ASSOCIATION BETWEEN GENETIC VARIATIONS IN SELECTED CELL RECEPTORS AND NUCLEAR TRANSCRIPTION FACTORS AND SUSCEPTIBILITY TO PAEDIATRIC *P. FALCIPARUM* SEVERE MALARIA ANAEMIA IN SIAYA COUNTY, WESTERN KENYA

BY

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DECLARATION

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

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DEDICATION

To my wife Quinter Atieno Tom for her support.

ABSTRACT

Plasmodium falciparum severe malaria aneamia [SMA, haemoglobin (Hb) <5.0 g/dL or Hb<6.0 g/dL with any parasite density] remains the most life-threatening malaria. With its immuno-genetic basis partially understood, efficacious therapeutics' development remains unachieved. Genetic susceptibility factors offer tools for unravelling molecular mechanisms involved. Cellular receptors pathways are crucial in immunological reactions leading to parasite clearance. However, contributions of genetic variations within immune cell receptors to SMA pathogenesis is partially understood. This study determined the role of polymorphisms in immune cell receptors i.e. Cluster of differentiation 40 (*CD40*), Interleukin-23 receptor (*IL-23R*) and Fc gamma receptors and their pathway genes; Nuclear factor of kappa light enhancer in B-cells (*NFκB1*) and its inhibitor IkappaB alpha (*NFκBIA*) on susceptibility to paediatric SMA in Siaya County, Western Kenya. Moreover, the *in-vitro* effects of *P. falciparum* haemozoin (*PfHz*) on expression of inflammatory mediators via CD40 pathway was also determined. Laboratory measures were determined in children (N=1,128, aged 6-36 months) at Siaya County Referral Hospital. Genotyping and gene expression was done using TaqMan® assays while inflammatory mediators' levels were determined by human cytokine 25-plex Ab Bead Kit. Odds ratios were computed by regression analysis controlling for confounders (age, HIV-1, bacteraemia, α-thalassemia and sickle-cell trait). Differences in the levels of inflammatory mediators between carriage and non-carriage of haplotypes were determined by Man-Whitney U-test/student's t-test. *CD40* -508G/173C/-1C (GCC) haplotype carriage had 69% protection against SMA [OR=0.31, 95% CI=0.14-0.67, *P*=0.003] while the carriage of GCT haplotype was associated with susceptibility to SMA [OR=5.24, 95% CI=3.31-8.82, *P*<0.001]. Carriage of GCC haplotype had increased IL-1β, IL-2 and MIP-1α (*P*=0.002, *P*=0.029 and *P*=0.048, respectively) while the carriage of GCT haplotypes had lower levels of IL-1β and IL-17, (*P*=0.017 and *P*=0.003, respectively). Analyses of *NFκB1* revealed that carriage of AT (-8079A/-3297T) haplotype was associated with risk of SMA (OR=1.58, 95% CI=1.13-2.33, *P*=0.008) while GC (-8079G/-3297C) was associated with 40% reduced risk of SMA (OR=0.60, 95% CI=0.42-0.86, *P*=0.005). The *NFκBIA* (-826A/-310A) AA haplotype carriage was associated with SMA risk (OR=1.60, 95% CI=1.02-2.47, *P*=0.042) while AG (-826A/-310G) conferred 43% protection from SMA (OR=0.57, 95% CI: 0.33-0.98, *P*=0.037). The carriage of *NFκBIA (*AG*)* (-826A/-310G) haplotype had increased levels of IL-10 and IP-10 compared to non-AG (*P*=0.050 and *P*=0.016, respectively). Combined genotypes showed that *NFκB1*-8079AA/*NFκBIA*-826GA was associated with susceptibility to SMA (OR=2.31, 95% CI=1.30-4.08, *P*=0.004). *NFκB1*-3279CC/*NFκBIA*-826GG had 31% protection against SMA (OR=0.69, 95% CI=0.48-0.96, *P*=0.033) while the *NFκB1*-3279TT/*NFκBIA*-826GA was associated with increased risk of SMA (OR=2.77, 95% CI=1.10-6.97, *P*=0.031). Carriage of the *NFκB1*-3297CC/*NFκBIA*-310GG was associated with protection against SMA (OR=0.64, 95% CI=0.44-0.92, *P*=0.016) whereas *NFκB1*-3297TT/*NFκBIA*-310GG combination were two-fold susceptible to SMA (OR=2.10, 95% CI=1.32-4.10, *P*=0.002). Further IL-17 levels were positively correlated with haemoglobin levels (ρ=0.151, *P*=0.027). Expression levels of IL-1β, TNF-α and IL-6 where increased by *PfHz* (*P*<0.05). Both *IL-23R* rs1884444T/rs7530511T (TT) and *FcγRIIA*-131Arg/*FcγRIIIA*-176F/*FcγRIIIB*-NA2 haplotypes were associated with increased susceptibility to SMA (OR=1.12, 95% CI=1.07-4.19, *P*=0.030) and (OR=1.70; 95% CI=1.02-2.93; *P*=0.036), respectively. Results demonstrate that polymorphisms in immune cell receptors and their associated pathway genes condition SMA susceptibility and that *PfHz* is important modulator of expression of pro-inflammation *in-vitro*. Polymorphisms associated with malaria risk identified here are important markers of genetic risk factors to disease severity. Future genetic studies should investigate a panel of cell receptor help explicitly unravel the molecular mechanisms involved in cell signalling leading to disease phenotypes and therapeutics with the ability to counter the deregulatory effects of *PfHz* during infection.

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ACRONYMS AND ABBREVIATIONS

Arg	Arginine
cDNA	Complementary DNA
CD40	Cluster of differentiation 40
CD40L	Cluster of differentiation 40 Ligand
CM	Cerebral malaria
DNA	Deoxyribonucleic Acid
GR-β	Glucocorticoid receptor-beta
GWAS	Genome wide association studies
G6PD	Glucose-6-phosphate dehydrogenase deficiency
HDP	High density parasitemia (>10,000 parasitesμL^{-1})
His	Histidine
HIV-1	Human immunodeficiency virus-1
HWE	Hardy-Weinberg Equilibrium
Ig	Immunoglobulin
IL	Interleukin
LD	Linkage disequilibrium
MCHC	Mean corpuscular haemoglobin concentration
MCH	Mean corpuscular haemoglobin
MCV	Mean corpuscular volume
MIP-1α	Macrophage inflammatory protein-1 alpha
MMP	Matrix metalloproteases
mRNA	Message ribonucleic acid
NFκB1	Nuclear factor of kappa light enhancer in B-cells 1 gene
NFκBIA	Nuclear factor of kappa light enhancer in B-cells inhibitor alpha gene
NOS-2	Nitric oxide synthase-2

PBMCs	Peripheral blood mononuclear cells
PfHz	*Plasmodium falciparum*-derived haemozoin
RANTES	Regulated on activation, normal T-cell expressed and secreted
RBC	Red blood cells,
RDW	Red cell distribution width;
RPI	Reticulocyte production index
sCD40	Soluble CD40
SNPs	Single nucleotide polymorphisms
SMA	Severe malaria anaemia (Hb<5.0 g/dL or 6.0 g/dL with any density parasitemia)
SCRH	Siaya County Referral Hospital
TNF-α	Tumour necrosis factor-alpha
TNFR	Tumour necrosis factor receptor
WHO	World health organization
WBCs	White blood cells

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CHAPTER ONE: INTRODUCTION

1.1. Background information

One of the most prevalent parasitic infections in humans is *Plasmodium falciparum* malaria (WHO, 2000). According to WHO estimates, Africa contributes to 91% of malaria deaths worldwide (WHO, 2018). However, between 2000 and 2017, malaria incidence has declined by 22% (WHO, 2016). *P. falciparum*-related morbidity and mortality primarily occurs in immune-naïve infants and young children (Giha *et al.,* 2005; Marsh *et al.,* 1995). Severe malaria presents with overlapping clinical sequelae that include severe malaria anaemia (SMA), metabolic acidosis, respiratory distress, cerebral malaria (CM) and hypoglycaemia (Rowe *et al.,* 2009). Severe malaria anaemia defined by WHO as [(SMA; Haemoglobin Hb<5.0 gdL^{-1}, with any density parasitemia)] or by local modified definition as [SMA; Hb<6.0 g/dL with any parasite density, McElroy *et al.,* 2000] in children below 3 years from *P. falciparum* holo-endemic transmission areas such as Siaya County in western Kenya is a common clinical manifestation of severe *P. falciparum* malaria. It is characterized by complex immunological processes that result into; shortened lifespan of circulating erythrocytes (Looareesuwan *et al.,* 1987), haemolysis of uninfected erythrocytes (Ekvall *et al.,* 2001), structurally and functionally impaired erythrocytes (Selvam and Baskaran, 1996), enhanced erythrophagocytosis (Waitumbi *et al.,* 2000) and reduced erythropoiesis (Chang *et al.,* 2004). These contribute to decreased erythrocytes in circulation hence anaemia. However, the actual molecular pathological mechanisms that constitute these manifestations remain to be elucidated.

The pathophysiology of SMA is considered to be a multifactorial process which can develop due to pathogenic and/or protective mechanisms by the host due to infection (Kai and Roberts, 2008; Phillips and Pasvol, 1992). As depicted by earlier studies in western Kenya, it is

generally becoming accepted that SMA in children is due to an imbalance in the production of both pro-inflammatory and anti-inflammatory mediators, polymorphisms in immune regulatory genes and worsened in the presence of co-infections with HIV-1 and pathogenic bacteria (Anyona *et al.,* 2013; Munde *et al.,* 2017; Ouma *et al.,* 2008a; Ouma *et al.,* 2012; Ouma *et al.,* 2010). Specific molecular pathways that may be involved in regulating these complex networks of immune genes remain largely unexplored. Since clinical course of *P. falciparum* malaria varies between infected individuals and depends on host genetics and or parasite factors, studying the role of genetic polymorphisms within important immune cell receptors such as Cluster designation 40 (*CD40*), Interleukin-23 receptor, (*IL-23R*) and Fc gamma receptors (*FcγR*) genes may provide additional into their contribution to malaria pathogenesis. Signals from these receptors are mediated via nuclear factor of kappa light enhancer in B-cells (*NFκB1*) and its inhibitor *NFκBIA*. Therefore, investigation of additional polymorphisms within these transcription factors is important in providing further insights into the role played by the disease pathways evoked by *P. falciparum* infection.

Cluster of differentiation 40 (CD40) is a type I 50kDa cell surface trans-membrane glycoprotein which belongs to the tumour necrosis factor receptor (TNFR) superfamily because of its structural and molecular homology to the TNFR (Smith *et al.,* 1994). It is ubiquitously expressed by a wide range of immune cells e.g. B cells, macrophages, monocytes, and dendritic cells and also non-immune cells such as endothelial cells, keratinocytes platelets and fibroblasts (Bourgeois *et al.,* 2002; Schonbeck and Libby, 2001). The CD40 ligand (CD154) is mainly expressed in the activated but not resting T-cells, activated B cells and activated platelets. However, inflammatory response leads to its expression in monocytes and macrophages (Danese *et al.,* 2003; Henn *et al.,* 1998; Higuchi *et al.,* 2002). CD40 ligation to CD40L (CD154) is

required for B-cell growth and differentiation, immunoglobulin (Ig) class switching (Jabara *et al.,* 1990) and somatic hyper-mutation (Rousset *et al.,* 1991). Under both normal and inflammatory conditions, the CD40 signalling pathway has been shown to be vital in the survival of immune cells such as germinal centre B-cells, dendritic cells (DCs) and endothelial cells (Bishop *et al.,* 2007). In the context of CD40, most studies have mainly focused on auto-immune and inflammatory disease such as inflammatory bowel disease, asthma (Danese *et al.,* 2004), atherosclerosis (Yun *et al.,* 2014) and rheumatoid arthritis (Orozco *et al.,* 2010). However on the basis of infectious diseases, the role of CD40 has been investigated mainly in mice models as opposed to human for diseases such as Leishmaniasis in which mice that were deficient of CD40L failed to show protective immune response (Soong *et al.,* 1996). In addition, CD40 has been shown to control *T. cruzi* infection through inflammatory cascade that includes interleukin -12 (IL-12) and nitric oxide (NO_X) (Chaussabel *et al.,* 1999). Moreover, during autoimmunity or infection, CD40 B-cell signalling is essential for immunoglobulin class switching, somatic hyper-mutation, generation of memory B-lymphocytes and immunoglobulin (Ig) production (Danese *et al.,* 2004). Antibodies have been shown to confer immunity during malaria infection (Bouharoun-Tayoun and Druilhe, 1992).

