# ASSOCIATION BETWEEN NCR3 PROMOTER POLYMORPHISMS WITH *PLASMODIUM FALCIPARUM* MALARIA OUTCOMES IN PAEDIATRIC POPULATION (AGED BELOW 3 YEARS) IN SIAYA COUNTY IN WESTERN KENYA

**BY**

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## DECLARATION

**Declaration by the student:**

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

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**Declaration by the supervisors:**

This thesis has been submitted for examination with our approval as university supervisors.

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Finally, I would like to express my profound gratitude to my parents for their unfailing support, my brothers and sisters for their continuous encouragement and my friends for their spiritual support and motivation during my study, through the process of research and during thesis writing.

## DEDICATION

This thesis is dedicated to mum, dad, Edwin, Illiane, Jack, Lawino, Clare and William.

## ABSTRACT

Malaria is the main cause of paediatric morbidity and mortality in holoendemic areas. The populations in these areas have similar transmission intensity and infection rates of *Plasmodium falciparum* malaria but present different malaria outcomes. The cause of the different clinical outcomes is poorly understood. *Pf.* has exerted selective pressure on the human genome leading to genetic variability in the host's immune response genes mediating protection and susceptibility to *Pf.* malaria. Natural Cytotoxicity Triggering Receptor 3 directly interacts with ligands on parasitized Red Blood Cells activating natural killer cells to carry out cell-mediated cytotoxicity of *Pf.* pRBCs. However, the contribution of NCR3 promoter polymorphisms in conditioning malaria disease outcomes such as acquisition of parasitaemia, severe malaria anaemia (Hb<5.0 g/dL, any density parasitaemia) and high density parasitaemia (≥10000 parasites/µL) in paediatric population in a holoendemic area has not been elucidated. Therefore, the study determined the association between NCR3 (-412C/G and -172G/A) genotypes and haplotypes, and acquisition of parasitaemia, high density parasitaemia, and severe malaria anaemia in a paediatric population presenting at Siaya County Hospital. The study assayed archived blood spot samples (n = 612) of children (aged 3-36 months) presenting with severe malaria anemia and controls of similar age and gender. The samples were genotyped for NCR3 (-412C/G and -172G/A) polymorphisms using TaqMan real-time PCR technique. Haplotypes were constructed using HPlus Version 2.5. Logistic regression analyses controlling for the confounding effects of age, gender, HIV-1, sickle-cell anemia, G6PD and bacteremia, results showed that there was no association between NCR3 (-412C/G and -172G/A) genotypes/haplotypes with acquisition of parasitaemia. However, the NCR3 -412GG genotype was associated with HDP (GG, OR 0.469, 95% CI; 0.252-0.873, $P$ = 0.017) while the NCR3 -412CG genotype was associated with increased risk to SMA (CG, OR 1.636, 95% CI; 1.018-2.631, $P$ = 0.042). The carriage of CC (NCR3 -412C and -172C) haplotype was associated with a risk to HDP (OR 1.934, 95% CI; 1.104-3.389, $P$ = 0.021) while the GC (NCR3 -412G and -172C) haplotype was associated with increased susceptibility to SMA (Hb<5.0 g/dL) (OR 1.635, 95% CI; 1.015-2.634, $P$ = 0.043). Taken together, these results demonstrate that NCR3 (-412C/G and -172G/A) promoter variants condition malaria outcomes in paediatrics in a holoendemic area. Future studies should determine how other genetic factors work together with NCR3 promoter variants to condition malaria outcomes among paediatric population and this can provide an insight to the causal link as well as pharmaceutical interventions.

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## ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **µL** | Microlitre |
| **DBL-1α** | Duffy Binding Like-1 alpha |
| **DNA** | Deoxyribonucleic Acid |
| **EDTA** | Ethylenediamine tetraacetic acid |
| **G6PD** | Glucose-6-Phospate Dehydrogenase |
| **Hb** | Haemoglobin |
| **HBB** | Haemoglobin Beta |
| **HbC** | Haemoglobin C |
| **HbE** | Haemoglobin E |
| **HbS** | Haemoglobin Sickle |
| **HDP** | High Density Parasitaemia |
| **HIV** | Human Immuno-deficiency Virus |
| **iRBCs** | Infected Red Blood Cells |
| **MIF** | Macrophage Inhibitory Factor |
| **NCR3** | Natural Cytotoxicity-triggering Receptor 3 |
| **PCR** | Polymerase Chain Reaction |
| ***Pf.*** | *Plasmodium falciparum* |
| **pRBCs** | Parasitized Red Blood Cells |
| **SMA** | Severe Malaria Anaemia |
| **SNPs** | Single Nucleotide Polymorphisms |
| **TNF** | Tissue Necrosis Factor |
| **WHO** | World Health Organization |

## DEFINITION OF TERMS

**Genotype** – a set of genes responsible for a particular trait or the genetic constitution of an organism.

**Haplotype** – a group of alleles in an organism that are inherited together from a single parent or a set of linked single nucleotide polymorphism alleles that tend to always occur together i.e. that are associated statistically.

**High density parasitaemia** – parasitaemia of ≥10000 parasites/μL and above it, immune patients will exhibit symptoms (WHO, 2000a).

**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 (WHO, 2000a).

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

**Sub-microscopic parasitaemia** – refers parasitaemia below the limit of detection of microscopy or rapid diagnostic tests (RDT), but detectable using molecular or other highly sensitive diagnostic methods (WHO, 2017).

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# CHAPTER ONE

# INTRODUCTION

## 1.1 Background information

Human malaria is caused by intracellular obligate protozoan parasites of the genus *Plasmodium* and *Plasmodium falciparum* is the most virulent (WHO, 2008). In 2015, it was reported that 429,000 deaths from malaria globally occurred, with most deaths estimated to have occurred in the WHO African Region (92%) (WHO, 2016). It was also estimated that 70% of these deaths in the African region occur among children aged below 5 years and were attributed principally to *P. falciparum* malaria (99%) (WHO, 2016). Thus, *Pf.* malaria still remains the main cause of mortality in children.

As much as *P. falciparum* malaria may not display any symptoms, when they manifest, they do so as fever, chills, sweating, headache and muscle aches (Marsh *et al.,* 1995). Moreover, life-threatening indicators of malaria are hyper-parasitaemia, hypoglycemia, hyper-lactatemia, kidney failure, metabolic acidosis, cerebral malaria, severe malarial anemia, and respiratory distress (Marsh *et al.,* 1995). In areas where malaria prevalence is high and the infection starts early in life, high density parasitaemia (HDP≥10,000 parasites/µL) and severe malarial anaemia (Hb<5.0g/dL, any density parasitaemia) are the common clinical manifestations (Bloland *et al.,* 1999). Children age 3-36 months are most to malaria infection as maternal antibodies that confer protection begin to wane after 3 months and protective immunity to malaria begin to developed after 36 months (Obonyo, Vulule, Akhwale, & Grobbee, 2007).

Malaria exerts a great selective pressure on the human genome (Tishkoff & Williams, 2002) causing certain alleles to exist in high frequencies in areas where malaria infection is common. For instance, HbS allele causing sickle haemoglobin is observed to be of high frequency despite its effect on sickle-cell individuals (Flint, Harding, Boyce, & Clegg, 1998)

and in *HBB, Glu6Val* (HbS) (Flint *et al.,* 1998), *Glu6Lys* (HbC), and *Glu26Lys* (HbE) alleles protect against malaria (Tishkoff & Williams, 2002).

On the other hand, the genetic factors of the host influence malaria outcomes (Adel *et al*., 2011; Kwiatkowski, 2005; Mackinnon, Mwangi, Snow, Marsh, & Williams, 2005) and susceptibility to malaria infection (Verra, Avellino, Bancone, Mangano, & Modiano, 2008; Verra, Mangano, & Modiano, 2009; Weatherall, 2008).

Polymorphisms in innate immune response genes of the host have been shown to condition malaria outcomes. For instance, *TNF* gene polymorphisms have been associated with mild malaria and maximum parasitaemia (based on a logarithmic transformation of the highest parasitaemia in each individual) (Flori *et al.,* 2005) and IL-10 promoter variants -1,082G/-819C/-592C (GCC) haplotypes were associated with protection against SMA as individuals with the -1,082A/-819T/-592A (ATA) haplotype had an increased risk of SMA (Ouma, Davenport, Were *et al*., 2008).

Natural Cytotoxicity-triggering Receptor 3 (*NCR3*) gene is located in the MHC class III region in the highly polymorphic MHC locus, just 15 kb away from the *TNF* gene (Moretta *et al*., 2001) and the MHC locus has been linked to mild malaria attacks (Flori *et al.,* 2005). Furthermore, Natural Cytotoxicity-triggering Receptor 3 that is encoded by the *NCR3* gene facilitates cell cytotoxicity and production of interferon-γ upon contact with *Pf.* iRBCs (Artavanis-Tsakonas *et al*., 2003; Baratin *et al*., 2007). These studies suggest that *NCR3* gene polymorphisms, just like *TNF* gene polymorphisms, may condition malaria disease outcomes. NCRs also confer protective immune responses against intracellular pathogens such as influenza, hepatitis C (De Maria *et al.,* 2007), West Nile (Hershkovitz *et al.,* 2009), and Ebola (Fuller *et al.,* 2007) emphasizing their importance in conferring immunity to intracellular pathogens. However, the role of NCR3 promoter variants (-412C/G and -172G/A) in

conditioning malaria outcomes, especially in paediatric populations resident in holoendemic *P. falciparum* regions remains poorly understood.

Of the NCR3 (-412C/G and -172G/A) variants, NCR3 -412 polymorphism has been associated with the risk of developing mild malaria among siblings in malaria endemic areas of Burkina Faso (Delahaye, Barbier, Fumoux, & Rihet, 2007). However, in holoendemic areas of western Kenya, no studies have described the effect of polymorphic variants of NCR3 promoter (-412C/G and -172G/A) on malaria outcomes in a paediatric population.

The international haplotype map project describing the common patterns of human genetic variation and their effects on health and disease flagged NCR3 -412C/G and -172G/A as having allele frequencies of over 10% in a holoendemic malaria transmission area (HapMap, 2003). Moreover, high density parasitaemia (HDP≥10,000 parasites/µl) and severe malarial anaemia (Hb<5.0g/dL) are the common malaria disease outcomes in a holoendemic malaria transmission area such as in western Kenya (Bloland *et al.,* 1999). As such, this study investigated whether the NCR3 promoter polymorphisms (-412C/G and -172G/A) are associated with the acquisition of parasitaemia, susceptibility to severe malarial anemia (Hb<5.0g/dL, any density parasitaemia) and high density parasitaemia (HDP≥10000 parasites/µL) in a pediatric population resident in a holoendemic *P. falciparum* transmission area of Siaya County in western Kenya.

## 1.2 Statement of the problem

Malaria is a health burden on the paediatric population in holoendemic areas. The population in holoendemic areas experience similar transmission intensity and infection rates of *P.f* malaria but present different paediatric malaria outcomes. The cause of differential clinical outcomes in these areas remains poorly understood. The causative agent, *Plasmodium falciparum,* exerts a strong selective pressure on the human genome especially on the host immune response genes leading to gene variants that may condition malaria outcomes. As a result, various genetic adaptations to malaria have arisen and these polymorphic variants of immune response genes have been demonstrated to condition other malaria outcomes. Although genetic polymorphisms have been associated with malaria outcomes, there has been limited investigations on class III region particularly the polymorphisms of the *NCR3* gene promoter region. Therefore, this study focused on the association between *NCR3* gene promoter variants -412C/G and -172G/A and malaria outcomes common in holoendemic areas so as to answer the question of why malaria outcomes manifest differently in children exposed to the same transmission intensity and infection rates.

## 1.3 Objectives of the study

### 1.3.1 General objective

To determine the association between NCR3 (-412C/G and -172G/A) genotypes and haplotypes, and malaria outcomes in a paediatric population in Siaya County in western Kenya.

### 1.3.2 Specific objectives

i. To determine the association between NCR3 (-412C/G and -172G/A) genotypes and haplotypes, and acquisition of parasitaemia in paediatric population in Siaya County in western Kenya.

ii. To determine the association between NCR3 (-412C/G and -172G/A) genotypes and haplotypes, and high density parasitaemia (HDP≥10,000 parasites/μL) in paediatric population in Siaya County in western Kenya.

iii. To determine the association between NCR3 (-412C/G and -172G/A) genotypes and haplotypes, and SMA (Hb<5.0g/dL) in paediatric population resident in Siaya County in western Kenya.

### 1.3.3 Null hypothesis

i. There is no association between NCR3 (-412GC and -172AG) genotypes and haplotype, and acquisition of parasitaemia in paediatric population in Siaya County in western Kenya.

ii. There is no association between NCR3 (-412GC and -172AG) genotype and haplotypes, and high density parasitaemia (HDP≥10,000 parasites/μL) in paediatric population in Siaya County in western Kenya.

iii. There is no association between NCR3 genotypes and haplotypes, and SMA (Hb<5.0g/dL) in paediatric population in Siaya County in western Kenya.

### 1.4 Significance of the study

Based on the results of the study, the following are likely to be the beneficiaries:

i. Genetic epidemiologists of malaria are likely to benefit. This is because some NCR3 -412C/G and -172G/A genotypes and haplotypes have been associated with malaria outcomes i.e. NCR3 GG genotype and the CC (NCR3 -412C and -172C) haplotype were associated with HDP (≥10,000parasites/μL). Such findings are important as they may explain 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.

ii. The Ministry of Health is also likely to benefit. Children <3 years of age have been found highly susceptible to severe malaria anaemia in holoendemic areas leading to high morbidity and mortality rates (Obonyo *et al*., 2007). Genotypes and haplotypes that are associated with SMA (Hb≤5.0g/dL, any density parasitaemia) for instance NCR3 -412 CG genotype and GC (NCR3 -412C and -172C) haplotype could be screened in malaria naïve children to identify those who are vulnerable as a health policy for further interventions to prevent them from being susceptible to SMA.

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

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Pathogenesis of *P. falciparum* malaria

Malaria is a life threatening disease caused by parasites of the *Plasmodium* species and is transmitted through the bite of infected female anopheles mosquitoes (WHO, 2014). The pathogenic process of malaria occurs in the asexual blood stage of the parasite, when merozoites invade and develop in erythrocytes (Louis, Michael, & Genevieve, 1994). *Plasmodium* spp. require specific ligand-receptor interactions which activate the process of entry into red blood cells (Cowman & Crabb, 2006). Multiple ligands stored in the micronemes and rhoptries are released from these secretory organelles to bind receptors of the RBCs, then the parasite undergoes reorientation, putting its apex onto the host cell membrane forming a tight junction (Cowman, Drew, & Jake, 2012).

The merozoites enter the RBC through the tight junction which moves across the surface to the posterior where a parasitophorous vacuole is formed in the host cell, a boundary between the host and the parasite creating an environment for survival and replication(Soldati-Favre, 2008). The merozoites then grow, replicate and rapture RBCs releasing merozoites which invade healthy RBCs (Soldati-Favre, 2008). When RBCs get infected, they express parasite-encoded proteins on their surfaces and when raptured, parasite products which cause malaria symptoms are released (Miller, Baruch, Marsh, & Doumbo, 2002).

Following an infection, the innate immunity responds first to the invading pathogen influencing the development of acquired immunity (Riley & Stewart, 2013) that has been shown to curtail the initial growth of blood-stage parasites of the *Plasmodium* species upon infection (Langhorne, Ndungu, Sponaas, & Marsh, 2008). Therefore, the focus of this study

was on the paediatric population because they are especially vulnerable since their naturally acquired immunity to malaria is still naïve.

Among the immune cells, the NK cells become activated early during malaria infection to confer innate immunity (Orago & Facer, 1991) and *ex vivo*, they are directly activated by malaria antigens (Theander *et al.*, 1987). They form direct and stable interactions with pRBCs (Artavanis-Tsakonas *et al*., 2003). Following the interaction, NK cells are among the first blood cells to produce IFN-γ as an immune response to *P. falciparum* infected RBCs (Artavanis-Tsakonas *et al*., 2003; Baratin *et al*., 2007).

