ASSOCIATION BETWEEN NATURAL CYTOTOXICITY TRIGGERING RECEPTOR-3 POLYMORPHISMS (-172A/G AND -412C/G) AND INTERFERON-GAMMA LEVELS IN CHILDREN AGED 3-36 MONTHS WITH SEVERE *PLASMODIUM FALCIPARUM* ANAEMIA AT SIAYA COUNTY REFERRAL HOSPITAL IN WESTERN KENYA

BY

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DECLARATION

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

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DEDICATION

To my beloved wife Roselyne Akoth and children Kezziah, Enoch, Esther and Blessings in appreciation of their love, encouragement and endurance during the time of this study.

May the Almighty God bless you.

ABSTRACT

In sub-Saharan Africa, *Plasmodium falciparum* infection is a major cause of morbidity and mortality mostly among children under five years. In Kenya, severe malaria anaemia prevalence is at 19%. In malaria holo-endemic region of western Kenya, *P. falciparum* infections account for major proportion of children with anaemia that leads to severe malaria anaemia (SMA, Hb<6.0g/dL with any parasite density). Children in these areas exhibit similar transmission intensity and infection rates of *P. falciparum* malaria with different malaria outcomes. The reason(s) for these varied clinical outcomes is poorly defined. Natural Cytotoxicity Triggering Receptor 3 directly interacts with Duffy binding-like-1 alpha (DBL-1α) peptides on parasitized red blood cells (PRBCs) thus activating natural killer cells to carry out cell-mediated cytotoxicity of *Pf.* PRBCs releasing interferon gamma (IFN-γ), a major cytokine that activates phagocytes during malarial infection. In particular, *NCR3* plays important role in pathogen recognition. Previous studies associated *NCR3* with pathogenesis of different infectious diseases, *P. falciparum* malaria included. However, the association between polymorphic variants of *NCR3* promoter and pathogenesis of SMA and peripheral-IFN-γ levels especially in paediatric populations exposed to *P. falciparum* in holoendemic transmission regions such as western Kenya has not been reported. As such, this case-control study, investigated the association between *NCR3* polymorphisms, susceptibility to SMA and IFN-γ levels; Specifically, the study determined the association between *NCR3* -172A/G (rs986475) and *NCR3* -412C/G (rs2736191) polymorphic variants and susceptibility to SMA; association between *NCR3* -172A/G (rs2736191) and *NCR3* -412C/G (rs986475) genotypes/haplotypes and IFN-γ levels and comparision of IFN-γ levels in children (n=455) aged below 3 years with SMA cases (n=254) and without SMA, controls (n=201) were targeted at Siaya County Referral Hospital (SCRH) of western Kenya. Prior to administration of any medication, haematological and parasitological parameters were determined in all the participants. *NCR3* (-172A/G and -412C/G*)* genotypes were determined using TaqMan high throughput assays while IFN-γ levels were determined using cytokine 25-plex Ab Bead Kit. Bivariate logistic regression analysis controlling for confounding factors such as age, HIV-1, bacteraemia, α-thalassemia and sickle-cell trait revealed that children with *NCR3* -172G/-412C haplotype carriage were nearly five times at an increased risks of developing SMA (OR; 4.977, 95% CI, 1.494-16.580, P = 0.009), while carriers of *NCR3* -172A/-412C and *NCR3* -172G/-412G haplotypes did not alter susceptibility to SMA (OR; 0.133, 95% CI, 0.011-1.610, P = 0.113) and (OR; 0.543, 95% CI, 0.227-1.300, P = 0.171), respectively. Children with *NCR3* -172G/-412C haplotye carriage showed significant difference in peripheal IFN-γ levels (P< 0.0001). Carriage of the genotype GG relative to genotype AG showed significant differences in IFN-γ levels (P=0.006). There were no significant difference in peripheral IFN-γ levels between SMA and non-SMA (P=0.173). Collectively, these results demonstrate that haplotypes of *NCR3* are important in conditioning susceptibility to SMA pathogenesis and changes in the peripheral IFN-γ levels in immune-naïve children naturally exposed to *P. falciparum* infection. These findings on the role of *NCR3* receptors in SMA pathogenesis may be used in informing crucial decisions for designing and developing therapeutics for children in *P. falciparum* holoendemic region of western Kenya.

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ABBREVIATIONS AND ACRONYMS

CD	Cluster of Differentiation
CM	Cerebral Malaria
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
GPI	Glycosylphosphatidylinositol
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon Gamma
µL	Microlitre
MIDAS	Multiallelic Interallelic Disequilibrium Analysis Software
NCRs	Natural Cytotoxicity Receptors
NKs	Natural Killer
NO	Nitric Oxide
PAMPs	Pathogen Associated Molecular Patterns
PCR	Polymerase Chain Reaction
SMA	Severe Malarial Anaemia
SNPs	Single Nucleotide Polymorphisms
TNF	Tumour Necrosis Factor
TGF	Transforming Growth Factor
WHO	World Health Organization

DEFINITION OF TERMS

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

Case-Control Study- A study design which involves identification of cases (outcome/disease) condition,then match with controls and look backwards and assess those with and without the outcome/disease.

Genotype – a set of genes that determines the expression of a particular for a particular trait or charcteristics of an organism.

Haplotype – A Combination of alleles in an organism that are transmitted together from a single parent.

High density parasitaemia – parasitaemia of ≥10000 parasites/μL and above it, immune patients will exhibit symptoms.

Holoendemic – an area is holoendemic when essentially all individuals in a population are infected and symptoms of the disease do not appear equally across the age groups as the young are most likely to express pathogenic responses while the older hosts will carry the disease asymptomatically due to adaptive immunity.

Low density parasitaemia – parasitaemia of >1000 parasites/μL in an endemic area.

Polymorphisms – variations at single positions in the DNA sequence among individuals.

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 parasites transmitted via a bite of an infected female *Anopheles* mosquito and it affects red blood cells (RBCs) and hepatocytes (WHO, 2016). Five species of *Plasmodium* genus are known to cause malaria in humans. These include *P. falciparum*, *P. ovale*, *P. malariae*, *P. vivax* and *P. knowlesi* (Cox-Singh *et al.*, 2008; Daneshvar *et al.*, 2009; Sabbatani *et al.*, 2010). *Plasmodium falciparum* is the most virulent and prevalent parasite in human population (Cox *et al.*, 2007; Snow *et al.*, 2005; WHO., 2010). In 2017, WHO estimated 219 million cases of malaria and 435,000 deaths worldwide of which, Africa accounted for 92% of the malaria cases. Children under 5 years are particularly susceptible to malaria infections, illness and death. It is estimated that malaria killed 266,000 children under-five years of age globally of which 93% were in Africa (WHO., 2016), and a disproportionately 99.7% *P.falciparum* high share of the global malaria burden (WHO, 2018).

In *P. falciparum* holoendemic transmission regions of western Kenya, life-threatening pediatric malaria manifests majorly as severe malaria anaemia (defined as having Hb≤6.0 g/dL with any density parasitemia). This definition of SMA is based on previous large-scale investigation of more than 14,000 longitudinal Hb distributions in an aged and geographically–matched reference children population in western Kenya (McElroy *et al.*, 1999). Severe malaria anaemia remains an important childhood health burden in sub-Saharan Africa due lack naturally acquired immunity against malaria (Breman *et al.*, 2001). The mortality rate in children with malaria-related severe malaria anaemia (SMA) is about 8.6% compared with 3.6% in children with severe anaemia due to other causes (Newton *et al.*, 1997). SMA immunopathogenesis

remains multifaceted and the mechanisms that promote erythropoietic dysfunction are not completely understood (Ong'echa *et al.,* 2011). The mechanisms implicated in the pathogenesis of SMA includes erythrocyte lysis and splenic phagocytosis, increased sequestration of parasitized red blood cells, immune-mediated erythrocyte destruction, bone marrow dyserythropoiesis, ineffective erythropoiesis and lower erythroblast proliferative rates and numbers (Wickramasinghe and Abdalla, 2000). Besides, other studies have shown that, a part from the high density parasitemia (HDP, ≥10,000 parasites/µl) infections, SMA is exacerbated with co-infections HIV-1 and bacteria diseases (Anyona *et al.,* 2011; Newton *et al.,* 1997; Otieno *et al.,* 2006b).

Severe malaria anaemia pathogenesis is conditioned by variability in the promoter and/or coding region(s) of inflammatory genes (Ouma *et al.,* 2008a). For example mild malaria and maximum parasitaemia have been shown to be linked with TNF gene polymorphis (Flori *et al.,* 2005) and IL-10 Promoter variants -1,082G/-592C (GCC) haplotypes were associated with protection against SMA while carriers of haplotype -1082A/-819T/-592A (ATA) had an increase risk of SMA (Ouma *et al.,* 2008b) Inflammatory responses to diseases is a complex process involving several body systems. To effectively combat malaria, a well-timed and proportional release of a type 1 pro-inflammatory mediator's response of interleukin (IL)-12, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α is required to minimize parasitemia and preserve erythropoiesis (Perkins *et al.,* 2011). The response in this phase should be followed by an equally timely abrogation by type 2 cytokines such as IL-10, transforming growth factor (TGF)-β, and IL-4, to prevent inflammatory host damage. Many studies done in *P. falciparum*-SMA holoendemic regions have shown elevated levels of key pro-inflammatory responses such as IL-12, TNF-α and IFN-γ released in early stages of a *P. falciparum* infection through their ability to

stimulate phagocytes, monocyte and macrophage activation thus controlling parasitaemia (Perkins DJ. *et al.,* 2011). High levels of circulating IFN-γ have been shown to be associated with enhanced SMA severity in paediatric population in western Kenya (Ouma *et al.,* 2011) and in a Thai adults study which showed elevated IFN-γ levels in patients with complicated malaria at the initial stage of the disease (Tangteerawatana *et al.,* 2007) further illustrating the role of IFN-γ in malaria pathogenesis.

Uncontrolled production of IFN-γ and TNF-1α is associated with anaemia (Lyke *et al.,* 2004; Perkins *et al.,* 2000). Overproduction of mediators such as TNF-α, IFN-γ and nitric oxide (NO) along with sustained phagocytic cell activation has been associated with the severity of *P. falciparum* infections in children (Biemba *et al.,* 1998; Cordery, 2007; Lyke, 2004; Perkins, 2000). Moreover, overproduction of the mediators promotes development of malaria anaemia mainly through dyserythropoiesis, erythrophagocytosis and bone marrow suppression (Clark and Cowden, 2003). In addition, IFN-γ Th1 and TNF-1α responses have also been shown to control parasitaemia in children and adults (Kremsner *et al.,* 1995). Similarly, earlier studies done in Papua New Guinea showed that early production of malaria-specific IFN-γ leads to immunity against clinical malaria (D'Ombrain *et al.,* 2007) while a study conducted by Torre *et al.,* 2002, showed that in the acute phase of uncomplicated *P. falciparum* malaria, elevated levels of IFN-γ limits progression to clinical malaria. In addition, studies done by Horowitz *et al.,* 2010 demonstrated that rapid IFN-γ production inhibits intra-erythrocytic replication of malaria parasite preventing the onset of clinical malaria further demonstrating the critical role of IFN-γ in malaria pathogenesis.

At the genetical level, studies have shown association between polymorphic variability in genes and the functional changes in levels of pro-inflammatory and hematopoietic mediators

such as IL-10, IL-1β, IFN-γ and stem cell growth factors (Anyona *et al.,* 2011; Ouma *et al.,* 2008a; Ouma *et al.,* 2010). For example, a study illustrated that, in children with SMA, IL-10 promoter polymorphic variability, -1,082G/-819C/-592C (GCC) haplotype, were associated with protection against disease and that individuals with the -1,082A/-819T/-592A (ATA) haplotype had an increased risk of SMA and reduced circulating IL-10 levels (Ouma *et al.,* 2008a). Indeed, a susceptibility locus for mild malaria has been mapped to the class III region of MHC and TNF polymorphisms have been associated with mild malaria (Delahaye *et al.,* 2007).

