtained from venous blood and stored at -80°C. Batch analysis was performed to re-strict experimental variability between assays. Circulating IFN-γ concen-trations were determined using the Human Cytokine 25-plex Antibody (Ab) Bead kit (BioSource International). Briefly, the wells were pre-wetted by adding 200 μL of working wash solution into designated wells then incubated for Incubate plate 30 seconds at room temperature. The working wash solution was then aspirated from the wells using the vacuum manifold. The bead solution was then vortexed for 30 seconds, then sonicated again for 30 seconds. 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 then added and beads allowed to soak for 20 seconds. The wells were then 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 were 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 sample. The filter plates were then covered with aluminium foil and incubated for 2 hours at room temperature on orbital shaker at 500 rpm. After incubation, the plated were washed using 200μL of wash solution.

To the washed plates, 100μL of 1X 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 1X streptavidin-rpe was added and incubated the plate for 30 minutes at room temperature on an orbital shaker 500 rpm. The liquid in the wells was removed by vacuum aspiration manifold and then beads by adding 200μL working wash solution to the wells allow the beads 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 Cor-poration) and analyzed using the Bio-plex Manager software (Bio-Rad Laboratories). The detection limit for IFN-γ was 2.0 pg/ml.

### 3.5.0. Statistical analyses

All statistical analyses were performed using SPSS (Version 19.0). Chi-square analyses were used to examine differences between proportions. Quantitative comparisons involving unpaired data were made using Mann–Whitney U test and Kruskal-Wallis test. FcγRIIIA-176F/V and TLR-9 (-1237T/C) SNPs combination were constructed using HPlus software program (Version 2.5). The association between genotypes/SNP combination and SMA was determined by multivariate logistic regression, controlling for the confounding

effects of age, gender, HIV-1 status, sickle cell trait, G-6-PD and bacteraemia. Statistical significance levels were set at $p \leq 0.05$ and all tests of significance were two-sided.

### 3.6.0. 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 (See Appendix 2). Informed consent to participate in the study was obtained from parents/guardians of the children. Treatment of patients whether they consented to participate in study or not was provided.

# CHAPTER FOUR

## 4.0. RESULTS

### 4.1. Clinical, demographic and laboratory characteristics of the study participants.

A cross-sectional analysis in children (n=301, 3-36 months) presenting with acute *P. falciparum* malaria (any density parasitemia) was performed. Clinical stratification of the study groups was done based on previous geographical definition (McElroy *et al.*, 1999), i.e. non-severe malaria (non-SMA; Hb≥6.0g/dL; n=163) and severe malaria (SMA; Hb<6.0g/dL; n=138). The distribution of gender, parasitemia (parasites/µL), proportions of those with high density parasitemia (HDP≥10,000 parasites/µL) and axillary temperature (°C) were not significantly different between the groups ($p$=0.668, $p$=0.508, $p$=0.456 and $p$=0.109, respectively; Table 1). Children presenting at hospital with SMA were younger in age relative to those with non-SMA ($p$=0.010). With reference to previous grouping, Hb (g/dL) concentration and erythrocyte counts ($x10^{12}$/L) were lower in SMA group ($p$<0.001) for the two clinical parameters (Table 1).

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

| Characteristics | non-SMA (Hb≥6.0g/dL) | SMA (Hb<6.0g/dL) | *p*-value |
|---|---|---|---|
| No. of participants (n=301) | 163 | 138 | |
| Gender, n (%) | | | |
| Male | 82 (50.3) | 66 (47.8) | 0.668[a] |
| Female | 81 (49.7) | 72 (52.2) | |
| Age, months | 11.0 (10.0) | 8.0 (8.0) | **0.010[b]** |
| Hemoglobin level, g/dL | 7.9 (3.0) | 4.9 (1.0) | **<0.001[b]** |
| Parasite density, parasite/μL | 18957.0 (43921.5) | 17261.5 (36272.0) | 0.508[b] |
| HDP (≥10,000 parasites/μL no. %) | 106/163 (65.0) | 84/138 (60.9) | 0.456[a] |
| Red Blood Counts, $x10^{12}$/L | 3.7 (1.2) | 2.0 (0.8) | **<0.001[b]** |
| Axillary temperature, °C | 37.6 (2.0) | 37.4 (2.0) | 0.109[b] |

Data are the median (interquartile range; IQR) unless otherwise stated. Children with parasitemia (n=301) 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=163) and SMA (n=138). HDP: (high density parasitemia). [a] Statistical significance was determined by the $\chi^2$ test. [b] Statistical significance was determined by the Mann-Whitney U test. Values in bold are statistically significant at $p \leq 0.05$.