The CD40 activation stimulates the NFκB pathway which results into regulation of specific gene targets for release of pro-inflammatory cytokines (Bourgeois *et al.,* 2002). Moreover, CD40 stimulation by its ligand i.e. CD40L has been shown to induce translocation of the NFκB p65 into the nucleus from cytoplasm in neutrophils (Song *et al.,* 2011b). These studies underscore the important immune functions of the CD40 pathway in inflammatory conditions and are worth studying in the context of SMA. However, CD40 signalling pathway still remains largely unexplored with regards to infectious diseases including *P. falciparum* malaria. This

study determined the association between *CD40* promoter polymorphisms (-508G/A, rs1800686, -173C/T rs752118 and -1C/T rs1883832) and outcome of *P. falciparum* malaria in children aged 6-36 months residing in Siaya County, western Kenya.

Nuclear factor of kappa light enhancer in B-cells (NFκB) and its inhibitor IkappaB alpha (IκB-α) play important roles in infectious disease pathogenesis through their ability to regulate the molecular pathways that results in the production of soluble immune modulators such as pro-inflammatory cytokines (Ghosh *et al.,* 1998; Siebenlist *et al.,* 1994). NFκB dependent signalling in macrophages has been shown to influence production of pro-inflammatory cytokines as a result of *P. falciparum* haemozoin and glycosylphosphatidylinositol (GPI) stimulation (Tachado *et al.,* 1996; Zhu *et al.,* 2005). Increased expression of NFκB p65 has been shown in severe *P. falciparum* malaria in Thai adults (Viriyavejakul *et al.,* 2014). Monocyte phagocytosis of *P. falciparum* haemozoin has been shown to evoke the production of pro-inflammatory molecules through the nuclear factor (NFκB) pathway. Engagement of the NFκB pathway during this process is shown by the translocation of the NFκB complex to the nuclear, phosphorylation and resultant degradation of the inhibitory IkB-alpha protein (Aldieri *et al.,* 2003; Nair *et al.,* 2006; Prato *et al.,* 2010). Aberrancy in the function of the NFκB has been linked to susceptibility to infectious disease (Gerondakis *et al.,* 1999; Perkins, 2000). Studies in mice deficient of specific *NFκB1* gene demonstrated enhanced susceptibility to bacterial and parasitic infections (Caamano *et al.,* 1999; Grigoriadis *et al.,* 1996; Sha *et al.,* 1995). Immune deregulations have been suggested to be partly a consequence of decreased expression of NFκB in peripheral blood mononuclear cells (Torres *et al.,* 2013). These studies show that NFκB signalling pathway could be involved in malaria pathogenesis. However, the potential role of NFκB in paediatric populations in which SMA is the common clinical phenotype remains unknown. This study

therefore determined the association between the genotypic and haplotypic structures in *NFκB1* (-3297 C/T, rs980455 and -8079G/A, rs747559) and *NFκBIA* (-826G/A, rs2233406 and -310G/A rs2233409) and severe malaria anaemia and whether these variations are associated with changes in the circulating levels of IL-17, IL-10 and IP-10 produced during malaria infection.

The main metabolite that has been established to be associated with immunosuppression in children with SMA is the *P. falciparum*-derived haemozoin (*PfHz*) (Giribaldi *et al.,* 2004; Keller *et al.*, 2006). In addition, *PfHz*-loaded monocytes have been associate with SMA in children from western Kenya holo-endemic transmission area (Novelli *et al.,* 2010). These studies clearly illustrate that *PfHz* is a pathological factor in SMA. Even though *PfHz* is an important factor in SMA, the cellular signalling mechanism lead to SMA remains to be established. To provide the evidence that CD40 signalling pathway maybe involved in *PfHz*-mediated SMA via stimulation of production of pro-inflammatory cytokines, the current study investigated whether the CD40 and signalling pathway is associated with SMA by measuring the expression levels of selected CD40 downstream pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) when PBMCs from malaria naïve individuals were challenged with physiological doses (concentrations, 10.0μg/mL) of *PfHz*.

Interleukin-23 receptor (IL-23R) is the receptor for IL-23 and, thus, is involved in the T-helper 17 (Th17) cell-mediated inflammatory reactions (Chen *et al.,* 2007; Volpe *et al.*, 2008). Th17 cells are pro-inflammatory $CD4^+$ effecter T-cells that have been shown to mediate inflammatory process by secreting increased levels of IL-17, a pro-inflammatory cytokine in response to stimulation of cells that express IL-23R (Romagnani, 2008). The IL-23R is involved in many important biological functions, for instance Th17 cell-mediated immune response, tumour promoting pro-inflammatory processes (Volpe *et al.*, 2008). These studies point out at

important functions of the IL-23R signalling pathway and therefore its role in immune mediated reactions. In a well-designed biological study, polymorphic variation rs10889677CC carriage of the *IL-23R* have been shown to be associated with reduced T-regulatory cells and faster proliferation n of T-cells (Zheng *et al.*, 2012). This clearly establishes that the genetic variants that affect the efficacy of the immune system are likely to be a risk factor in inflammatory diseases. Therefore, variants within this receptor especially the exonic variants would influence outcome of *P. falciparum* malaria. However, no investigations have explored the potential role of IL-23R polymorphisms in paediatric populations in which SMA is the most common clinical phenotype. The study therefore, in addition, determined the association between the genotypic and haplotypic structures in *IL-23R* (94G/T; rs1884444 and 53219C/T; rs7530511) and paediatric severe malaria anaemia.

Fragment crystallisable gamma receptors (FcγRs) are integral in linking both humoral and cellular immunity as it allows interaction between specific antibodies and effector cells (Raghavan and Bjorkman, 1996). The human FcγR mediates phagocytic function of monocytes, macrophages and neutrophils. The presence of *FcγRIIA*-131Arg/131His and *FcγRIIIA* (-176F/V) polymorphism affects the binding to the IgG_1 and IgG_3 (Warmerdam *et al.*, 1990). *FcγRIIIB* are found on the polymorphonuclear leucocytes and has two allotypic forms i.e. neutrophil antigens (NAs) 1 and 2 which differ in minor amino acids at position 65 and 82 in two extra-glycosylation sites in NA2 hence different binding affinities. This leads to preferential parasite clearance from circulation. Currently, no studies have been performed on extensive haplotypes analysis of the role of polymorphisms in *FcγR* genes in a well phenotyped cohort of children with *P. falciparum* malaria. The current study in addition determined the association between *FcγRIIA* (-131Arg/His), *FcγRIIIA* (176F/V) and *FcγRIIIB*-NA1/NA2 haplotypes and SMA, and the

influence of these haplotypes on peripheral parasite burden during acute *falciparum* infections in well phenotyped cohort of children from a *P. falciparum* holo-endemic transmission area western in Kenya.

The unifying hypothesis for selection of these cell receptors genes NFκB signalling pathway genes in the current study is that these molecular signalling pathway has been shown to modulate production of pro-inflammatory mediators that influence outcome of inflammatory diseases. Since receptors' regulatory pathway remains largely unexplored in the context of severe malaria anaemia especially in holo-endemic areas, and considering their important biological function in initiating signal transduction in immune response, the current study determined the potential role of genetic polymorphisms in the *CD40, NFκB1, NFκBIA, IL-23R* and *FcγR* genes in *P. falciparum* severe malaria anaemia in children of age 6-36 months who are resident of holo-endemic transmission area of Siaya County, western Kenya.

1.2. Statement of the Problem

In *P. falciparum* holo-endemic transmission areas, such as Siaya County, western Kenya, severe malaria is a predominant cause of under-five morbidity and mortality (Amek *et al.*, 2014). Since most malaria control methods have failed and there is emergence of increased risk of resistance to conventional malaria drugs, there is urgent need to develop long-lasting malaria vaccines and other therapeutics. Understanding of molecular/genetic mechanism of SMA is a prerequisite for rationale based therapeutic design. However, the lack of adequate knowledge on the molecular/genetic mechanisms presents a significant limitation to the development of more effective prophylactic and therapeutics.

1.3. General objective

To determine the association between genetic variations in selected cell receptors and nuclear transcription factors genes and susceptibility to paediatric *P. falciparum* severe malaria anaemia in Siaya County, Western Kenya.

1.3.1. Specific objectives

i. To determine the association of between *CD40* promoter polymorphism (-508G/A, rs1800686; -173C/T rs752118 and -1C/T rs1883832) and susceptibility to severe malaria anaemia and the alterations in malaria associated pro-inflammatory cytokines.

ii. To determine the association between *NFκB1* (-8079G/A, rs747559 and -3297 C/T rs980455) and *NFκBIA* (-826G/A, rs2233406 and -310G/A rs2233409) variations and susceptibility to severe malaria anaemia and alterations in malaria associated IL-17, IL-10 and IP-10 levels.

iii. To determine *in-vitro* the effect of *PfHz* on the expression of CD40 downstream pro-inflammatory cytokines.

iv. To determine association between the haplotypes of non-synonymous mutations within *IL-23R* (94G/T; rs1884444 and 53219 C/T rs7530511) and susceptibility to severe malaria anaemia in a *P. falciparum* holo-endemic transmission area of Kenya.

v. To determine the association between *FcγRIIA*-131Arg/His, *FcγRIIIA*-176F/V and *FcγRIIIB*-NA1/NA2 genetic polymorphisms and susceptibility to severe malaria anaemia in children in western Kenya.

1.3.2. Null Hypothesis

i. There is no association between *CD40* promoter polymorphism (-508G/A, rs1800686; -173C/T rs752118 and -1C/T rs1883832) and susceptibility to severe malaria anaemia and alterations in malaria associated pro-inflammatory cytokines.

ii. There is no association between *NFκB1*, -8079G/A rs747559 and -3297 C/T rs980455) and *NFκBIA* (-826G/A, rs2233406 and -310G/A rs2233409) variations and susceptibility to severe malaria anaemia and alterations in malaria associated IL-17, IL-10 and IP-10 levels.

iii. There is no *in-vitro* effect of *PfHz* on the expression of CD40 downstream pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α).

iv. There is no association between the haplotypes of non-synonymous mutations within *IL-23R* (94G/T; rs1884444 and 53219 C/T rs7530511) and susceptibility to severe malaria anaemia in a *P. falciparum* holo-endemic transmission area of Kenya.

v. There is no association between *FcγRIIA*-131Arg/His, *FcγRIIIA*-176F/V and *FcγRIIIB*-NA1/NA2 genetic polymorphisms and susceptibility to severe malaria anaemia in children in western Kenya.

1.4. Significance of the study

Earlier studies have demonstrated that children between 7 to 24 months who reside in *P. falciparum* holo-endemic areas such as Siaya County, western Kenya are most susceptible to SMA (Bloland *et al.,* 1999; McElroy *et al.,* 2000). The choice of children aged between 6-36 months was to capture the vulnerable group as well as when they acquire immunity against clinical disease. Siaya County is a *P. falciparum* holo-endemic transmission area (Ong'echa *et al.,* 2006). Moreover, since ~94% of the participants belong to the Luo ethnic group (Ong'echa *et al.,* 2006), this approach provided a homogenous population suitable a genetic-based study of this nature. From this cohort of children, the study identified significant effects of genetic variations i.e. haplotypes, genotypes and genotype combinations in cell receptors (*CD40, IL-23R* and *FcγRs*) and their downstream transcription factors (*NFκB1* and *NFκBIA*). Furthermore, the

study provides evidence that the *P. falciparum*-derived haemozoin (*PfHz*) is an important modulator of the CD40 pathway which affects a number of downstream genes involved in production of reactive oxygen species (ROS), pro-inflammation, immunoglobulin class switching and B-cell maturation geared towards infection clearance. This provide a basic information required in design of therapeutics that can target the CD40 pathway hence reducing SMA in immune naïve populations.