The Natural Cytotoxicity-triggering Receptor 3 (*NCR3*) gene encoding a natural killer cell receptor, is located in the peak region of linkage and is 15 kb distal to TNF (Delahaye *et al.,* 2007) thus predicting possibility of *NCR3* gene polymorphisms conditioning malaria disease outcomes. The human Natural Cytotoxicity Receptors (NCRs) are germ line-encoded receptors composed of three receptors, namely NKp46 (NCR1, CD335), NKp44 (NCR2, CD336), and NKp30 (NCR3, CD337). They play important roles in the activation of cytotoxicity and also mediate the production of pro-inflammatory cytokines by NK cells (Hudspeth. *et al.,* 2013). Recent work, however, has established that NCRs can also generate inhibitory responses under certain circumstances (Delahaye *et al.,* 2011).

Natural killer cell activation by pathogens via NCRs appears not only to be limited to viruses but it has also been reported for intracellular bacteria and parasites. The Duffy binding-like (DBL)-1 alpha domain of *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) of the malaria parasite has been shown to bind and activate NK cells through NKp46 and NKp30 (Mavoungou *et al.,* 2007). Many studies conducted on NCRs have illustrated protective immunity against intracellular parasites like hepatitis C (De Maria *et al.,* 2007), influenza (Mandelboim *et al.,* 2001) and Ebola (Fuller *et al.,* 2007) demonstrating its importance in

conferring immunity to intracellular pathogens. In malaria endemic area of Burkina Faso, studies on the adults population showed an association of *NCR3* -412C/G with parasitaemia or mild malaria (Baaklini *et al.,* 2017). In the current study, *NCR3* promoter multi-site haplotypes was used since they are highly informative allelic markers that can reveal associations with disease outcomes not easily identifiable with single polymorphisms and combinations of different functional polymorphic alleles in a haplotype which may demonstrate how polymorphisms interact to amplify, or moderate their individual effects (Ouma *et al.,* 2008a). However, empirical evidence on the role of polymprphic variability in *NCR3* promoter on SMA in a paediatric population in a holoendemic area such as Siaya County, in western Kenya, is lacking.

1.2. Problem Statement for the Study

Severe malaria anaemia (SMA) is one of the complications associated with *P. falciparum* infection leading to childhood morbidity and mortality in malaria holoendemic area of western Kenya. Genetically, *P. falciparum* infection has exerted selective pressure causing genetic variability in the host's immune response genes, leading to the progressive accumulation of genetic adaptations in these populations further conditioning different malaria clinical outcomes. However, molecular factors explaining as to why some children develop SMA disease and others do not, yet, they are all exposed to the same transmission intensity are not known. A clear understanding and identification of molecular factors which contribute to the modulation of SMA pathogenesis is an essential step in designing of a vaccine and/or therapeutic interventions against the disease sequeleae.

Previous studies have shown that *NCR3* plays important role in conferring protective immune responses by triggering NK-cell mediation against intracellular pathogens and the

pathogenesis by diseases such as in Ebola, influenza, West Nile and hepatitis C viruses. However, the role of polymorphic variants on the *NCR3* promoter on peripheral IFN-γ levels during SMA pathogenesis in naïve children in a *P. falciparum* holoendemic region in western Kenya, remains largely unexplored. As such, the association between the *NCR3* variants (-172A/G and-412C/G), susceptibility to SMA and role in peripheral IFN-γ levels was investigated in the current study. This was to establish whether they alter susceptibility to SMA through differential IFN-γ levels in children below 3 years of age, presenting at Siaya County Referral Hospital, in western Kenya.

1.3. Objectives

1.3.1. General objective

To investigate the association between *NCR3* polymorphisms and susceptibility to SMA and IFN-γ levels in children below 3 years of age with acute malaria presenting at Siaya County Referral Hospital, western Kenya.

1.3.2. Specific objectives

1. To determine the association between *NCR3* (-172A/G and -412C/G) genotypes/haplotypes and SMA in children presenting with acute malaria at Siaya County Referral Hospital, western Kenya.

2. To determine the association between *NCR3* (-172A/G and -412C/G) genotypes/haplotypes and IFN-γ levels in children presenting with acute malaria at Siaya County Referral Hospital, western Kenya.

3. To compare differences in peripheral IFN-γ levels in children with SMA and non-SMA presenting with acute malaria at Siaya County Referral Hospital, western Kenya.

1.4. Null hypotheses

1. There is no association between *NCR3* (-172A/G and -412C/G) genotypes/haplotypes and SMA in children presenting with acute malaria at Siaya County Referral Hospital, western Kenya.

2. There is no association between *NCR3* (-172A/G and -412C/G) genotypes/haplotypes and IFN-γ levels in children presenting with acute malaria at Siaya County Referral Hospital, western Kenya.

3. There is no difference in IFN-γ levels between children with SMA and non-SMA presenting with acute malaria at Siaya County Referral Hospital, western Kenya.

1.5. Significance of the Study

Plasmodium falciparum-SMA complications is still a major public burden leading to child morbidity and mortality in holoendemic area of western Kenya. From this study results therefore,the following are likely to be beneficiaries:

i. The results may be beneficial to Ministry of Health. Siaya County in western Kenya ia a holoendemic area and children between 3months -3 years of age have been found highly susceptible to severe malaria anaemia leading to high morbidity and mortality rates (Obonyo *et al.,* 2007). In this study children having *NCR3* (-412C/G) genotypes and *NCR3* (-172G/-412C) haplotypes were associated with susceptibility to development of SMA (Hb≤6.0g/dL, any density parasitaemia). Health Ministry may use this to develop a policy for screening

and earlier identification of all children between 3-36 months who are vulrable to *P.falciparum* infection due to their naïve protective immunity for management intervention.

ii. Molecular epidemiologists may find this findings beneficial. The findings that co-inheritance of variations in *NCR3* (-172A/G and -412C/G) polymorphisms conditions changes in the peripheral IFN-γ levels in children with severe malaria anaemia. The finding is vital will provide insight into the reasons explaing why paediatrics below age 3-36 months who are infected for the first time with malaria develop different malaria outcomes in an area with the same transmission intensity and infection rates of malaria.

iii. Researchers/pharmaceutical firms are also likely to benefit from the findings of the study. The findings may inform the development of therapeutics or vaccines either directly or indirectly to protect malaria naïve children or to treat SMA in those who are infected.

CHAPTER TWO

LITERARURE REVIEW

2.1. Mechanisms of Severe *Plasmodium falciparum* Malaria

In high malaria transmission regions, numerous mechanisms have been implicated in SMA pathogenesis which includes reduced erythropoietin activity, erythrophagocytosis, pro-inflammatory cytokines and increased erythrocyte destruction due to parasites, antibody and complement lysis (el Hassan *et al.,* 1997; Stoute *et al.,* 2003). Other mechanisms involves reapeated cycles of red blood cells invasion, replication and erythrocytic bursting by *P.falciparum*-parasites (Wickramasinghe and Abdalla, 2000). Previous studies have shown phagocytosis and complement activation induction by ring surface protein 2/Recombinant Rhoptry-associated Protein 2 (RSP-2/RAP-2) tagged to the normal erythrocytes and erythroid precursor cells in the bone marrow of malaria anaemic patients as a mechanism of unparasitized erythrocytes and dyserythropoiesis destruction (Layez *et al.,* 2005).

Mice model studies also shown that anti-erythrocyte auto-antibodies may be involved in the destruction of uninfected erythrocytes (Helegbe *et al.,* 2009). It has also been shown that release of *P. falciparum* glycosylphosphatidylinositol (GPI) inserted into unparasitized erythrocyte membranes causes its recognition by circulating anti-GPI antibodies resulting in subsequent elimination (Brattig *et al.,* 2008). Reduction in regulation upon activation, normal T-cell expressed and secreted (RANTES) levels due to naturally-acquired hemozoin by monocytes could also contribute, in part, to suppression of erythropoiesis in children with malarial anaemia (Were *et al.,* 2006). In addition, hemozoin acquisition by phagocytes has been shown to suppress cellular immunity and enhance malaria disease severity (Casals-Pascual *et al.,* 2006; Lyke *et al.,* 2004). In the event of *P. falciparum* malaria infection, both the innate and adaptive

immunity come into play, with the former responding first followed by the latter (Riley and Stewart, 2013). The body immunity responds first by combating the initial infection of blood-stage parasites of *plasmodium* species (Langhorne *et al.,* 2008).

During malaria infection, natural killer cells (NK cells) play a special role in immunity. They become activated early in infection conferring the innate immunity (Orago and Facer, 1991). One of the mechanism underlying NK cells cytotoxicity in *P. falciparum* malaria, is the recognition of the DBL1α domain of the parasitic antigen PfEMP1 by the NKp30 activating receptor, resulting in parasitized red blood cells lysis (Mavoungou, 2007). This interraction leads to immune response to *P. falciparum* infected RBCs secreting cytokines such as Interferon gamma (IFN-γ), tumor necrosis factor (TNF-α) and G-CSF upon activation by dendritic cells (Artavanis-Tsakonas *et al.,* 2003; Baratin *et al.,* 2007). Moreover, NK cells have been shown to produce immune chemokines such as macrophage inflammatory factor (MIP-1α/β) and RANTES (Fauriat *et al.,* 2010). Additionally, NK cells on activation may also release cytotoxic elements such as granzymes and perforins which leads to eliminate identified *Pf.* iRBCs (Artavanis-Tsakonas *et al.,* 2003). Taken together, these studies demonstrate the vital role played by the immune system in the elimination of pathogens and associated infected cells such as *P.falciparum*-iRBCs. In early childhood, adaptive immune system is naive hence the altenative; innate immunity plays a central role in elimination of malaria parasites. Early, innate mechanism include,, NK cells activated via *NCR3* to remove *P.falciparum*-iRBCs from the body.

To uncover the causes of different malaria disease outcomes in paediatric population exposed to the same *P.falciparum* infections and transmission intensity in the holoendemic region, this study investigated the role of *NCR3* polymorphisms. Natural Cytotoxicity-triggering Receptor 3 encodes for *NCR3* gene that facilitates cell cytotoxicity and production of interferon-γ upon

contact with *Pf.* iRBCs (Artavanis-Tsakonas *et al.,* 2003; Baratin *et al.,* 2007). Natural Cytotoxicity Receptor 3 gene polymorphisms may condition severe malaria anaemia (SMA) pathogenesis by mediating phagocytosis of red blood cells and triggering cytokine production. In this study, consistent with two separate studies conducted in Burkina Faso and Congo (Baaklini *et al.,* 2017; Delahaye *et al.,* 2007), the carriage of -412C genotype replicated significant increased numbers of clinical malaria episodes compared to the non-carriers. In this current study,the proportions of individual genotypes among children presenting with non-SMA (n = 254) versus SMA (n = 201) were comparable. Polymorphism of *NCR3* -172 was identified only in one family in the Burkina Faso study (Delahaye *et al.,* 2007). This rare allele showed a frequency of <1% in Burkina Faso and too small power of association analysis leading to its exclusion from further analyses. In this current study, it was expected that the same outcome is replicated and the distributions of the haplotype carriers in the clinical groups was expected to be statistically comparable. Carriage of *NCR3* promoter haplotypes (-172G/-412C) was expected to condition SMA susceptibility in children presenting at Siaya County Refferal Hospital where *P. falciparum* transmission is holoendemic.