### 4.2. Associations between FcγRIIIA -176 F/V and TLR-9 (-1237 T/C) genotypes and severe malaria anemia

The investigation on the association between individual genotypes of FcγRIIIA -176 F/V and TLR-9 (-1237 T/C) and susceptibility to SMA was determined using multivariate logistic regression analyses while controlling for age, gender, HIV-1 status, sickle cell trait (HbAS), bacteraemia and G-6-PD deficiency (Aidoo *et al.*, 2002; Otieno *et al.*, 2006; Were *et al.*, 2011). As shown in Table 2, there were no significant statistical associations between the variations at individual loci of FcγRIIIA (-176 F/V) and TLR-9 (-1237 T/C) and susceptibility to SMA.

**Table 2: Association between FcγRIIIA -176 F/V and TLR-9 (-1237 T/C) genotypes and severe malarial anemia (SMA)**

| | SMA (Hb level ≤6.0g/dL) | | |
|---|---|---|---|
| Genotypes | OR | 95% CI | *p-value* |
| FcγRIIIA -176 F/V | | | |
| FF | 1.00 (reference) | | |
| FV | 0.88 | 0.53-1.45 | 0.163 |
| VV | 0.58 | 0.24-1.40 | 0.235 |
| TLR9(-1237T/C) | | | |
| TT | 1.00 (reference) | | |
| TC | 0.67 | 0.41-1.10 | 0.110 |
| CC | 0.72 | 0.31-1.68 | 0.450 |

Children with acute malaria (n=301) 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 G-6-PD deficiency. The reference groups in the multivariate logistic regression analysis were the homozygous wild-type genotypes.

### 4.3. Associations between FcγRIIIA -176 F/V, TLR9 -1237 T/C SNPs combinations and severe malaria anemia

Prior to determining the associations between the SNPs combinations and the circulating IFN-γ levels, a multivariate logistic regression analysis controlling for covariates (Aidoo *et al.*, 2002; Otieno *et al.*, 2006; Were *et al.*, 2011) was performed to determine whether these SNPs combinations are associated with SMA. The analysis results demonstrated that carriers of -176V/ -1237T (VT) SNPs combinations were twice at an increased risk of developing SMA (OR, 2.04, 95%CI, 1.19-3.50, *p*=0.009) relative to non-carriers while the carriers of -176V/ -1237C (VC) were 64% protected against risk of SMA (OR, 0.36, 95%CI, 0.20-0.64, *p*=0.001; table 3). Further analysis however did not reveal any association between the -176F/ -1237T (OR, 0.94, 95%CI, 0.52-1.68, *p*=0.830) and the -176F/ -1237C (OR, 1.30, 95%CI, 0.80-2.10, *p*=0.288) SNPs combinations and SMA (Table 3).

**Table 3: Association between FcγRIIIA -176 F/V and TLR9 -1237 T/C SNPs combinations and severe malaria anemia (SMA)**

| SNPs Combination | SMA(Hb<6.0g/dL) | | |
|---|---|---|---|
| | OR | 95% CI | *p-value* |
| FcγRIIIA-176F/TLR9-1237T | 0.94 | 0.52-1.68 | 0.830 |
| FcγRIIIA-176F/TLR9-1237C | 1.30 | 0.80-2.10 | 0.288 |
| FcγRIIIA-176V/TLR9-1237T | 2.04 | 1.19-3.50 | **0.009** |
| FcγRIIIA-176V/TLR9-1237C | 0.36 | 0.20-0.64 | **0.001** |

Children with acute malaria (n=301) 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, sickle cell trait (HbAS), bacteremia, and G-6-PD deficiency. The reference groups in this multivariate logistic regression analysis were those without the respective SNPs combinations. Values in bold are statistically significant at $p \leq 0.05$.