In addition to producing IFN-γ, they also produce other cytokines, and chemokines as immune responses against pathogen infected cells (Vivier, Tomasello, Baratin, Walzer, & Ugolini, 2008). The cytokines they produce include tumor necrosis factor (TNFα) and G-CSF whereas the chemokines they produce include macrophage inflammatory factor (MIP-1α/β) and RANTES (Fauriat, Long, Ljunggren, & Bryceson, 2010). NK cells are also very cytotoxic due to granzymes and perforins that are released when these cells are activated (Biassoni *et al*., 2001). These NK cell activities enable them to eliminate recognized pathogen-infected cells such as *Pf.* iRBCs effectively.

These studies show that in the case of paediatric population, in the absence of adaptive immunity, the innate immunity is responsible for clearing of parasites and infected RBCs in order to eliminate disease. In addition, the NK cells that are activated by NCR3 early in innate immunity play a critical role in the elimination of malaria from the body. Thus the study narrowed down on NCR3 polymorphisms to find out what causes the different outcomes in paediatric population exposed to the same infection rates and transmission intensity of malaria.

## 2.2 Role of Natural cytotoxicity-triggering receptor 3 in malaria

Natural cytotoxicity receptor 3 is a type 1 trans-membrane receptor (Li, Wang, & Mariuzza, 2011) and is expressed constitutively on the surface of NK cells (Moretta *et al*., 2001). Structurally, they comprise of an extracellular Ig domain and a trans-membrane region with adaptor molecules (Pende *et al.*, 1999). Following the interaction of NCR3 with their ligands, these adaptor molecules promote intracellular signal transmission through immune-receptor tyrosine-based activating motifs (ITAMs) that activate NK cells (Pessino *et al.*, 1998). This activation of NK cells through NCR3 (NKp30 or CD337) occurs independent of the MHC molecules (Moretta *et al*., 2001).

NK cells can be activated due to the presence of *P. falciparum* asexual blood stages in two ways: direct recognition of the parasitized RBCs by NK cell receptors and the release of cytokines (Korbel, Finney, & Riley, 2004). Parasite-derived proteins are inserted on the infected RBC membrane surface in knob-like protrusions (Kyes, Horrocks, & Newbold, 2001). These protruding knobs consist of a Duffy Binding-like α domain (DBL1-α) that provides anchorage for *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP-1) (Howard *et al.*, 1988). In direct recognition of pRBCs, NCR3 binds DBL1-α specifically in the absence of accessory molecules (Mavoungou, Held, Mewono, & Kremsner, 2007).

*Pf* infected erythrocytes are targeted for cell-mediated cytotoxicity and NK cells directly destroy them via cell-mediated cytotoxicity (Mavoungou, Luty, & Kremsner, 2003). NK cells and cytotoxic T lymphocytes (CTLs) are the key players in cell-mediated cytotoxicity of which NK cells performs it in the innate immune system while CTLs in adaptive immune response (Trinchieri, 1989). The NK cells being the effectors of innate immunity and the

recognition of pRBCs by NCR3 activates them, the role of *NCR3 gene* promoter was linked to the different outcomes of malaria displayed by paediatrics in a holoendemic area.

### 2.3 The role of promoter variability in conditioning malaria outcomes

There is increasing evidence showing that variability in the promoter region of immune and inflammatory genes affect malaria disease outcomes. In the case of inflammatory genes, it has been shown that the disease process of malaria is influenced by polymorphisms in the promoter and/or coding regions (Burgner *et al*., 2003; Hobbs *et al*., 2002; Ouma, Davenport, Were *et al*., 2008). The R/R131 genotype of Fcγ receptors that binds IgG1 and IgG3 (Bouharoun-Tayoun, Oeuvray, Lunel, & Druilhe, 1995) is associated with protection against intermediate levels of parasitaemia (>5,000 parasites/μL) (Shi *et al*., 2001) and was associated with protection against HDP (>10,000 parasites/μL) in a holoendemic *P. falciparum* transmission area of western Kenya (Ouma *et al.,* 2006).

Polymorphic variability in the IL-10 promoter (-1,082G/A, -819T/C and -592A/C) is associated with susceptibility to SMA and functional changes in circulating IL-10, TNF, and IL-12 concentrations in children (aged <3 years) in a holoendemic *P. falciparum* transmission area (Ouma, Davenport, Were *et al.,* 2008). Polymorphic variability in the IL-1β promoter (-31C/T and -511G/A) is also associated with increased susceptibility to SMA and functional changes in circulating IL-1β concentrations (Ouma, Davenport, Were *et al.,* 2008).

Polymorphism at the macrophage migration inhibitory factor (MIF) -173 is associated with HDP (≥10,000 parasites/μL), SMA (Hb<5.0g/dL) and functional changes in the production of MIF in response to stimulation by malaria parasite products (*pf*Hz) in children with malaria (Awandare *et al.,* 2006). With respect to *NCR3* gene, NCR3 -412 locus has been associated with parasitaemia and mild malaria in adult population in malaria endemic areas of Bukina

Faso (Afridi, Atkinson, Garnier, Fumoux, & Rihet, 2012). These evidences show that polymorphisms of the *NCR3* gene promoter may also be associated malaria outcomes, and may explain the different malaria outcome observed in paediatrics exposed to the same transmission intensity and infection rates of malaria.

NCR3 variants -412C/G and -172G/A have been reported by International Haplotype Map project to have a mutant allele frequencies of over 10% in reference African Yoruba population in a holoendemic malaria transmission area (dbSNP: http://www.ncbi.nlm.nih.gov/SNP/, HAPMAP: http://www.hapmap.org/index.html.en) making NCR3 -412C/G and -172G/A variants of special interest in areas where malaria is holoendemic. However, despite the increased variation in these positions, their association with malaria outcomes remains unknown. Despite the fact that NCR3 binds neo-antigens expressed on iRBC activating NK cells, HDP (≥10,000 parasites/µL) and SMA (Hb<5.0 g/dL) are the most common malaria outcomes in holoendemic areas and the fact that pediatric populations are at an increased risk of these disease outcomes, the association between NCR3 -412C/G and -172G/A genotypes and haplotypes and malaria disease outcomes in paediatric population in holoendemic regions such as in Siaya County in western Kenya, remains unestablished.

**2.4 Malaria disease outcomes**

The pathogenic and protective outcomes of malaria are influenced by the interaction between the host's immune cells and the parasite (Abdalla & Pasvol, 2004). In holoendemic transmission areas, the common clinical outcomes of malaria are high density parasitaemia (HDP≥10,000 parasites/µL) and severe malaria anaemia (Hb<5.0 g/dL, any density

parasitaemia) (Bloland *et al.*, 1999). Children have been reported to mostly display SMA in holoendemic malaria regions (Novelli *et al*., 2010).

By definition, anaemia is the presence of low Hb levels in relation to age, gender, and physiological status of an individual in a defined geographical context (Murrray, 1996). In sub-Sahara Africa, children below 5 years with Hb<11.0g/dL are considered anemic (Murrray, 1996). Moreover, in areas of high prevalence of malaria and HIV in sub-Sahara Africa, other factors such as frequent infections, deficiency of micronutrients and improper feeding practices may predispose infants to anaemia (Lartey, 2008). As a result, the WHO more specifically defines anaemia caused by severe malaria (SMA) as Hb<5.0g/dL or haematocrit<15% in the presence of any density parasitaemia (WHO, 2000a).

The exact definition of SMA may differ depending on the geographic region. For instance, a previous study that carried out >14 000 longitudinal Hb measurements in children (aged<48 months) in holoendemic western Kenya (McElroy *et al*., 1999) defined SMA as Hb<6.0 g/dL. However, the World Health Organization (WHO) definition of SMA of (Hb < 5.0 g/dL) was used in this study to give it a global context.

Aetiology of SMA includes dyserythropoiesis (Phillips *et al.*, 1986), lysis of infected and uninfected RBCs, splenic sequestration of RBCs (Buffet, Safeukui, Milon, Mercereau-Puijalon, & David, 2009), and bone marrow suppression (Abdalla, Weatherall, Wickramasinghe, & Hughes, 1980). SMA may also occur as a result of co-infections with HIV(Otieno, Ouma, Ong'echa *et al*., 2006), bacteremia(Berkley *et al*., 2005), hookworms and chronic malaria transmission (Davenport *et al*., 2010). Since the effect of co-infections were controlled for in the analyses in the current study, the key factors causing SMA were confined to parasite and host-driven haemolysis together with dyserythropoiesis.

Studies have identified polymorphisms in immune response genes that have significant relationships with both the risk of developing SMA and functional changes in their respective gene products i.e. variation in the Macrophage Inhibitory Factor (MIF) promoter influences susceptibility to SMA and peripheral production of MIF (Awandare *et al*., 2006). The IL-1β promoter haplotype -31C/ -511A (CA) was associated with increased risk of SMA (Hb < 6.0 g/dL) and reduced circulating IL-1β levels (Ouma, Davenport, Awandare *et al*., 2008).

Another study on the relationship between common IL-10 promoter variants (-1,082G/A, -819T/C, and -592A/C), SMA (Hb < 6.0 g/dL), and circulating inflammatory mediator levels (i.e., IL-10, TNF-α, IL-6 and IL-12) in Kenyan children in a holoendemic region showed that -1,082G/ -819C/ -592C (GCC) haplotype was associated with protection against SMA and increased IL-10 production. Individuals with the -1,082A/ -819T/ -592A (ATA) haplotype had an increased risk of SMA and reduced circulating IL-10 levels (Ouma, Davenport, Were *et al.,* 2008). These previous observations raised the prospects that the *NCR3* gene promoter polymorphisms may also be associated with SMA.

The earlier studies suggest that promoter polymorphisms of innate immune response genes can either lead to susceptibility to SMA or protection against SMA in children (aged 3-36 months) in a holoendemic area in western Kenya. As such, NCR3 -412C/G and -172G/A genotypes and haplotypes were associated with SMA (Hb<5.0g/dL) to investigate why there is differential development of disease by some paediatric population as much as they get the same malarial exposure in holoendemic region of western Kenya. The current study therefore focused on SMA in children (aged 3–36 months) since they are highly susceptible to severe anaemia in the study area (Obonyo *et al.*, 2007).

In adults with *Pf.* malaria, a high density parasitaemia of 100,000 parasites/µL is associated with severe clinical illnesses, complications and mortality (Hassan & Tariq, 2008) but a parasite density below this level is well tolerated by adults without individuals developing any complications in holoendemic areas (Sowunmi, Walker, & Salako, 1992). On the other hand, the clinical manifestations in children reach their highest in those below 5 years of age but decreases later, whereby children are parasitaemic but asymptomatic (Sowunmi *et al*., 1992). This observation is seen because of the development of immunity to infection as children grow older (Prybylski, 1999). It is on this background that the association between NCR3 (-412C/G and -172G/A) genotypes and haplotypes and malaria disease outcomes in a paediatric population in Siaya County in western Kenya, was investigated.

The confounding effects were controlled for because they have been demonstrated to cause anaemia that is different from SMA: glucose-6-Phosphate Dehydrogenase deficiency RBCs to breakdown prematurely causing haemolytic anaemia (Cappellini & Fiorelli, 2008), HIV-1 (+) children with *P.f* malaria have been shown to display more profound anaemia and they had a significantly more mortality than HIV-1 (-) *P.f* (+ ) children (Otieno, Ouma, Ong'echa *et al*., 2006), bacteremia has also been associated with developing low Hb concentrations compared wth abacteremia children with malaria (Were *et al*., 2011) and children with homozygous sickle-cell trait develop low Hb concentrations (Aidoo *et al*., 2002). Age was controlled for since younger children are more vulnerable to SMA while gender was controlled for because it has been shown that male children are more likely to be hospitalized (Ong'echa *et al*., 2006).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Study Area

Blood spot samples used in the present study were obtained from a larger study carried out among a paediatric population in Siaya County, western Kenya (Figure 1). Siaya County Hospital is the major government hospital for the population living in Siaya County as well as the neighboring counties (Ong'echa *et al.,* 2006). In the study area, malaria is the primary cause of childhood morbidity and mortality, whereas SMA and high density parasitaemia are the most common clinical outcomes (Bloland *et al.,* 1999; Ong'echa *et al.,* 2006).

The mosquito vectors in this area are commonly *Anopheles gambiae s.s, Anopheles arabiensis,* and *Anopheles funestus* (Beach *et al.,* 1993) and *Pf.* malaria transmission is holoendemic (Ong'echa *et al*., 2006) and its prevalence is approximately 83% in children below 4 years of age, with severe disease manifesting as severe anemia and/or high-density parasitaemia (HDP) (Obonyo *et al.*, 2007).

Siaya County was chosen for this study since it's a holoendemic malaria transmission area and all ages risk exposure to malaria but the paediatric population is mostly susceptible to malaria disease as they lack acquired immunity to malaria obtained from repeated exposures (WHO, 2015). This population from holoendemic regions of western Kenya suffers from SMA and HDP (≥10,000 parasites/μL) as the most common clinical manifestations in this area (Bloland *et al*., 1999).

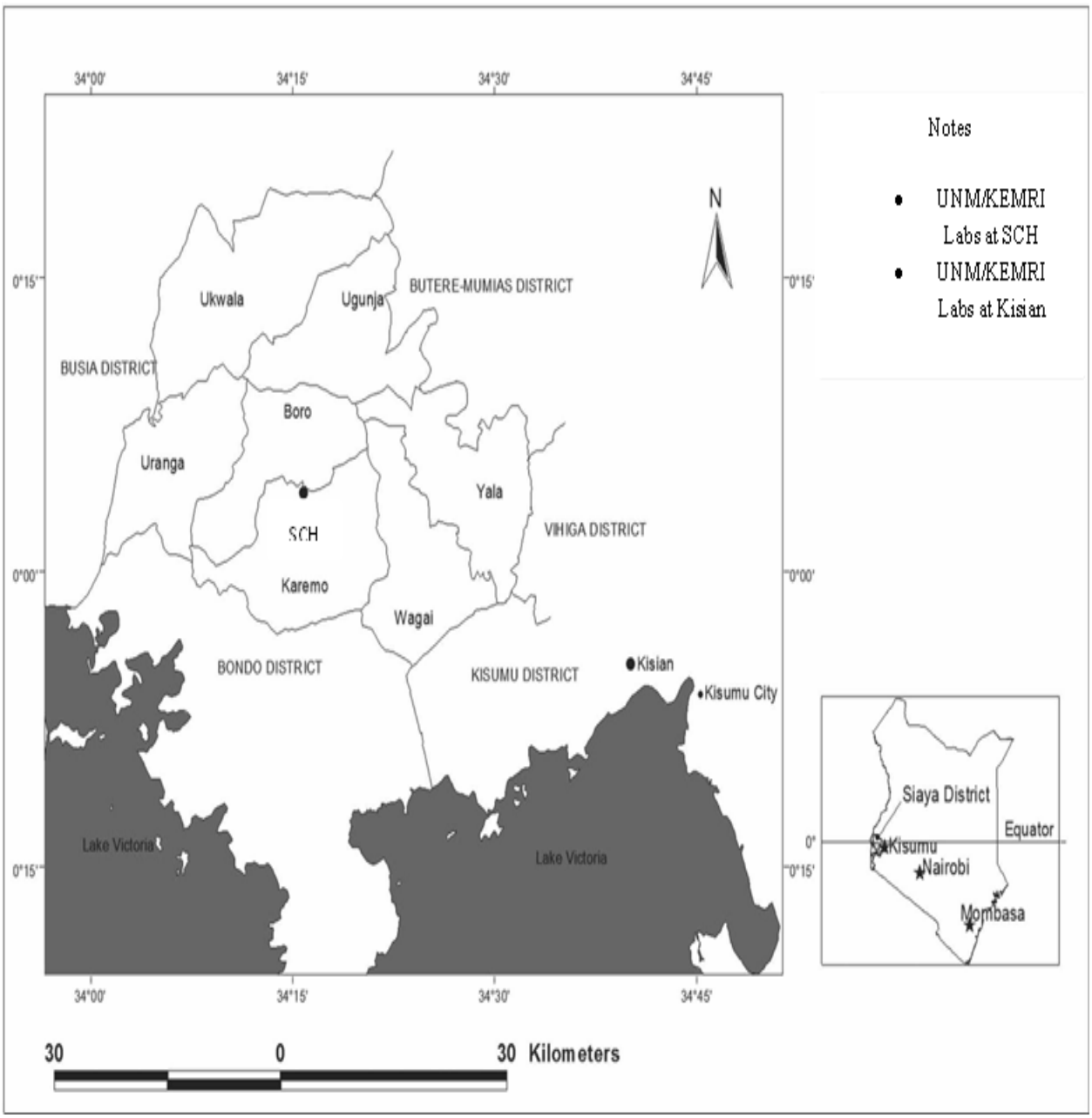


**FIGURE 3.1:** Location of Siaya District (currently Siaya County) in western Kenya, adopted from (Ong'echa *et al.*, 2006).