Even though previous studies on the role of IFN-γ SMA pathogenesis generated dissimilar results, in this current study, it was expected that there was no differences in the concentrations of circulating IFN-γ across *NCR3* (-172A/G and -412G/C) genotypes. Nevertheless, C carriage in heterozygous GC haplotype was expected to generate significant differences while G carriage in homologous GG haplotype was expected to generate comparable results. In addition, more previous studies also generated conflicting results on the role of IFN-γ in SMA pathogenesis, for example a study in Papua New Guinea (D'Ombrain *et al.,* 2007) shown early IFN-γ production as protective against malaria while a study conducted in sourthen

Ghana reported production of IFN-γ as associated with reduced clinical malaria and fever (Dodoo *et al.,* 2002). In this current study, it was expected that children with SMA to show significant difference in the peripheral IFN-γ level with the non-SMA group. It was therefore, expected that incase of predictions not met, then, the explaination of the differences could be attributed to either differences in the study design and geographical regions.

2.2. Natural Cytotoxicity Receptors and Severe Malaria Pathogenesis

Natural cytotoxicity receptors are type I transmembrane glycoproteins which transduce either activation or inhibitory signals, directly or through associated adaptor proteins, to dynamically regulate the activation state of NK cells and homeostasis. They were named in accordance with their molecular weight on SDS-PAGE (NKp30, NKp44, and NKp46). Earlier studies showed *NCR3* receptors play important roles in the activation of cytotoxicity and mediating the production of pro-inflammatory cytokines by NK cells (Hudspeth *et al.,* 2013). Natural cytotoxicity receptors (NKp30; CD337) has been shown to play important roles in crosstalk between NK cells and dendritic cells (DCs) by promoting both the maturation of and the cytotoxicity of immature DC (Ferlazzo *et al.,* 2002; Vitale *et al.,* 2005). Surface expression levels of NKp30 and NKp46 can be upregulated by IFN-α, IL-2, and prolactin and down regulated by cortisol and methylprednisolone (Bozzano *et al.,* 2011; Mavoungou, 2007; Vitale *et al.,* 2005). In addition, both receptors are also commonly down-regulated in "adaptive" or "memory-like" NK cells that are found in some cytomegalovirus-infected individuals (Cox-Singh *et al.,* 2008; Schlums *et al.,* 2015). Natural cytotoxicity receptors strongly activate NK cell cytotoxicity against cancer and various pathogens including HIV-1, influenza, hepatitis viruses and *Plasmodium falciparum* (Mandelboim *et al.,* 2001; Mavoungou, 2007; Vieillard *et al.,* 2005).

In humans, *NCR3* protein is encoded by the *NCR3* genes that may aid natural killer cells in tumour lysis. The encoded protein interacts with CD3-zeta (CD247), a T-cell receptor. A single nucleotide polymorphism in the 5' untranslated region of this gene has been associated with mild malaria susceptibility. Mutations on isoforms of NKp30 in exon 4 leads to three splice variants as NKp30a, NKp30b and NKp30c. Binding of NKp30a and NKp30b with their putative ligands and on iDCs produces IFN-γ and TNF-α whereas NKp30c is immunosuppressive (Delahaye *et al.,* 2011). Interaction of NKp30 on NK cells with autologous DCs contributes to establish important links between innate and adaptive immune responses through a process that requires both NK cell-DC cellular contacts and secretion of IFN-γ and TNF-α (Cooper *et al.,* 2004; Degli-Esposti and Smyth, 2005; Ferlazzo *et al.,* 2002; Gerosa *et al.,* 2002; Raulet, 2004; Vivier, 2008). Interferon gamma is produced by natural killer and αβ-T cells, as well as the regulatory γδ-T cells, during the early phases of the immune response to a malaria infection (Artavanis-Tsakonas *et al.,* 2003; D'Ombrain *et al.,* 2007).

In malaria parasite, the Duffy binding-like (DBL)-1 alpha domain of *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) is expressed on infected erythrocytes and is a positive effector of NK cells. Direct recognition by the NKp30 results in more efficient killing of parasitized erythrocytes by NK cells (Mavoungou, 2007). However, the functional roles of polymorphic variants within the *NCR3* promoter in conditioning susceptibility to SMA in paediatric populations resident in *P. falciparum* holoendemic transmission areas such as in western Kenya remain largely unexplored. As such, the association between the *NCR3* variants (-172A/G and -412C/G) and their haplotypes, susceptibility to SMA and functional role in interferon gamma levels was investigated in children below 3 years of age residing in a *P. falciparum*-SMA holoendemic region in Siaya County of western Kenya.

Expression of distinct NKp30 isoforms is currently of great interest since NCR is involved in DC-to-NK cell crosstalk (Vitale *et al.,* 2005) and can lead to tumour cell recognition (Fiegler *et al.,* 2013; Pende *et al.,* 1999; Pogge von strandmann *et al.,* 2007) and can influence the prognosis of different infectious diseases (Marras *et al.,* 2014). Six splice variant transcripts have been identified with most highly expressed *ncr3* variants designated as a, b, and c and encodes for NKp30 proteins with an extracellular V-type Ig domain, while d, e, and f isoforms encode NKp30 receptors possessing a C2-type Ig domain that lacks 25 amino acids (Nalabolu *et al.,* 1996; Neville and Campbell, 1999).

2.3. Interferon-gamma in Pathology and Protection in Malaria

Interferon gamma (IFN-γ) is chemo-attractant type II multifunctional cytokine produced by T cells (αβ and γδ) and NK cells (Miller *et al.,* 2009). It plays a central role in immunoregulatory function through pro-inflammatory immune response on type-1 helper (Th1) CD4+ T-cells, effector CD8+ T-cells, and Natural Killer (NK) T-cell innate-type lymphocytes (Artavanis-Tsakonas *et al.,* 2003). In humans, IFN-γ has been associated with both beneficial and detrimental effects (Perkins DJ. *et al.,* 2011). It appears to play a protective role in children and adults during the early stages of a *P. falciparum* infection by stimulating monocyte/macrophage activation and aid in controlling parasitemia (Kremsner *et al.,* 1995). Over-production of these innate inflammatory mediators is also associated with anaemia (Lyke *et al.,* 2004; Perkins *et al.,* 2000). Interferon-gamma can also work to upregulate the expression of both MHC class I and MHC class II, thereby facilitating the presentation of antigen and driving the adaptive immunity (Lammas *et al.,* 2000). On the other hand, IFN-γ has the ability to regulate the amount of TNF-α made by macrophages/monocytes, the amount of IL-12 and the amount of IFN-γ itself, all of which act cyclically and stimulate each other's release. Many

studies conducted have shown varied outcomes of IFN-γ levels as a result of infection with *P. falciparum* malaria. For example, earlier studies done in Papua New Guinea showed that early production of malaria-specific IFN-γ leads to immunity against clinical malaria (D'Ombrain *et al.,* 2007) and another study showed that in the acute phase of uncomplicated *P. falciparum* malaria, elevated levels of IFN-γ limits 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. In contrary,higher levels of circulating IFN-γ have been shown to be associated with enhanced SMA severity in paediatric population in western Kenya (Ouma *et al.,* 2011) and in adult population in Thai studies of patients with complicated malaria at the initial stage of the disease than uncomplicated malaria (Tangteerawatana *et al.,* 2007) illustrating the role IFN-γ in malaria pathogenesis.

Since the functional role of *NCR3* modulates IFN-γ levels in SMA disease, pathogenesis remains unexplored in children populations elsewhere and in holoendemic regions. The current study, therefore, investigated the relationship between the IFN-γ levels and *NCR3* (-172A/G and -412C/G) polymorphisms and modulation of SMA pathogenesis in case of any alteration in the production of IFN-γ levels.

CHAPTER THREE

MATERIAL AND METHODS

3.1. Study Site

This study was conducted at Siaya County Referral Hospital (SCRH), in Siaya county, western Kenya (Appendix I). Siaya County Referral Hospital is a rural government hospital located in a *P. falciparum* holoendemic area with residents receiving approximately 100-300 infective mosquito bites per annum (Bloland *et al.,* 1999; Ong'echa *et al.,* 2006). The inhabitants of this study area are predominantly Luo (> 96%), making the population culturally homogeneous and desirable for genetic based studies (Bloland *et al.,* 1999; Otieno *et al.,* 2006b). It is borders Busia,Kakamega, Kisumu and Vihiga counties. It is approximately 1520 sq^2 km in size and lies between Latitude 0º 26' to 0º 18' North and Longitude 33º 58' East and 34º 33' West. The county lies at an altitude of between 1140m and 1430m above the sea level, annual rainfall range of 800 to 200mm withand an average annual temperatures range from 15 ºC to 30ºC (RoK, 2000). The area experience seasonal rainfalls in April to August and November to January resulting into intense malaria transmission (Beier *et al.,* 1994). Mosquito vectors in this area are predominantly *Anopheles gambiae* s.s., *Anopheles arabiensis* and *Anopheles funestus* (Minakawa *et al.,* 2012). Severe malaria anaemia and hyperparasitemia *Plasmodium falciparum*-associated severe malarial anaemia (SMA, hemoglobin (Hb) <6.0 g/dL) and hyperparasitemia are the most common clinical outcome of severe malaria and primary cause of peadriatic morbidity and mortality (Bloland *et al.,* 1999; Otieno *et al.,* 2006a). Malaria prevalence in Siaya County is 21% (Obonyo *et al.,* 2007).

3.2. Study Design and Patient Population

3.2.1. Recruitment of Study participants

In this case-control study, parasitemic children (n=455) samples presenting with acute malaria were used. Recruitment of the children was done as per previously established protocols (Ong'echa *et al.,* 2006). Children of both sexes (males = 239 and females = 216) aged 3–36 months visiting SCRH with symptoms of malaria for the first time were enrolled in the study based on a questionnaire and existing medical records.

Children with acute malaria were stratified into two categories namely; SMA group defined by a positive smear for asexual *P. falciparum* (Hb < 6.0 g/dL and any density parasetaemia) and non-severe malarial anaemia (non-SMA) group defined as a positive smear for asexual *P. falciparum* parasitemia (of any density) and Hb ≥ 6.0 g/dL with Oral/axillary temperature > 37.5 °C or two of the following symptoms namely; poor feeding, nausea/vomiting, diarrhoea, myalgias and headache.

3.2.2. Screening and Enrollment

Recruitement of children with SMA(cases) which are children with positive smear for asexual *P. falciparum,* parasitemia (of any density) and Hb<6.0 g/dL and non-SMA (controls) group, which are children with positive smear for asexual *P. falciparum,* parasitemia (of any density) and Hb≥6.0 g/dl (McElroy *et al.* 1999) followed a two-phase tier of screening and enrolment process. Parents or legal guardians received detailed explanation about the study. Decision to enrol in the study was, therefore, arrived at after prior screening of the child for HIV-1 and signing of informed consent. The study questionnaires and written informed consent were administered in the language of choice (i.e. English, Kiswahili or Dholuo). Prior to provision of treatment or any supportive clinical care at enrolment, <3.0mls of venous blood samples were

asceptically collected in the ethylenediaminetetraacetic acid (EDTA) vacutainer tubes. Blood samples were used for malaria diagnosis, haematological indices measurements, HIV-1 testing, Glucose-6-phosphste dehydrogenase (G6PD) deficiency and sickle cell trait and bacterial blood culture isolation.

All enrolled children with malaria disease were treated immediately following the Kenya Ministry of Health (MOH) guidelines. This included administration of oral artemether/lumefantrine (Coartem®) for uncomplicated malaria or intravenous quinine and or blood transfusion, when ordered by doctor) for severe malaria patients.

3.2.3 Inclusion criteria

In this study, the inclusion criteria were: Children of both sexes aged ≥3 months and ≤36 months and presenting with malaria parasitaemia (any density) and Hb<11.0g/dL during their initial hospitalization at SCRH; Parent/guardians of children willing and able to sign the consent form.

3.2.4. Exclusion criteria

In this study, the exclusion criteria were: positive blood smears with non-*P. falciparum* species, diagnosis with cerebral malaria (though rare in holoendemic area), previous hospitalization (for any reason), and/or reported or documented use of anti-malarial drugs two weeks prior to enrolment into the study and clinical evidence of acute respiratory infection.