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## 4.4. Associations between FcγRIIIA -176 F/V and TLR-9 (-1237 T/C) SNPs combinations and Hb levels (g/dL)

To further confirm whether the SNPs combinations associated with susceptibility to SMA alter susceptibility through changes in haemoglobin, the haemoglobin levels were compared against the different SNPs combinations. The results as shown in table 4 revealed that -176V/-1237T SNPs combination are associated with low Hb levels [median (IQR); 5.70g/dL (3.00)], $p$=0.014 relative to non-carriers. The carriers of -176V/-1237C had significantly higher levels of Hb [median (IQR); 6.70g/dL (3.00)], $p$=0.002 compared to those without the SNPs combination. However, the Hb levels were comparable between the -176F/-1237T and -176F/- 1237C SNPs combination.

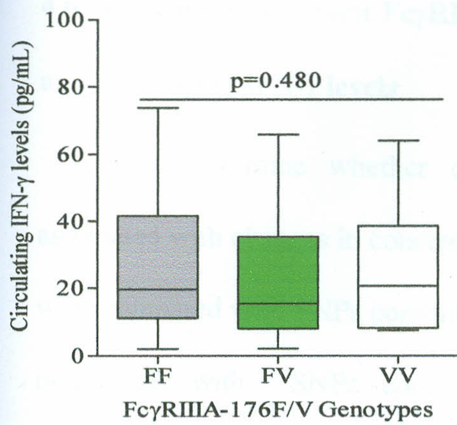
**Table 4. Associations between FcγRIIIA -176 F/V and TLR-9 (-1237 T/C) SNPs combinations and Hb levels (g/dL)**

| | | Haemoglobin level (g/dL) | |
|---|---|---|---|
| SNPs combination | | Median (IQR) | *p-value* |
| -176F/-1237T (n=241) | 1 | 6.20 (3.00) | 0.588[b] |
| | 0 | 6.25 (3.00) | |
| -176F/-1237C (n=115) | 1 | 5.90 (3.00) | 0.064[b] |
| | 0 | 6.30 (3.00) | |
| -176V/-1237T (n=80) | 1 | 5.70 (3.00) | **0.014[b]** |
| | 0 | 6.30 (3.00) | |
| -176V/-1237C (n=74) | 1 | 6.70 (3.00) | **0.002[b]** |
| | 0 | 5.60 (3.00) | |

Hb levels are medians (IQR) and the comparisons between carriers and non-carriers of the SNPs combinations were computed using Mann-Whitney U test. 1=carrier of SNPs combination, 0=non-carrier. [b]Statistical significance determined by the Mann-Whitney U test. Values in bold are statistically significant at $p \leq 0.05$.

### 4.5. Associations between circulating IFN-γ and FcγRIIIA (-176 F/V), TLR-9 (-1237 T/C) genotypes

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 genotype groups. As presented in Figure 1 (A) and (B), there were no significant differences in the concentrations of circulating IFN-γ across the genotypes of both FcγRIIIA (-176 F/V) ($p$=0.480) and TLR-9 (-1237 T/C) ($p$=0.559). The distribution of IFN-γ in the FcγRIIIA -176 F/V genotype were; FF (n=77) [median (IQR); 19.6 (30.6)], FV (n=53) [median (IQR); 15.9 (28.1) and VV (n=9) [median (IQR); 19.6 (27.3) while the distribution of IFN-γ in TLR-9 (-1237 T/C) genotype were; TT (n=65) [median (IQR); 16.2 (34.6), TC (n=65) [median (IQR); 19.6 (33.6) and CC (n=9) [median (IQR); 14.0 (27.5)]. These results show that the individual polymorphisms in FcγRAIII-176F/V and TLR-9 (-1237T/C are not associated with production of IFN-γ in this population.