### 3.2 Study Design and patient population

#### 3.2.1. Recruitment of study participants

The study used archived blood samples from a case-control study collected in 2012. In the study, children were recruited at Siaya County Hospital as per previously established protocols (Ong'echa *et al.*, 2006). Children (aged 3-36 months) of both sexes were recruited in Siaya County Hospital in western Kenya during their first hospitalization for the treatment of malaria using questionnaires and existing medical records. The participants had positive smears for asexual *P. falciparum,* with acquired parasitaemia, high density parasitaemia (HDP≥10,000 parasites/μL) and severe malarial anaemia (Hb<5.0 g/dL, any density parasitaemia). Controls were children of similar age and same gender with non-parasitaemic and non-severe malaria anemia. The presence of parasitaemia was determined by the following features: a positive blood smear showing asexual *P. falciparum* malaria and oral/axillary temperature >37.5 °C or two of the following: nausea/vomiting, diarrhoea, headache, myalgia, poor feeding.

#### 3.2.2. Screening and enrolment

The following screening process was used to target selected groups of parasitaemic and non-parasitaemic children and children with SMA and non-SMA. The recruitment process involved screening and enrolment. The parent/legal guardian of the child received an explanation of the study before informed consent was obtained for all participants. It was clarified that enrolment decision was to be made after initial HIV-1 screening of the child and obtaining an informed consent (Appendix 2). Questionnaires were used to collect relevant demographic and clinical information. Written informed consent was administered in the participant's language of choice (i.e. English, Kiswahili or Dholuo).

Venous blood samples (<3.0 mL) were collected in Ethylenediamine tetraacetic acid (EDTA) containing tubes after screening for grouping of participants, prior to any treatment intervention or any supportive care. Blood samples were used for malaria diagnosis, complete hematological profile measurements, HIV testing, bacterial culture and genetic analyses. Based on the HIV-1 test results, malaria parasitaemia, and haemoglobin (Hb) status, children that satisfied all inclusion criteria were enrolled into the study. All participants promptly received appropriate antimalarial treatment and required supportive therapy as per the Kenya Ministry of Health (MoH) guidelines.

The children with acute malaria were stratified into non-severe malarial anemia (non-SMA) group: Children with a positive smear for asexual *P. falciparum*, parasitaemia (of any density) and Hb≥5.0 g/dL and severe malarial anemia (SMA): Children with a positive smear for asexual *P. falciparum*, parasitaemia (of any density) and Hb<5.0 g/dL (WHO, 2000a).

### 3.2.3. Inclusion criteria

Malaria parasitaemia (any density) and non parasitaemic controls, and SMA (Hb<5.0g/dL) and non-SMA controls; aged ≥3 months and ≤36 months; has been resident in the study area for the last 3 years; parent/guardian who were willing and able to sign consent form; parent/guardian who were able to keep schedule and study appointments; parent/guardian living ≤15km to the hospital; ability to provide two contacts familiar with the child's whereabouts during the study period according to previously established protocols (Ong'echa *et al*., 2006).

### 3.2.4. Exclusion criteria

Children with cerebral malaria; history of any HIV-1 related symptoms such as oral thrush; clinical evidence of acute respiratory infection; prior hospitalization; intent to relocate during the study period; unwillingness of the parent or guardian to enroll the child in the study according to previously established protocols (Ong'echa *et al*., 2006).

## 3.3 Sample size

The study analyzed all available blood spot samples (n=612). Healthy controls were 151 and parasitaemic blood spot samples were 461. The parasitemic children were further stratified into SMA (n=112) and non-SMA (n=349). The effect size was determined to measure the significance of the difference between the groups by quantifying the size of the difference between two groups (Coe, 2002). The choice of the appropriate kind of effect size measurement is determined by the test statistic of the hypothesis testing procedure (Lieber R.L, 1990) and Odds Ratio (OR) is the effect size measurement for logistic regression (Ialongo, 2016).

Since the effect size is an estimate, its confidence interval (CI) was given as well i.e. the narrower the interval the more precise the estimate (Simundic, 2008). This CI quantifies the margin of error and provides the same information contained in the significance test i.e. 5% significance level is equal to 95% confidence interval (Coe, 2002). Thus, the CI gave the amount of variation in the effect size estimate if new samples of the same size are repeatedly taken. In each association test of the current study, the effect size was estimated as the odds ratio that gave 95% confidence interval in the results and the statistical power for the association was also determined.

### 3.4 Collection and processing of blood samples

#### 3.4.1 Collection of infected blood samples

Thick and thin blood smears were prepared from heel or finger prick blood sample and stained with Giemsa to determine the parasite density. Following microscopic confirmation of the diagnosis of *P. falciparum* parasitaemia, 3mL venous blood was collected into EDTA vacutainers (Becton Dickinson, USA). About 100uL of the blood sample was bloated onto FTA Classic® cards (Whatman Inc., Clifton, NJ, USA), air dried, and stored with desiccants at room temperature for use in DNA extraction.

#### 3.4.2 Calculating parasitaemia level

Parasite count was performed on thick blood smears stained with Giemsa and examined under oil immersion. Starting at the top most part of the film, the field with a good number of white blood cells was observed and counting started. A multiple type tally counter (The Tally Counter Co. Ltd, United Kingdom) was used to count parasites and white blood cells simultaneously by clicking on the assigned key as parasites or white blood cells as observed. Two tally counters were used, one for the WBCs and the other for parasites. After counting, the parasite density was counted on the basis of the patient's actual white cell count (WHO, 2009). The following formula was used to calculate parasite density:

$$\textit{Pf.}\text{ per }\mu\text{L of blood} = \frac{\textit{Pf.}\text{ Count x WBCs}}{\text{Number of WBCs counted}}$$

#### 3.4.3 Hematological measurements

Haemoglobin levels and complete blood counts were determined using the Beckman Coulter ACT diff2™ (Beckman-Counter Corporation, Miami, FL, USA). SMA was delineated from other anaemia promoting conditions: HIV-1, bacteraemia and sickle cell traits. HIV

infections were determined serologically (Unigold and Determine) and discordant results were confirmed through HIV-1 proviral DNA PCR testing, while bacteraemia was determined using the Wampole Isostat Pediatric 1.5 System as per previous work (Were *et al*., 2006). Sickle cells status was determined by alkaline cellulose acetate electrophoresis on Titan III plates (Helena BioSciences, Sunderland, United Kingdom) (Appendix 3) according to the manufacturer's instructions. G6PD deficiency was determined by a fluorescent spot test using the manufacturer's methods (Trinity Biotech Plc., Bray, Ireland) (Appendix 4).

### 3.4.4 DNA extraction

Genomic DNA was extracted using the Gentra System extraction protocol (Gentra System Inc., Minneapolis, MN, USA) according to the manufacturer's recommendations. The following procedures were followed to extract DNA from the FTA cards: The thermal cycler was preheated at 99$^{o}$C. Three millimeters of the disc was punched from sample collection filter paper (FTA) and placed into 0.2mL tube. To the tube, 50µL of DNA purification solution 1 was added and incubated at room temperature (25$^{o}$C) for 15 minutes. The solution was pipetted up and down twice then discarded. The washing step was repeated twice. DNA solution 1 was added again and incubated 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$^{o}$C in the thermal cycler for 15 minutes to release DNA and then cooled at room temperature. The eluted DNA was transferred into a clean tube and then stored at -20$^{o}$C until use. To confirm the presence of DNA, human glyceraldehyde-3-phospate dehydrogenase (hG3PDH), a house-keeping gene was amplified.

### 3.4.5 Genotyping of NCR3 -412C/G (rs2736191) and -172G/A (rs11575837) variants

The NCR3 variants (-412C/G and -172G/A) were genotyped using allele-specific PCR amplification. This was performed using a high-throughput TaqMan® SNP genotyping assay technology (Applied Biosystem, Foster city, CA, USA) which accurately discriminates alleles using allele-specific fluorochrome labeled probes. The primer sequence used were forward (5'-GATGGGTCTGGGTACTGGTG-3') and reverse (5'-GGGATCTGAGCAGTGAGGTC-3') primers as previously described (Delahaye *et al*., 2007). For genotyping of NCR3 variant -412C/G, a master mix containing 5.0µL of TaqMan® genotyping mix, 0.5µL of SNP assay mix, 3.5µL of PCR grade water and 1.0µL of DNA was added into each micro-well and genotyped using the following cycling parameters; pre-PCR hold stage was done at 60ºC for 30 seconds, hold stage at 90ºC for 10 minutes, cycling stage at 95ºC for 15 seconds and annealing at 60ºC for 1 minute. The hold stage to the annealing stage was repeated 45 times. For genotyping of NCR3 -172G/A), the same reaction volumes and cycling parameters was used.

### 3.5 Data management and analysis

Data were entered into a Microsoft Excel 2010 spreadsheet. The data was analyzed using SPSS (Version 23.0). Chi-square analyses were used to determine the frequencies. NCR3 (-412C/G and -172G/A) haplotypes were constructed using HPlus (Version 2.5). The association between NCR3 (-412C/G and -172G/A) genotypes and haplotypes, and malaria disease outcomes was determined by binomial logistic regression, controlling for the confounding effects of age, gender, G6PD (Preuss, Jortzik, & Becker, 2012), sickle-cell trait (Aidoo *et al*., 2002), HIV-1 status (Otieno, Ouma, Ong'echa *et al*., 2006), and bacteremia (Were *et al*., 2011). Critical significance levels was set at $p \leq 0.05$ and all tests of significance were two-sided.

### 3.6 Ethical Considerations

The study was approved by the Ethics Review Committee of the Kenya Medical Research Institute (Appendix 1). Informed written consent was obtained from the parent or legal guardian of all children participating in the study (Appendix 2). The risks and benefits of the study were outlined in the consent forms (Appendix 2).

# CHAPTER FOUR

# RESULTS

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

The study analyzed 612 blood spot samples of children aged 3‑36 months. Clinically, the study participants were grouped into three categories i.e., healthy controls (n=151), severe malaria anemia (Hb<5.0 g/dL, n=112) and non-SMA (Hb≥5.0 g/dL, n = 349). SMA and non-SMA children were grouped according to the WHO definition of SMA (Hb<5.0 g/dL) (WHO, 2000b). A summary of demographic, clinical, and laboratory characteristics of the parasitaemic study participants is shown in Table 4.1.

There were more healthy females [83 (55.0%)] than males and more females who were parasitic had SMA [61 (54.5%)]. Children with SMA were younger (age in months) [median (IQR); 8.0 (8.0)] than those in the non-SMA group [median (IQR); 10.0 (8.0)]. Even though it was not statistically significant, the proportion of participants with high-density parasitaemia (HDP) was higher in non-SMA children than in SMA children (58.9% in SMA and 64.5% in non-SMA, $P$ = 0.290). Similarly, the distributions of carriers of sickle cell traits, glucose-6-phosphate (G6PD) deficiency, bacteremia and HIV were comparable among healthy, non-SMA and SMA groups ($P$=0.001, $P$=0.378, $P$=0.575 and $P$=0.033, respectively).

**TABLE 4.1: Demographic, clinical, and laboratory characteristics of the study participants**

| Characteristics | Non-Parasitic | Parasitic | |
|---|---|---|---|
| | Healthy controls n=151 | SMA (Hb < 5.0 g/dL) n=112 | Non-SMA (Hb ≥ 5.0 g/dL) n=349 |
| Gender, n (%) | | | |
| Males | 68 (45.0) | 51 (45.5) | 193 (55.3) |
| Females | 83 (55.0) | 61 (54.5) | 156 (44.7) |
| Age (Months) | 8.0 (9.0) | 8.0 (8.0) | 10.0 (8.0) |
| HDP, n (%) (≥10,000 parasites/uL) | __ | 66/112 (58.9) | 225/349 (64.5) |
| Sickle cell trait, n (%) | | | |
| Normal | 114 (77.0) | 104 (92.9) | 297 (85.3) |
| Carriers | 31 (20.9) | 8 (7.1) | 51 (14.7) |
| Sickle | 3 (2.0) | 0 (0.0%) | 0 (0.0) |
| G6PD, n (%) | | | |
| Normal | 115 (87.1) | 100 (93.5) | 283 (87.6) |
| Intermediate | 10 (7.6) | 4 (3.7) | 18 (5.6) |
| Deficient | 7 (5.3) | 3 (2.8) | 22 (6.8) |
| Bacteremia, n (%) | | | |
| Absence | 100 (71.4) | 79 (76.7) | 230 (71.7) |
| Presence | 40 (28.6) | 24 (23.3) | 91 (28.3) |
| HIV, n (%) | | | |
| Negative | 141 (93.4) | 102 (91.1) | 337 (96.8) |
| Positive | 10 (6.6) | 10 (8.9) | 11 (3.2) |

Data are presented as the median (interquartile range) and n (%) unless stated otherwise. The children (n = 612) were categorized as healthy controls (n=151), SMA (n=112) and non-SMA (n =349) according to WHO definition of SMA (Hb< 5.0 g/dL, with any density parasitaemia). Abbreviations: G6PD-Glucose-6-Phaspahte dehydrogenase, HDP-high density parasitaemia and HIV-Human Immunodeficiency Virus.

### 4.2 NCR3 -412 C/G and -172G/A allele frequencies

The NCR3 -412 C/G and -172G/A allele frequencies were determined using the Hplus 2.5. The overall allele frequency of the NCR3 -412C/G genotype was: C allele (n = 859, 0.702) and G allele (n = 365, 0.298) whereas the overall allele frequency of the NCR3 -172G/A genotype was: C allele (n = 1189, 0.971) and T allele (n = 35, 0.029) (Table 4.2).

**TABLE 4.2: NCR3 -412 C/G and -172G/A allele frequencies**

| Genotypes | Coding | Occurrences | Frequency |
|---|---|---|---|
| NCR3 (-412C/G) | | | |
| Wild | C | 859 | 0.702 |
| Variant | G | 365 | 0.298 |
| Missing | | 0 | 0.000 |
| NCR3 (-172G/A) | | | |
| Wild | C | 1189 | 0.971 |
| Variant | T | 35 | 0.029 |
| Missing | | 0 | 0.000 |

The allele frequencies were determined using Hplus 2.5.

### 4.3 Distribution of NCR3 -412C/G and -172G/A genotypes in the study groups

Prior to establishing the association between the NCR3 -412C/G and -172G/A genotypes and malaria disease outcomes, the distribution of the NCR3 loci were examined across the different groups: NCR3 -412C/G and -172G/A. The prevalence of NCR3 -412C/G genotypes was CC [57.0% (healthy controls), 42.0% (SMA) and 54.4% (non-SMA)], CG [33.1% (healthy controls), 42.0% (SMA) and 33.2% (non-SMA)] and GG [9.9% (healthy controls), 16.1% (SMA) and 12.3% (non-SMA)]. The prevalence of NCR3 -172G/A genotypes was CC [93.4% (healthy controls), 95.5% (SMA) and 95.4% (non-SMA)], CT [6.6% (healthy controls), 4.5% (SMA) and 3.4% (non-SMA)] and TT [0.0% (healthy controls and SMA) and 3.4% (non-SMA)].