3.3. Sample size determination

A total of 455 samples of parasitemic children aged between 3-36 months with clinical symptoms of *P. falciparum* at the SCRH were used. The sample size (n=455) was based on actual number of children below 3 years that were admitted with clinical symptoms of malaria.

Study previously conducted using the same paediatric populations by (Ouma *et al.,* 2008a) had showed that the magnitude of significant allele frequency differences ranged from 0.10 to 0.13 between SMA and non-SMA arms. In this study, an allele frequency difference of 0.10 (e.g. 0.15 in SMA cases *vs.* 0.25) in non-SMA controls, was chosen as the smallest difference to distinguish from chance variation between the groups.

The Bonferroni correction for 2 different loci would require a per-comparison alpha of 0.004 for sample size calculations, based on Dupont and Plummer (Dupont and Plummer, 1990). However, given the increased power provided by the procedure, it is estimated that sample sizes based on a $\alpha \leq 0.05$ will provide 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.

$$n = \left(\frac{r+1}{r}\right)\frac{(\bar{p})(1-\bar{p})(Z_{\beta} + Z_{\alpha/2})^2}{(p_1 - p_2)^2}$$ (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_{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 study Ouma *et al.,* (2008a) (Ouma *et al.*, 2008a), to detect an odds ratio of 2, equal number of cases and controls was used. Therefore r=1. To determine proportion of children with malaria and have the genotypes understudy determined,

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

$$p = \frac{2.0(0.25)}{(0.25)(2.0-1)+1} = \frac{0.5}{1.25} = 0.40$$

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, this current study had 201 cases (SMA) and 254 non-SMA (controls) children enrolled.

3.4. Study Design

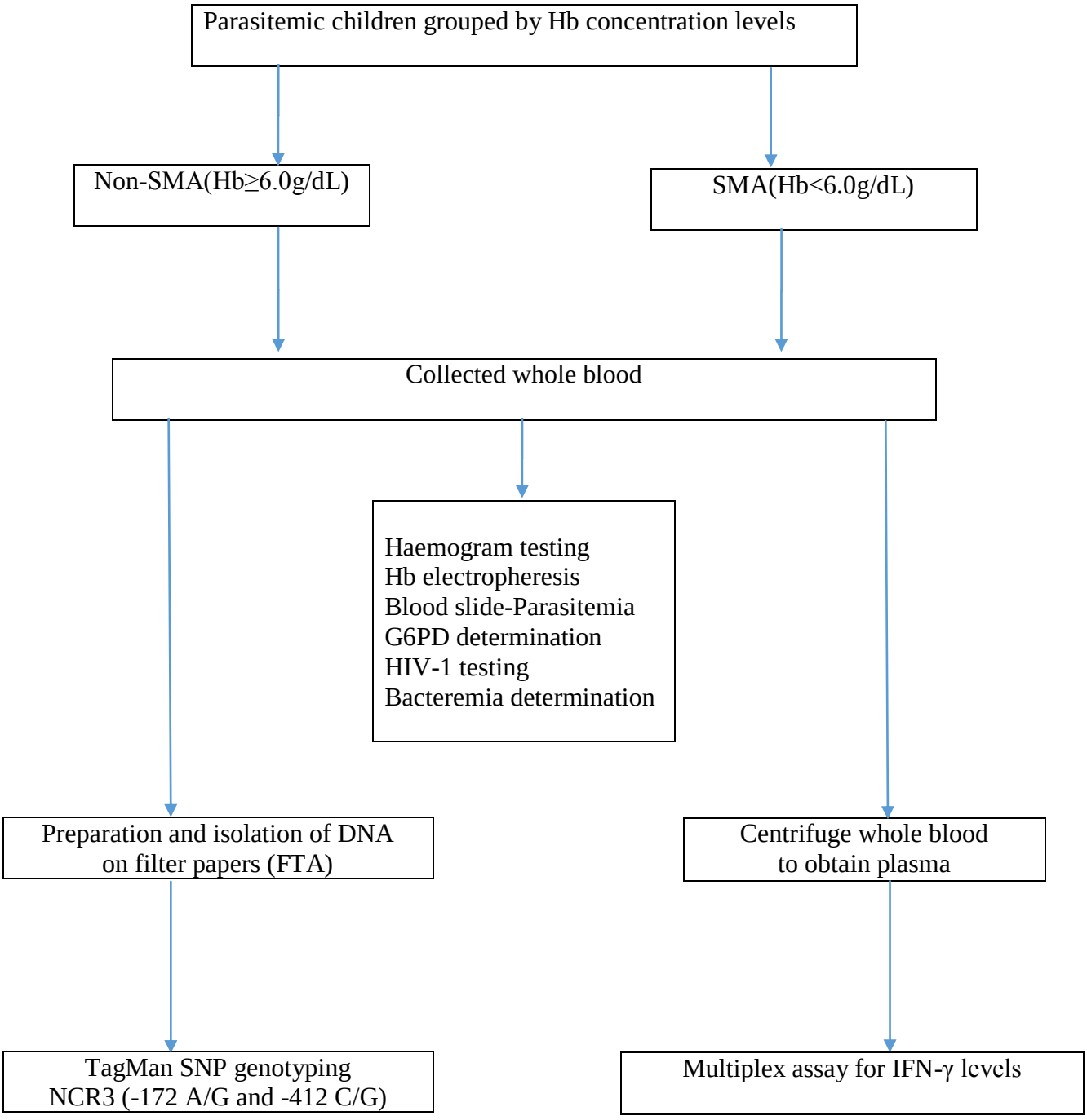


Figure 3. 1. Flow chart showing the experimental design of the study

3.5. Laboratory procedures

3.5.1. Malaria diagnosis

To determine presence of *P. falciparum* parasitaemia (per µL), a 10% Giemsa-stained thick and thin blood smears for 15 minutes were prepared from <100µL of finger or heel prick blood a septically collected. Giemsa stained smears were examined per 300 white blood cells using a high power magnification (X100 oil immersion objective) microscope and parasitemia (µL) estimated using absolute total WBC count from Hemogram results. Formulae: Parasite density/µL =WBC count/ µL x trophozoites/300.

3.5.2. Haematological indices measurement.

Red blood cells (RBCs), Red blood cells distribution width (RDW), Hemoglobin(Hb), Hematocrit (HCT), Mean cell Haemoglobin Concentration (MCHC), White blood cells (WBCs) and Lymphocytes haematological indices were determined by analyzing of EDTA-collected blood samples using a Beckman Coulter AcT Diff2™ (Beckman-Coulter Inc., Miami, FL, USA).

3.5.3. Determination of Bacteremia, HIV-1, Glucose-6-Phosphate Dehydrogenase Deficiency and Sickle Cell Trait

The confounding effects were controlled for because they have been shown to individually able to cause anaemia other than that due to SMA. Human Immunodeficiency Virus-1 (+) infected children have been shown to display more profound anaemia and they had a significantly more mortality than HIV-1 (-) *P.f* (+) children (Ongecha *et al.* 2006). Presence of HIV-1 exposure and infection was determined by two serologically test kits; namely Determine™ and Unigold™ test kits and HIV-1 proviral DNA PCR testing was done to confirm positive serological test (Otieno *et al.*, 2006b).

Bacteremia has also been associated with developing low Hb concentrations compared with abacteremia children with malaria (Were *et al.*, 2011). It's presence was determined using the Wampole Isostat Pediatric 1.5 system (Wampole Laboratories, Town, Country) and blood was processed following manufacturer's instructions. API biochemical galleries (BioMerieux Inc) and/or serology were used in the identification of blood- borne bacterial isolates.

Glucose-6-phosphate dehydrogenase deficiency leads to prematuarly breakdown of RBCs causing haemolytic anaemia (Cappellini & Fiorelli, 2008). Glucose-6-phosphate dehydrogenase deficiency was determined by fluorescence spot test as per the manufactures procedures (Trininity Biotech Plc.,Bray,Ireland). Briefly a hemolyzed blood sample spot was made onto a filter paper. A solution composed of oxidized NADP ($NADP^+$) and glucose-6-phosphate dehydrogenase was added and samples excited with UV light at 340nm. Samples were categorized based on the presence or absence of flourescene emmission as either normal (high emission), intermediate (moderate emission) or deficient (lack of emission).

Carriage of homozygous sickle-cell trait have been show to develop to low Hb concentrations (Aidoo *et al*., 2002). In this current study,presence of Sickle cell trait (HbAS) was determined by cellulose acetate electrophoresis using Titan III plates following the manufacturers procedures (Helena laboratories). Briefly, Hemolysed blood samples and AFSC controls were spotted onto the acetate paper and electrophoresis with alkaline buffer pH 8.6 used to separate different haemoglobin variants. Staining of plates was done using Ponceau S stain and Hemo AFSC control used in identification of various types of Hb.

3.5.4. Deoxyribonucleic Acid extraction and Amplification

Genomic DNA was extracted from the FTA classic® cards using Gentra System (Gentra System Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly,

three millimeters of the disc were punched from sample collection filter paper (FTA) and placed into 0.2mL tube as the heatblock was being preheated at 99^{o}C. 50µL of DNA purification solution 1 was added to the tube and incubated at room temperature (25^{o}C) for 15 minutes. The solution was pipetted up and down twice then discarded. The washing step was repeated twice. DNA solution 1 was again added and incubated at room temperature for 15 minutes followed immediately by up and down pipetting. For elution, 70µL of DNA elusion solution 2 was added and incubated at 99^{o}C in the preheated heat block for 15 minutes to release DNA and then cooled at room temperature. The eluted DNA was aliquoted into a clean and sterilized 1.5mL eppendorff tube then stored at -20^{o}C until use.

Presence of DNA in the eluted samples was done by amplifying the human 3 phosphate dehydrogenase (hG3PDH), a house-keeping gene using the PTC 100 programmable thermal controller (MJ Research Inc.). In brief, the master mix per 20 µL reaction contained 200 µM of dNTPs, 10x buffer, 50Mm MgCl, 100 µM of each primer (forward and reverse), 1U/ µL of Taq polymerase and 1µL of the test sample.

The conditions were as follows; initial denaturation at 94^{o}C for 2 minutes: Denaturation at 94^{o}C for 45 seconds: Annealing at 62^{o}C for 45 seconds: Extension at 72^{0}C for 7minutes. This were repeated for 40 cycles: Final extension at 72^{0}C for 7minutes. Once amplification was completed; gel electrophoresis was performed to visualize 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 in the wells along the DNA molecular weight marker. This was run for 2 hours at 80 volts with results viewed under ultraviolet (UV) source and captured pictures stored on a flash drive or printed out for documentation.

3.5.5. Genotyping of *NCR*-3 (-172 A/G, rs2736191 and -412C/G, rs986475) variants

Natural trigerring cytotoxicity receptor-3 (-172A/G and -412C/G) SNPs were genotyped using Applied Biosystems SNP-specific primer-probe combinations (TaqManW SNP Genotyping Assays, assay ID; C_16286876_10; rs2736191 and C_7514908_10; rs986475) respectively, on the Applied Biosystems StepOnePlus™ Real- Time PCR System (Applied Biosystems, Foster City, CA,USA), following the manufacturer's guidelines. Briefly, genotyping of *NCR3* variants was done by preparation of 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 added into each micro-well and genotyped using following cycling parameters as; 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. *NCR3* (-172A/G and -412G/C) polymorphisms was genotyped using PCR.