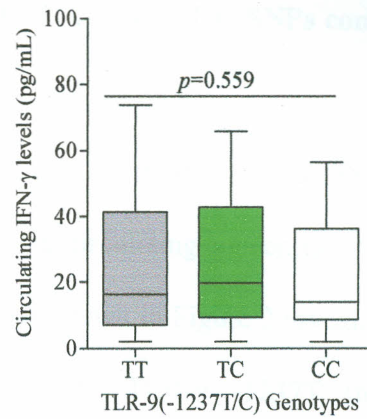




**Figure 1.** **(A)** **(B)**

**Figure 1 (A) and (B): Association between circulating IFN-γ and FcγRIIIA (-176 F/V), TLR-9 (-1237 T/C) genotypes.**

Data are represented in box-plots. The boxes represent interquartile range; the line through boxes is the median while the whiskers show the $10^{th}$ and the $90^{th}$ percentiles. Across group comparisons were determined using Kruskal-Wallis test.

### 4.6. Associations between FcγRIIIA -176 F/V, and TLR9 -1237 T/C SNPs combinations and circulating IFN-γ levels

To determine whether co-inheritance of these receptors polymorphisms were associated with changes in concentrations of IFN-γ levels, circulating concentrations of IFN-γ were compared with SNPs combinations. The results as shown in Figure 2 revealed that, the individuals with SNPs combination; FcγRIIIA -176V/ TLR-9 -1237T (n=35) had significantly higher levels of IFN-γ [median (IQR); 19.6 pg/mL (32.1)], than those without this combination [median (IQR); 13.4 pg/mL (16.9)], $p=0.011$. However, there were no significant differences in the concentration of IFN-γ was observed in individuals with or without the other three SNPs combinations i.e. FcγRIIIA -176F/ TLR-9 -1237T (n=120) ($p$=0.450), FcγRIIIA -176F/ TLR-9 -1237C (n=45) ($p$=0.775) and FcγRIIIA -176V/ TLR-9 -1237C (n=33) ($p$=0.188) and their respective non-carriers.

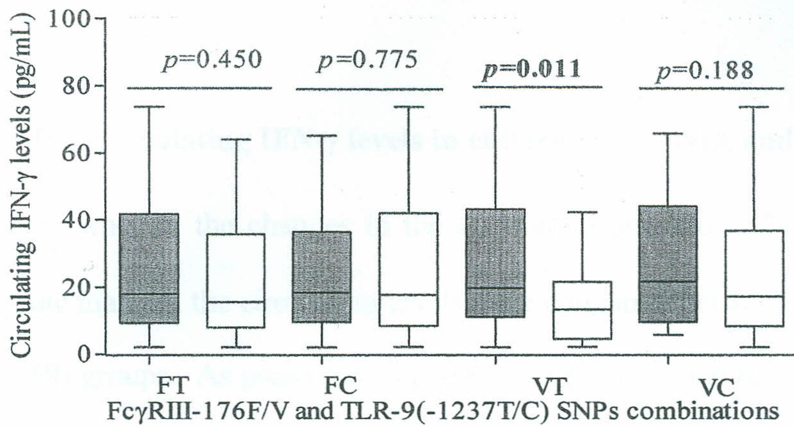



**Figure 2. Associations between FcγRIIIA -176 F/V, and TLR9 -1237 T/C SNPs combinations and circulating IFN-γ levels.**

Data are represented in box-plots. The boxes represent interquartile range; the line through boxes is the median while the whiskers show the $10^{th}$ and the $90^{th}$ percentiles. Pair-wise comparisons between those with SNPs combinations and those without the SNPs combinations was done using Mann-Whitney U test at $p \leq 0.05$. Shaded boxes show children with the indicated SNPs combinations while white boxes show those without the SNPs combination. These results revealed that the FcγRIIIA-176V/ TLR-9 -1237T SNPs combination are associated with increased levels of circulating IFN-γ levels.