The Hardy-Weinberg Equilibrium frequency of the NCR3 -412C/G genotypes were CC (n = 323, 0.528), CG (n = 213, 0.348) and GG (n = 76, 0.124) (Table 4.2). There was significant departure from Hardy Weinberg Equilibrium in the overall distribution (HWE, $\chi 2 = 17.371$, $P < 0.0001$) pointing out the presence of evolutionary influences that cause the allele and genotype frequencies to change from generation to generation in the study population. The

overall frequency of the NCR3 -172G/A genotypes were CC (n = 581, 0.949), CT (n = 27, 0.044) and TT (n = 4, *P*=0.007) (Table 4.3). There was significant departure from Hardy Weinberg Equilibrium in the overall distribution (HWE, $\chi 2 = 25.936$, $P < 0.001$).

**TABLE 4.3: Distribution of NCR3 -412C/G and -172G/A genotypes in the study groups**

| Genotypes | n (%) | Healthy Controls n=151 | SMA (Hb < 5.0 g/dL) n=112 | Non-SMA (Hb ≥ 5.0 g/dL) n=349 | Genotype Frequency (HWE) | *P*-value (HWE) |
|---|---|---|---|---|---|---|
| NCR3 -412C/G | | | | | | |
| Wild type (CC) | 323 (52.8) | 86 (57.0) | 47 (42.0) | 190 (54.4) | 0.528 | |
| Heterozygous(CG) | 213 (34.8) | 50 (33.1) | 47 (42.0) | 116 (33.2) | 0.348 | **<0.001**[c] |
| Mutant (GG) | 76 (12.4) | 15 (9.9) | 18 (16.1) | 43 (12.3) | 0.124 | |
| NCR3 -172G/A | | | | | | |
| Wild type (CC) | 581 (94.9) | 141 (93.4) | 107 (95.5) | 333 (95.4) | 0.949 | |
| Heterozygous (CT) | 27 (4.4) | 10 (6.6) | 5 (4.5) | 12 (3.4) | 0.044 | **<0.001**[c] |
| Mutant (TT) | 4 (0.7) | 0 (0.0) | 0 (0.0) | 4 (1.1) | 0.007 | |

Data are presented as [n, (%)] for proportions of NCR3 promoter variants −412GC and −172AG within the healthy controls (n = 151), SMA (n = 112) and non-SMA (n = 349) groups in children in western Kenya. HWE, Hardy-Weinberg Equilibrium. Significant statistical result is indicated in bold. [c]Hardy-Weinberg Equilibrium Chi-square ($\chi 2$) test. Statistical significance was set at $P \leq 0.05$.

### 4.4 Distribution of NCR3 -412 C/G and -172G/A haplotypes in the study groups

Before determining the associations between the haplotypes and acquired parasitaemia, HDP (≥10,000parasites/μL) and SMA (Hb<5.0 g/dL), the overall distribution of the different haplotypes was determined: CC, (n = 527/612) were 86.1%, CT, (n = 31/612) were 5.1% and GC, (n = 289/612) were 47.2%. The distribution of the haplotypes in the study groups were as follows (Table 4.4); CC (healthy controls, n = 134/151, 88.7%; SMA, n = 93/112, 83.0%; non-SMA, n = 300/349, 86.0%), CT (healthy controls, n = 10/151, 6.6%; SMA, n = 5/112, 4.5%; non-SMA, n = 16/349, 4.6%) and GC (healthy controls, n = 65/151, 43.0%; SMA, n = 65/112, 58.0%; non-SMA, n = 159/349, 45.6%). The frequency of the GC haplotype was

significantly higher in the SMA (n = 65/112, 58.0%) relative to non-SMA (n = 159/349, 45.6%) and healthy control (n = 65/151, 43.0%) groups (*P* = 0.035); Table 4.4.

**TABLE 4.4: Distribution of NCR3 -412 C/G and -172G/A haplotypes in the study groups**

| Haplotypes | Healthy Controls n=151 | SMA (Hb < 5.0 g/dL) n=112 | Non-SMA (Hb ≥ 5.0 g/dL) n=349 | *P*-value ($\chi 2$) |
|---|---|---|---|---|
| CC, n (%) | | | | |
| Presence | 134 (88.7) | 93 (83.0) | 300 (86.0) | 0.414 |
| Absence | 17 (11.3) | 19 (17.0) | 49 (14.0) | |
| CT, n (%) | | | | |
| Presence | 10 (6.6) | 5 (4.5) | 16 (4.6) | 0.603 |
| Absence | 141 (93.4) | 107 (95.5) | 333 (95.4) | |
| GC, n (%) | | | | |
| Presence | 65 (43.0) | 65 (58.0) | 159 (45.6) | **0.035** |
| Absence | 86 (57.0) | 47 (42.0) | 190 (54.4) | |

Haplotypes were constructed based on the NCR3 -412 C/G and -172G/A variants using the HPlus version 2.5 software. Significant statistical power is indicated in bold. Distributions of haplotypes compared between the healthy controls, SMA and non-SMA groups using a $\chi 2$ test.

### 4.5 Association between NCR3 -412C/G and -172G/A genotypes and acquisition of parasitaemia

A logistic regression analysis was carried out using the most common genotype as the reference group while controlling for the confounding effects of age, gender, G6PD deficiency, sickle-cell trait, HIV and bacteremia, as these confounders have been shown to independently alter malaria disease outcome in this population. Relative to the wild type

NCR3 genotypes (-412, CC and -172, CC), there was no significant association between the individual promoter variants and acquisition of parasitaemia at -412 C/G (CG, OR 1.630, 95% CI; 0.789-3.371, *P* = 0.187) and (GG, OR 1.492, 95% CI; 0.937-2.378, *P* = 0.092). At -172G/A, the CT genotype showed no significant association with acquisition of parasitaemia (CT, OR 0.424, 95% CI; 0.174-1.036, *P* = 0.060) (Table 4.5).

**TABLE 4.5: Association between NCR3 -412C/G and -172G/A genotypes and acquisition of parasitaemia**

| **Genotypes** | **OR** | **95% CI** | ***P*-value** |
|---|---|---|---|
| NCR3 -412 C/G, (n) | | | |
| CC (267) | 1.000 (Reference) | | |
| CG (186) | 1.630 | 0.789-3.371 | 0.187 |
| GG (59) | 1.492 | 0.937-2.378 | 0.092 |
| NCR3 -172 G/A, (n) | | | |
| CC (487) | 1.000 (Reference) | | |
| CT (22) | 0.424 | 0.174-1.036 | 0.060 |
| TT (3) | ( ) x | ( ) x | ( ) x |

Logistic regression analysis was used to determine the odds ratio (OR) and 95% Confidence Interval (CI), controlling for confounding effects of age, gender, G6PD deficiency, sickle-cell trait, HIV and bacteremia. ( ) x shows the number was too small to generate any association. The homozygous wild-type genotypes were used as the reference group for the logistic regression analyses.

### 4.6 Association between NCR3 -412C/G and -172G/A genotypes and high density parasitaemia

The association between NCR3 -412C/G and -172G/A genotypes and high density parasitaemia was determined using a logistic regression analysis using the wild type in each variant as the reference group. Relative to the wild type NCR3 genotypes (-412, CC and -172, CC), there was no significant association between the individual promoter variants and high

density parasitaemia at -412C/G (CG, OR; 0.791, 95% CI; 0.508-1.232, *P* = 0.300). However, there was a significant association between GG genotype and high density parasitaemia (GG, OR; 0.469, 95% CI; 0.252-0.873, *P* = 0.017) (Table 4.6). There was also no significant association between the individual promoter variants and high density parasitaemia at -172 G/A (CT, OR 0.838, 95% CI; 0.266-2.638, *P* = 0.763 and (TT, OR 1.186, 95% CI; 0.104-13.569, *P* = 0.891) (Table 4.6).

**TABLE 4.6: Association between NCR3 -412C/G and -172G/A genotypes and high density parasitaemia**

| **Genotypes** | **OR** | **95% CI** | ***P*-value** |
|---|---|---|---|
| NCR3 -412 C/G, (n) | | | |
| CC (215) | 1.000(Reference) | | |
| CG (154) | 0.791 | 0.508-1.232 | 0.300 |
| GG (53) | 0.469 | 0.252-0.873 | **0.017** |
| NCR3 -172 G/A, (n) | | | |
| CC (377) | 1.000(Reference) | | |
| CT (13) | 0.838 | 0.266-2.638 | 0.763 |
| TT (3) | 1.186 | 0.104-13.569 | 0.891 |

Logistic regression analysis was used to determine the odds ratio (OR) and 95% Confidence Interval (CI), controlling for confounding effects. Significant statistical power was indicated in bold. The homozygous wild-type genotypes were used as the reference group for the regression analyses.

### 4.7 Association between NCR3 -412C/G and -172G/A genotypes and SMA (Hb<5.0g/dL)

The association between NCR3 -412C/G and -172G/A genotypes and SMA was determined using NCR3 genotypes (-412, CC and -172, CC) in each variant as the reference group. There was significant association between the individual promoter variants with SMA (Hb<5.0 g/dL) at -412C/G (CG, OR 1.636, 95% CI; 1.018-2.631, *P* = 0.042) as those with CG

genotype were 1.6 times more likely to develop SMA relative to those with CC genotype. In contrast, there was no significant association between GG and acquisition of parasitaemia (GG, OR 1.783, 95% CI; 0.932-3.412, *P*=0.081). Likewise, there was no significant association between the individual promoter variant at -172 G/A (CT, OR 1.472, 95% CI; 0.433-5.011, *P* = 0.536) and SMA (Hb<5.0 g/dL) (Table 4.7).

**TABLE 4.7: Association between NCR3 -412C/G and -172G/A genotypes and SMA (Hb≤5.0 g/dL)**

| **Genotypes** | **OR** | **95% CI** | ***P*-value** |
|---|---|---|---|
| NCR3 -412 C/G, (n) | | | |
| CC (237) | 1.000 (Reference) | | |
| CG (162) | 1.636 | 1.018-2.631 | **0.042** |
| GG (60) | 1.783 | 0.932-3.412 | 0.081 |
| NCR3 -172 G/A, (n) | | | |
| CC (377) | 1.000 (Reference) | | |
| CT (13) | 1.472 | 0.433-5.011 | 0.535 |
| TT (3) | ( ) x | ( ) x | ( ) x |

Logistic regression analysis was used to determine the odds ratio (OR) and 95% Confidence Interval (CI), controlling for confounding effects of age, gender, G6PD deficiency, sickle-cell trait, HIV and bacteremia. ( ) x shows the number was too little to generate any meaningful association. Significant statistical power was indicated in bold. The homozygous wild-type genotypes were used as the reference group for the logistic regression analyses.

### 4.8 Association between NCR3 -412C/G and -172G/A haplotypes and acquisition of parasitaemia

The association between the NCR3 -412C/G and -172G/A promoter haplotypes and acquisition of parasitaemia while controlling for confounding effects of age, gender, G6PD deficiency, sickle-cell trait, HIV and bacteremia revealed the following: Relative to the non-

CC, non-CT and non-GC groups, the presence of the haplotypes was not associated with acquisition of parasitaemia, that is CC (NCR3 -412C and -172C) (OR 0.791, 95% CI; 0.411-1.525, *P* = 0.485), CT (NCR3 -412C and -172T) (OR 0.511, 95% CI; 0.216-1.206, *P* =0.125) and GC (NCR3 -412G and -172C) (OR 1.524, 95% CI; 0.990-2.345, *P* = 0.055) (Table 4.8).

**TABLE 4.8: Association between NCR3 -412C/G and -172G/A haplotypes and acquisition of parasitaemia**

| **Haplotypes** | **OR** | **95% CI** | ***P*-value** |
|---|---|---|---|
| **-412**C/**-172**C (446) | 0.791 | 0.411-1.525 | 0.485 |
| **-412**C/**-172**T (25) | 0.511 | 0.216-1.206 | 0.125 |
| **-412**G/**-172**C (245) | 1.524 | 0.990-2.345 | 0.055 |

Logistic regression analysis were used to determine the odds ratio (OR) and 95% CI, controlling for age, gender, G6PD deficiency, sickle-cell trait status and presence of bacterial infections. The absence of the haplotype was used as the reference groups in each row.

### 4.9 Association between NCR3 -412C/G and -172G/A haplotypes and high density parasitaemia (HDP≥10,000 parasites/μL)

Relative to the non-CC group, carriage of the CC (NCR3 -412C and -172C) haplotype was 1.9 times more likely to increase susceptibility to high density parasitaemia (OR 1.934, 95% CI; 1.104-3.389, *P* = 0.021). However, the other haplotypes; CT (*P* = 0.832) and GC (*P* =0.052) were not significantly associated with high density parasitaemia (Table 4.9).

**TABLE 4.9: Association between NCR3 -412C/G and -172G/A haplotypes and high density parasitaemia (HDP≥10,000 parasites/µL)**

| Haplotypes | OR | 95% CI | *P*-value |
|---|---|---|---|
| CC (363) | 1.934 | 1.104-3.389 | **0.021** |
| CT (16) | 0.893 | 0.314-2.539 | 0.832 |
| GC (197) | 1.528 | 0.996-2.345 | 0.052 |

Logistic regression analysis were used to determine the odds ratio (OR) and 95% CI, controlling for age, gender, G6PD deficiency, sickle-cell trait status and presence of bacterial infections. The absence of the haplotype was used as the reference groups.

**5.0 Association between NCR3 -412C/G and -172G/A haplotypes and SMA (Hb≤5.0 g/dL, any density parasitaemia)**

A further analysis controlling for the potential confounders was used to determine the association between the NCR3 -412C/G and -172G/A promoter haplotypes and SMA. The analysis (Table 4.10) revealed that relative to the non-GC group, carriage of the GC (NCR3 -412G and -172C) haplotype was associated with 1.6 times increased susceptibility to SMA (OR 1.635, 95% CI; 1.015-2.634, $P = 0.043$). However, the other haplotypes; CC ($P = 0.537$) and CT ($P = 0.753$) were not associated with susceptibility to SMA; Table 4.10.

**TABLE 4.10: Association between NCR3 -412C/G and -172G/A haplotypes and SMA (Hb≤5.0g/dL)**

| Haplotypes | OR | 95% CI | *P*-value |
|---|---|---|---|
| CC (340) | 0.811 | 0.418-1.575 | 0.537 |
| CT (16) | 1.210 | 0.369-3.961 | 0.753 |
| GC (197) | 1.635 | 1.015-2.634 | **0.043** |

Logistic regression analysis were used to determine the odds ratio (OR) and 95% CI, controlling for age, gender, G6PD deficiency, sickle-cell trait status and presence of bacterial infections. The absence of the haplotype was used as the reference groups in each row.

# CHAPTER FIVE

# DISCUSSION

## 5.1 Association between NCR3 (-412C/G and -172G/A) genotypes and haplotypes, and acquisition of parasitaemia

Results from the current study showed no significant association between NCR3 -412C/G and -172G/A genotypes and haplotypes, and the acquisition of parasitaemia. These observations underscore one important point: that the host genetics may not regulate the acquisition of parasitaemia in this population. However, the genes may set in at play following the acquisition of parasitaemia and dictate whether or not a child in this population develops severe disease. The fact that these promoter polymorphisms do not predispose one to acquisition of parasitaemia concurs with a report that the genetics of the host may not dictate how one acquires parasitaemia but once parasitaemia is acquired, it dictates whether or not one gets severe disease (Kwiatkowski, 2005). Moreover, polymorphic variability in innate immune response gene promoters has also been shown to condition malaria disease outcomes (Flori *et al.,* 2005). In the case of the NCR3 haplotypes, the current observation is consistent with some promoter variants of the immune response genes i.e. IL-10 promoter variants -1,082G/-819C/-592C (GCC) haplotypes, which were shown not to be associated with parasitaemia acquisition in the current population (Ouma, Davenport, Were *et al*., 2008).

NCR3 -412 loci has been associated with parasitaemia and mild malaria in adult population in malaria endemic areas of Bukina Faso (Afridi *et al.,* 2012). However, in a paediatric population in a holoendemic area in western Kenya, the current study found no significant association between NCR3 -412C/G and -172G/A genotypes and haplotypes, and the acquisition of parasitaemia. (Delahaye *et al*., 2007) showed that NCR3 -412G>C

polymorphism was associated with mild malaria. For example, NCR3 -412C carriers had more frequent mild malaria attacks than NCR3 -412GG individuals while NCR3 -412C and a haplotype containing NCR3 -412C were significantly associated with increased risk of mild malaria. Differences in current versus previous studies may be attributed to differences in study design and population. As much as the previous study focused on family-based approaches, the current study only focused on paediatric populations. Furthermore, the populations in the previous study presented with mixed clinical presentations as opposed to the current study in which the most severe forms of disease is SMA and HDP.