3.5.6. Quantification of Interferon-gamma levels

Plasma samples were obtained by spinning the collected EDTA venous blood which were stored at -80^{o}C until assay. Interferon-gamma concentrations were determined using the cytokine 25-plex antibody bead kit, Hu (BioSource^{TM} International) following the manufacturer's protocol. Batch analyses were done to restrict experimental variability between the assays. In summary, 200µl of working wash solution was added into the designated well plates to pre-wet them and then incubate at room temperature for 30 seconds. Using the vacuum manifold, aspiration of the working wash solution from the wells was done. Vortexing followed by sonication of the bead solution was done for 30 seconds each. About 25µl of bead solution was then dispensed into each well and immediate foiling against light to prevent photo-bleaching

done. Addition of 200µl of working wash solution into the well plates and then beads allowed to soak for 20 seconds. Aspiration using vacuum manifold was done again as above. Once again, a repeat washing step was performed and the filter well plates bottom were blotted on a clean adsorbent paper towel to enable complete removal of residual liquid. About 50µl of incubation buffer was then added into each well. To the sample wells, 50µl of assay diluent was added followed by 50µl of sample. On the other hand, 100µl of standard dilution was added to well designated for standard curves. Using aluminium foil paper, the filter plates were covered and then incubated at room temperature for 2 hours at 500rpm on orbital shaker. On completion of the incubation period, the plates were washed by addition of 200µl of washed solution to each well.

To each washed well plates, 100µL of 1X biotinylated anti-IFN-γ antibody was added followed by 1 hour incubation at room temperature with constant agitation on orbital shaker at 500 rpm to allow beads remain in suspension. About 200µL of wash solution was added in all the wells soaking the beads then aspirated using vacuum manifold. After aspiration of wash solution,100µL of 1x streptavidin-RPE was added and then plates incubated for 30 minutes at room temperature on an orbital shaker 500 rpm. Vacuum manifold aspiration step was performed again and then addition of 200µL working wash solution to the wells was done to soak the beads for 10 seconds, and then aspirated using the vacuum manifold. Washing step was performed two times. Reading of the plates were done using the Luminex 100™ system (Luminex Cor-poration) and analyzed using Bio-plex Manager software (Bio-Rad Laboratories). The detection limit for IFN-γ was be 2.0 pg/ml.

3.6. Data Statistical Analyses

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) software package version 20.0 (IBM SPSS Inc., Chicago, IL, USA). Chi-square ($\chi 2$) analyses were used to compare proportions. Mann-Whitney U test was used for comparisons of demographic and clinical characteristics between the two clinical arms. The association between *NCR3* (-172A/G and -412C/G) genotypes/haplotypes and SMA was determine using bivariate logistic regression analysis controlling for confounding effects of age, gender, bacteremia, HIV-1, G6PD deficiency and sickle cell trait (HbAS). *NCR3* (-172A/G and -412C/G) allele frequencies, consistency and/or deviations from Hardy-Weinberg Equilibrium (HWE) were determined using the web-based site given emerald.turfts.eduAQ3/~court01/Documents/Court%20lab%20-%20calcutor. Haplotypes were constructed using HPlus software program (Fred Hutchinson Cancer Research Centre) and there frequencies estimated based on the Bayesian algorithm. Differences in the levels of peripheral IFN-γ between genotypes was done using ANOVA and Student t-test where applicable. Statistical significance were set at $P \leq 0.05$.

3.7. Ethical Considerations

To conduct this study approval was first provided by the School of Graduate Studies of Maseno University (SGS) (Appendix II). The Kenya Medical Research Institute (KEMRI) National Ethical Review committee provided the Ethical approval (Appendix III). To participate in the study, the parents or guardians' informed consent were obtained. Blood collection was carried out by trained and qualified phlebotomists, to reduce risks of bleeding incidents. Study participation was voluntary; participants were allowed to withdraw at any time during the study. This was the study's drawback since withdrawal lead to reduction of enrolled

participant. To ensure confidentiality, all the samples collected were coded for using the study identification number. No samples were labelled with the participant's name. Data were stored in a computer in the Excel spreadsheat and had a password accessable only to the study investigator.

CHAPTER FOUR

RESULTS

4.1. Clinical, Demographic and Laboratory characteristics of study participants.

Analysis of study samples from a case-control study of children (aged 3–36 months, n=455) presenting with acute *P. falciparum* malaria (any density parasitemia) was conducted. Clinically, the study participants were stratified into two main categories namely:

Non-SMA; participants having hemoglobin of ≥ 6.0 g/dL; any density parasitemia (n=254) and SMA participants having hemoglobin of < 6.0 g/dL; any density malaria parasitemia, (n=201) according to SMA definition based on age and geographically matched Hb concentrations (McElroy *et al.,* 1999). The clinical, demomographic and laboratory characteristics are summarized in Table 4.1.

Children in SMA and non-SMA clinical groups were comparable in gender, axillary temperature (^{o}C), parasitemia density (parasites/µl) and G6PD deficiency (P = 0.638, P = 0.076, P = 0.172 and P = 0.348) respectively. These (except temperature) were controlled for in the analyses since the variables significantly influence susceptibility to severe malaria disease. However, analyses of children presenting with SMA revealed a less proportion of sickle-cell carriers (HbAS) compared to non-SMA group (7.5% vs. 17.6% ; P=0.006). In addition, children with SMA were significantly younger in age [median (IQR); 8.0 (7.0)] relative to those with non-SMA [median (IQR); 10.0 (9.0)], (P<0.001). Furthermore, analyses on hematological indices revealed that, Red Blood Cells (RBCx10^{12}/µL), Hemoglobin (Hb), Hematocrit (HCT) and Mean cell Haemoglobin Concentration (MCHC) were lower in children with SMA group [median, (IQR); 2.2 (0.9); 5.0 (1.0); 15.9 (4.6) and 31.6 (3.3)] compared with the non-SMA

group [median, (IQR); 3.9 (1.1); 8.7 (3.0); 26.4 (8.1) and 32.7 (1.9)]; $P<0.001$, respectively in all cases.

Similarly, white blood cells (WBC $x10^3/\mu L$), red blood cell distribution width (RDW) and lymphocytes were all higher in children with SMA [median, (IQR); 13.2 (7.9); 23.2 (4.8) and 6.4 (4.6)] relative to those of non-SMA [median,(IQR); 10.9 (5.6); 20.2 (4.3) and 5.3 (2.9)]; $P\leq0.001$, respectively in all cases.

Table 4. 1. Clinical, Demographic and Laboratory characteristics of the study participants

Characteristics	Clinical Groups		
	Non-SMA ($Hb \geq 6.0 gdL^{-1}$) n=254	SMA ($Hb < 6.0 gdL^{-1}$) n=201	*P*-value
Sex, n (%)			
Male	136 (53.5)	103 (51.2)	0.638[a]
Female	118 (46.5)	98 (48.8)	
Age, (months)	10.0 (9.0)	8.0 (7.0)	**0.001**[b]
Axillary Temperature, (ºC)	37.0 (2.0)	37.0 (2.0)	0.076[b]
Heamatological Indices			
Hemoglobin, (g/dL)	8.7 (3.0)	5.0 (1.0)	**<0.001**[b]
Hematocrit, %	26.4 (8.1)	15.9 (4.6)	**<0.001**[b]
RBC, ($\times 10^{12} \mu L^{-1}$)	3.9 (1.1)	2.2 (0.9)	**<0.001**[b]
RDW, %	20.2 (4.3)	23.2 (4.8)	**<0.001**[b]
MCHC,g/dl	32.7 (1.9)	31.6 (3.3)	**<0.001**[b]
WBC ($\times 10^{3} \mu L^{-1}$)	10.9 (5.6)	13.2 (7.9)	**<0.001**[b]
Lymphocytes, ($\times 10^{3} \mu L^{-1}$)	5.3 (2.9)	6.4 (4.6)	**<0.001**[b]
Parasitological indices			
Parasite density (μL^{-1})			
LDP, n (%)	88 (34.6)	83 (41.3)	0.172[b]
HDP, n (%)	166 (65.4)	118 (58.7)	
Genetic factors			
Sickle cell trait, n (%)	44 (17.6)	15 (7.5)	**0.006**[a]
G6PD deficiency, n (%)	15 (6.4)	7 (3.7)	0.348[a]

Data are presented as the median (interquartile range; IQR) or N(%). Children were categorized into non-SMA (Hb≥6.0g/dL, with any density parasitaemia) and SMA (Hb<6.0g/dL, with any density parasitaemia) were stratified according to a modified definition of SMA based on age-and geographically matched Hb concentrations (McElroy *et al.,* 1999). [a]Statistical significance was determined by the Chi-square (χ^2) analysis. [b]Statistical significance was determined using Mann-Whitney U test. *Abbreviations*: G6PD:Glucose-6-phosphate dehydrogenase, LDP:Low density parasitemia, HDP:High density parasitemia; MCH:Mean corpuscular haemoglobin concentration; RBC:Red blood cells; RDW:Red cell distribution width and WBC:White blood cells. Values in bold are significant *P*-values at a cut-off of $P \leq 0.05$.

4.2. Distribution of *NCR3* (-172 A/G and -412 C/G) genotypes within the study groups

In Table 4.2, the overall frequency of the -172 A/G genotypes were presented as GG (n=434, 0.954), GA (n=17, 0.037) and AA (n=4, 0.009). The combined overall distribution of genotypes in the clinical groups displayed significant departure from Hardy-Weinberg Equilibrium (HWE, $\chi 2$=41.166, P<0.001). The distribution of -172 A/G variant in the non-SMA (n=254) and SMA (n=201) and the associated genotypic frequencies within the clinical groups were GG (non-SMA, n=224, 96.1%; SMA, n=190, 94.5%), GA (non-SMA, n=7, 2.8%; SMA,n=10, 5.0%) and AA (non-SMA, n=3, 1.2%; SMA, n=1.0, 0.5%). These distributions revealed no significant difference between the non-SMA and SMA groups (P = 0.349).

The overall genotypic frequency of the -412 C/G genotypes is presented in Table 4.2 as follows; CC (n=233, 0.512), CG (n=161, 0.354) and GG (n=61, 0.134). Significant departure from HWE was displayed for this variant in the overall genotypic population distribution (HWE, $\chi 2$=13.826, P=0.001). The distribution of -412 C/G variant and the genotypic frequencies within the clinical groups were CC (non-SMA, n=132, 52.0%; SMA, n=101, 50.2%), CG (non-SMA, n=90, 35.4%; SMA, n=71, 35.3%) and GG (non-SMA, n=32, 12.6%; SMA, n=29, 14.4%) which were comparable between the two clinical arms (P=0.841).

Table 4. 2 Distribution of *NCR3* (-172 A/G and -412 C/G) genotypes within the study groups

Genotypes	n (%)	N (%) with genotype in group[a]		*P*-value[b]	HWE (SMA+non-SMA)[c]
		Non-SMA (Hb≥6.0gdL^{-1}) n=254	**SMA** (Hb<6.0gdL^{-1}) n=201		
***NCR3* -172 (A/G)**					
GG, n (%)	434 (95.4)	244 (96.1)	190 (94.5)		
GA, n (%)	17 (3.7)	7 (2.8)	10 (5.0)	0.349[b]	**<0.001[c]**
AA, n (%)	4 (0.9)	3 (1.1)	1 (0.5)		
***NCR3* -412 (C/G)**					
CC, n (%)	233 (51.2)	132 (52.0)	101 (50.2)	0.841[b]	
CG, n (%)	161 (35.4)	90 (35.4)	71 (35.4)		**0.001[c]**
GG, n (%)	61 (13.4)	32 (12.6)	29 (14.4)		

[a]Data are presented as n (%) for proportion of *NCR3* (-172 A/G and -412 C/G) genotypes within the non-SMA (n=254) and SMA (n=201) groups. Children with parasitemia were categorized on the basis of presence or absence of severe malarial anaemia SMA (defined as Hb<6.0 g/dL, with any density parasitemia). [b]Statistical significance determined by Chi-squire ($\chi 2$) analysis. [c]HWE: Hardy-Weinberg Equilibrium. Values in bold are significant *P*-values at a cut-off of $P \leq 0.05$.