## 4.7. Functional role of circulating IFN-γ levels in 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=70) and non-SMA (n=69) groups. As presented in Figure 3, the results demonstrate that children with SMA had significantly higher levels of circulating IFN-γ plasma concentrations [median (IQR); 21.5 (34.9) pg/mL] compared to non-SMA [14.8 (27.1) pg/mL], ($p$=0.008). These results indicate a potential role of elevated IFN-γ in the development of SMA.

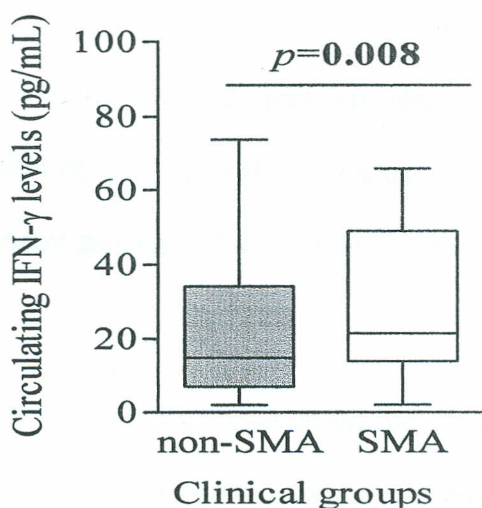



Figure 3. **Functional role of circulating IFN-γ levels in children with SMA and non-SMA.**

Data are represented as box-plots. The boxes represent interquartile range; the line through boxes is the median while the whiskers show the $10^{th}$ and the $90^{th}$ percentiles. Difference in circulating IFN-γ levels were considered significant at $p \leq 0.05$ using Mann-Whitney U test.

# CHAPTER FIVE

## 5.0. DISCUSSION

To describe the role of cell surface receptors in susceptibility to child severe malaria anemia, a cross-sectional analysis of the impacts of FcγRIIIA -176 F/V and TLR-9 (-1237 T/C) promoter variants in a phenotypically well-defined cohort of children aged 3-36 months resident in a *P. falciparum* holoendemic transmission region was performed. The results presented here demonstrate that carriage of the FcγRIIIA -176V and TLR-9 (-1237C) (VC) confers protection against SMA (Hb<6.0 g/dL) (McElroy *et al.*, 1999) and is associated with significantly higher Hb levels in this population while the carriage of FcγRIIIA -176V and TLR-9 (-1237T) (VT) predicts susceptibility to SMA and produces significantly higher levels of circulating IFN-γ. Consistent with previous observations, children with SMA had significantly high levels of circulating IFN-γ (Ouma *et al.*, 2011). In this cross-sectional study, however, individual genotypes did not show any association with SMA and circulating levels of IFN-γ.

Since FcγRIIIA is mainly expressed on the macrophages and monocytes, they play a primary role in phagocytosis of both parasitized and unparasitized erythrocytes and induction of pro-inflammatory cytokines, which play a role in SMA pathogenesis (Brattig *et al.*, 2008). Of all FcγRs, FcγRIIIA is thought to play an important role in immune complex clearance (Daeron, 1997). The intermediate affinity of this receptor on monocytes makes it ideally suited as a capture receptor to facilitate clearance of immune complexes (Clarkson *et al.*, 1986). Consistent with a previous study in Thai adults (Omi *et al.*, 2002), FcγRIIIA -176 F/V polymorphism failed to show any association with SMA in this study. The study moreover,

did not find any association between circulating IFN-γ levels and the individual genotypes. However, it is important to note that this polymorphism influences preferential binding of immunoglobulins (Ig)G in which the FcγRIIIA (-176V/V) has higher binding affinity to $IgG_1$ and $IgG_3$ which are associated with low parasitemia and low risk of malaria (Aribot *et al.*, 1996; Wu *et al.*, 1997). Further, the increased binding affinity of the FcγRIIIA -176V allele has been observed to enhance more robust downstream functional effects such as antibody dependent cell-mediated toxicity of natural killer (NK) cells (Koene *et al.*, 1997). Consistent with functional role for FcγRIIIA in clearance of immune complexes (ICs), the lower IgG binding allele -176F has mainly been associated with susceptibility to systemic lupus erythromatous (SLE) in several case-control studies in multiple ethnic groups (Caucasian and African-American) (Li *et al.*, 2009). In chidren with SMA, monocytes respond differently to *P falciparum* by overexpression of FcγRIIIA, which could increase erythrophagocytosis of both parasitized and non-parasitized erythrocytes and production of TNF-α which may enhance SMA (Ogonda *et al.*, 2010).