### 5.2 Association between NCR3 (-412C/G and -172G/A) genotypes and haplotypes, and high density parasitaemia

Although the proportion of NCR3 -412GG genotype was the least among the genotypes among HDP individuals (29/291) it had a statistically significant association with HDP (HDP≥10,000 parasites/µL). These results suggest that after the acquisition of parasitaemia, the NCR3 -412GG genotype comes into play and predisposes children aged 3-36 months to high density parasitaemia. On the other hand, NCR3 -412CG genotype was not significantly associated with HDP. These findings may describe what happens in malaria naïve paediatrics who rely solely on the innate immunity for protection in a primary infection. It has previously been established that NCR3 activate NK cells (Moretta *et al*., 2001) and it has further been shown that NK cells from malaria naïve adult donors display differences in the production of NK cell mediated IFN-γ with varied levels of NK cell activation, a phenomenon attributed to genetic variability (Artavanis-Tsakonas & Riley, 2002).

A previous family-based association study has reported no association between NCR3 -412 polymorphisms and maximum parasitaemia, in a malaria endemic area of Burkina Faso

(Delahaye *et al*., 2007). The different result from that of the current study may be attributed to the differences in malaria transmission rates between the study areas and the study participants. For example, the current study was done in a holoendemic malaria transmission area in participants aged 3-36 months while in the previous study, the malaria transmission of study area was endemic and the mean age of participants was 12.1±6.2 years. Furthermore, NCR3 -172 alleles were found to be rare (frequency<1%) and were exluded from further analysis (Delahaye *et al*., 2007) but in the current study, NCR3 -172 polymorphisms were associated with HDP but no association between the individual promoter variants and high density parasitaemia at -172 G/A was observed suggesting that NCR3 -172 G/A genotypes may not be linked to HDP.

Another study in the same malaria transmission area and in the same study population as that of the current study associated polymorphism in the promoter of Macrophage Inhibitory Factor gene with HDP (HDP≥10,000 parasites/μL) (Awandare *et al*., 2006) showing that promoter polymorphisms of immune response genes may condition HDP. The C allele was also observed to exist in high frequency, an observation similar to that of the current study.

Among the NCR3 (-412C/G and -172G/A) haplotypes, the CC (NCR3 -412C and -172C) haplotype was significantly associated with high density parasitaemia, a haplotype which happens to have the highest occurrence and frequency in the study participants and the frequency of individual alleles in the -412 and -172 loci, 0.702 and 0.971, respectively. This result is important because in holoendemic areas, clinical illnesses associated with malaria reach their peak in children under 5 years of age and decline substantially later where the children may be parasitaemic without febrile illness (Sowunmi *et al*., 1992). Moreover, it was observed that the CC haplotype was associated with HDP but not with SMA, supporting

the fact that high density parasitaemia and severe malaria anaemia are not significantly associated.

Since the alleles in a haplotype are inherited together from a single parent (Cox, Moore, & Ladle, 2016), and with increasing age, individuals in a holoendemic area display no signs and symptoms of malaria even-though they have high *Pf.* parasite densities (Sowunmi *et al*., 1992), it is hypothesized that the CC haplotype predisposes children (3-36 months), with naïve immunity to HDP. This early predisposure may allow their immune system to interact with the parasite at tolerable levels without necessarily developing symptomatic malaria.

### 5.3 Association between NCR3 (-412C/G and -172G/A) genotypes and haplotypes, and severe malaria anaemia

Previous studies (Awandare *et al*., 2006; Ouma, Davenport, Awandare *et al*., 2008; Ouma, Davenport, Were *et al*., 2008) investigating the association of promoter polymorphisms of immune response genes and SMA have demonstrated an increased likelihood that NCR3 gene promoter polymorphisms could be associated with SMA. In the current study, there was a significant association between the -412CG genotype and SMA (Hb≤5.0g/dL, any density parasitaemia) since heterozygotes were more susceptible to SMA than the mutants (GG). SMA (Hb≤5.0g/dL, any density parasitaemia) may occur in the presence of any density parasitaemia (WHO, 2000a) and even though the NCR3 -412 mutants (GG) were significantly associated with HDP, they were not significantly associated with SMA.

A previous study carried out in the same study area on the same study population showed that SMA (Hb≤5.0 g/dL, any density parasitaemia) is responsible for high mortality in paediatrics admitted because of malaria (Obonyo *et al*., 2007). Identifying genes that condition severe malaria anaemia may provide a clear understanding of the genetic pathways that condition

susceptibility to or protection against SMA. For example, immunological studies in the same area and study population demonstrate that promoter polymorphisms of immune response genes condition SMA (Anyona *et al*., 2011; Munde *et al*., 2012; Okeyo *et al*., 2013; Ouma, Davenport, Were *et al*., 2008). The current study adds knowledge to the complex gene pathways that condition SMA in paediatrics in a holoendemic area.

The GC haplotype was higher in parasitaemic individuals (77.5%) than in healthy controls and the most frequent in SMA children (58%) compared to the non-SMA group and the healthy controls. These results show that individuals with GC haplotype are more likely to have HDP and develop SMA. The current study infers that presence of the GC (NCR3 -412G and -172C) haplotype was associated with susceptibility to SMA and that the GC haplotype could modulate the progression of malaria to SMA in children. A further study is thus required to ascertain if carriers of the GC haplotype are predisposed to SMA beyond the age of 36 months.

Haplotypes show how allele combinations of different functional polymorphic alleles interact in amplifying or moderating their individual effects (Ouma, Davenport, Were *et al*., 2008). For instance, the C allele had an allele frequency of 0.971 at -172 G/A locus and an allele frequency of 0.702 at the -412 C/G locus, and their combination, CC haplotype was of 83% of SMA individuals. However, the carriage of the CC haplotype did not alter susceptibility to malaria. The current study postulates that this observation may be due to the fact that the combination of C alleles moderates their individual effects. Therefore a common disease may not be necessarily caused by a common variant. On the other hand, the G allele had an allele frequency of 0.298 at the -412 C/G locus but when combined with C allele with a frequency of 0.971, the carriage of the GC haplotype which is 58% of SMA individuals, predisposed carries to SMA.

A previous study has demonstrated that deleterious alleles are maintained at low frequencies by purifying selection (Goldstein *et al.*, 2013) whereby variants that cause disease are selected against (Bulmer, 1971). However, the rate of polymorphisms are higher than purifying selection and all deleterious variants are not completely removed (Charlesworth, 2000) resulting in abundance of rare variants (1 in 17 bases) that are geographically localized (Nelson *et al.*, 2012). In the current study, the carriage of CT haplotype (5% among SMA individuals) was not associated with SMA. This observation may be as a result of the alleles being kept at low frequency by purifying selection.

It was observed that NCR3 -172G/A genotypes showed a consistent pattern of no significant association with acquisition of parasitaemia, high density parasitaemia and severe malaria anaemia. Nevertheless, NCR3 -172G/A genotypes have been reported to occur in high frequencies in a malaria holoendemic area (HapMap, 2003). This study therefore postulates that another evolutionary force apart from malaria may be responsible for this occurrence of NCR3 -172G/A variants in a holoendemic area.

In the case of haplotypes, it was noted that the C allele from the NCR3 -172G/A genotype was present in the NCR3 (-412C/G and -172G/A) haplotypes that were associated with malaria outcomes that is, CC (-412C and -172C) haplotype and GC (-412G and -172C) haplotype. This observation suggests that at the NCR3 -172 loci, the C allele is passed to the offsprings and it conditions malaria outcomes: HDP and SMA in children when combined with the C and G alleles, respectively.

Functional analysis of the *NCR3* gene promoter polymorphisms would provide functional effects of the promoter polymorphisms that will inform the results of the study by giving a causal relationship between the polymorphisms and malaria outcomes. A study carried out in children mean age 4.34 (SD=2.55) has demonstrated that NCR3 -412 C/G polymorphisms

affect NCR3 expression, altering resistance to malaria (Baaklini *et al*., 2017). This study raises prospects that NCR3 polymporhisms associated with malaria outcomes in this study might have causal relationship with malaria outcomes.

# CHAPTER SIX

# SUMMARY OF FINDINGS, CONCLUSION AND RECOMMENDATIONS

## 6.1 SUMMARY OF FINDINGS

The objectives of this study were to determine the association between NCR3 (-412C/G and -172G/A) genotypes and haplotypes, and malaria outcomes in paediatric population in western Kenya. Results from the study showed that there is no statistically significant association between NCR3 -412C/G and -172G/A genotypes and haplotypes, and the acquisition of parasitaemia, the NCR3 -412GG genotype, the least frequent among the genotypes in HDP individuals, had a statistically significant association with HDP and the CC (NCR3 -412C and -172C) haplotype was also significantly associated with high density parasitaemia. Finally, the -412CG genotype was associated with SMA and the GC (NCR3 -412G and -172C) haplotype was associated with increased susceptibility to SMA.

## 6.2 CONCLUSION

i. The first hypothesis was supported since there was no association between NCR3 -412C/G and -172G/A genotypes and haplotypes, and the acquisition of parasitaemia. Therefore, NCR3 -412C/G and -172G/A polymorphisms may affect the predisposition of the children to disease following acquisition but they independently may not alter acquisition of parasitaemia.

ii. The null hypothesis of second objective was rejected. NCR3 GG genotype and the CC (NCR3 -412C and -172C) haplotype were associated with HDP implying that malaria naïve paediatrics with the GG genotype and the CC haplotype are susceptible to HDP.

iii. The alternative hypothesis of the third objective was accepted since NCR3 -412 CG genotype and GC (NCR3 -412G and -172C) haplotype was associated with SMA. Therefore malaria naïve paediatrics with the CG genotype and GC haplotype from parents are susceptible to malaria. Children <3 years of age paediatric population highly susceptible to severe anaemia, in the study area showing that SMA is found almost in 89% of the cases (Obonyo *et al*., 2007). The findings of the current study may inform the development of management policies to reduce the burden of SMA in children.

## 6.3 RECOMMENDATIONS FROM CURRENT STUDY

In light of the findings of this study, it is recommended that results of this study should inform the genomic epidemiology of malaria as they provide information about NCR3 -412C/G and -172G/A genotypes and haplotypes that predispose children 3-36 months to severe forms of malaria (HDP and SMA) after acquisition of parasitaemia and explaining the development of HDP and SMA in some paediatrics and not others, who are exposed to the same malaria transmission intensity and infection rate in a holoendemic area.

## 6.4 SUGGESTIONS FOR FUTURE STUDIES

The following recommendations for future studies are based on the study findings:

1. Analyses should be done on the NCR3 -412C/G and -172G/A genotypes and haplotypes with significant association with malaria outcomes to determine the functional effects of the polymorphisms located within the promoter of *NCR3* gene.

These functional effects will provide insights on the causal links between polymorphisms and malaria outcomes.

2. Future studies should expand the search for other genes by genome wide association studies or candidate haplotype approach to determine how other genetic factors work together with NCR3 promoter vaariants since malaria is multi-factorial.

3. NCR3 -172G/A genotypes were not associated with HDP or SMA despite being of high frequency in a malaria holoendemic area. Therefore, a study should be carried out to investigate the driving force that causes this phenomenon in a holoendemic malaria transmission area.

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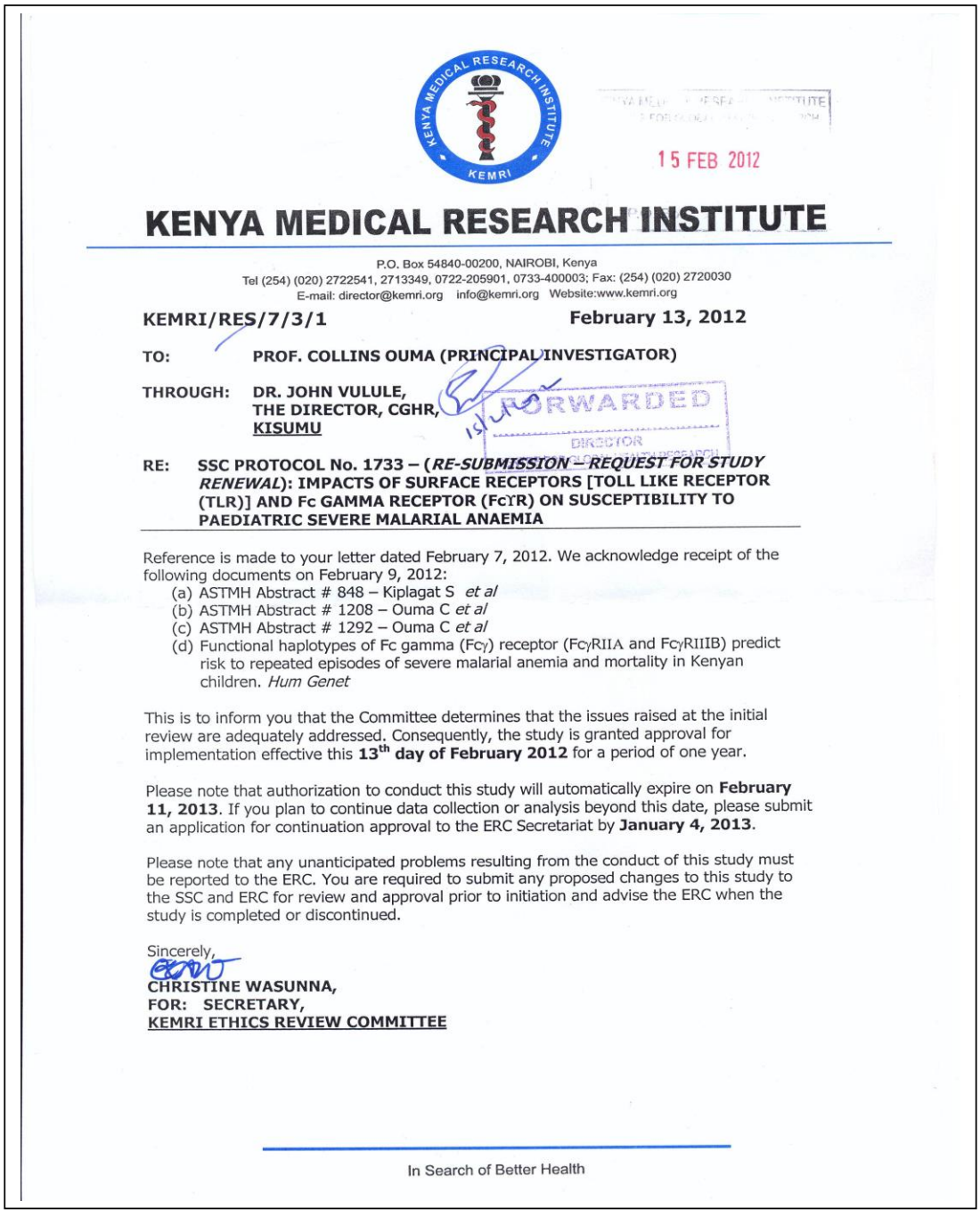
# APPENDICES

## Appendix 1: Research Approval



KENYA MEDICAL RESEARCH INSTITUTE

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 (FcϒR) 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

## Appendix 2: Consent forms

### Consent Form 1a: Written consent for parent/guardian of child in malarial anemia hospital-based prospective study

The Kenya Medical Research Institute (KEMRI) and the University of New Mexico are doing a research study at the Siaya District Hospital (SDH). Dr. Douglas Perkins of University of New Mexico would like your child to be in the study since you live in an area where nearly all children get malaria. The title of the study is "The genetic basis of severe malarial anemia." This research study is funded by the National Institutes of Health (NIH) in the United States. We want to learn how children fight malaria during the first three years of life. We might learn ways to help improve treatment or prevention of malaria.