4.3. Association between *NCR3* (-172 A/G and -412 C/G) genotypes and severe malaria anaemia

To determine the role of individual *NCR3* genotypes in conditioning susceptibility to SMA, bivariate logistical regression analyses were carried out using the wild type in each variant as the reference group while controlling for the confounding effects of age, gender, bacteremia, G6PD deficiency and sickle-cell trait. Relative to the wild type (-172,GG), the AA genotype were too few in number and could not generate any meaningful statistical association by the software. However, GA genotype analysis showed no statistical difference; *P*=0.418. Further, analysis of the *NCR3* (-412 C/G) genotype; GC relative to the wild type (-412,CC) demonstrated statistical significance; *P*=0.043. The GG genotype relative to the wild type (-412,CC) demonstrated no statistical difference in the individual genotypes; *P*=0.883 (Table 4.3).

Table 4. 3. Association between NCR3 (-172 A/G and -412 C/G) genotypes and severe malaria anaemia

Genotypes	Non-SMA	SMA	Association with SMA(Hb<6.0g/dL)[a]		
			Odds Ratio (OR)	95% CI	*P*-value
***NCR3* -172 (A/G)**					
GG, (n=434)	244	190	1.00 (Reference)	-	-
GA, (n=17)	7	10	1.572	0.526-4.704	0.418
AA, (n=4)	3	1	x	x	
***NCR3* -412 (C/G)**					
CC, (n=233)	132	101	1.00 (Reference)	-	-
CG, (n=161)	90	71	0.254	0.067-0.957	**0.043**
GG, (n=61)	32	29	0.928	0.345-2.495	0.883

Children with acute malaria (n= 455) were stratified as non-SMA (Hb ≥ 6.0 g/dL) and SMA (Hb< 6.0g/dL;with any densisty parasitaemia) according to SMA definition based on age and geographically matched Hb concentration (McElroy *et al.*, 1999). Odds ratios (OR) and 95% confidence intervals (CIs) were determined using bivariate logistic regression controlling for age, gender, HIV-1 infection, sickle cell trait (HbAS), bacteremia and G6PD deficiency. $P \leq 0.05$ is considered statistically significant. The reference groups in the bivariate logistic regression analysis were homozygous wild-type genotypes. x shows that the genotypes were too few to generate any meaningful association by the software.

4.4. Distribution of *NCR3* (-172 A/G and -412 C/G) haplotypes within the study groups

Construction of *NCR3* (-172 A/G and -412 C/G) haplotypes variants was preceded by determination of the overall distributions of different haplotypes. Distribution of the carriage of haplotypes within the study groups were compared. As shown in Table 4.4, the overall percentage distribution of haplotypes carriage were as follows: -172G/ -412G (GG, n=222/455, 48.8%), -172A/ -412C (AC, n=21/455, 4.6%) and -172G/ - 412C (GC, n=387/455, 85.1%).

The distribution of the haplotype carriers in the clinical groups were as follows: GG (non-SMA, n=122/254, 48.0%; SMA, n=100/201, 49.8%; AC (non-SMA, n=10/254, 3.9%; SMA, n=11/201, 5.5%). GC (non-SMA, n=219/254, 86.2%; SMA, n=168/201, 83.6%. The distributions of the haplotype carriers in the clinical groups showed comparable distributions (P=0.716; P=0.438 and P = 0.433), respectively.

Table 4. 4. Distribution of *NCR3* (-172 A/G and -412 C/G) haplotypes within the study groups

Haplotypes		Non-SMA (Hb≥6.0g/dL) n (%)	SMA (Hb<6 .0g/dL) n (%)	*P*-value
-172 G/-412C	1	219 (86.2)	168 (83.6)	0.433[a]
	0	35 (13.8)	33 (16.4)	
-172 G/-412 G	1	122 (48.0)	100 (49.8)	0.716[a]
	0	132 (52.0)	101 (50.2)	
-172 A/-412 C	1	10 (3.9)	11 (5.5)	0.438[a]
	0	244 (96.1)	190 (94.5)	

Haplotypes were constructed based on the *NCR3* variants using HPlus software. 1 represents carriers of haplotypes, while 0 represents non-carriers. Distributions of haplotypes were compared between the SMA and non-SMA groups using Chi-square ($^{a}\chi 2$) test. $P \leq 0.05$ cut-off values is considered significant.

4.5. Association between *NCR3* (-172 A/G and -412 C/G) haplotypes and severe malaria anaemia

Association between haplotypes and SMA was performed using bivariate logistic regression analyses, controlling for potential confounders. The analysis results as shown in Table 4.5 revealed that carriers of *NCR3* (-172 G/-412 C) haplotype were almost five times at an increased risks of developing SMA (OR; 4.977, 95% CI, 1.494-16.580, *P*=0.009). Further analysis however, demonstrated that the haplotypes of *NCR3 (*-172 A/-412 C) and (-172 G/ -412 G) (OR; 0.133, 95% CI, 0.011-1.610, *P*=0.113) and (OR; 0.543, 95% CI, 0.227-1.300, *P*=0.171) respectively, did not alter susceptibility to SMA.

Table 4. 5. Association between NCR3 (-172 A/G and -412 C/G) haplotypes and severe malaria anaemia

Haplotypes	**Association with SMA (Hb≤6.0g/dl)**		
	Odds Ratio (OR)	**95% CI**	***P*-value**
-172G/ -412G	0.543	0.227-1.300	0.171
-172A/ -412C	0.133	0.011-1.610	0.113
-172G/ -412C	4.977	1.494-16.580	**0.009**

Bivariate logistic regression analyses were used to determine the odds ratio (OR) and 95% CI, controlling for age,HIV-1, gender, G6PD deficiency, sickle-cell trait status and bacteraemia (Otieno *et al.,* 2006b; Were *et al.,* 2011). The non-carriers haplotype were used as reference groups for the analyses in the regression analyses. $P \leq 0.05$ is considered statistically significant.

4.6. Association between *NCR3* (-172A/G and -412G/C) genotypes and IFN-γ levels

To explore whether these genotypes were associated with changes in circulating IFN-γ levels, peripheral IFN-γ concentration levels were compared within the individual genotypes of *NCR3* (-172 A/G and -412 C/G). In Figure 4.1 (a), the results revealed that there were no statistically significant difference in normalized IFN-γ levels across the *NCR3* -412G/C genotypes at the $P<0.05$ level for the three groups as determined by one-way ANOVA [$F(2, 217)=0.948$, $P=0.389$)]. However, due to a lower prevalence of homozygous AA individuals in the samples archived for the *NCR3* -172A/G genotypic group, the software could not generate the normalized IFN-γ levels. As such, a bivariate analysis comparision of the normalized IFN-γ levels in the two available *NCR3* -172A/G genotypes GG;n =210; [mean (SEM) [2.92 (±0.05)] relative to the GA carriers n=8 [mean (SEM) [3.52 (±0.15)]; $P= 0.006$] was performed [Figure. 4.1 (b)]. Results revealed that the normalized IFN-γ levels were significantly different between the genotypes ($P=0.006$; Student's *t* test).

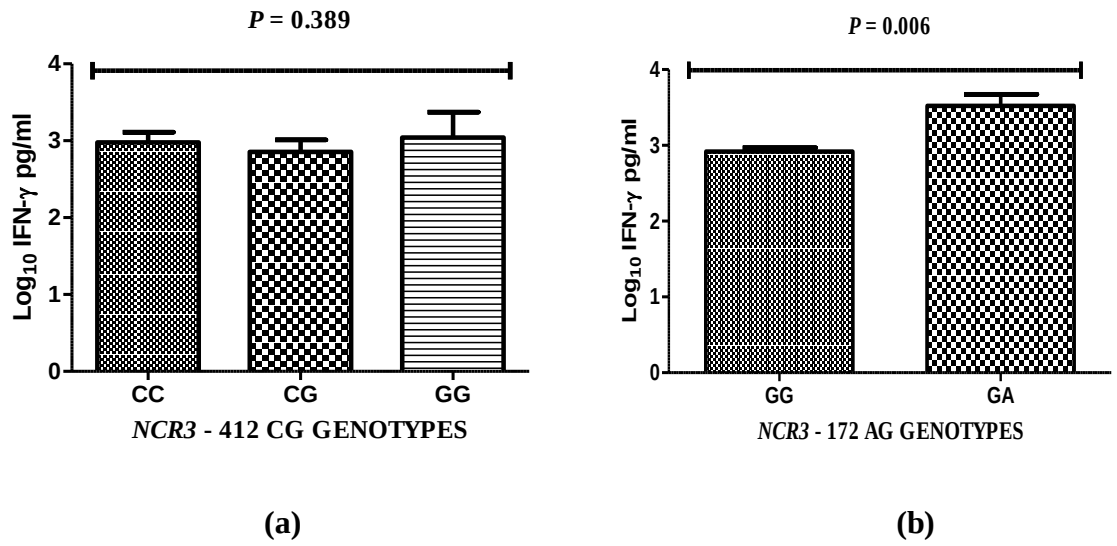
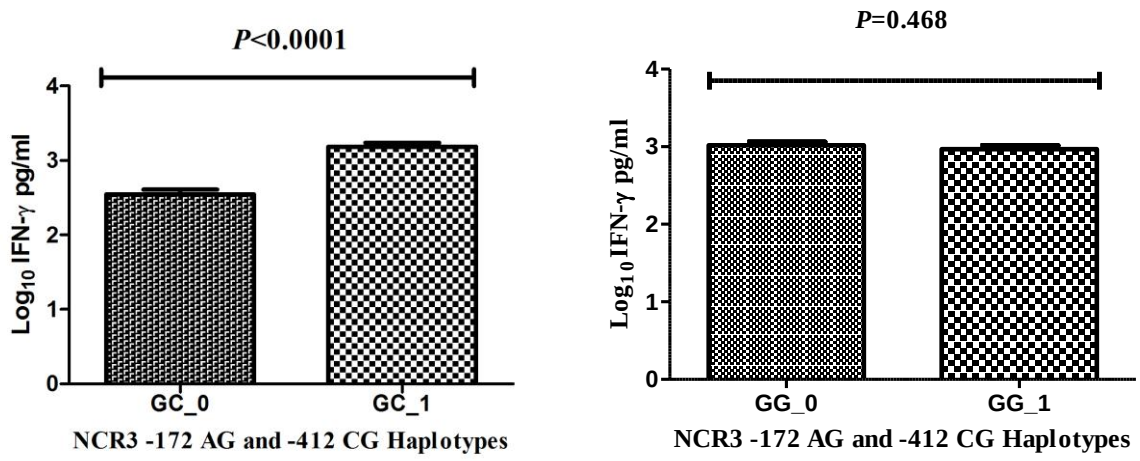


Figure 4. 1. Association between NCR3 (-172A/G and -412G/C) genotypes and IFN-γ levels

Peripheral IFN-γ levels were stratified according to *NCR3* -412G/C (a) and -172A/G (b) genotypes. Statistical significance across the *NCR3* -412G/C genotypes was determined by one-way ANOVA, while bivariate comparisons for *NCR3* -172A/G were performed using two-tailed unpaired Student's *t* test with Welch's correction at a 95% confidence interval. Error bar represents standard error of means (SEM).

4.7. Association between *NCR3* (-172 A/G and -412 C/G) haplotypes and IFN-γ levels

Further analyses on the association between *NCR3* (-172A/G and -412C/G) haplotypes and normalized peripheral IFN-γ levels were explored. As shown in the Figure 4.2 (a), participants with the -172C/-412G haplotype carriers showed significant difference in normalized IFN-γ levels; GC non- carriers (GC_0) [mean (SEM) [2.55 (±0.06)] relative to the GC carriers (GC_1), [mean (SEM) [3.19 (±0.05)]; $P<0.0001$]. In Figure 4.2 (b), the results of participants with the -172G/-412G haplotype showed comparable peripheral IFN-γ levels, (non-GG_0 carriers) [mean (SEM) [3.02 (±0.05)] relative to the (GG_1 carriers) [mean (SEM) [3.00 (±0.05)]; $P=0.468$]. Haplotypes of -172 C/-412 A could not be compared due to lack of corresponding peripheral IFN-γ levels data values for the CA_1 carriers.