There is increasingly accumulating evidence of the role of TLR-9 polymorphisms in clinical malaria (Leoratti *et al.*, 2008; Campino *et al.*, 2009; Papadopoulos *et al.*, 2010; Zakeri *et al.*, 2011). Consistent with this study, two separate studies, carried out in Brazil and Iran, have recently revealed no impact of TLR-9 (-1237T/C) promoter polymorphism on susceptibility to mild malaria in their respective populations (Leoratti *et al.*, 2008; Zakeri *et al.*, 2011). Moreover, the TLR9 (-1237TT) genotype has been associated only with low parasitemia but no effect on susceptibility to clinical malaria in Ghanaian children aged 3-11 years (Omar *et al.*, 2012). Investigations in the Gambian and Malawian children less than 5 years characterized by mixed clinical phenotypes (cerebral malaria and/or severe malaria anemia) did not show any association between TLR-9 (-1237T/C) polymorphisms and severe

malaria (Campino *et al.*, 2009). However, a study in Ugandan children (aged 4-12 years), showed that TLR-9 (-1237CC) genotype was associated with elevated levels of plasma IFN-γ and enhanced cerebral malaria (Sam-Agudu *et al.*, 2010). Previous studies in transfected human cells indicated that signalling and ultimate production of cytokines is a function of TLR SNPs (Schröder *et al.*, 2005). Moreover, studies have revealed individuals infected by malaria have up-regulated TLR-9 and elevated IFN-γ, and that mice with TLR-9 gene knockout have reduced levels of IFN-γ in response to *Plasmodium chabaudi* AS (Franklin *et al.*, 2009). The discrepancies observed between this study and others may in part be explained by the difference in clinical phenotypes since in this study population, the main phenotype is SMA while the earlier studies were based on cerebral malaria and heterogeneous populations. In addition, pathways of TLR-9 signalling involve polymorphisms in the downstream molecules, for instance NF-κB and MyD88 (Leoratti *et al.*, 2008) that were not investigated in the current study. Furthermore, due to high prevalence of malaria in this population and TLRs only act in recognition and induction of immune response, it could be argued that TLRs are not the primary determinants of clinical outcome of malaria. Additional genes that may significantly alter TLR pathways and alter malaria disease susceptibility should be investigated to determine their impact.

Since susceptibility to severe malaria occurs through multi-factorial, complex and rather contradictory selective pressure (Balaresque *et al.*, 2007), cross-SNPs combinations between FcγRIIIA (-176 F/V) and TLR-9 (-1237 T/C) were constructed, in an attempt to further decipher the immunogenetic impacts of co-inheritance of these receptor SNPs combinations on susceptibility to SMA. Based on results from this study, children who were carriers of the -176V/ -1237C (VC) SNPs combination were found to be 64% protected from the development of SMA compared to non-carriers. Consistent with this observation, carriers

of this combination also had higher levels of haemoglobin, suggesting a potential protective role against SMA pathogenesis through increased erythropoietic responses. This current study results also demonstrated that SMA in this population is characterised in part by elevated circulating IFN-γ levels as previously shown (Ouma *et al.*, 2011). Furthermore, the -176V/-1237T SNPs combination which was associated with an increased risk to SMA was also associated with higher circulating IFN-γ levels. This is not surprising given that elevated circulating IFN-γ levels is associated with SMA in this population. As such, any gene combination that may be associated with higher circulating IFN-γ levels may promote SMA. The study underscores the importance of the use of cross-SNPs combinations in genetic association studies of infectious diseases such as malaria because it reveals associations that are not identifiable with just single gene polymorphisms since any disease outcome(s) is dictated by genes functioning in concert (Adler *et al.*, 1993).