This research study will look at how your child gets malaria. This means that we will need to see your child at hospital many times. We will give you a schedule. We ask that you bring your child back to SDH for follow-up visits every three months for three years after you enroll your child in the study. The study ends on 31 June 2011. We are also taking blood from healthy children that go to the clinic for their vaccinations. This will let us compare healthy kids with those that have malaria.

The overall study will last five years. We will enroll and follow-up children in years 2-5. We ask that your child be in the study until they complete three years of follow-up visits from the date that your child is enrolled in the study. That date is the date you sign the consent form below.

**First Hospital Contact for Malaria:** If you choose for your child to be in this research study, we will ask you about your child's health and give your child an exam. Based on the screening result, we know that your child has malaria and low blood. To check for malaria and low blood, we will get blood (several drops) by sticking your child's heel or finger with a small needle. If your child has malaria, we may draw a small amount of blood from a vein in your child's arm (about 1 teaspoonful) to test how your child's body fights malaria. The stool sample will be used to check for hookworm (another parasite that can cause low blood). If your child has hookworm, we will refer him or her for treatment from the MOH at the hospital to get rid of the parasite. You may not want your child to have treatment at SDH. You may go to any other health facility you choose, such as, the Kisumu District Hospital or the Provincial District General Hospital in Kisumu but will only provide the cost of the health care if it is done at SDH. If your child is healthy, we ask that you bring your child back to hospital at the first sign of a fever.

Since your child has malaria, signs of low blood, and maybe other illnesses, we will refer your child for treatment by the MOH at this time**.** All tests that are not covered for free by the MOH will be provided by the research study for the children who are enrolled. The MOH will perform all treatments based on the results of the tests. If your child is admitted to

hospital, we will check to see if he/she still has malaria or low blood. This will be done on Days 3 and 7 (or daily, if they need closer attention to treat their illness. We will collect blood (several drops) by sticking your child's heel or finger with a small needle.

**Follow-up visit (Day 14):** On Day 14, after your initial visit, you will need to bring your child back to the SDH. We will take a few drops of blood by sticking your child's heel or finger with a small needle. This is to check for malaria and signs of low blood. If your child becomes very ill before the Day 14 visit, you should immediately take your child to pediatric ward at SDH.

If your child still has malaria parasites in his or her blood on Day 14, we will refer your child for treatment at SDH. If your child is still having low blood, we will refer your child for treatment of anemia at SDH. If your child needs treatment, we will follow your child once every week after treatment until there is no parasite in your child's blood and/or low blood. Since we will want to see your child back at the hospital, we will pay for the fare to cover the cost of round-trip transport between the hospital and your home. If you do not bring your child to hospital as planned, a member of our study team will visit you at your home. We will see if you still want to keep your child in the study. We ask that you bring your child back to hospital at the first sign of a fever so that we can monitor your child's sickness.

**Follow-up visits (every 3 months for the duration of the study):** If you join the research study, we will give you a schedule of when to return to hospital for follow-up visits. This is so we can watch the health of your child. We ask that you bring your child back to SDH for these visits every three months for three years after the date your child is enrolled in the study. We will pay for your trip both to and from the hospital for these visits. If you do not bring your child to SDH, a member of our study team will visit you at your home. They will do this to see if you still want your child in the study. During the follow-up visits, we will check your child for malaria and low blood. To do this, we will get blood (several drops) by sticking your child's heel or finger with a small needle. If your child has malaria or low blood, we will refer your child for treatment to the MOH staff at SDH. If your child gets a fever or signs of malaria at any time, you should bring your child to the hospital to get checked.

Only a person trained to get blood will take blood from your child. If your child has no problems with bleeding, taking blood should not cause harm. There may be a small bruise or short time of discomfort when we do the finger- or heel-stick or take blood from a vein in your child's arm. Taking blood from a child with low blood could make the low blood problem worse. To avoid this, we will take only an amount of blood that is safe. The amount of blood we take is based on your child's age and size. Taking blood could cause vitamin and iron loss in your child. This could make the low blood problem worse. To avoid this, we will give your child vitamins that have iron and folate in them. If your child has a problem with the blood draw, Dr. Benjamin Esiaba, of the MOH, will treat the problem at SDH.

Also, since we did screening for HIV/AIDS, the HIV results from your child are known. This information will be used to see if HIV exposure affects the severity of malaria in your child.The results of the HIV test will be kept private to the extent allowed by law. Receiving your child's HIV test results can be stressful. However, knowing the tests results will benefit your child, because you will be able to act to protect your child's health. If your child's blood tests positive for HIV, we would like you to talk with a doctor.

Benefits for your child in this study are access to HIV testing and counseling. If your child has malaria, you will be referred to MOH doctors to provide appropriate treatment. You can also get drugs for your child from the MOH if your child has HIV. Other benefits include getting vitamins with iron for your child that may improve his or her health status. This research study may help us find new treatments for malaria in the future.

If you do not want your child to be in the research study, your child will still get the best possible medical care at the hospital. You can leave the study at any time. If your child joins the study, but you have questions or change your mind later, you can contact any investigator of this research study and we will remove your child from the research study. If you have questions about HIV or AIDS, you are free to ask the HIV/AIDS counselors. If you have questions about the study or feel you have been harmed, you can contact Dr. Douglas Perkins (KEMRI/University of New Mexico, PO Box 1578, Kisumu, Kenya, telephone: 0733360098). You can also contact Dr. John Michael Ong'echa (KEMRI/University of New Mexico, PO Box 1578, Kisumu, Kenya, telephone: 0733447920). You can also contact Dr Benjamin Esiaba (telephone 321055/321554) at SDH, Nyanza Province, P.O. Box 144, Siaya. In the United States, Dr. Perkins address is: University of New Mexico, MSC10-5550, 1 University of New Mexico, Albuquerque, NM, 87131 (telephone 505-272-6867, e-mail dperkins@salud.unm.edu). . For questions or problems about your rights as a research subject, please call or write: The National/KEMRI Ethical Review Committee, PO Box 54840, Nairobi, telephone: 02-20722541.

**Parent/guardian's name: _____Child's name:_____**

(Please Print) (Please Print)

**Date: _____** **Study# :_____**

Parent/guardian's statement:

□ The above research study has been explained to me. The consent form has been read to me or I have read the consent form. My questions have been answered to my satisfaction. I have received a copy of this form. I was told that being in this research study is my choice. I was told that my child cannot be in the research study without my consent. I agree for my child to take part in this research study. By signing this form, I give my consent for my child to join this research study.

**Signatures:**

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

**Witness Signature: _____ Date: _____**

**Parent/guardian' thumbprint: _____**

**********

**INVESTIGATOR'S CERTIFICATION**

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

**Investigator's signature: _____ Date: _____**

**Consent form 1b: Written consent for storage of samples collected from children**

As explained above in form 1a, we will do several tests on your child's blood for our research study. We can only do some of the tests at the hospital now. To do other tests, we will need to store part of your child's blood. The blood will be stored in the freezers at SDH and/or at the KEMRI laboratory in Kisumu. We will also store some data (facts) about your child, such as age and health status. Later on, we will need to do more tests on the stored blood at SDH and/or at the KEMRI/University of New Mexico laboratory in Kisumu. If the machines needed for the tests are not in these places, the tests may be done at the University of New Mexico in the United States. We will not ship your child's samples to the United States without approval from KEMRI Ethical Review Committee (ERC). These tests will be directly related to the research study described above. The test results will tell us how your child fights malaria. We will keep track of your child's sample with a unique number that matches your child with your child's blood sample (linkage code). We will report back to you any clinically relevant (important) results that we find from doing the tests. Dr. Douglas Perkins from the University of New Mexico and Dr. John Michael Vulule, KEMRI/University of New Mexico, Kisumu, will be in charge of and have access to these records. If we need to do any other test in the stored samples, not included in the current study, we will get full approval from KEMRI Scientific Steering Committee (SSC) and ERC and the University of New Mexico HHRC.

If you decide you do not want any samples stored, your child will still get the best possible medical care here at hospital. Your child can still be a part of this research study. If you agree to this, but then have questions or want to withdraw from the research study later, you can do so by contacting the research study investigators. Any further questions can be addressed to Dr. Douglas Perkins (KEMRI/University of New Mexico, PO Box 1578, Kisumu, Kenya, telephone: 0733360098) or Dr. John Michael Ong'echa (KEMRI/University of New Mexico, PO Box 1578, Kisumu, Kenya, telephone: 0733447920).

**Parent/guardian's Name: _____Child's Name:_____**

(Please Print) (Please Print)

**Date: _____** **Study# :_____**

**Parent/guardian's statement:**

□ The above aspect of the research study has been explained to me. The consent form has been read to me. My questions have been answered to my satisfaction. I was told that being in this aspect of the study is my choice. **I agree** to allow storage of my child's blood sample for the research study described above. I have received a copy of this form.

**Signatures:**

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

**Witness signature: _____ Date:_____**

**Investigator's signature: _____ Date: _____**

**Parent/guardian' thumbprint: _____**

*For questions or problems about your rights please call or write: The National/KEMRI Ethical Review Committee, PO Box 54840, Nairobi, telephone: 02-20722541.*

**Consent form 1c: Written consent of parent/guardian of child to allow use of child's blood specimens for the malaria genetic studies.**

As part of the research study outline above "The genetic basis of severe malarial anemia", the Kenya Medical Research Institute (KEMRI) and the University of New Mexico are studying how inherited factors (genes) affect malaria. The aim of this study is to learn how these genes in your child's body fight malaria. We also want to know how genes in malaria parasites affect your child's body. We will test the genes using blood taken from your child. We hope that learning about genes will help treat and prevent malaria.

Since you let your child take part in this research study, we will take blood from your child. We would like you to let us store some of the blood and facts about your child, such as age and health status. We will check the G6PD and sickle cell status of your child now. We will give you the results when you return for the follow-up visit two weeks after your first visit. These tests will help us learn what kind of response your child may have to malaria and malaria treatments. This may help us to find the reason why your child has anemia. We will test other genes in your child's body later. We may also test the genes in the malaria parasites later. So this may need longer storage of your child's blood. Your child's blood and facts will be stored at Siaya District Hospital (SDH) and/or University of New Mexico Mexico/Kenya Medical Research Institute (KEMRI) in Kisian. The machines that are needed to do some of the tests may not be available at SDH or University of New Mexico/KEMRI. If they are not, the tests may be done at the University of New Mexico in the USA. No tests will be done for any other disease. We will keep track of your child's sample with a unique number that matches your child with your child's blood sample (linkage code). We will report back to you any clinically relevant (important) results that we find from doing the tests. Dr. Douglas J. Perkins (University of New Mexico) and Dr. John Michael Ong'echa (KEMRI/University of New Mexico, Kisumu), will be in charge of and have access to these records. If we need to do any other test in the stored samples not included in the current study, we will get full approval from KEMRI Scientific Steering Committee (SSC) and ERC, and the University of New Mexico HHRC-IRB.

If you want, you can agree that we store your child's blood for these tests. If you refuse, you can still be part of the main study. You can tell us any time during the next 4 years if you don't want us to keep your child's blood for these tests. No matter what you decide, your child will still get the best possible medical care here at the hospital. If you have any questions or want to withdraw later, you can contact the study members listed below (Drs. Perkins and Ong'echa, and Vulule). If you ask us, we will remove your child and his/her sample from the study. If you have questions or feel you have been hurt you can contact Dr. Douglas J. Perkins (KEMRI/University of New Mexico, P.O. Box 1578, Kisumu, Kenya, telephone: 0733360098) or Dr. John Vulule (KEMRI, P. O. Box 1578, Kisumu, Kenya, telephone: 2022923/24). You can also contact Dr. John M. Ong'echa (KEMRI/University of

New Mexico, P.O. Box 1578, Kisumu, Kenya, telephone: 0733447920). For questions or problems about your rights please call or write: The National/KEMRI Ethical Review Committee, P. O. Box 54840, Nairobi, telephone: 02-20722541.

**Parent/guardian's Name: _____Child's Name:_____**

(Please Print) (Please Print)

**Date: _____** **Study #:_____**

**Parent/guardian's statement:**

□ The above study has been explained to me. The consent form has been read to me and my questions have been answered to my satisfaction. I have been told that taking part is voluntary. **I agree** to allow my child's blood sample to be stored for genetic tests. I have received a copy of this form.

□ The above study has been explained to me. The consent form has been read to me and my questions have been answered to my satisfaction. I have been told that taking part is voluntary. **I do not agree** to allow storage of my child's blood sample for genetic testing. I have received a copy of this form.

**Signatures:**

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

**Witness signature: _____** **Date:_____**

**Investigator's signature: _____** **Date: _____**

**Parent/guardian' thumbprint: _____**

*For questions or problems about your rights please call or write: The National/KEMRI Ethical Review Committee, P. O. Box 54840, Nairobi, telephone: 02-20722541.*

**********

**INVESTIGATOR'S CERTIFICATION**

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

**Investigator's signature: _____ Date: _____**

## Appendix 3 Helena Titan® IV Citrate Haemoglobin Electrophoresis Protocol

# HELENA TITAN® IV CITRATE HEMOGLOBIN ELECTROPHORESIS

The Helena Titan® IV Citrate Hemoglobin Electrophoresis Procedure is intended as a qualitative procedure for the identification of human hemoglobins.

**SUMMARY**

Hemoglobins (Hb) are a group of proteins whose chief functions are to transport oxygen from the lungs to the tissues and carbon dioxide in the reverse direction. They are composed of polypeptide chains called globin, and iron protoporphyrin heme groups. A specific sequence of amino acids constitutes each of four polypeptide chains. Each normal hemoglobin molecule contains one pair of alpha and one pair of non-alpha chains. In normal adult hemoglobin (HbA), the non-alpha chains are called beta. The non-alpha chains of fetal hemoglobin are called gamma. A minor (3%) hemoglobin fraction called $HbA_2$ contains alpha and delta chains. In a hereditary inhibition of globin chain synthesis called thalassemia, the non-alpha chains may aggregate to form HbH (β4) or Hb Bart's (γ4).

The major hemoglobin in the erythrocytes of the normal adult is HbA and there are small amounts of $HbA_2$ and HbF. In addition, over 400 mutant hemoglobins are now known, some of which may cause serious clinical effects, especially in the homozygous state or in combination with another abnormal hemoglobin. Wintrobe[1] divides the abnormalities of hemoglobin synthesis into three groups;

(1) production of an abnormal protein molecule (e.g. sickle cell anemia),
(2) reduction in the amount of normal protein synthesis (e.g. thalassemia), and
(3) development anomalies (e.g. hereditary persistence of fetal hemoglobin, HPFH).

The two mutant hemoglobins most commonly seen in the United States are HbS and HbC. Hb Lepore, HbE, HbG-Philadelphia, HbD-Los Angeles, and HbO-Arab may be seen less frequently.[2] Electrophoresis is generally considered the best method for separating and identifying hemoglobinopathies. The protocol for hemoglobin electrophoresis involves the use of two systems.[3-8] Initial electrophoresis is performed in alkaline buffer. Celluloseacetate is the major support medium used because it yields rapid separation of HbA, HbF, HbS and HbC and many other mutants with minimal preparation time. However, because of the electrophoretic similarity of many structurally different hemoglobins, the evaluation must be supplemented by citrate agar electrophoresis which measures a property other than electrical charge. This simple procedure requires only minute quantities of hemolysate to provide highly specific (but not absolute) confirmation of the presence of HbS, HbC and HbF, as well as several other abnormal hemoglobins.

**PRINCIPLE**

Very small samples of hemolysates prepared from whole blood are applied to the Titan® IV Citrate Agar Plate. The hemoglobins in the samples are separated by electrophoresis using citrate buffer, pH 6.0 to 6.3 and are stained with an o-Dianisidine or o-Tolidine staining solution. Separation of hemoglobins under these conditions depends both on the location of the substituted residue and on its electrophoretic charge. The method is based on the complex interactions of the hemoglobin with the electrophoretic buffer (acid pH) and the agar support.