(a) (b)

Figure 4.2 .Association between *NCR3* (-172A/G and -412CG) haplotypes and Interferon-gamma levels

Normalized IFN-γ levels were stratified according to *NCR3* (-412C/G and -172A/G) haplotyic categories (Figure *a* and *b*). Bivariate comparisons were performed using two-tailed unpaired Student's *t* test with Welch's correction at a 95% confidence interval. Error bars represents standard error of means (SEM). *Abbreviations*: GC_1: Carriage of GC haplotype; GC_0: Non-carriage of GC haplotype;GG_1: Carriage of GG haplotype and GG_0:-Non-carriage of GG haplotype. Haplotypes of -172 C/-412 A could not be compared since the CA_1 carriers had no corresponding peripheral IFN-γ levels data values.

4.8. Comparision of Interferon-gamma levels in children with Severe Malaria Anaemia and non-Severe Malaria Anaemia

To determine whether changes in peripheral levels of IFN-γ plays a role in the severity of acute malaria, the peripheral levels were investigated and compared between non-SMA (HB≥6.0g/dL, n= 104) and SMA (HB<6.0g/dL, n=116) clinical groups. As indicated in Figure 4.3, the results shows that there were no significant difference between the clinical groups, SMA, [mean (SEM) [2.88 (±0.06)] and non-SMA, [mean (SEM) [3.01 (±0.08)] , P = 0.173. It's quiet important to point out that, the reduction in sample numbers used in analysis were attributed by failure to collect additional blood samples at the screening stage. This was attributed to the fact that children were either too sick or extreme anaemia to allow re-bleeding, which could have constituted study ethical violation.

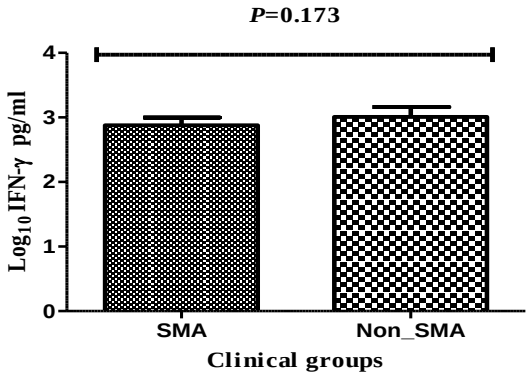


Figure 4.3. Comparision of peripheral Interferon-gamma levels between children with Severe Malaria Anaemia and non-Severe Malaria Anaemia

Bivariate analysis of normalized peripheral IFN-γ levels (arbitrary units [AU]) in the non-SMA (HB ≥ 6.0 g/dl; n = 104) and SMA (HB < 6.0g/dl ;n =116) groups was performed. Bivariate comparisons were performed using two-tailed unpaired Student's *t* test with Welch's correction at a 95% confidence interval. Error bars represents standard error of means (SEM). The peripheral IFN-γ levels were comparable between the two clinical groups (P=0.173).

CHAPTER FIVE

DISCUSSION

The genetic mechanisms involved in susceptibility to multifactorial diseases such as SMA among immune-naïve children residing in *P. falciparum* holo-endemic areas remains partially understood. Using whole genome scans, a number of previously unknown candidate genetic markers associated with SMA have been identified. To further provide more insights into the understanding of this complex molecular mechanism, this study determined the associations between variations in cell receptors to immune molecules i.e. *NCR3* and SMA, a common clinical outcome of severe malaria in children from *P. falciparum* holo-endemic transmission areas was done.

5.1. Association between *NCR3* (-172A/G and -412C/G) genotypes/haplotypes and severe malaria Anaemia

To determine the role of individual *NCR3* genotypes/haplotypes in conditioning susceptibility to SMA, bivariate logistical regression analyses were carried out using the wild type in each variant as the reference group, while controlling for the confounding effects of age, gender, HIV-1, bacteremia, G6PD deficiency and sickle-cell trait because earlier studies have enumerated them as factors which individually alters susceptibility to SMA (Aidoo *et al.,* 2002; Otieno *et al.,* 2006b; Were *et al.,* 2011). Results obtained from this study of parasitemic children (n=455) aged 3-36 months, demonstrated that carriage of the *NCR3* (-412C/G) (CG) genotypes had 75% reduced odds of developing SMA (Hb<6.0g/dL) while *NCR3* (-172A/G*)* polymorphism relative to the wild type (-172,GG) did not reveal any association with the SMA (Hb<6.0g/dL). Previous data on *NCR3* (-412C/G) polymorphisms reported by others authors, obtained results

that were largely similar with each other. A study conducted in Burkina Faso reported that *NCR3* (-412C/G) polymorphism was associated with the risk of developing mild malaria attack but had no association with maximum parasitemia, which was based on the highest parasitemia in each individual during the study (Delahaye *et al.,* 2007). Similarly, a previous study confirmed that *NCR3* (-412C/G)-C carriers had significantly increased number of mild malaria episodes compared to the non-carriers in a Congolese cohort and further investigated it's potential of cis-regulatory effect (Baaklini *et al.,* 2017). Consistent with the Burkinabe and Republic of Congo population studies, the current case-control study reveal a similar pattern despite the differences in endemicities between the current study site in western Kenya and the two endemic malaria study regions in Burkina Faso and Repulic of Congo. In addition, the results were congruent despite the fact that the populations in the previous studies had different genetic and ethnic background as well as use of different study stratification and sample sizes. In conclusion, this might be a pointer that these genes may be of interest especially in relation to protection against development of disease once parastaemia is acquired. The fact that it protected against mild malaria in the previous studies could mean that the protective mechanisms associated with the gene could be beginning much earlier when populations acquire parasitemia and further eliminate the processes leading to development of severe disease. Mechanistic studies need to be designed across populations resident in different endemic areas to test this hypothesis.

Since haplotypes are important markers that often reveal how combinations of different functional polymorphic alleles interact to amplify or moderate disease outcome that are unidentified with SNPs (Ouma *et al.,* 2008a; Wilson *et al.,* 2005) haplotype between *NCR3* (-172A/G and -412C/G) were constructed (using HPlus software) so as to identify whether co-

inheritance of these-SNP combinations could influence susceptibility to SMA. In the current study, analyses using biavariate logistic regression, controlling for cofounders, revealed that children with -172C/-412G (CG) haplotype carriers are nearly five times at an increased risk of developing SMA compared to non-CG carriers using modified definition of SMA (Hb<6.0g/dl, and any density parasitemia). Even though the mechanism through which (GC) haplotype carriage increases susceptibility to SMA was not not determined in this study, the presence of (CG) haplotype may be promoting non-erythropoietic responses in children with *P. falciparum* malaria. The presence of this haplotype could creat an alteration of *NCR3* (-172A/G and -412C/G) promoter binding of transcription factors to *NCR3* (-172A/G and -412C/G) promoter region thus affecting downstream processes associated with non-responses to erythropoietic activities. A study of an independent population living in Central Africa, Congo demonstrated an association of *NCR3* (-412C/G) with mild malaria with evidence of its effect on the promoter activity and on the binding of nuclear proteins, including transcription factors, such as STAT4 and RUNX3 (Baaklini *et al.,* 2017). This observation confirms the findings in this study, that this haplotype could confer genetic predisposition to SMA in children naturally exposed to *P. falciparum* in holoendemic region of western Kenya.

5.2. Association between *NCR3* (-172A/G and -412C/G) genotypes/haplotypes and IFN-γ levels

Since variation in the *NCR3* genotypes/haplotypes may influence production of peripheral IFN-γ levels, by interacting with NK association between *NCR3* (-172A/G and -412C/G) genotypes/haplotypes and peripheral IFN-γ levels were explored. Results demonstrated that -172A/G genotypes and -172G/-412C haplotypes were significantly associated with elevated levels of peripheral IFN-γ. Conversely, prior analysis in the study demonstrated that *NCR3* (-

172A/G) genotype is not associated with susceptibility to SMA suggesting that its presence could confer protection role against pathogenesis of SMA through increased peripheral IFN-γ levels. Data on previous studies revealed different and largely conflicting immunogenetic outcome towards severe disease. For example, a study on Thai adults (Omi *et al.*, 2002) demonstrated that genotypic and haplotypic variation of IL12Bpro and IL12B 3' UTR on the adult population influences susceptibility to SMA and functional changes in circulating IL-12p40 and circulating IFN-γ levels. In addition, a study conducted on children (Ouma *et al.*, 2011) also demonstrated that FcγRIIIA 176V/TLR9-1237T (VT) SNPs combination which were associated with an increased risk of SMA was also associated with higher peripheral IFN-γ levels. Furthermore, two separate studies performed in Malawian and Gambian children (less than 5 years of age) with mixed clinical infection of cerebral and severe malaria anaemia did not show any association between TLR9 (-1237TC) polymorphisms and severe malaria (4 to 12 years). Again, study conducted by (Munde *et al.*, 2012) on FC gamma and Toll-like receptors failed to demonstrate existence of any association between the circulating IFN-γ levels and the individual genotypes.

In the present study, haplotypic carriage of -172G/-412C (GC) demonstrated statistical significant difference in normalized peripheral levels of IFN-γ suggesting that it plays an important role in production of elevated levels of IFN-γ in children with severe malaria infection. This study therefore hypothesizes that, the carriage of GC haplotypes enhances higher levels of peripheral IFN-γ that increases susceptibility towards SMA development. Even though the mechanism through which carriage of GC haplotype increases susceptibility to SMA remains less understood, it may be hypothesized that presence of the GC haplotype could be promoting non-erythropoietic responses in children infected with *P. falciparum* malaria through alteration

of IFN-γ promoter binding transcription factors to the IFN-γ promoter region. However, carriage of -172G/-412G and -172A/-412C haplotypes revealed no association with susceptibility to SMA.

5. 3. Comparision of Peripheral IFN-γ levels in children with SMA and non-SMA

Several previous studies have reported varied outcomes in the role of IFN-γ levels in the pathogenesis of SMA. For example, in malaria endemic region of Papua New Guinea showed high early IFN-γ levels production conferring protection against symptomatic malaria episodes in children aged between 5 to 14 years (D'Ombrain *et al.,* 2007). In addition, an earlier study carried out by Dodoo *et al.,* (2002) in a *P. falciparum* malaria endemic transmission region of southern Ghana reported that production of malaria-specific IFN-γ was associated with reduced risk of clinical malaria and fever. Taken together, these studies illustrates that elevated circulating IFN-γ levels production is associated with reduced clinical malaria. Nevertheless, other studies have shown association between higher levels of IFN-γ and severe malaria. For example, a study in Uganda reported a positive association between elevated IFN-γ levels and cerebral malaria (Sam-Agudu *et al.,* 2010). Similarly, a previous report by (Ong'echa *et al.,* 2011), on a population of children resident in western Kenya also showed that increased IFN-γ levels was a positive predictor of SMA. However, in this current study, children with SMA and non-SMA had comparable peripheral IFN-γ levels, a finding not consistent with a previous study conducted in same population (Ouma *et al.,* 2011). The differences in the current study results with those of the previous studies carried out in the same population could likely be attributed to differences in the stratification of the clinical 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), the populations were further stratified into several sub-groups i.e. into non-SMA (Hb levels of 6.0 to 10.9 g/dl; n=37) and uncomplicated malaria (UM)

(Hb levels of >11.0 g/dl; *n*=31) for the least-angle regression (LAR) analyses. Furthermore, potential underlying genetic variations linked to production of IFN-γ levels may potentially contribute to differences in functional changes during disease in the population and these underlying factors were never controlled for as a variable in the LAR analyses. The varied findings underscores the continuous complexity in analysis of date associated with the determination of host immune responses against severe malaria. Even though previous studies have shown that IFN-γ is involved in alternative activation of macrophages, a mechanism which is specialized for defense against extracellular pathogens (Martinez *et al.,* 2009), it is worth noting that *P. falciparum* is an obligatory intracellular pathogen (Breman *et al.,* 2004), and thus, the defense mechanism provided by alternative activation of macrophages may not absolutely aid in the defense against it. Furthermore, differences in the current results versus previous findings could be due to geographical and demographic differences which may concomitantly be accompanied by various selective pressures (Ayimba *et al.,* 2011; Quelhas *et al.,* 2012). For instance, the current pediatric population is naturally exposed to additional conditions such as other parasitic infections, which may independently alter IFN-γ production.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1. Summary of findings

The results of this study demonstrate that individual genotype of *NCR-3* (-412 C/G) (CG) had 75% lower odds of developing SMA (Hb <6.0g/dL). Similarly, the study results further showed that carriers of the -172G/-412C (GC) haplotypes were nearly five times at an increased risk of developing SMA (Hb < 6.0g/dL). In addition, the results also demonstrated that there were significant differences between *NCR3* -172 A/G genotypes and normalized peripheral IFN-γ levels. Further analysis, revealed that children with -172G/-412C (GC_1 carriers) haplotypes had a significantly higher peripheral IFN-γ levels than those of -172G/-412C (GC_0 carriers). However, there were no significant differences in peripheral IFN-γ levels between SMA and non-SMA children in this study population.