CHAPTER TWO: LITERATURE REVIEW

2.1. Mechanisms of severe *P. falciparum* malaria

Severe malaria anaemia (SMA) has been characterized by a shortened lifespan of circulating erythrocytes (Looareesuwan *et al.,* 1987), haemolysis of uninfected erythrocytes (Ekvall *et al.,* 2001; Gyan *et al.,* 2002), structurally and functionally impaired erythrocytes (Selvam and Baskaran, 1996), reduced erythropoiesis (Chang *et al.,* 2004), and enhanced erythro-phagocytosis (Waitumbi *et al.,* 2000). Naturally acquired immunity in children who are exposed to malaria occurs slowly during childhood and involves both anti-disease and anti-parasitic components in which children first develop anti-disease (clinical) immunity prior to acquiring the immunological mechanisms necessary to control parasitemia (al-Yaman *et al.,* 1996; Di Perri *et al.,* 1995). Since most malaria control methods have failed and emergence of increased resistance to conventional drugs, there is urgent need to develop a long-lasting vaccine. Development of these vaccines requires better understanding of the molecular mechanisms involved in acquired immunity to malaria. Therefore, molecular based studies are required to help discern how genetic variations impact on outcome of malaria.

This study determined the role of promoter polymophisms of *CD40*, nuclear factor of kappa light enhancer in B-cells (*NFκB1*), and its inhibitor IkappaB alpha (*NFκBIA*), *IL-23R* and *FcγRs* in children aged 6-36 months with *P. falciparum* malaria from Siaya County, western Kenya. In addition, *in-vitro* mechanism of the CD40 signal transduction pathway was performed to determine the effect of *P. faciparum*-dereived haemozoin (*PfHz*) on selected pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) deregulated in SMA.

2.2. The cluster designation 40 (*CD40*) and its role in inflammatory diseases

Cluster of designation 40 (*CD40*) is a member tumour necrosis factor receptor 5 (TNFR5) superfamily protein expressed on a number of cells including dendritic cells, basophiles, eosinophils, macrophages, monocytes, B-cells and non-hematopoietic cells (Tan *et al.,* 2002; van Kooten and Banchereau, 2000). When stimulated by *CD40*L expressed on the surface of $CD4^+$ T-cells, it induces antigen presenting cells (APCs) to secrete Th1 inflammatory cytokines e.g. IFN-γ and IL-12, up-regulates antigen presentation and increased surface expression of co-stimulatory molecules such as CD80 and CD86 (Cella *et al.,* 1996). The IFN-γ and IL-12 have been linked to protection against *P. falciparum* malaria (Keller *et al.,* 2006; McCall and Sauerwein, 2010; Oyegue-Liabagui *et al.,* 2017). Consistent with these, *CD40* initiates and advances both cellular and adaptive immunity including development of memory B-cell, $CD4^+$ T-cell dependent class switching of immunoglobulin (IgG) and formation of germinal center (Carlring *et al.,* 2011; Elgueta *et al.,* 2009). When interaction between *CD40* and *CD40*L is inhibited in mice models, a lower mortality rate was reported on infection with *P. berghei,* suggesting a pivotal role of *CD40* in malaria severity (Piguet *et al.,* 2001). *In-vitro* models have also revealed that immunoglobulins cannot be generated in response to MSP-*Pf*EB200 antigens expressed during erythrocytic stage of *P. falciparum* in absence of *CD40*-*CD40*L interaction (Garraud *et al.,* 2002). These observations point out to the important roles of CD40.

2.2.1. The *CD40* polymorphisms role in inflammatory diseases

CD40 gene is mapped to chromosome 20(q12–q13.2) and contains eight introns and nine exons (Park *et al.,* 2007). Despite *CD40* gene standing out as a multi-potent immune-modulator in malaria aetio-pathogenesis, few studies have examined the role of genetic polymorphisms that exist in it. Polymorphisms in *CD40* have been identified mostly in autoimmune and inflammatory diseases such as Graves' disease, multiple sclerosis, rheumatoid arthritis, diabetes,

cerebral infraction and systemic lupus erythematous (Chen *et al.,* 2015; Li *et al.*, 2013; Li *et al.,* 2012; Wagner *et al.,* 2014; Wu *et al.,* 2016; Zhang *et al.,* 2013). In the context of malaria, a study in Amazonian Brazil region did not show any association between *CD40*-1C/T with susceptibility to *P. vivax* infection (Capobianco *et al.,* 2013). Additionally the CD40-1C>T has been shown to be associated with chronic HBV infection (Chen *et al.,* 1999). Therefore, the current study carried out an elaborate genetic analysis of three SNPs within the *CD40* gene i.e. *CD40*-508G/A, -173C/T and-1C/T) to determine the associations between its genotypes and haplotypes with severe malaria anaemia in children residents of *P. falciparum* holo-endemic transmission area in Kenya and whether these polymorphisms influence circulating levels of inflammatory mediators during *P. falciparum* infection.

2.3. The nuclear factor of kappa light enhancer in B-cells (NFκB) and nuclear factor of kappa B inhibitor alpha (IκBα)

Genes that regulate transcription of immune genes such as the nuclear factor of kappa light enhancer in B-cells (NFκB) also play important roles in infectious disease pathogenesis through their ability to activate and regulate immune response through production of pro-inflammatory molecules (Baeuerle and Henkel, 1994; Ghosh *et al.,* 1998). Members of the NFκB family include p50/p105, p65/RelA, c-Rel, RelB and p52/p100 but the main form of NFκB is a heterodimer of the p50 and p65/RelA subunits, which are encoded by the genes *NFκB1* and *NFκB2*, respectively (Chen *et al.,* 1999). The nuclear factor of kappa light polypeptide gene enhancer in B-cells (*NFκB1*) is located at chromosome 4q24 (Le Beau *et al.,* 1992; Mathew *et al.,* 1993). In normal cells, the NFκB binds to its inhibitor IkappaB alpha (IκB-α) and is inactive but when IκB is activated through phosphorylation and proteasome degradation (Karin and Ben-Neriah, 2000), the NFκB is released and trans-located to the nucleus where it initiates transcription of target genes based on the stimuli (Gilmore, 2003). NFκB is only active

during inflammation and its overlapping sites can be found at the regulatory sites of various genes (Zhang and Fuller, 1997) hence it is a central point of cellular stimulation activation.

2.3.1. The role of nuclear factor of kappa light enhancer in B-cells (NFκB) and nuclear factor of kappa B inhibitor alpha (IκB-α) in infectious and inflammatory diseases

In infectious diseases such as malaria and some bacterial infections, the hallmark of inflammatory responses is a consequence of NFκB activation (Lavon *et al.,* 2000; Mason *et al.,* 2004; Punsawad *et al.,* 2012; Tripathi *et al.,* 2009). Studies in mice that are deficient of *NFκB* gene demonstrate enhanced susceptibility to many bacterial and parasitic infections (Caamano *et al.,* 1999; Grigoriadis *et al.,* 1996; Sha *et al.,* 1995). It has been demonstrated *in-vitro* that *P. falciparum*-derived haemozoin (*PfHz*) induces NFκB translocation into the nucleus in a time-dependent fashion (Kempaiah *et al.,* 2016).

The involvement of the NFκB in immunity to infectious diseases and its effector function has been widely shown by mice studies involving gene-deletions. Studies in mice that are deficient of specific NFκB gene demonstrate enhanced susceptibility to many bacterial and parasitic infections (Caamano *et al.,* 1999; Grigoriadis *et al.,* 1996; Sha *et al.,* 1995). However, the basis for the increased susceptibility to infection is often uncertain because NFκB is involved in many aspects of the development and function of immune cells (Biron and Gazzinelli, 1995; Sanjabi *et al.,* 2000; Xie *et al.,* 1994). *P. falciparum* glycosylphosphatidylinositol (GPIs) and haemozoin have been shown to elicit expression of pro-inflammatory cytokines by macrophages through NFκB-dependent signalling pathway (Tachado *et al.,* 1996; Zhu *et al.,* 2005). This may imply that the pathological mechanisms involved in *P. falciparum* malaria due to imbalance of pro-inflammatory molecules are ultimately mediated through the NFκB signalling pathway. It is worth to note that decreased expression of NFκB p65 sub-unit has been shown to suggest a role

in immune deregulation (Torres *et al.,* 2013). In addition NFκB p65 expression was shown to be significantly increased in individuals with severe *P. falciparum* malaria in Thai adults (Viriyavejakul *et al.,* 2014). These studies together show the importance of the NFκB in the modulation of immunity.

2.3.2. The role of polymorphisms in nuclear factor of kappa light enhancer in B-cells (NFκB) and nuclear factor of kappa B inhibitor alpha (IκBα) and their association with inflammatory disease

The *NFκB1* gene is mapped on 4q23-q24 while its main inhibitor, IκBα, is encoded by *NFκBIA* genes located on chromosome 14q13. The commonly studied *NFκB1* insertion–deletion (−94ins/delATTG) polymorphism has been linked to mortality in sepsis and decreased expression of NFκB protein (Adamzik *et al.,* 2012). In addition, combined genotype analysis of *NFκB1*-94ins/del ATTG and *NFκBIA* 3'UTR A/G has revealed that the ins/ins/GG is protective against Hashimoto thyroiditis disease (Koc *et al.,* 2014). Earlier studies have shown an association between polymorphisms in *NFκBIA*-420AA with Crohn's disease susceptibility (Klein *et al.,* 2004) while the *NFκBIA* -826 AA polymorphism and -826G/-550A/-519C haplotype have been demonstrated to be associated with increased risk of rheumatoid arthritis (Lin *et al.,* 2007). However, no current study has reported associations between genetic variations in *NFκB1* and its inhibitor *NFκBIA* with severe malaria anaemia a common infectious childhood disease in western Kenya. Given the important roles of the NFκB pathway genes in immune responses, the current study carefully selected and investigated the association between The *NFκB1 (*rs747559; -8079A/G*;* rs980455; -3297C/T) and *NFκBIA* (rs2233406; -826A/G, rs2233409; -310A/G), their haplotypes and genotype combinations and susceptibility to severe

malaria anaemia in children from western Kenya a *P. falciparum* holo-endemic transmission area.

2.3.3. The nuclear factor of kappa light enhancer in B-cells (NFκB) and production of pro-inflammatory cytokines

The nuclear transcription factors regulate the production of many mediators of immune function, including pro-inflammatory cytokines, chemokines, and adhesion molecules (Ghosh *et al.,* 1998; Siebenlist *et al.,* 1994). Specifically, it has been demonstrated that NFκB-dependent *PfHz* induction leads to the production of inflammatory cytokines (Jaramillo *et al.,* 2003). Similarly, genome-wide expression profiles in PBMCs of clinical malaria patients has revealed enhanced inflammatory cytokine and unregulated signalling via NFκB pathway (Ockenhouse *et al.,* 2006). Since one of the hallmarks of *P. falciparum* severe anaemia is the deregulated production of inflammatory mediators (Mackintosh *et al.,* 2004; Perkins *et al.,* 2011) and production of most of these mediators are regulated via the NFκB signalling pathway, it is possible that genetic polymorphisms in the promoter region of *NFκB1* and *NFκBIA* influence SMA outcome. These demonstrate the importance of NFκB pathway in malaria immune-pathogenesis. In this regard to further investigate the role of *NFκB1* and *NFκBIA* polymorphisms in this paediatric population, the study determined the differences in the levels of polymorphism mediated alterations in IIL-10 and IP-10.

2.4. The *in-vitro* impact of *P. falciparum* haemozoin (*PfHz*) on expression of pro-inflammatory cytokines

One of the key metabolites associated with immune deregulation or immunosuppression, aberrant cytokine production and SMA is the *P. falciparum* haemozoin (*PfHz*) (Giribaldi *et al.,* 2004; Keller *et al.,* 2006). Studies have revealed that when circulating monocytes and

neutrophils acquire haemozoin (Hz), there is enhanced malaria severity (Arese and Schwarzer, 1997; Casals-Pascual *et al.,* 2006; Luty *et al.,* 2000). *PfHz* loaded monocytes have been associate with SMA in children from western Kenya holo-endemic transmission area (Novelli *et al.,* 2010). Murine models have shown that during *P. falciparum* malaria infection, there occurs changes in monocyte function for example antigen presentation and microbe killing ability (Ho and Webster, 1989). Monocyte loaded with haemozoin have been shown to impair the expression of major histocompatibility complex class II antigen, CD54, and CD11c (Schwarzer *et al.,* 1998). These studies clearly show the pathological role of *PfHz* in SMA. However, the cellular signalling mechanism that results into the changes in inflammatory mediator deregulation, dyserythropoiesis and subsequent SMA remains unresolved. Hence, the current study determined the effect expression levels of downstream molecules of CD40 pathway i.e. interleukin-1 beta (IL-1β), Interluekin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) on stimulation of CD40 signalling pathway with *PfHz* to help understand in part the mechanisms of *PfHz* mediated immune deregulations observed during SMA.