**REAGENTS**

1. **Titan® IV Citrate Agar Plates (Cat. No. 2400)**
   **Ingredients:** Plates contain 1.5% agar (w/v) in 0.03 M citrate buffer with thimerosal added as a preservative.
   **WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY.**
   **Preparation for Use:** The plates are ready for use as packaged.
   **Storage and Stability:** Plates should be stored flat at 2° to 8°C and are stable until the expiration date indicated on the label. Store in the protective packaging in which the plates are shipped. **DO NOT FREEZE THE PLATES OR EXPOSE THEM TO EXCESSIVE HEAT**.
   **Signs of Deterioration:** The plates should have a smooth, clear surface. Discard the plates if they appear cloudy, show fungal or bacterial growth, or if they have been exposed to freezing (a cracked or bubbled surface) or excessive heat (a dried, thin surface).
2. **Citrate Buffer (Cat. No. 5121)**
   **Ingredients:** Each package of Citrate Buffer contains sodium citrate and citric acid.
   **WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST.**
   **Preparation for Use:** Dissolve one package of buffer in 1000 mL of deionized water. The buffer is ready for use when all material is dissolved and completely mixed.
   **Storage and Stability:** The packaged buffer should be stored at room temperature (15° to 30°C) and is stable until the expiration date indicated on the package. Diluted buffer is stable for one month at 2° to 8°C.
   **Signs of Deterioration:** Do not use packaged buffers if the material shows signs of dampness or discoloration. Discard diluted buffer if it becomes turbid.
3. **Hemolysate Reagent (Cat. No. 5125)**
   **Ingredients:** Hemolysate Reagent is an aqueous solution of 0.005 M EDTA and 0.07% potassium cyanide as hemoglobin preservatives.
   **WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT PIPETTE BY MOUTH. HARMFUL IF SWALLOWED**.
   **Preparation for Use:** The reagent is ready for use as packaged.
   **Storage and Stability:** The reagent should be stored at room temperature (15° to 30°C) and is stable until the expiration date indicated on the vial.
   **Signs of Deterioration:** The reagent should be a clear, colorless solution.
4. **Stains**
   **a. o-Dianisidine (Cat. No. 5036)**
   **Ingredients:** 0.2% (w/v) 3,3 dimethoxybenzidine in methanol after reconstitution.
   **WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. CARCINOGEN. DO NOT INGEST. AVOID CONTACT WITH SKIN.** The reagent is highly toxic and can cause skin irritation. Should reagent come into contact with skin, wash with copious amounts of water. Harmful if swallowed.
   **Preparation for Use:** Dissolve one vial of stain with 1 L methanol.
   **Storage:** The stain should be stored at room temperature (15° to 30°C) and is stable until the expiration date on the vial.

**Signs of Deterioration:** The reagent should be light yellow-brown. Discard reagent if it becomes dark brown and/or contains precipitate.

**b. o-Tolidine (Cat. No. 5041) (may be substituted for o-Dianisidine)**

**Ingredients:** 0.2% (w/v) o-Tolidine in methanol after reconstitution.

**WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. SUSPECTED CARCINOGEN. DO NOT INGEST. AVOID CONTACT WITH SKIN.** The reagent is highly toxic and can cause skin irritation. Should the reagent come into contact with skin, wash with copious amounts of water. Harmful if swallowed.

**Preparation for Use:** Dissolve the contents of one vial with 1 L methanol.

**Storage and Stability:** The stain should be stored at room temperature (15° to 30°C) and is stable until the expiration date on the vial.

**Signs of Deterioration:** The reagent should be light yellow-brown. Discard reagent if it becomes dark brown and/or contains precipitate.

## SPECIMEN COLLECTION AND PREPARATION

**Specimen:** Whole blood collected in tubes containing EDTA is the specimen of choice.

**Specimen Preparation:** Specimen hemolysates are prepared as outlined in the STEP-BY-STEP METHOD.

**Specimen Storage:** Whole blood samples may be stored up to one week at 2° to 8°C.

## PROCEDURE

**Materials required:** The following materials required for the procedure are available from Helena Laboratories.

| Item | Cat. No. |
|---|---|
| Zip Zone® Applicator | 4080 |
| Zip Zone® Sample Well Plate (2) | 4081 |
| Titan IV Aligning Base | 4083 |
| Titan Gel Electrophoresis Chamber | 4063 |
| Microdispenser and Tubes | 6008 |
| Zip Zone® Sponge Wicks | 9014 |
| Titan Plus Power Supply | 1504 |
| Titan® IV Citrate Agar Plates | 2400 |
| Citrate Buffer | 5121 |
| AFSC Hemo Control | 5331 |
| o-Dianisidine | 5036 |
| o-Tolidine | 5041 |
| Hemolysate Reagent | 5125 |
| Blotters | 5034 |
| Helena Marker | 5000 |

**Materials needed but not supplied:**

Hydrogen peroxide (3%)

Glacial acetic acid (Dilute 5 parts with 95 parts deionized water, to yield 5% solution.)

Absolute Methanol

1% Sodium nitroferricyanide

**SUMMARY OF CONDITIONS**

| | |
|---|---|
| Plate | Titan® IV Citrate Agar |
| Buffer | Citrate Buffer diluted to 1000 mL |
| Sample volume (Hemolysate) | 5µL |
| Application point | Anode |
| Number of applications | one (1) or two (2) |
| Electrophoresis time | 45 minutes |
| Voltage | 50 V |
| Staining time | 5 to 10 minutes |

## STEP-BY-STEP METHOD

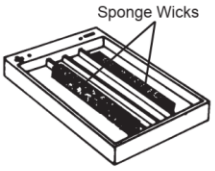
**A. Preparation of Titan® IV Citrate Agar Plate**

1. Remove the Titan® IV Citrate Agar Plate from the refrigerator and allow the plate to come to room temperature (15 to 30°C) while preparing the patient samples.
2. Remove the plate from the plastic bag and properly identify it by marking with a marker on the plastic support backing of the agar filled half of the plastic plate. Place the mark in one corner so that it will be aligned with sample No. 1.

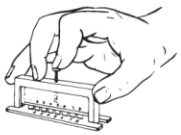
**B. Preparation of Patient Sample and the Control**

1. To prepare a hemolysate of the patient sample, add one (1) part of whole blood to 19 parts Hemolysate Reagent. Alternatively, if removal of denatured hemoglobins from the sample is deemed necessary; washed cells should be used.
   a. Centrifuge the blood sample at 3500 RPM for 5 minutes.
   b. Remove the plasma from the sample and wash the red blood cells in 0.85% saline (v/v) three times. After each wash, centrifuge the cells for 10 minutes at 3500 RPM.
   c. Add 1 volume deionized water and 1/4 volume toluene (or carbon tetrachloride) to the washed red cells. Vortex at high speed for one minute. Centrifuge the samples at 3500 RPM for 10 minutes.
   d. If toluene is used, the top layer in the tube will contain cell stroma and should be removed with a capillary pipette before proceeding to the next step. The clear middle layer contains the desired sample. If carbon tetrachloride is used, all red cell waste material will be contained in the bottom of the tube after centrifugation.
   e. Filter the clear red solution through two layers of Whatman #1 filter paper.
2. Prepare the AFSC Hemo Control by adding one (1) part of the control to one (1) part Hemolysate Reagent.
3. Mix all hemolysate preparations well. Cover the tubes and allow to stand for five (5) minutes.

**C. Preparation of Titan Gel Chamber**

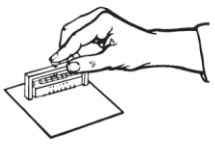



1. Pour approximately 100 mL of Citrate Buffer into each outer section of the Titan Gel Chamber.
2. Wet two sponge wicks in the buffer, and place the sponges in each outer compartment so that the top surface protrudes approximately 2 mm above the inner chamber ridges. (Care should be taken to rinse all buffer from the sponges before each use.) Gently press the sponge to assure complete saturation with buffer.

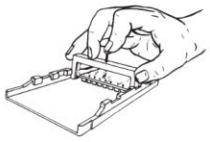


**D. Sample Application**

1. Mix the hemolysate solutions once more to ensure complete lysis.
2. Place 5 µL of each prepared hemolysate (patient and control) in separate wells of the Zip Zone® Sample Well Plate using the Microdispenser. Cover the Sample Well Plate with a glass slide if the samples are not used within 2 minutes.
3. To prime the Zip Zone® Applicator, quickly press the tips into the sample wells 3 or 4 times and apply to a blotter. Priming the applicator makes the second loading more uniform. Do not load the applicator again at this point, but proceed quickly to the next step.



4. Remove the cover from the Titan® IV Citrate Agar Plate. Position the plate in the Titan IV Aligning Base. The identification mark should be aligned with sample No. 1.
5. If desired, the spring can be removed from the applicator, allowing the applicator to rest upon the agar without cutting into it. To apply the sample to the plate, press the applicator tips into the sample wells 3 or 4 times and promptly transfer the applicator to the first set of stanchions on the Titan IV Aligning Base. Gently press the applicator tips down onto the gel surface. Allow the samples to soak into the agar for about one minute. To run 16 samples on one plate, use a second Zip Zone® Sample Well Plate and fill the wells with a second set of hemolysates (patient and control). Using a clean Zip Zone® Applicator, place the applicator in the second set of stanchions on the Titan IV Aligning Base and apply the samples to the plate in the same manner as before.



**E. Electrophoresis of the Sample Plate**

1. Quickly put the plate, agar side down, in the Titan Gel Chamber so that the agar layer makes good contact with the top surface of the sponges. The first application point should be nearest the anode (+).
2. Place the lid on the chamber and ensure that it is completely seated.
3. Electrophorese for 45 minutes at 50 volts. Electrophoresis time may be increased to 60 minutes, if additional separation of HbS from the application point is desired.

**F. Visualization of the Hemoglobin Bands**

1. Prepare the staining solution while electrophoresis is in progress. The reagents in this staining solution should be kept in separate bottles, mixed just prior to use, and discarded after each use. Prepare the staining solution as follows:
   - 5 mL 0.2% o-Dianisidine (o-Tolidine may be substituted)
   - 10 mL 5% acetic acid
   - 1 mL 3% hydrogen peroxide
   - 1 mL 1% sodium nitro ferricyanide
2. Upon completion of electrophoresis, remove the plate from the chamber and place on the counter top, agar side up.
3. Puddle the stain over the entire surface of the plate and stain for 5 to 10 minutes. Plates may also be immersed in the stain, but a greater volume of stain is required.
4. The hemoglobins present in the patient samples should be identified by comparison to the migration pattern of the AFSC Hemo Control. For immediate visualization, pour off the stain.
5. If permanent storage is desired:
   (1) Wash in 5% acetic acid for 30 minutes.
   (2) Rinse in deionized water for 10 minutes.
   (3) Hold the plate under gently running water.
   (4) Cut the agar in half, then slide a 3 x 5 card under the stained half of the agar and remove it from the holder.
   (5) Flood the agar surface with 2% glycerol for 35 minutes.
   (6) Tilt and drain the plate onto a blotter for 2 minutes.
   (7) Lay the plate on a fresh blotter, then dry at 50°C for 1 hour and 20 minutes or dry at 37°C for 3 to 4 hours.
   (8) Add an I.D. label.

**Stability of End Product:** The unpreserved plates are stable for three months if kept tightly closed. Dried plates are stable indefinitely.

**Quality Control:** The Helena AFSC Hemo Control (Cat. No. 5331) should be run on each Titan® IV Citrate Agar Plate. The control verifies all phases of the procedure and acts as a marker to aid in the identification of the hemoglobins in the unknown samples.

**RESULTS**

Figure 1 illustrates how a comparison of Citrate Agar and Cellulose Acetate plates can eliminate possible hemoglobins. Figure 2 lists the relative mobilities of various hemoglobin mutants on cellulose acetate and citrate agar plates.

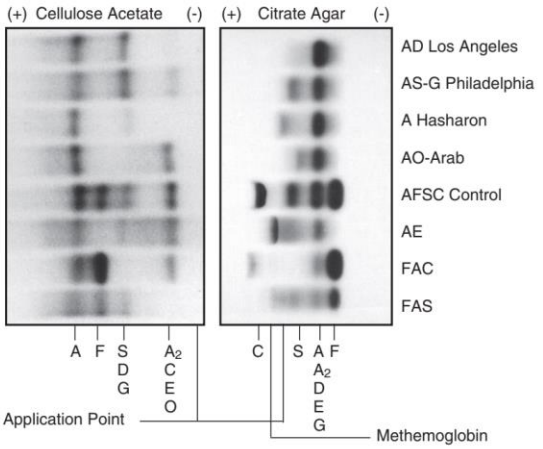



Figure 1. Electrophoretic Mobilities of Hemoglobins on Titan® III Cellulose Acetate and on Titan® IV Citrate Agar.

| | Cellulose Acetate | | | | | Citrate Agar | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | + | 0 A | -2.6 F | -5.2 S | -10 $A_2$ | – | -4.4 F | -0 A | +5.8 S | +10 C | + |
| S | | | | -5.2 | | | | | +5.8 | | |
| C | | | | | -10 | | | | | +10 | |
| *E | | | | | -10 | | | 0 | | | |
| Lepore | | | -5 | | | | | 0 | | | |
| G Philadelphia | | | -5.2 | | | | | 0 | | | |
| D Punjab | | | -5.2 | | | | | 0 | | | |
| O-Arab | | | | | -9.7 | | | +1.7 | | | |
| *Hasharon | | | | -5.5 | | | | +6.25 | | | |
| H | +8.5 | | | | | | | 0 | | | |
| Constant Spring | | | | | -11.9 | | | 0 | | | |
| Malmo | +0.5 | | | | | | | -1.1 | | | |
| $A_2$' | | | | -11.1 | | | | 0 | | | |
| Wood | 0 | | | | | | | -2.25 | | | |
| Barts | +7.6 | | | | | | -4.0 | | | | |
| *Köln | | | | | -7.5 | | | -2.5 | | | |
| N Baltimore | +6.6 | | | | | | | 0 | | | |
| ASG Philadelphia | 0 | | -5.2 | | -10 | | | 0 | +5.8 | | |
| J Oxford | +4.6 | | | | | | | 0 | | | |
| J Baltimore | +4.3 | | | | | | | 0 | | | |
| *Tacoma | +0.9 | | | | | | | 0 | | | |
| *Lufkin | | +3.2 | | | | | | 0 | | | |
| *Camperdown | | | | | | | | 0 | | | |
| K | +0.8 | | | | | | -4.0 | | | | |
| Hope | +0.8 | | | | | | -4.0 | | | | |
| Camdem | | +1.6 | | | | | | -2.8 | | | |
| New York | | +1.5 | | | | | | 0 | | | |
| *G San Jose | | | -3.6 | | | | | +7.5 | | | |
| C Harlem | | | | | -10.0 | | | +5.8 | | | |

O Arab - Migration varies on citrate agar from Hemoglobin A through Hemoglobin S.
J Baltimore - Trait is approximately 50% of the total.
J Oxford - Trait is approximately 25% of the total.
*Unstable hemoglobin
D Los Angeles and D Punjab are the same hemoglobin.
C Harlem and Georgetown are the same hemoglobin.
Köln is broadly smudged on both media possibly due to instability.

Figure 2. Relative Electrophoretic Mobilities of Hemoglobins on Cellulose Acetate and Citrate Agar.[9]

## REFERENCE VALUES

At birth, the majority of hemoglobin in the erythrocytes of the normal individual is fetal hemoglobin, HbF. Some of the major adult hemoglobin, HbA, and a small amount of $HbA_2$, are also present. At the end of the first year of life and through adulthood, the major hemoglobin present is HbA with up to 3.5% $HbA_2$ and less than 2% HbF.

## INTERPRETATION OF RESULTS

### Hb Electrophoresis

Citrate agar electrophoresis is a necessary followup test for confirmation of abnormal hemoglobins detected on cellulose acetate. Hemoglobins are genetically controlled, and the presence of abnormal hemoglobins is often associated with functional, physical and morphologic abnormalities in the erythrocyte, as well as pathological manifestations, such as hemolytic anemia.

### Sickle Trait

This is a heterozygous state showing HbA and HbS and a normal amount of $HbA_2$ on cellulose acetate. Results on citrate agar show hemoglobins in the HbA and HbS migratory positions (zones).

### Sickle Cell Anemia

This is a homozygous state showing almost exclusively HbS, although a small amount of HbF may also be present.

### Sickle-C Disease

This is a heterozygous state demonstrating HbS and HbC.