6.2. Conclusion

1. The results demonstrated that carriage of *NCR3* -172G/-412C (GC) haplotype and *NCR3* (-412 C/G) genotype is associated with susceptibility to SMA development in paediatric population at Siaya County Refferal Hospital.
2. Co-inheritance of variations in *NCR3* (-172A/G and -412C/G) polymorphisms conditions changes in the peripheral IFN-γ levels in children with severe malaria anaemia.
3. In this paediatric population of children three years and below presenting at SCRH in western Kenya, peripheral IFN-γ levels are similar between SMA and non-SMA groups.

6.3. Recommendations from current study

From the findings of this current study, it is recommended that these results should inform malaria immunogenetics since they enumerate vital information about *NCR3* -412C/G and -

172A/G genotypes and haplotypes which predisposes children between 3-36 months to severe malaria which further explains the SMA developments in some paediatrics and not others even though they are exhibiting similar malaria transmission intensity and infection rate in a holoendemic area of Siaya County Referral Hospital, in western Kenya.

6.4. Suggestion for future studies

1. To decipher the impact of surface receptors in development of innate immunity against malaria and disease outcome, immunonogenetic studies involving exclusive panel of receptor polymorphisms are warranted to determine if the responses characterized here may provide an immune-genetic basis for vaccine development that modulate receptor functions.
2. To enhance knowledge on the pathological basis of elevated peripheral IFN-γ levels in children residenting of *P.falciparum* holoendemic transmission regions westen Kenya, designing of more immunological studies should be considered.
3. Further studies are needed to explore the complex interaction between other genes in malaria signaling pathways and *NCR3* gene polymorphisms in the risk of malaria, especially in ethnically dispersed populations.

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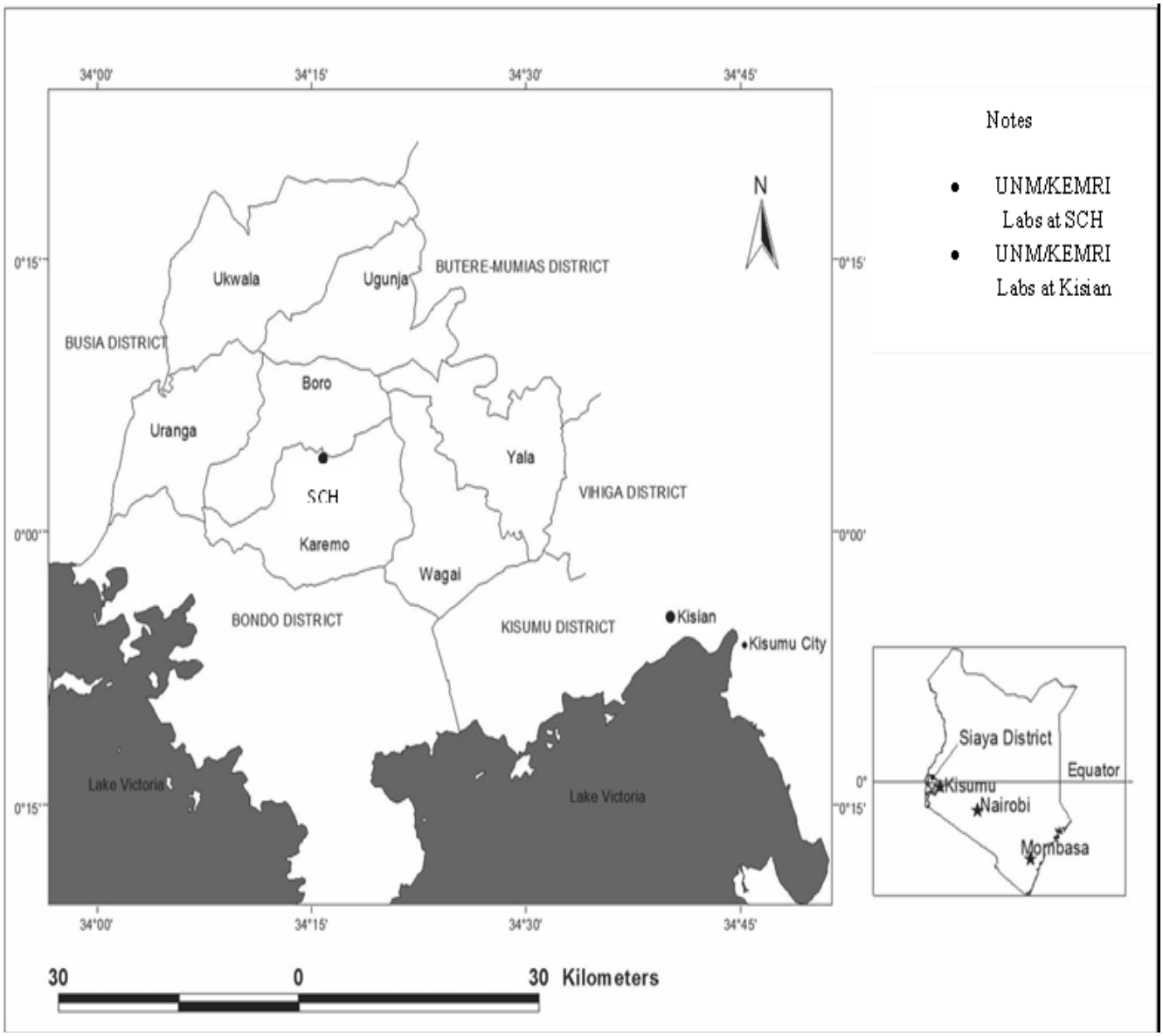
APPENDICES

Appendix I: Map of the study area



Location of Siaya County in western Kenya, adopted from (Ongecha *et al.* 2006)

Appendix II: School of Graduate Studies Research Proposal Approval Letter

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES
Office of the Dean

Our Ref: MSC/00103/2014

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

Date: 5th October, 2018

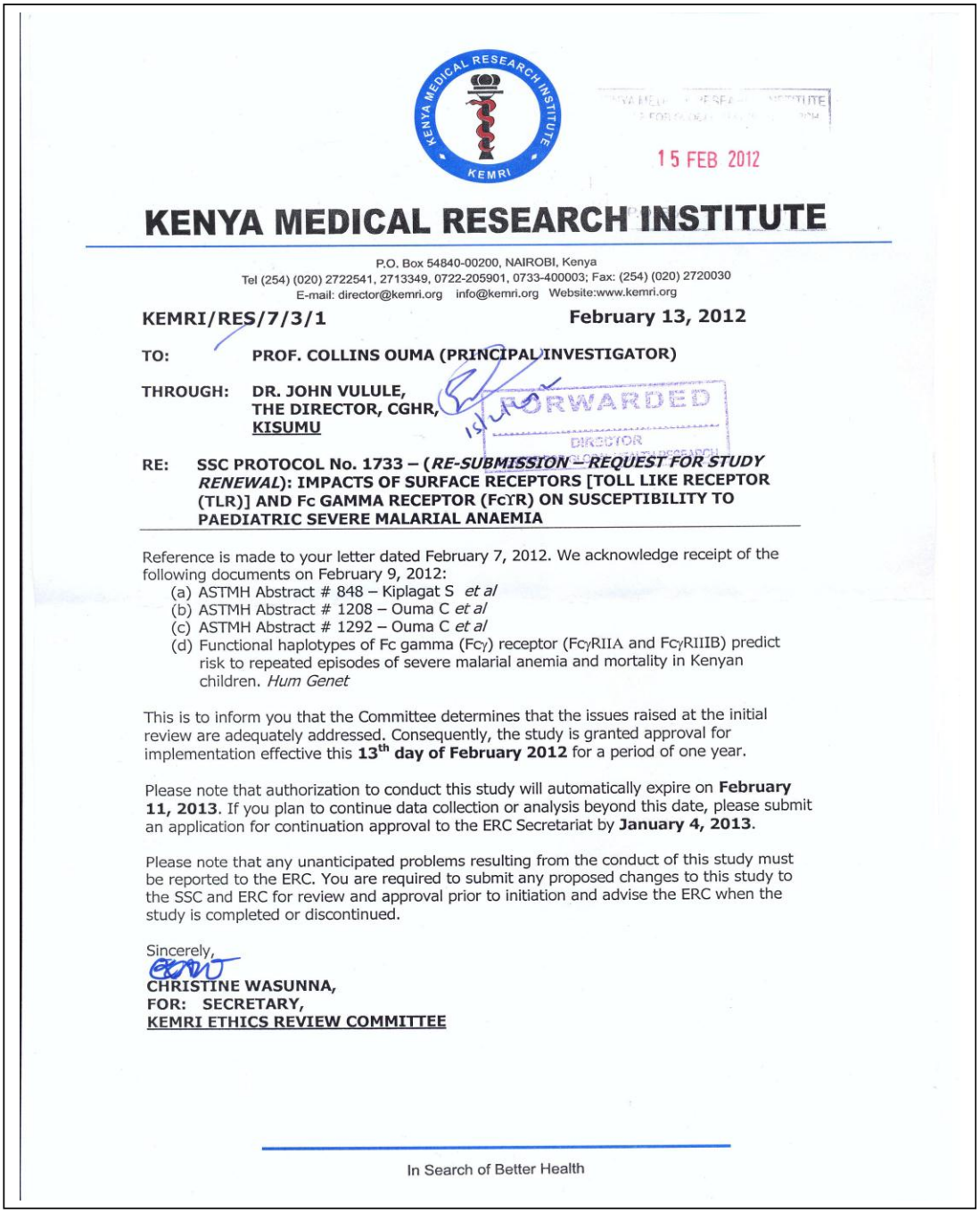
TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR JOAB DIRO MIDDII —MSC/00103/2014

The above named is registered in the Master of Science in Medical Biotechnology in the School of Public Health and Community Development, Maseno University. This is to confirm that his research proposal titled "*Association Between Genetic Differences in Natural Cytotoxicity Triggering Receptor-3 (NCR3-172A/G and NCR3-142C/G) and Susceptibity to Severe Malaria Anaemia in Children Presenting at Siaya County Referral Hospital.*" 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

Appendix III: Research Approval Letter



15 FEB 2012

KENYA MEDICAL RESEARCH INSTITUTE

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

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

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

FORWARDED
DIRECTOR

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

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

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

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

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

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

Sincerely,

CHRISTINE WASUNNA,
FOR: SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

In Search of Better Health