2.5. Interleukin-23 receptor (*IL-23R*) role in infectious and inflammatory diseases

Interleukin-23 receptor (IL-23R) engagement by IL-23 promotes T-helper 17 (Th17) cell-mediated inflammatory reactions (Chen *et al.,* 2007; Volpe *et al.,* 2008). Th17 cells are pro-inflammatory $CD4^+$ effector T-cells that mediate inflammatory process by secreting IL-17 (Onishi and Gaffen, 2010). Binding of IL-23 to IL-23R activates signal transduction via the Janus kinase (JAK)–signal transducer activator of transcription (STAT3/4), and NFκB pathways (Cho *et al.,* 2006). IL-17 is produced by activated T-cells and is involved in priming of T-cells through its ability to stimulate macrophages and some epithelial cells to produce pro-inflammatory mediators [e.g., IL-1, IL-6, TNF-α, NOS-2, metallo-proteases (MMP) known to be

important in inflammatory diseases (Kolls and Linden, 2004; Nakae *et al.,* 2003). IL-17 is also important in linking immune responses with erythropoiesis through its ability to enhance proliferation of erythroid precursor cells (Jovcic *et al.,* 2007; Jovcic *et al.,* 2004; Jovcic *et al.,* 2001). Although IL-17 has not previously been explored during malaria mono-infection in the cohort of children investigated here, a study in this population has shown that IL-17 has a significant positive association with Hb in malaria-infected children with HIV-1 (Davenport *et al.,* 2012) and that IL-17 is elevated in children with *falciparum* malaria and bacteraemia co-infections (Davenport *et al.,* 2016). In addition, IL-23 is elevated in children with SMA (Ong'echa *et al.,* 2008). Thus, the IL-23/IL-17 cytokine axis appears to be important in mediating the development of inflammatory reactions in children that develop SMA.

2. 5.1. Polymorphisms in *IL-23R* gene and effect on susceptibility to inflammatory mediated diseases

Investigations have further shown that in polygenic infectious diseases such as malaria, pathogenesis is influenced by genetic variation in regulatory and/or coding regions of inflammatory mediators and conserved molecular pattern receptors (Hobbs *et al.,* 2002; Munde *et al.,* 2012; Ouma *et al.,* 2008b; Tishkoff and Williams, 2002). As such, an improved understanding of SMA pathogenesis can be achieved through identification of polymorphisms in genes that mediate the development of severe disease. Although IL-23 receptor variation has not been explored in malaria, carriage of rs10889677CC in the *IL-23R* was associated with increased risk of cancer in Chinese populations (Zheng *et al.,* 2012). Additional studies have provided evidence on the important role of the IL-23/Th17 axis on immune-mediated diseases such as Crohn's disease (CD) (Duerr *et al.,* 2006), psoriasis (Capon *et al.,* 2007; Cargill *et al.,* 2007) and ankylosing spondylitis (AS) (Rueda *et al.,* 2008). Collectively, these previous studies show that

variations in *IL-23R* influence immune responses and thereby mediates the risk of inflammatory diseases. Despite its potential importance, the role of non-synonymous *IL-23R* polymorphisms has not been explored in the context of susceptibility to SMA. To address this gap-in-knowledge, the current association between the genotypic and haplotypic structures of non-synonymous *IL-23R* variants (i.e., rs1884444 G/T and rs7530511 C/T) and susceptibility to SMA in children (6-36 months) resident of *P. falciparum* holo-endemic region of western Kenya.

2.6. Fragment crystallisable gamma receptors *(FcγRs)* gene and its biological functions

The Fc gamma Receptors (FcγRs) are cell surface proteins expressed on the surface of antigen presenting cells (APCs) which function to help in recognition and elimination of invading pathogens (Cabrera *et al.,* 2004; Marsh *et al.,* 1989). FcγRs bind to Fc portion of the immunoglobulin G (IgG) and thereby link antigen recognition by antibodies with cell-based effector mechanisms (Hulett and Hogarth, 1994; Unkeless *et al.,* 1988). Human *FcγR* genes form a clustered gene family, which are mapped to chromosome 1q and are located at q21-q23 (Daeron, 1997; Indik *et al.,* 1995; Ravetch and Kinet, 1991). They are involved in the clearance of immune complexes, phagocytosis of antibody-coated pathogens, enhancement of antigen presentation, secretion of reactive oxygen intermediates (ROI), antibody-dependent cellular cytotoxicity (ADCC), and cytotoxicity of antibody-coated tumour cells (Fanger *et al.,* 1989; Morganelli *et al.,* 1992). The structural heterogeneity of FcγR isoforms contributes to differences in preferential binding affinity to the sub-classes of human IgG, distinct signal transduction pathways, and cell type-specific distributions (Salmon and Pricop, 2001).

2.6.1. Biological impact of FcγRs polymorphisms

The binding of immunoglobulin domains to Fc receptors on target cells is important to initiate immunological defence against pathogens including antigen presentation, phagocytosis,

cytotoxicity, induction of inflammatory processes and modulation of immune responses (Pleass and Woof, 2001). Therefore, FcγRs are important in providing a significant link between the humoral and cellular immunity by bridging the interaction between specific antibodies and effector cells (Raghavan and Bjorkman, 1996). Previous studies demonstrate that polymorphic variability in these receptors is an important determinant of susceptibility to infections (van de Winkel and Capel, 1993; van der Pol and van de Winkel, 1998) and that the efficacy of the cellular immune response is influenced by *FcγR* polymorphisms, and consequently, influence clinical outcomes for infectious diseases' such as malaria (Lehrnbecher *et al.,* 2000; van der Pol and van de Winkel, 1998). The human FcγRIIA mediates phagocytic function of monocytes, macrophages and neutrophils. The presence of *FcγRIIA*-131Arg/131His polymorphism affects the binding to the IgG_1 and IgG_3 (Warmerdam *et al.,* 1990). As revealed by Warmerdam and colleagues (Warmerdam *et al.,* 1991), the *FcγRIIA*-131Arg allele is associated with low binding affinity leading to reduced phagocytic activity and immune complex clearance. *FcγRIIIA* is an activating receptor with two co-dominantly expressed alleles, the 176V and the 176F that differ in an amino acid at position 176 in the extracellular domain Valine (V) or Phenylalanine (F), respectively, (Ravetch and Kinet, 1991). This dimorphism in the amino acid influences the binding of the immunoglobin G (IgG) subtype, with the 176V variant having higher binding affinity for monomeric forms of IgG_1 and IgG_3, as compared to the 176F (Koene *et al.,* 1997) which is potentially important in infectious disease immunity.

On the surface of polymorphonuclear leucocytes, the most abundantly expressed FcγRs is the *FcγRIIIB*. These receptors exhibits two allotypic forms i.e. neutrophil antigens (NAs) 1 and 2 which differ in minor amino acids at position 65 and 82 in two extra-glycosylation site in NA2 (Bux *et al.,* 1999; Ory *et al.,* 1989) with different binding affinities. The NA2/NA2 allotype is

associated with low immunoglobulin-mediated phagocytosis (Bredius *et al.,* 1994; Salmon *et al.,* 1990). The phagocytosis of IgG_1-and IgG_3-opsonized immune complexes is more efficient on neutrophils bearing *FcγRIIIB*-NA1 relative to *FcγRIIIB*-NA2 (Bredius *et al.,* 1994).

2.6.2. Fc gamma receptors *(FcγRs)* polymorphisms in malaria

A number of genetic association studies have provided evidence that polymorphic variation in FcγRs have a strong effect on susceptibility to inflammatory mediated diseases (Chen *et al.,* 2006; Omi *et al.,* 2002; Ouma *et al.,* 2012; Schuldt *et al.,* 2010; Wu *et al.,* 1997). Even though FcγRs are important in the immune response to infection, the effect of its haplotypes on susceptibility to SMA in immune-naive children remain largely undetermined. In the present study, the association between *FcγRIIA*, *FcγRIIIA* and *FcγRIIIIB* haplotypes and SMA, and the influence of these haplotypes on peripheral parasite burden during *P. falciparum* infections were determined in a well phenotyped cohort of children (aged 6-36 months) from a *P. falciparum* holo-endemic transmission area western in Kenya.

CHAPTER THREE: MATERIALS AND METHODS

3.1. Study site

The study was conducted in Siaya County Referral Hospital (SCRH) in western Kenya. (Appendix 1). An earlier studies by (Obonyo *et al.,* 2007) at this site reported that the percentage of children aged below 5 years who were hospitalised with SMA was 18%. In addition, it has been demonstrated that despite concerted malaria control strategies, this region continues to show an increasing trend of infection (Okiro *et al.,* 2010). Moreover, SCRH is the major government referral hospital for the population living in the *P. falciparum* holo-endemic transmission region of Siaya County, western Kenya. The intensity of malaria transmission in this holo-endemic region is experienced during the seasonal rainfalls in April to August and November to January (Beier *et al.,* 1994). Inhabitants of the study area are predominantly of the Luo ethnic tribe (>96%), with the population being genetically homogeneous hence suitable for genetic study (Bloland *et al.,* 1999; Ong'echa *et al.,* 2006). It is approximately 1520 km^2 in size and lies between Latitude 0º 26' to 0º 18' North and Longitude 33º 58' East and 34º 33' West, (Appendix 1).

3.2. Study design and patient population

3.2.1. Recruitment of study participants

This was a retrospective case-control study using persevered samples of children of aged6-36 months. Children of both sexes were recruited at SCRH in western Kenya during their initial hospitalization for treatment of malaria using a questionnaire and existing medical records. Enrolment was confined to children less than 3 years of age. Case were children within with positive smear for asexual *P. falciparum* parasitaemia of any density and haemoglobin, (Hb<5.0 g/dL and Hb<6.0 g/dL). Controls were children of similar age and same gender with non-severe

malaria anaemia *P. falciparum* malaria (two of the following: nausea/vomiting, diarrhoea, headache, myalgia, poor feeding) accompanied by a positive blood smear showing asexual *P. falciparum* parasitaemia and Hb>5.0 g/dL or Hb>6.0 g.dL).

3.2.2. Screening and enrolment

Recruitment followed a two-phase tier of screening and enrolment. The parent/legal guardian of the child received a detailed explanation of the study. Enrolment decisions were made after an initial HIV-1 screening of the child and a signed informed consent, which included the authority to publish the findings. Questionnaires and written informed consent were administered in the language of choice (i.e. English, Kiswahili or Dholuo) (Appendix 2). Children with acute malaria were stratified into two categories according to the WHO criterion of classification SMA: non-severe malaria anaemia (non-SMA) group defined as a positive smear for asexual *P. falciparum* parasitemia (of any density) and Hb≥5.0 gdL^{-1}; and SMA group defined by a positive smear for asexual *P. falciparum* parasitemia (of any density) and Hb<5.0 gdL^{-1} (WHO 2000). For further regression analysis to include the regional perspective of SMA, children were also classified as non-SMA; Hb≥6.0 gdL^{-1} and SMA; Hb<6.0 gdL^{-1} based on sex, age and geographically matched measurements of over 14,000 Hb concentrations of children from western Kenya (McElroy *et al.,* 2000). Venous blood samples (<3.0 mL) were collected into EDTA-containing vacutainer tubes at the time of enrolment, prior to any treatment interventions or supportive care. Blood samples were used for malaria diagnosis, complete haematological profile measurements, HIV testing, bacterial culture. Participants were treated according to the Ministry of Health (MOH)-Kenya guidelines.

3.2.3. Eligibility criteria

Upon enrolment into the study, HIV-1 status, parasitaemia and haematological measurements of the child were determined. The children with acute malaria were then stratified into two categories of SMA and non-SMA based on both local and the WHO definition of SMA.

3.2.4. Inclusion criteria

Malaria parasitaemia (any density) and Hb<11.0g/dL; age ≥6 months and≤36 months; parent/guardian willing and able to sign consent form; able to keep schedule and study appointments; able to provide two contacts familiar with the child's whereabouts during the study period.

3.2.5. Exclusion criteria

Children with CM (a rare manifestation in this holo-endemic area); 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 to enrol child in the study.