### Sickle Cell - Thalassaemia Disease

This condition shows HbA, HbF, HbS and $HbA_2$.
In Sickle Cell $\beta^{\circ}$-Thalassemia HbA is absent.
In Sickle Cell $\beta^{+}$-Thalassemia HbA is present in reduced quantities.
Thalassaemia-C Disease
This condition shows HbA, HbF, and HbC. C Disease This is a homozygous state showing almost exclusively HbC.
Thalassaemia Major
This condition shows HbF, HbA and $HbA_2$.

## LIMITATIONS

Some abnormal hemoglobins have similar electrophoretic mobilities and must be differentiated by other methodologies such as solubility tests and sickling or heat tests.

**Further testing:**

1. Globin chain analysis (both acid and alkaline) and structural studies may be necessary for positive identification.[5]
2. Anion exchange column chromatography is the most accurate method for quantitating $HbA_2$. Recommended are Sickle-Thal Quik Column® Method (Cat. No. 5334) for quantitation of $HbA_2$ in the presence of HbS, or the Beta-Thal $HbA_2$ Quik Column® Method (Cat. No. 5341). $HbA_2$ quantitation is one of the most important diagnostic tests in the diagnosis of β-thalassemia trait.
3. Low levels of HbF (1% to 10%) may be accurately quantitated by radial immunodiffusion using the Helena HbF-QUIPlate Procedure (Cat. No. 9325).

## REFERENCES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4. Center for Disease Control, Laboratory Methods for Detecting Hemoglobinopathies, U.S. Department of Health and Human Services/Public Health Service, 1984.
5. Schneider, R.G., Methods for Detection of Hemoglobin Variants and Hemoglobinopathies in the Routine Clinical Laboratory, CRC Critical Reviews in Clinical Laboratory Sciences, 1978.
6. Schneider, R.G., et al., Abnormal Hemoglobins in a Quarter Million People, Blood, 48(5):629-637, 1976.
7. Huisman, T.H.J. and Schroeder, W.A., New Aspects of the Structure, Function, and Synthesis of Hemoglobins, CRC Press, Cleveland, 1971.
8. Schmidt, R.M., et al., The Detection of Hemoglobinopathies, CRC Press, Cleveland, 1974.
9. Personal communication from Dr. Virgil Fairbanks.


**Titan® IV CITRATE HEMOGLOBIN ELECTROPHORESIS**

The following items, needed for the performance of the Titan® IV Citrate Hemoglobin Electrophoresis Procedure, must be ordered individually.

| Item | Cat. No. |
|---|---|
| Titan® IV Citrate Agar Plates | 2400 |
| Citrate Buffer | 5121 |
| AFSC Hemo Control | 5331 |
| o-Dianisidine | 5036 |
| o-Tolidine | 5041 |
| Hemolysate Reagent | 5125 |
| Blotter Pads (76 x 102 mm) | 5034 |
| Helena Marker | 5000 |
| Zip Zone® Applicator | 4080 |
| Zip Zone® Sample Well Plate (2) | 4081 |
| Titan IV Aligning Base | 4083 |
| Titan Gel Electrophoresis Chamber | 4063 |
| Microdispenser and Tubes (5 uL) | 6008 |
| Zip Zone® Sponge Wicks | 9014 |
| Titan Plus Power Supply | 1504 |

For Sales, Technical and Order Information and Service Assistance, call 800-231-5663 toll free.



HELENA LABORATORIES
Beaumont, Texas USA 77704

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2/14 (5)

# Appendix 4 Glucose-6-Phosphate Dehydrogenase (G-6-PDH) Deficiency Protocol

Trinity Biotech

## Glucose-6-Phosphate Dehydrogenase (G-6-PDH) Deficiency

REF 203-A

| | |
|---|---|
| Pour d'autres langues | Para outras línguas |
| Für andere Sprachen | Για τις άλλες λώσσες |
| Para otras lenguas | För andra språk |
| Per le altre lingue | For andre språk |
| Dla innych języków | For andre sprog |

www.trinitybiotech.com

### INTENDED USE

**Trinity Biotech** Glucose-6-Phosphate Dehydrogenase reagents are for the qualitative, visual fluorescence screening of G-6-PDH in whole blood. Samples which have been determined deficient or intermediate should be assayed by a quantitative G-6-PDH method such as **Trinity Biotech** Procedure No. 345.

### BACKGROUND AND PRINCIPLE OF TEST

G-6-PDH deficiency in red cells has been demonstrated to be the basis for certain drug-induced haemolytic anaemias.[1] Tarlov et al.[1] points out the importance of identifying individuals with this biochemical defect as an aid in the selection of therapeutic agents. Severe haemolytic anaemia may result in these individuals when they are given many commonly used drugs. The majority of subjects who have demonstrated G-6-PDH deficiency are clinically normal until exposed to one of several oxidant drugs (anti-malarial drugs, sulfa drugs, ascorbic acid and others).[2] This defect should be considered whenever an otherwise unexplained case of haemolytic anaemia is encountered.

Red cell G-6-PDH deficiency has been found in about 13% of African-American males and in about 3% of African-American females. The incidence is also high among other racial and ethnic groups, such as Sardinians, Greeks and Sephardic Jews.[1,3,4]

For semi-quantitative purposes, Beutler[3] first suggested estimating glucose-6-phosphate dehydrogenase (G-6-PDH, EC 1.1.1.49) in terms of visual appearance of fluorescence in red cell-substrate mixtures. The **Trinity Biotech** procedure is a modification of the Beutler revised method,[5,6] involving the reaction:

Glucose-6-Phosphate + NADP (Not Fluorescent) —G-6-PDH→ 6-Phosphogluconate + NADPH (Fluorescent)

The test is performed by incubating a small amount of blood with glucose-6-phosphate and nicotinamide adenine dinucleotide phosphate (NADP). Drops of the mixture are removed at 5-minute intervals, spotted on filter paper and then viewed under long-wave ultraviolet light. Fluorescence is clearly evident in mixtures prepared from normal blood, whereas deficient samples yield little or no fluorescence.

### REAGENTS

**TRIZMA® BUFFER SOLUTION, 1 x 12 ml, 203-2A**
TRIZMA® Buffer, 100 mmol/L, pH 7.8 and preservative.

**G-6-PDH SUBSTRATE, 5 x 2 ml, 203-2B**
Glucose-6-Phosphate (4 µmol), NADP (1.6 µmol), Glutathione, oxidized (1.6 µmol), and lytic agent.

### PRECAUTIONS

Glucose-6-Phosphate Dehydrogenase Deficiency reagents are "For *in vitro* diagnostic use". Normal precautions exercised in handling laboratory reagents should be followed. Dispose of waste observing all local, state and federal laws.

### PREPARATION

G-6-PDH Substrate solution is prepared by reconstituting G-6-PDH Substrate vial, Catalogue No. 203-2B, with 2.0 ml TRIZMA® Buffer Solution, Catalogue No. 203 2A. Allow to stand for 1-2 minutes and then mix by inversion.

### STORAGE AND STABILITY

Store G-6-PDH Substrate refrigerated (2-8°C). Reagent label bears expiration date.

Store TRIZMA® Buffer Solution at room temperature or refrigerated. Discard if turbidity develops.

G-6-PDH Substrate solution is stable for at least 2 weeks stored frozen, 1 week stored refrigerated (2-8°C), or up to 4 hours at room temperature (18-26°C).

### DETERIORATION

If a dried spot of G-6-PDH Substrate solution exhibits fluorescence when viewed under long-wave ultraviolet light, or blood-reagent spots prepared from normal specimens yield dull fluorescence, the reagent may have deteriorated and should be discarded.

### OPTIONAL REAGENTS

**G-6-PDH CONTROLS**

G-6-PDH Control Normal, 6 x 0,5 ml, G6888
G-6-PDH Control Intermediate, 6 x 0,5 ml, G5029
G-6-PDH Control Deficient, 6 x 0,5 ml, G5888

Lyophilized preparations containing G-6-PDH in a stabilized human red cell haemolysate base.

G-6-PDH Controls are POTENTIALLY BIOHAZARDOUS MATERIALS. Source materials from which these products were derived were found negative for HBsAg and for antibodies against HCV and HIV by approved test methods. However, since no test method can offer complete assurance that infectious agents are absent, these products should be handled observing the same safety precautions employed when handling any potentially infectious material.

### SPECIMEN COLLECTION AND STORAGE

It is recommended that specimen collection be carried out in accordance with CLSI document M29-A3.[7] No known test method can offer complete assurance that human blood samples will not transmit infection. Therefore, all blood derivatives should be considered potentially infectious.

Whole blood collected with ethylenediaminetetraacetic acid (EDTA), heparin or acidcitrate-dextrose (ACD) is satisfactory. Red cell G-6-PDH is stable in whole blood for one week refrigerated (2-8°C), but is unstable in red cell haemolysates.[2] Freezing of blood is not recommended.

### INTERFERING SUBSTANCES

The results of this assay should be interpreted with the haematologic status of the individual in mind at the time of testing.

Leukocytes and platelets may be rich in G-6-PDH and may cause some interference in the assay if present beyond normal levels.[8] Therefore, higher than normal levels of G-6-PDH could occur in cases of leukocytosis and thrombocytosis. In cases where the clinical picture fits G-6-PDH deficiency and the assay is not conclusive, the treating clinician may, on an individual basis, want to request a repeat of the assay with the buffy-coat removed before preparing the red cell haemolysate.

Young red blood cells (reticulocytes) have higher G-6-PDH levels than mature erythrocytes. A false-negative result, indicating the sample is normal, may be obtained in individuals with an anaemia and resulting reticulocytosis. Therefore the screening test should be performed after resolution of reticulocytosis resulting from a haemolytic episode or other causes.[9] The clinician should decide the time interval to wait for testing since it will depend on individual circumstances.

We are not aware of any drugs which may interfere with the test.

### PROCEDURE

**MATERIALS REQUIRED BUT NOT PROVIDED**

A long-wave ultraviolet light in a viewing box or a darkened room is needed.
A suitable lamp is the General Electric No. F15T8-BL, 15W, black light, which emits light between 320-420 nm
A short-wave ultraviolet light should not be used
Conventional or automatic pipettes are needed that reliably deliver 0.01, 0.2 and 2.0 ml
Pasteur pipettes or small glass rods are used to transfer reaction mixture
Whatman No. 1 filter paper
37°C Water bath
Timer
Recently drawn normal whole blood sample stored in refrigerator (less than one week)

**PROCEDURE**

1. Into tube (e.g., 13 x 100 mm) labeled NORMAL, add 0.2 ml G-6-PDH Substrate solution and 0.01 ml recently drawn normal blood. Mix by swirling and promptly transfer a drop of mixture to filter paper (Whatman No. 1). Identify spot on filter paper as "Zero-Time Normal". Place NORMAL tube in 37°C water bath and record time.
   **Note**: Spot sizes should be approximately 1/2 inch in diameter.
2. Into tube labeled TEST, add 0.2 ml G-6-PDH Substrate solution and 0.01 ml blood sample to be tested. Mix by swirling and promptly transfer a drop of mixture to filter paper. Identify spot on filter paper as "Zero-Time Test". Place TEST tube in 37°C water bath and record time.
3. Transfer additional drops of NORMAL and TEST to filter paper 5 and 10 minutes after "Zero-Time" applications. Label spots with appropriate times and allow to dry for 15-20 minutes.
4. Visually inspect dried spots under long-wave ultraviolet light. Record fluorescent intensity (absent, weak, moderate or strong) of each sample at 5 and 10 minutes.

**Notes**:
1. Because of the rapid speed of reaction, "Zero-Time" spots may exhibit traces of fluorescence.
2. Fluorescent spots are stable for up to two weeks stored in a plastic bag with desiccant in the refrigerator at 2-8°C.
3. In the absence of a recently drawn blood sample required for the Normal tube in Step 1 of the Procedure, you may substitute G-6-PDH Normal Control, Catalogue No. G 6888.

### QUALITY CONTROL

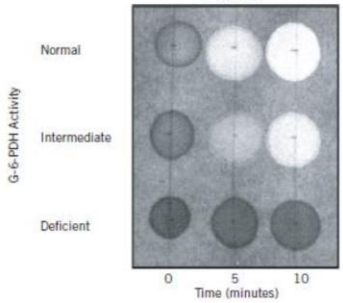
Samples with normal G-6-PDH and with G-6-PDH deficiency should be included with each group of assays to ensure reliable test performance. A sample with intermediate G-6-PDH activity could also be included. G-6-PDH Control Normal, Catalogue No. G 6888, G-6-PDH Control Intermediate, Catalogue No. G 5029, and G-6-PDH Control Deficient, Catalogue No. G 5888, are suitable for this purpose. They are lyophilized human blood preparations with known levels of G-6-PDH activity.

### RESULTS

The test is designed to distinguish normal from grossly deficient samples. By visually comparing the amount of fluorescence in the 5 minute spots of the sample with that of a normal sample, samples with intermediate deficiencies may also be discerned.

Typical results are shown in Figure 1 below:

FIGURE 1




A normal sample will demonstrate moderate to strong fluorescence after 5 minutes, and strong fluorescence after 10 minutes.

An intermediate level sample, will generally demonstrate weak fluorescence after 5 minutes and moderate fluorescence after 10 minutes.

A grossly deficient sample will reveal very faint or no fluorescence even after 10 minutes.

It is recommended that samples which have been determined as deficient or intermediate by this procedure be assayed by a quantitative G-6-PDH technique such as **Trinity Biotech** No. 345.

## LIMITATIONS

The test is designed to distinguish normal and intermediate from grossly deficient samples and should not be used to assess the degree of deficiency. It is recommended that samples which have been determined as deficient or intermediate by this Procedure be assayed by a quantitative G-6-PDH technique such as **Trinity Biotech** Procedure No. 345.

## EXPECTED VALUES

| G-6-PDH Activity | Fluorescence |
|---|---|
| **Normal** | Moderate or strong fluorescence is observed after 5 minutes and strong fluorescence after 10 minutes. |
| **Intermediate** | Weak fluorescence is observed after 5 minutes and moderate fluorescence after 10 minutes. |
| **Deficient** | Weak or no fluorescence is observed after both 5 minutes and 10 minutes. |

Blood samples from 24 clinically healthy adults showed moderate to strong fluorescence after 5 minutes and strong fluorescence after 10 minutes. Blood samples from 15 donors with intermediate G-6-PDH showed weak fluorescence after 5 minutes and moderate fluorescence after 10 minutes. Blood samples from five known G-6-PDH deficient individuals showed very faint or no fluorescence after 5 minutes and 10 minutes.[10]

## PERFORMANCE CHARACTERISTICS

### CORRELATION

71 samples including normal, intermediate, and deficient enzyme levels were assayed simultaneously by G-6-PDH Deficiency Screening Kit No. 202 and modified Kit No. 203-A. All samples were identified similarly by the two test kits.

### REPRODUCIBILITY STUDIES

Normal, deficient, and intermediate samples were assayed on three occasions over a period of several days. Results obtained for each of the samples were identical for the replicate assays.

## REFERENCES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9. *Glucose-6-Phosphate Dehydrogenase Deficiency*. IN Hematology, WJ Williams, E Beutler, AJ Erslev, RW Rundles, Editors, McGraw Hill, 1972, p 397.
10. Data obtained by **Trinity Biotech**.


## ORDERING INFORMATION

| KIT | | |
|---|---|---|
| **Catalogue No.** | | **203-A** |
| Maximum Assays | | 50 |
| **Contents – Catalogue Numbers** | | |
| TRIZMA® Buffer, 203-2A | | 12 ml |
| G-6-PDH Substrate, 203-2B | | 5 x 2 ml |
| Reagents available in kit only and cannot be purchased individually. | | |
| **OPTIONAL REAGENTS** | | |
| **Catalogue No.** | **Item** | **Quantity** |
| G 6888 | G-6-PDH CONTROL NORMAL | 6 x 0,5 ml |
| G 5029 | G-6-PDH CONTROL INTERMEDIATE | 6 x 0,5 ml |
| G 5888 | G-6-PDH CONTROL DEFICIENT | 6 x 0,5 ml |

## GUIDE TO SYMBOLS