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 5-14 years from malaria endemic region of Papua New Guinea (D'Ombrain *et al.*, 2008). An additional study in holoendemic perennial *P. falciparum* malaria transmission area 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 association between higher levels of IFN-γ with severe malaria. For instance, a study in Uganda reported positive association between increased IFN-γ levels and CM severity (Sam-Agudu *et al.*, 2010). The current study, demonstrates that children with SMA had significantly higher IFN-γ concentrations, a finding consistent with a previous study in the same population (Ouma *et al.*, 2011). Even

though not explicitly explored, the pathogenic mechanisms of elevated IFN-γ in SMA may in part be as a consequence of over-stimulation of monocytes by IFN-γ to secret TNF-α (Oswald *et al.*, 1992). This over-stimulation may lead to over-production of toxic oxides and free radicals, such as reactive oxygen species ($H_2O_2$ and iNOS) by liver cells against intra-hepatic parasites and erythrocytic-stage parasite (Nussler *et al.*, 1993; Tsuji *et al.*, 1995; Su and Stevenson, 2000; Wang *et al.*, 2009), as well as enhanced phagocytic activities of monocytes/macrophages against parasitized and non-parasitized erythrocytes (Naotunne *et al.*, 1991). Over-production of these free radicals during *P. falciparum* malaria has also been proposed to cause damage of red blood cells and inhibits erythropoiesis leading to anemia (Descamps-Latscha *et al.*, 1987). Moreover, overproduction of IFN-γ, also promote enhanced malarial anemia pathogenesis through bone marrow suppression, dyserythropoiesis, and erythro-phagocytosis (Clark and Cowden, 2003). However, for enhanced immunity to be accomplished, milieus of both pro-inflammatory and anti-inflammatory cytokines balance are involved (Dodoo *et al.*, 2002) and should be considered in future study designs. It would be scientifically plausible to explore how different cytokine milieu in relation to IFN-γ levels and IgG production promote the development of SMA over time in this pediatric population resident in western Kenya. This approach will address the inherent limitation in examining cytokine production at a single time point (time of admission) and in circulation rather than in the local microenvironments which in essence complicates the clear understanding of the exact role of immune mediators such as IFN-γ in SMA (Mirghani *et al.*, 2011).

## CHAPTER SIX

### 6.0. CONCLUSIONS

In summary, these results demonstrate that:

i. Co-inheritance of the FcγRIIIA -176V/ TLR-9 -1237C SNPs combination is associated with protection against SMA while carriage of FcγRIIIA -176V/ TLR-9 -1237T SNPs combination is associated with susceptibility to sever malaria anemia. However, individual FcγRIIIA -176F/V and TLR-9 -1237T/C genotypes are not associated with severe malaria anemia in this population.

ii. FcγRIIIA -176V/ TLR-9 -1237T SNPs combination is associated with increased IFN-γ production in children presenting with acute malaria.

iii. Elevated IFN-γ enhances severe malaria anemia in children resident in *P. falciparum* holoendemic transmission areas of western Kenya.

### 6.1. RECOMMENDATIONS

i. To exhaustively describe the impacts of surface receptors in development of naturally acquired immunity against malaria, further longitudinal studies aimed at examination of an inclusive panel of receptor polymorphisms that influence innate immune response and disease outcome are required as this may provide an immuno-genetic basis for the development of vaccines that modulate receptor functions.

ii. Since FcγRIIIA (-176V/V) has higher binding affinity to $IgG_1$ and $IgG_3$ which are associated with low parasitemia and low risk of malaria, studies aimed at exploring

this model to test whether carriage of this variant is associated with higher IgG binding in children naturally exposed to *P. falciparum* malaria in holoendemic region of western Kenya, in which the primary clinical outcome of severe malaria is SMA should be performed.

iii. Immunological studies should be designed to help decipher the pathological basis of elevated levels of IFN-γ in severe malaria anemia in pediatric populations who are residents of *P. falciparum* holoendemic transmission areas such as western Kenya.

iv. 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.

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