3.3. Sample size determination

The current study recruited children (aged 6-36 months) that presented with clinical symptoms of malaria at SCRH. According to previous reports (Dupont and Plummer, 1990), the Bonferroni correction for 2 different loci would require a per-comparison alpha of 0.004 for sample size calculations. However, given the increased power provided by the procedure (Dupont and Plummer, 1990), it is estimated that sample sizes based on an $\alpha \leq 0.01$ provides a balance between Type I and Type II errors. Based on this rationale, it is conservatively estimated that to be able to achieve 80% power with a Type I error rate of $\alpha \leq 0.05$, allele frequency difference of 0.2 between cases and controls was required. Therefore the following

formulae by Whitley and Ball (2000) was used. (Whitley and Ball, 2002).

$$N = (\frac{r+1}{r})\frac{(\bar{p})(1-\bar{p})(Z_{\beta} + Z_{\alpha/2})^2}{(p_1 - p_2)^2}$$

Where

N is the sample size

r is the ratio of controls to cases.

$\bar{p}$ is a measure of variability (similar to the standard deviation)

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

Z_{β} represents the desired power (typically 0.84 for 80% power)

Z_{α} represents confidence interval (typically 1.96 for 95% CI)

The proportion of children with SMA in this holo-endemic region according to previous studies is about 20% (P_1=0.2) of all malaria admissions (Obonyo *et al.,* 2007). Based on previous study (Ouma *et al.,* 2008b), to detect odds ratio of 2 (OR=2), ratio of cases to control should be ≥1. Using ration of r=1, the proportion of the control group i.e. P_2 is given by;

$$OR = \frac{p_2(1-p_1)}{p_1(1-p_2)}$$

$$2 = \frac{p_2(1-0.2)}{0.2(1-p_2)}$$

$$p_2 = 0.35$$

The average proportion of children exposed to *P. falciparum* malaria and possess the genotypes is given by; $\bar{p} = \frac{p_2 + rp_1}{(1+r)} = \frac{0.51}{2} = 0.26$

Therefore, $N = (\frac{1+1}{1})\frac{(0.26)(1-0.26)(0.84+1.96)^2}{(0.21-0.35)^2} = 154$

3.4. Collection and processing of blood samples

Haemoglobin levels and complete blood counts were determined using the Beckman Coulter ACT diff2™ (Beckman-Counter Corporation, Miami, FL, USA). To determine parasitemia, 10% Giemsa-stained thick and thin blood smears were prepared and examined under a microscope at high power magnification. *P. falciparum* parasites per 300 white blood cells (WBCs) were determined, and parasitemia (per µL) was estimated using the total WBC count. In order to delineate severe anaemia caused by malaria versus other anaemia-promoting conditions, human immunodeficiency virus (HIV)-1, bacteraemia, glucose-6-phosphate dehydrogenase deficiency (G6PD), α-thalassemia and sickle-cell trait (HbAS) were determined in study participants. The effects of these parameters on disease severity outcomes were statistically controlled in the regression analysis. Pre- and post-test HIV counselling was provided for all participants. HIV-1 exposure and infection were determined serologically (i.e., Unigold™ and Determine™) and discordant results confirmed through HIV-1 proviral DNA PCR testing, according to previously published methods (Otieno *et al.*, 2006). Bacteraemia was determined by microbial cultivation according to standard methods (Perkins *et al.*, 2011). The presence of the sickle cell trait (HbAS) was determined by cellulose acetate electrophoresis (Helena Bio-Sciences, Oxford, United Kingdom), Appendix 3, while G6PD deficiency was determined by a fluorescent spot test using the manufacturer's methods (Trinity Biotech Plc., Bray, Ireland), Appendix 4.

3.4. Functional analysis and genotyping of selected genetic variations

3.4.1. Functional analysis and the selection of *CD40* promoter single nucleotide polymorphisms (SNPs)

The promoter polymorphisms SNPs were selected from a subset of transcriptomics data which showed *CD40*, *NFκB1* and *NFκBIA* were differentially expressed in SMA versus non-SMA. Since existence of functional variants within gene regulatory regions [promoter and/or 5' untranslated regions (5'UTR)] has the ability to significantly alter transcriptional activity and translational initiation (Chorley *et al.*, 2008; Cookson *et al.*, 2009), those potential SNPs that are in regulatory regions were selected and *in-silico* Transcription Factor Binding Sites (TFBSs) performed according to (Piechota *et al.*, 2010). From the genome array of the *CD40* haploblock spanning (84kb), the array composed of 46 SNPs. Based on TFBSs analysis and MAF (>5%), top three *CD40* variants [i.e. rs1800686; -508G/A, rs752118; −173C/T and rs1883832; -1C/T] were selected for validation. The analysis showed that presence C allele at -1 locus on *CD40* (rs1883832) results into the binding of Yin Yang 1 (YY1), Enkephalin Transcription Factor -1 (ENKTF-1), and X-box binding protein1 (XBP1) while the presence of allele T abolishes the binding these transcription factors. The C allele at the -173 (rs752118) locus YY1 and peroxisome proliferator-activated receptor alpha (PPAR-α) while the T allele leads to binding of XBP1. Analysis of the transcription factors that bind in the presence of G allele at the -508 (rs1800686) locus revealed the binding of Guanine nucleotide binding proteins alpha (G-α), Nuclear factor kappa B (NFκB), and c-E-twenty-six transformation-specific (c-ETS) while the minor allele A allows for the binding of CCAAT/enhancer-binding protein beta (C/EBP-β). In summary, the identified transcription factors reveal that, presence of mutant alleles within the regions may have profound impact on *CD40* function, (Appendix 5).

3.4.2. Functional analysis of and selection of *NFκB1* and *NFκBIA* SNPs

The *NFκB1* variants [i.e. rs747559; -8079 G/A and rs980455; -3297C/T] and *NFκBIA* [i.e. rs2233406; -826G/A, rs2233409; -310G/A] were tested. Via TFBS analysis, presence G allele at -8079 locus on *NFκB1* (rs747559) results into the binding of glucocorticoid receptor-beta (GR-β), CCAAT/enhancer-binding protein alpha and beta (C/EBP-α and β), POU class 2 homeobox 2 (POUF2) and X-box binding protein1 (XBP1) while in the presence of the A allele; only the GR-β binds. The C allele at -3297 of *NFκB1* locus (rs980455) leads to binding of chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1) while in the presence of the minor allele, COUP-TF1 and GR-α bind. Analysis of the *NFκBIA* rs2233406 locus showed that the major allele (G) at the -826 locus leads to binding of GR-β while the minor allele (A) causes the binding of Foxhead box protein 3 (FOXP3), Homeobox D10 (HOXD10) and GR-β. The major allele (G) for the *NFκBIA* rs2233409 (-310G/A) locus showed the binding of Activating enhancer binding Protein 2-alpaha (AP-2A). However, the minor allele (A) at this locus showed additional transcription factor binding i.e. FOXP3, Vitamin D receptor (VDR) and Peroxisomal targeting signal import receptor 1(PXR-1). These *in-silo* analyses point to the prospect that presence of either the wild type or the mutant alleles may have profound impact of the function of the gene (Appendix 5).

After TFBS analysis, the three *CD40* variants [[i.e.: rs1800686; -508G/A; assay ID C_7499626_10, rs752118; −173C/T), assay ID: C_594685_10, and rs1883832; -1C/T), assay ID: C_11655919_20]; two *NFκB1* i.e. rs747559; -8079G/A*;* assay ID: C_804250_10) and rs980455; -3297C/T, assay ID: C_8935018_10 and two *NFκBIA* i.e. rs2233406; -826G/A assay ID; C_73867_10 and rs2233409; -310G/A assay ID: C_15945891_10] which had possible effect on transcription factors binding were genotyped.

3.4.3. Selection of non-synonymous mutation within *IL-23R* and *FcγRs*

Non-synonymous mutations are important in determining the binding affinity of receptors due to alterations of amino acid sequences. In addition, selection of *IL-23R* (rs1884444 G/T, (assay ID: C__11728603_10) and *IL-23R* rs7530511C/T (assay ID: C__2990018_10) and *FcγRIIA*-131Arg/His (rs1801274, assay ID: C__9077561_20) and *FcγRIIIA* 176F/V (rs396991, assay ID: C__25815666_10) non-synonymous mutation was based on their ability to alter respective amino acids sequences.

3.4.4. Genotyping selected single nucleotide polymorphisms (SNPs) and construction of haplotypes

Genomic DNA was extracted from buccal swabs using the MasterAmp™ Buccal swab DNA extraction kit (Epicentre Biotechnologies, Post Road, Madison, WI, USA), cleaned and concentrated where necessary using the genomic DNA clean and concentration kit (Zymo Research, Irvine, CA, USA). To achieve more DNA copies, Genomiphi™ V2 DNA Amplification Kit (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA) was used. Genotyping for all these selected polymorphisms except *FcγRIIIB*-NA1/NA2 genotyping was performed using TaqMan® 5' allelic discrimination Assay-By-Design high-throughput method based on the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). For genotyping of the *FcγRIIIB*-NA1/NA2 was performed using allele-specific sequence primers as described by (Bux *et al.,* 1995). After determination of genotype frequencies, the genotypes generated were then ran in HPlus software (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) to generate haplotype constructs.

3.5. Determination of *In-vitro* Effect of *P. falciparum*-Derived Haemozoin (*PfHz*) on the Levels of IL-1β, IL-6 and TNF-α via the CD40 Pathway

To determine the expression levels of CD40 downstream molecules (IL-1β, IL-6 and TNF-α) produced as a result of stimulation of the CD40 pathway in peripheral blood mononuclear cells (PBMCs), blood was obtained from n=2 malaria unexposed North American individuals. Venous blood (30.0 mL) was drawn into EDTA-containing vials and PBMCs prepared using ficoll/Hypaque method as described (Weinberg *et al.*, 1981). PBMCs were the cultured at 1×10^6 cells/mL in media and stimulated with small molecule inhibitor (SML1160, 100.0µM, Sigma Aldrich, Missouri, USA; inhibits the CD40 pathway] alone or with SML and *PfHz* (10.0 µg/mL). *PfHz* was isolated according to established laboratory protocol (Appendix 6). In addition, Anti-CD40 monoclonal antibody-S2C6 (2.5 µg/mL, MabTech Ab, West Street, Ohio, USA) alone or with *PfHz* were also used for stimulation. Both phosphate buffered saline (1XPBS Sigma Aldrich, Missouri, USA) and Dimethyl Sulfoxide (1.0% DMSO, Sigma Aldrich, Missouri, USA). The cells were stimulated with either the SML or Anti-CD40 for one hour prior to addition of the *PfHz*. Cultures were incubated in triplicates at 37°C in 5% CO_2 atmosphere for 4, 12, 24 and 48 hours. Cells were then collected at each time point and cell pellets collected for RNA isolation and determination of IL-1β, IL-6 and TNF-α expression levels. Using 1.0 µg/µL of total RNA, complementary DNA (cDNA) was prepared with the transcriptor first strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany). For measurement of gene expression levels, 0.5µg/µL of resulting cDNA was used for gene-specific TaqMan® qPCR assays. cDNA was amplified in duplicate with specific primer/probe sets for *NFκB1* and *NFκBIA* respectively (Hs015554410_m1, Hs00174128_m1 and Hs0098564_m1 [Applied Biosystems, Foster City, CA]). Beta-actin (β-actin), which is a constitutively expressed

housekeeping gene, was used as an endogenous control to normalize the gene expression data in a quantitative gene expression assay on the StepOnePlus™ Real-Time PCR System (ABI).

3.6. Determination of Peripheral Inflammatory Mediator Levels

Circulating levels of inflammatory mediators were measured using multiplex assay. Plasma samples obtained from venous blood were stored at -80ºC until use. To limit variations between measurements, batch analysis was performed. Inflammatory mediator's levels were determined by the Human Cytokine 25-plex Antibody Bead Kit (BioSource™ International, Camarillo, CA, USA) according to the manufacturer's instructions. Briefly, the wells were pre-wetted by adding 200 µL of working wash solution into designated wells then incubated for Incubate plate 30 seconds at room temperature. The working wash solution was then aspirated from the wells using the vacuum manifold. The bead solution was then vortexed for 30 seconds, then sonicated again for 30 seconds. 25µL of the bead solution was added into each well and the plate immediately protected from light to prevent photo-bleaching.

To the wells 200µL working wash solution was then added and beads allowed to soak for 20 seconds. The wells were then washed by aspirating the working wash solution from the wells with the vacuum manifold. The washing step was repeated once and the bottoms of the filter plate were blotted on clean paper towels to remove any residual liquid. To each of the wells 50 µL of incubation buffer was added. To the wells designated for standard curves 100µL of each standard dilution was added while to the wells designated for sample, 50µL of assay diluent was added followed by 50µL sample. The filter plates were then covered with aluminium foil and incubated for 2 hours at room temperature on orbital shaker at 500 rpm. After incubation, the plated were washed using 200µL of wash solution.

To the washed plates, 100µL of 1X biotinylated antibody was added to each well and then incubated for 1 hour at room temperature on an orbital shaker at 500 rpm to keep the beads suspended during incubation. After incubation 200µL of wash solution was added to each well to soak the beads then aspirated using vacuum manifold. To each of the wells, 100µL of 1X streptavidin-RPE was added and incubated the plate for 30 minutes at room temperature on an orbital shaker 500 rpm. The liquid in the wells was removed by vacuum aspiration manifold and then beads by adding 200µL working wash solution to the wells allow the beads to soak for 10 seconds, and then aspirated using the vacuum manifold. Washing step was repeated twice. The plates were read on a Luminex® 100™ system (Luminex® Corporation, Austin, TX) and data was analysed using the Bio-plex manager software (version IS.2.3; Bio-Rad Laboratories, Hercules, CA, USA). The analytes detection limits were: 15pgmL^{-1} for IL-1β, 6pgmL^{-1} for IL-2, 15pgmL^{-1} for IL-10 and IP-10, and 10pgmL^{-1} for both MIP-1α/CCL3 and IL-17. These inflammatory mediators were selected based on the presence of NFκB consensus binding sites within their promoters as this would influence the final gene products.

3.7. Statistical analyses

Chi-square (χ^2) analysis was used to determine differences between proportions. Mann-Whitney U test or student's t-test was used for comparisons of demographic and clinical characteristics between the clinical groups, inflammatory mediators, in carriage and non-carriage of respective haplotypes where appropriate. Genotype and haplotypic frequencies of *CD40, NFκB1, NFκBIA, IL-23R and FcγRs* polymorphisms were compared between non-SMA and SMA groups by using the χ^2 tests. The association between genotypes and/or haplotypes and SMA was determined using bivariate logistic regression, controlling for the confounding effects of age, sex, co-infections (HIV-1 and bacteraemia), G6PD deficiency, HbAS and alpha-

thalassemia status in the regression model at 95% confidence interval (CI). Correlation between haemoglobin levels and IL-17 was determined using spearman's correlation. All statistical significance were set at $P \leq 0.05$.

3.8. Ethical considerations

Approval to carry out this study was provided by the Kenya Medical Research Institute (KEMRI) Ethical Review Committee (Appendix 7). Confidentiality of patient information was ensured by ensuring restricted access to patient information and use of unique identifiers.

CHAPTER FOUR: RESULTS

4.1. General Clinical, Demographic and Laboratory Characteristics of all Study participants.

Children (n=1,128, aged 6-36 months) presenting with acute *P. falciparum* malaria (any parasite density) were recruited into the study. For general clinical characterization, the WHO definition of severe malaria anaemia, i.e. non-severe malaria anaemia (non-SMA; Hb≥5.0gdL^{-1}; n=916) and severe malaria anaemia (SMA; Hb<5.0gdL^{-1}; n=212). All the determined clinical, demographic and laboratory characteristics are summarized in Table 4.1. The proportions of sex were comparable between the clinical groups (P=0.487). Children in non-SMA group [median (IQR); 12.7 (10.7)] were older compared to those in SMA group [median (IQR); 10.1 (10.1), P=0.001]. The axillary temperature (ºC) at admission did not differ between SMA (median (IQR) 38.0 (10)] and non-SMA (median (IQR), 37.9 (1.0), P=0.210]. Children with non-SMA presented with lower respiration rate (median (IQR), 26.0 (14.0) relative to SMA (median (IQR), 32.0 (12.0), P<0.001]. Analysis of haematological parameters as expected based on clinical stratification revealed that the children with non-SMA had higher levels of haemoglobin (median (IQR), 7.1 (3.0) compared to SMA (median (IQR), 4.2 (1.0), P<0.001)]. Similarly, haematocrit was also higher in the non-SMA (median (IQR), 22.4 (8.3) than the SMA (median (IQR), 13.1 (3.2), P<0.001]. The levels of red blood cells (RBC) (× 10^{12}μL^{-1}) were relatively higher in non-SMA [median, (IQR) 3.4 (1.4)], than SMA [median (IQR), 1.8 (0.6)], P<0.001]. The red cell distribution width (RDW) was lower in children with non-SMA [median, (IQR) 21.4 (4.5)] compared to those with SMA group [median (IQR), 22.8 (6.0)], P<0.001]. There was higher mean corpuscular volume (MCV, fL) in SMA compared to non-SMA; median (IQR), 72.8 (12.0)

and median (IQR), 68.6 (12.3), respectively, $P<0.001$]. Mean corpuscular haemoglobin (MCH, fL/cell) was also lower in the non-SMA group [median (IQR), 22.0 (4.2)] compared to SMA group 22.6 (4.3), $P<0.001$]. Accordingly, the mean corpuscular haemoglobin concentration (MCHC, gdL^{-1}) was higher in the non-SMA category [mean (IQR) 32.2 (2.4)] than SMA [median (IQR), 31.9 (3.5)], $P=0.001$]. The proportion of children with reduced reticulocyte production index (RPI<2) was were also comparable between SMA group 192 (90.0%) relative to non-SMA 765 (86.1%) $P=0.089$. Children with non-SMA had lower counts of white blood cells WBC ($\times 10^3 \mu L^{-1}$); [median (IQR) 11.2 (5.6)] relative to those with SMA [median (IQR), 15.0 (9.9), $P<0.001$]. Similarly, there were lower counts of lymphocytes ($\times 10^3 \mu L^{-1}$) in non-SMA [median (IQR) 5.2 (3.6), while higher in SMA [median (IQR), 6.8 (5.3), $P<0.001$]. The monocytes counts ($\times 10^3 \mu L^{-1}$) were also higher in SMA [median, (IQR), 1.4 (1.5) than the non-SMA group [median (IQR), 0.9 (0.8), $P=0.001$]. There was, however, no differences in the counts of granulocytes ($\times 10^3 \mu L^{-1}$), between the children with non-SMA [median, (IQR), 5.2 (4.1)] vs SMA [median, (IQR), 5.5 (5.1)], $P=0.104$. In addition, the platelet counts ($\times 10^3 \mu L^{-1}$) were higher in the non-SMA group [median, IQR), 151.0 (120.0)] compared to SMA group [median (IQR), 142.0 (84.0), $P=0.027$]. Parasitemia did not differ between the non-SMA [median, IQR) 28737.9 (79160.8)] and SMA [median, IQR) 23313.4 (67350.8)], $P=0.113$]. Moreover, the proportion of children with high density parasitemia (HDP≥10,000 parasitesμL^{-1}) were comparable; non-SMA, 647 (70.6%) versus SMA, 136 (64.2%), $P=0.065$]. Examination of the distribution of the genetic factors that have been shown to influence malaria outcome (sickle cell trait, G6PD and alpha thalassemia) revealed that children with sickle cell trait were more in non-SMA 150 (16.4%) than SMA 19 (9.0%), ($P=0.007$) while G6PD and alpha thalassemia were comparable between the groups; G6PD in non-SMA 37 (4.4%) while in SMA 13 (6.5%),

(*P*=0.435) and for alpha thalassemia were in non-SMA 148 (18.5%) and in SMA 35 (17.8%), (*P*=0.689), respectively. Children with bacteraemia as a co-infection in malaria were also comparable between non-SMA 86 (9.4%) and SMA 20 (9.5%), (*P*=0.979). Similarly, the HIV-1 co-infections were also not different between the non-SMA 29 (3.2%) and SMA 11 (5.3%), (*P*=0.145).

Table 4. 1. General Clinical, Demographic and Laboratory Characteristics of the Study Participants

Characteristics	Non-SMA (Hb≥5.0gdL^{-1}) n=916	SMA (Hb<5.0gdL^{-1}) n=212	*P*-value
Sex, n (%)			
Male	452 (49.3)	99 (46.7)	0.487[a]
Female	464 (50.7)	113 (53.3)	
Age, (months)	12.7 (10.7)	10.1 (10.1)	**0.001**[b]
Temperature, (ºC)	38.0 (1.0)	37.9 (1.0)	0.210[b]
Respiration rate, (breathsmin^{-1})	26.0 (14.0)	32.0 (12.0)	**<0.001**[b]
Haematological Parameters			
Haemoglobin, gdL^{-1}	7.1(3.0)	4.2 (1.0)	**<0.001**[b]
Haematocrit, %	22.4 (8.3)	13.1 (3.2)	**<0.001**[b]
RBC, (× 10^{12}µL^{-1})	3.4 (1.4)	1.8(0.6)	**<0.001**[b]
RDW, %	21.4 (4.5)	22.8 (6.0)	**<0.001**[b]
MCV, fL	68.6 (12.3)	72.8 (12.0)	**<0.001**[b]
MCH, fL/cell	22.0 (4.2)	22.6 (4.3)	**<0.001**[b]
MCHC, gdL^{-1}	32.2 (2.4)	31.9 (3.5)	**0.001**[b]
RPI<2, n (%)	765 (86.1)	192 (90.6)	0.089[a]
WBC (×10^3µL^{-1})	11.2 (5.6)	15.0 (9.9)	**<0.001**[b]
Lymphocytes, (×10^3µL^{-1})	5.2 (3.6)	6.8 (5.3)	**<0.001**[b]
Monocytes, (×10^3µL^{-1})	0.9 (0.8)	1.4 (1.5)	**0.001**[b]
Granulocytes, (×10^3µL^{-1})	5.2 (4.1)	5.5 (5.1)	0.104[b]
Platelet Counts, (×10^3µL^{-1})	151.0 (120.0)	142.0 (84.0)	**0.027**[b]
Parasitological indices			
Parasite density (µL^{-1})	28737.9 (79160.8)	23313.4 (67350.8)	0.113[b]
HDP, n (%)	647 (70.6)	136 (64.2)	0.065[a]
Genetic factors			
Sickle cell trait, n (%)	150 (16.4)	19 (9.0)	**0.007**[a]
G6PD deficiency, n (%)	37 (4.4)	13 (6.5)	0.435[a]
Alpha thalassemia, n (%)	148 (18.5)	35 (17.8)	0.689[a]
Co-infections			
Bacteraemia	86 (9.4)	20 (9.5)	0.979[a]
HIV-1	29 (3.2)	11 (5.3)	0.145[a]

Data are presented as the median (interquartile range; IQR) values unless stated otherwise. Study participants were categorized into non-SMA (Hb≥5.0gdL^{-1}, with any density parasitaemia and SMA (Hb<5.0gdL^{-1}, with any density parasitaemia). [a] Statistical significance was determined by the Chi-square (χ^2) analysis. [b] Statistical significance was determined using Mann Whitney test. Abbreviations: G6PD; Glucose-6-phospahate dehydrogenase; HDP: High density parasitemia; HIV-1: Human Immunodeficiency Virus-1; MCV; Mean corpuscular volume; MCH; mean corpuscular haemoglobin: MCHC; mean corpuscular haemoglobin WBC concentration; RBC-Red blood cells, RDW; Red cell distribution width; RPI: Reticulocyte production index; White blood cells.

4.2. CD40 Variations and SMA

4.2.1. Proportional distributions of *CD40* genotypes in the study groups

Based on clinical classification of study participants, the distributions of *CD40* SNPs within these groups were determined. There were 806 (88.0%) for -508GG, 96 (10.5%) for -508GA and 14 (1.5%) for -508AA in the non-SMA group while 192 (90.6%) for -508GG, 11 (5.2%) for -508GA and 9 (4.2%) for -508AA in SMA. These distributions showed significant differences between non-SMA and SMA, P=0.003, (Figure 4.1A). Furthermore, the -508G/A SNP revealed that the overall major allele (G) frequency was 0.93 while the minor allele (A) frequency was 0.07. The alleles showed a significant deviation from the Hardy–Weinberg Equilibrium [HWE, (χ^2=70.0, P<0.001)]. The frequency of the *CD40*-173C/T were as follows in non-SMA; -173CC 808 (88.3%), -173CT 92 (10.0%) and -173TT 16 (1.7%) whereas in the SMA group were; -173CC 193 (91.0%), -173CT 5 (2.4%) and 14 (6.6%). These proportions revealed that the -173CT were higher in the non-SMA as compared to SMA unlike the -173TT which were higher in SMA relative to non-SMA, P<0.001, (Figure 4.1B). For the -173C/T variation, the overall major allele (C) frequency was 0.94 while the minor allele (T) frequency was 0.06. The alleles showed a significant deviation from the Hardy–Weinberg Equilibrium [HWE, (χ^2=67.5, P<0.001)]. The proportions of *CD40*-1C/T in non-SMA were -1CC; 867 (94.7%), -1CT; 44 (4.8%) and –1TT; 5 (0.5%) while in SMA, -1CC were 165 (77.8%), -1CT; 36 (17.0%) and -1TT; 11 (5.2%), (Figure 4.1C). These results revealed significant differences in proportional distributions of -1CT and -1TT between non-SMA and SMA groups, P<0.001. The overall major allele (C) frequency was 0.95 while the minor allele (T) frequency was 0.05. The alleles showed a significant deviation from the Hardy–Weinberg Equilibrium [HWE, (χ^2=69.6, P<0.001).

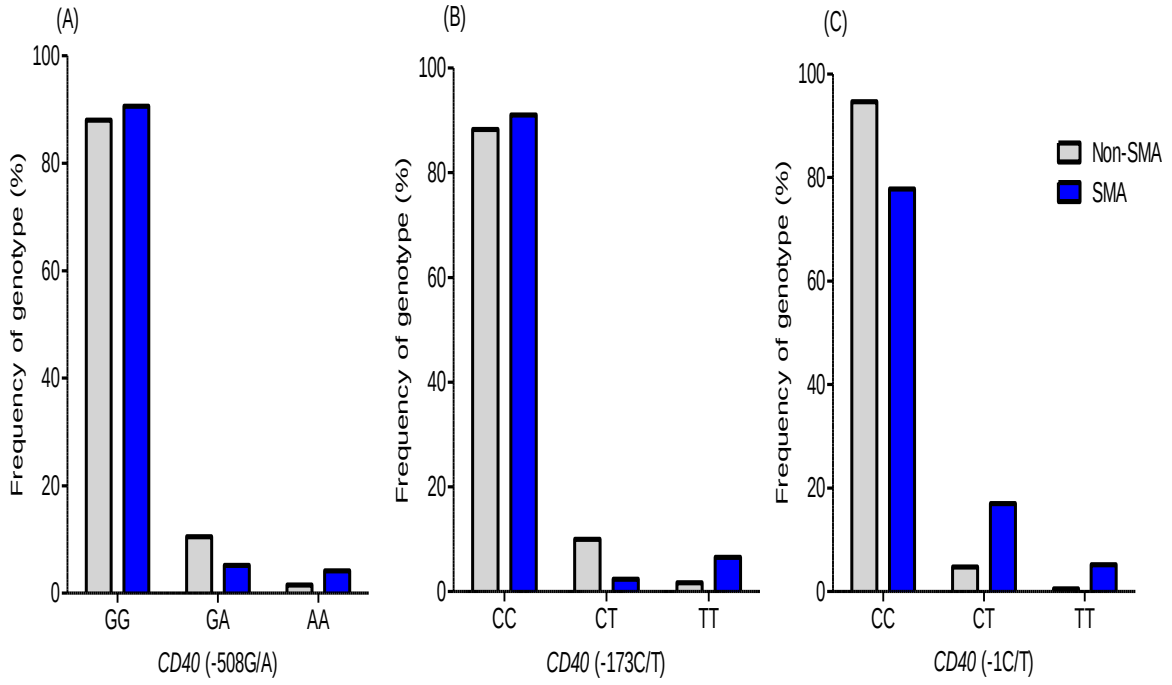

Figure 4. 1. Proportional distributions of *CD40* genotypes in the study groups

Data are presented as percentage of children within the study groups with respective genotypes. Children with parasitemia were categorized on the basis of presence or absence of SMA. Statistical significance was determined by the χ^2 analysis.

4.2.2. Association between *CD40* Variations and Severe Malaria Anaemia

On the determination of the proportional distributions within the study groups, the associations between these polymorphisms and SMA using different genetic models (i.e. dominant, additive and recessive models of each genotype) were determined. Bivariate logistic regression analysis in a model which involved controlling for anaemia promoting factors; age, sex, co-infection (HIV-1 status and bacteraemia), alpha-thalassemia, sickle cell trait (HbAS) and G6PD deficiency (Otieno *et al.*, 2006; Shah *et al.*, 2016; Wambua *et al.*, 2006a; Wambua *et al.*, 2006b; Were *et al.*, 2011) was used. Using the *CD40*-508GG as the reference in the dominant model, the *CD40*-508 (GA+AA) showed no association with SMA [OR=0.72, 95% CI=0.43-1.19, P=0.198]. The additive model of *CD40*-508G/A revealed that *CD40*-508GA (heterozygous) was associated with protection against SMA [OR=0.39, 95% CI=0.15-0.85, P=0.020], and was in agreement to its higher proportions in non-SMA whereas the carriage of the *CD40*-508AA under this model was associated with risk of SMA [OR=2.29, 95% CI=1.18-4.44, P=0.013]. In the recessive model, the carriage of the *CD40*-508AA revealed a strong association with susceptibility to SMA [OR=3.26, 95% CI=1.35-7.84, P=0.008]. The *CD40*-173C/T variations dominant model showed that there was no association between the *CD40*-173 (CT+TT) [OR=0.71, 95% CI=0.42-1.19, P=0.196]. While considering the additive model, the carriage of the heterozygous *CD40*-173CT genotype was associated with 77% protection against SMA [OR=0.23, 95% CI=0.11-0.49, P<0.001] consistent with its distribution in non-SMA while the existence of *CD40*-173TT was associated with risk to SMA [OR=4.97, 95% CI=1.96-12.62, P=0.001]. In the recessive model, the carriage of the *CD40*-173TT *versus* -173(CC+CT) revealed 4.8-folds risk to SMA [OR=4.82, 95% CI=2.23-10.42, P<0.001]. In the dominant model of *CD40*-1C/T using *CD40*-1CC as the reference, carriage of CT+TT revealed a

significant association with the risk of SMA [OR=5.27; 95% CI=3.03-8.20, *P*<0.001]. The additive model revealed that the *CD40*-1CT was associated with high risk of SMA [OR=11.26, 95% CI=3.82-33.23, *P*<0.001]. With only sixteen (16) homozygous variant *CD40*-1TT, no significant association with SMA susceptibility (OR=2.53, 95% CI=0.79-8.09, *P*=0.117) was detected. Using the local definition of SMA (Hb<6.0 g/dL) further regression analysis revealed that the carriage of *CD40* -508 GA and the AA genotypes were associated with protection (OR=0.61, 95% CI=0.47-0.92, *P*=0.041) and risk (OR=1.63, 95% CI=1.05-2.96, *P*=0.038), respectively. Carriage of the *CD40*-173 CT (OR=0.53, 95% CI=0.32-0.78, *P*=0.027) and the *CD40* -173 TT (OR=2.51, 95% CI=2.01-7.62, *P*=0.011) were associated with protection and risk of SMA, respectively. As shown when SMA of Hb<5.0 g/dL was used, the carriage of *CD40*-1 CT showed association with risk to SMA with modified definition (OR=8.01, 95% CI=1.50-21.81, *P*<0.001) as shown in Table 4.2.

Table 4. 2. Association between *CD*40 Variations and Severe Malaria Anaemia

		SMA (Hb<5.0 g/dL)			SMA (Hb<6.0 g/dL)		
Genetic Models	**Variation**						
***CD*40 -508 G/A**		**OR**	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**
Dominant	GG	Ref					
	GA+AA	0.72	0.43-1.19	0.198	0.82	0.67-2.11	0.251
Additive	GG	Ref					
	GA	0.39	0.15-0.85	**0.020**	0.61	0.47-0.92	**0.041**
	AA	2.29	1.18-4.40	**0.013**	1.63	1.05-2.96	**0.038**
Recessive	GG+GA	Ref					
	AA	3.26	1.35-7.84	**0.008**	1.89	1.20-3.82	**0.001**
***CD*40 -173 C/T**							
Dominant	CC	Ref					
	CT+TT	0.71	0.42-1.19	0.196	0.93	0.67-2.16	0.372
Additive	CC	Ref					
	CT	0.23	0.11-0.49	**<0.001**	0.53	0.32-0.78	**0.027**
	TT	4.97	1.96-12.62	**0.001**	2.51	2.01-7.62	**0.011**
Recessive	CC+CT	Ref					
	TT	4.82	2.23-10.42	**<0.001**	3.75	2.89-7.09	**0.017**
*CD*40 -1C/T							
Dominant	CC	Ref					
	CT+TT	5.27	3.03-8.20	**<0.001**	3.66	2.20-6.90	**0.021**
Additive	CC	Ref					
	CT	11.26	3.82-33.23	**<0.001**	8.01	1.50-21.81	**<0.001**
	TT	2.53	0.79-8.09	0.117	1.97	0.38-6.42	0.397
Recessive	CC+CT	Ref					
	TT	XX	XX	XX	XX	XX	XX

Children with parasitemia were categorized on the basis of presence or absence of SMA. Statistical significance determined by logistic regression analysis for dominant, additive and recessive models while controlling for age, bacteraemia and sickle cell trait (reduced model). *P*-values in bold were statistically significant at $P \leq 0.05$. XX; number not adequate to run in the regression model.

4.2.3. Distribution of *CD40* (G-508A/C-173T C-1T) haplotypes in the study groups

Haplotype construction based on the *CD40* polymorphisms construction generated 8 haplotypes. Five haplotypes with frequencies >1.4% within the population of study were selected as these were the most common in the population. Carriage of the most common GCC haplotype was higher in the non-SMA group 881 (96.2%) relative to the SMA group 185 (87.2%), while the non-carriage of this haplotype were lower in the non-SMA group 35 (3.8%) relative to the SMA group 27 (12.8%), $P<0.001$. The carriage of GTC haplotype were relatively lower in the non-SMA group 48 (5.2%) compared to the SMA group 47 (22.2%) while the non-carriage GTC were comparatively higher in non-SMA 868 (94.8%) than SMA group 165 (77.8%), $P<0.001$. There were, however, no differences between the distributions of the carriage and non-carriage of GTC haplotype in the study groups; GTC in non-SMA 31 (3.4%) and in SMA 7 (3.3%), non-GTC in non-SMA 885 (96.6%) and in SMA 205 (96.7%), respectively, $P=0.952$. The carriage of ACC haplotypes non-SMA group 32 (3.5%) were comparable to those in SMA group 3 (1.4%) while non-carriage of this haplotype in non-SMA was 884 (96.5%) and SMA 209 (98.6%). These distributions were comparable, however, between the two groups, $P=0.116$. The carriage of the ATC haplotype in non-SMA was 85 (9.3%) and in SMA 16 (7.5%) while non-carriage was 831 (90.7%) in non-SMA and SMA 196 (92.5%). These distributions were comparable between the two groups ($P=0.426$).

Further, assessments of whether the carriage and non-carriage of these haplotypes had differences in Hb levels (gdL^{-1}) was performed. Man-Whitney U test revealed that carriage of GCC haplotype had higher Hb levels (median, 7.1; IQR, 3.4) relative to non-GCC carriage (median, 5.1; IQR, 3.1), $P<0001$. Further, the carriage of the ACC haplotype had higher Hb levels (median, 8.8; IQR, 2.1) relative to non-carriage (median, 6.9; IQR, 3.4), $P<0.001$. Hb

levels were, however, not different between carriage of GTC haplotype (median, 5.9; IQR, 3.3) and non-GTC (median, 7.1; IQR, 3.4), P=0.114. The carriage of the ATC haplotype versus non-ACT also showed comparable Hb levels for ACT (median, 7.0; IQR, 2.8) and non-ATC (median, 7.0; IQR, 3.5), P=0.858. Moreover, carriage of GCT haplotype showed significantly lower Hb levels (median, 4.8; IQR, 1.4) compared to carriage of non-GCT (median, 7.3; IQR, 3.3), P<0.001 shown in Table 4.3.

Table 4. 3. Distribution of *CD40* (G-508A/C-173T/C-1T) haplotypes in the study groups.

***CD40* G-508A/C-173T/C-1T Haplotypes**		**Hb gdL^{-1} (median IQR)**	**P*a***	**Non-SMA (n=916)**	**SMA (n=212)**	**P*b***
GCC	Carriers (n=1066)	7.1 (3.4)	**<0.001**	881 (96.2)	185 (87.2)	**<0.001**
	Non-carriers (n=62)	5.1 (3.1)		35 (3.8)	27 (12.8)	
GCT	Carriers (n=95)	4.8 (1.4)	**<0.001**	48 (5.2)	47 (22.2)	**<0.001**
	Non-carriers (n=931)	7.3 (3.3)		868 (94.8)	165 (77.8)	
GTC	Carriers (n=38)	5.9 (3.3)	0.144	31 (3.4)	7 (3.3)	0.952
	Non-carriers (n=988)	7.1 (3.4)		885 (96.6)	205 (96.7)	
ACC	Carriers (n=35)	8.8 (2.1)	**<0.001**	32 (3.5)	3 (1.4)	0.116
	Non-carriers (n=991)	6.9 (3.4)		884 (96.5)	209 (98.6)	
ATC	Carriers (n=101)	7.0 (2.8)	0.858	85 (9.3)	16 (7.5)	0.426
	Non-carriers (n=925)	7.0 (3.5)		831 (90.7)	196 (92.5)	

Data presented are medians (IQR) for haemoglobin levels and as proportions for haplotypes (n, with percentages in parentheses). Values in bold depict statistical significance at $P \leq 0.05$. [a] Statistical significance was determined by Mann-Whitney U test. [b] Statistical significance was determined by Chi square (χ^2) tests.

4.2.4. Association between *CD40* (G-508A/C-173T/C-1T) haplotypes and SMA

Considering the distribution of these haplotypes within the study groups, their association with susceptibility to SMA was computed. In the reduced model with age, α-thalassemia and bacteraemia as covariates, consistent with its higher distribution in the non-SMA group, the GCC haplotype demonstrated 69% protection against SMA (Hb<5.0 g/dL), (OR=0.31, 95% CI=0.14-0.67, *P*=0.003). Further regression analysis revealed that carriage of the GCT haplotype was associated with susceptibility to SMA (OR=5.24, 95% CI=3.31-8.82, *P*<0.001). However, the carriage of ACC, (OR= 0.37, 95% CI=0.11-1.23, *P*=0.106) and GTC, (OR=0.97, 95% CI=0.42-2.27 *P*=0.951) did not show associations with susceptibility to SMA. Likewise, carriage of the ATC haplotype did not reveal any association with susceptibility to SMA (OR=0.75, 95% CI=0.43-1.33 *P*=0.325). Further regression analysis using Hb<6.0 g/dL, revealed that the GCC was associated with protection (OR=0.41, 95% CI=0.31-0.92, *P*=0.036) and that the GCT was associated with SMA risk (OR=3.81, 95% CI=2.31-6.11, *P*=0.012). However, the carriage of other haplotypes did not show significant association with SMA based on either definition (Figure 4.2).

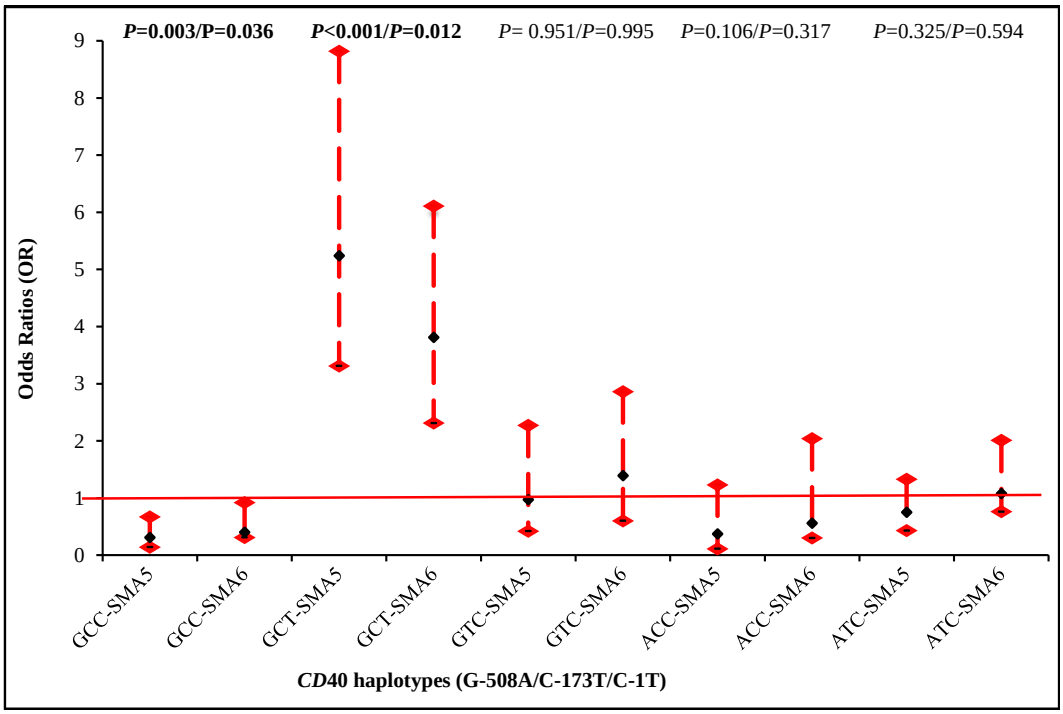

Figure 4. 2. Association between *CD40* (G-508A/C-173T/ C-1/T) haplotypes and severe malaria anaemia

Children with parasitemia were categorized on the basis of presence or absence of both (SMA Hb<5.0 g/dL and SMA; Hb<6.0 g/dL). Odds ratios (OR) and 95% confidence intervals (CI) were determined using bivariate logistic regression controlling for age, bacteraemia and sickle cell trait (reduced model). The reference groups in the bivariate logistic regression analysis were the non-carriers of the respective haplotypes. (n); number of participants with respective haplotype. *P*-values in bold indicate statistical significance at $P \leq 0.05$. SMA5= SMA; Hb<5.0 g/dL and SMA6=Hb<6.0 g/dL.

4.2.5. Association between *CD40* (G-508A/C-173T/C-1/T) haplotypes copies and SMA

Haplotype carriage may be defined as no haplotype, carriage of single copy and/or carriage of double copy. To further show the additive effect of haplotype carriage on SMA susceptibility, regression model was used to determine whether variation in the number of copies of each of the haplotype influenced susceptibility to SMA. Based on the WHO definition of SMA (Hb<5.0 g/dL), the results presented show that, carriage of one copy of the GCC is associated with reduced risk to SMA (OR=0.34, 95% CI=0.17-0.67, *P*=0.002) while carriage of two copies of GCC was more associated with protection from SMA (OR=0.24, 95% CI=0.13-0.45, *P*<0.001) further showing the additive effect of this haplotype in protection from SMA. For the GCT haplotype, carriage of single copy GCT was significantly associated with increased risk of SMA (OR=4.82, 95% CI=3.12-7.24, *P*<0.001). The low number of carriage of two copies of the GCT haplotype did not run in the model. On further analysis, carriage of a single copy GTC (n=34) haplotype was also not associated with SMA (OR=0.83, 95% CI=0.33-2.05, *P*=0.683) while association between carriage of two copies (n=4) and SMA could not be established in the model. Carriage of single copy of ACC haplotype showed no association with SMA (OR=0.49, 95% CI=0.15-1.66, *P*=0.251). The association between carriage of two copies of ACC and SMA could not be established in the model due to low numbers (n=3). The carriage of single copy of ATC (n=90) haplotype was not associated with SMA (OR=0.86, 95% CI=0.44-1.46, *P*=0.461) while carriage of two copies of ATC (n=11) did not run in the model. Further analysis based on the local definition of SMA (Hb<6.0 g/dL), revealed that the carriage of both single and double copy of the GCC haplotype is associated with SMA (*P*=0.032 and *P*=0.005 respectively. The carriage of single copy of GCT was associated with SMA (*P*=0.009). Carriage of other CD40 haplotypes did not reveal association with SMA as shown in Table 4. 4.

Table 4. 4. Association between haplotype copies and SMA

	SMA (Hb<5.0 gdL^{-1})			SMA (Hb<6.0 gdL^{-1})		
Haplotypes copies	**OR**	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**
GCC $_{(0)}$, (n=62)	Ref	-	-	Ref	-	-
GCC $_{(1)}$, (n=180)	0.34	0.17-0.67	**0.002**	0.59	0.34-0.9	**0.032**
GCC $_{(2)}$, (n=886)	0.24	0.13-0.45	**<0.001**	0.41	0.23-0.76	**0.005**
GCT $_{(0)}$, (n=1033)	Ref			Ref		
GCT $_{(1)}$, (n=81)	4.82	3.12-7.24	**<0.001**	2.57	1.63-5.70	**0.009**
GCT $_{(2)}$, (n=14)	XX	XX	XX	XX	XX	XX
GTC $_{(0)}$, (n=1090)	Ref			Ref		
GTC $_{(1)}$, (n=34)	0.83	0.33-2.05	0.683	0.54	0.11-1.54	0.819
GTC $_{(2)}$, (n=4)	XX	XX	XX	XX	XX	XX
ACC $_{(0)}$, (n=1093)	Ref	-	-	Ref	-	-
ACC $_{(1)}$, (n=32)	0.49	0.15-1.66	0.251	0.87	0.18-1.51	0.475
ACC $_{(2)}$, (n=3)	XX	XX	XX	XX	XX	XX
ATC $_{(0)}$, (n=1027)	Ref			Ref		
ATC $_{(1)}$, (n=90)	0.86	0.44-1.46	0.461	0.93	0.58-2.87	0.801
ATC $_{(2)}$, (n=11)	XX	XX	XX	XX	XX	XX

Data are presented as n (%) of children within the study groups. Children with parasitemia were categorized on the basis of presence or absence of SMA. Statistical significance were determined by logistic regression analysis controlling for age, bacteraemia and sickle cell trait (reduced model). *P*-values in bold were statistically significant at $P \leq 0.05$. XX; number not adequate to run in either regression model.

4.2.6. Comparison of inflammatory mediator levels between carriage and non-carriage of *CD40* haplotypes

To further investigate the potential mechanisms through which the carriage of the 'protective' GCC and 'susceptible' GCT haplotypes influence SMA based on their associations observed in the regression analysis, the differences in the peripheral levels of an array of inflammatory mediators between carriage and non-carriage of these haplotypes in children (n=400) devoid of co-infections were determined. On stratification of inflammatory mediators based on carriage of the GCC haplotype, the carriers of the haplotype had significantly higher circulating IL-1β levels [194.3 (193.3), versus non-carriage [124.45 (130.9)], P=0.002, while the carriage of GCT haplotype had lower levels of circulating IL-1β [108.9 (210.1) versus non-GCT [193.7 (187.1)], P=0.017. Furthermore, GCC haplotype carriage revealed association with higher levels of IL-2 [50.6 (75.6) versus non-GCC [22.2 (42.9)], P=0.029. However, there were no significant differences in the levels of IL-2 between GCT [52.6 (87.3] and non-GCT [44.5 (75.6)], P=0.783. Moreover, the carriage of GCC haplotype relative to non-GCC showed significant differences in the circulating levels of MIP-1α [80.7 (83.0)] versus [56.7 (60.4)], respectively, P=0.048. These levels were however, comparable in GCT [85.6 (105.4)] versus non-GCT [80.3 (79.8)], P=0.913. Additional analysis revealed that the 'susceptible' GCT haplotype had relatively lower levels of circulating IL-17; GCT [50.6 (13.9)] versus non-GCT [130.2 (23.7)], P=0.003 while the levels were comparable between GCC [69.6 (73.5)] versus non-GCC [50.2 (13.7)], P=0.258. Results presented here show that the carriage of the 'protective' GCC haplotype and carriage of the 'susceptible' GCT haplotypes are associated with altered levels of inflammatory mediators, consistent with previous studies in which demonstrated that these deregulations are associated with SMA (Davenport *et al.,* 2016; Ouma *et al.,* 2008a;

Were *et al.*, 2006). None of the other inflammatory mediators differed significantly between the carriage of GCC and GCT as compared to their respective non-carriage, (Figure 4.3 A-D).

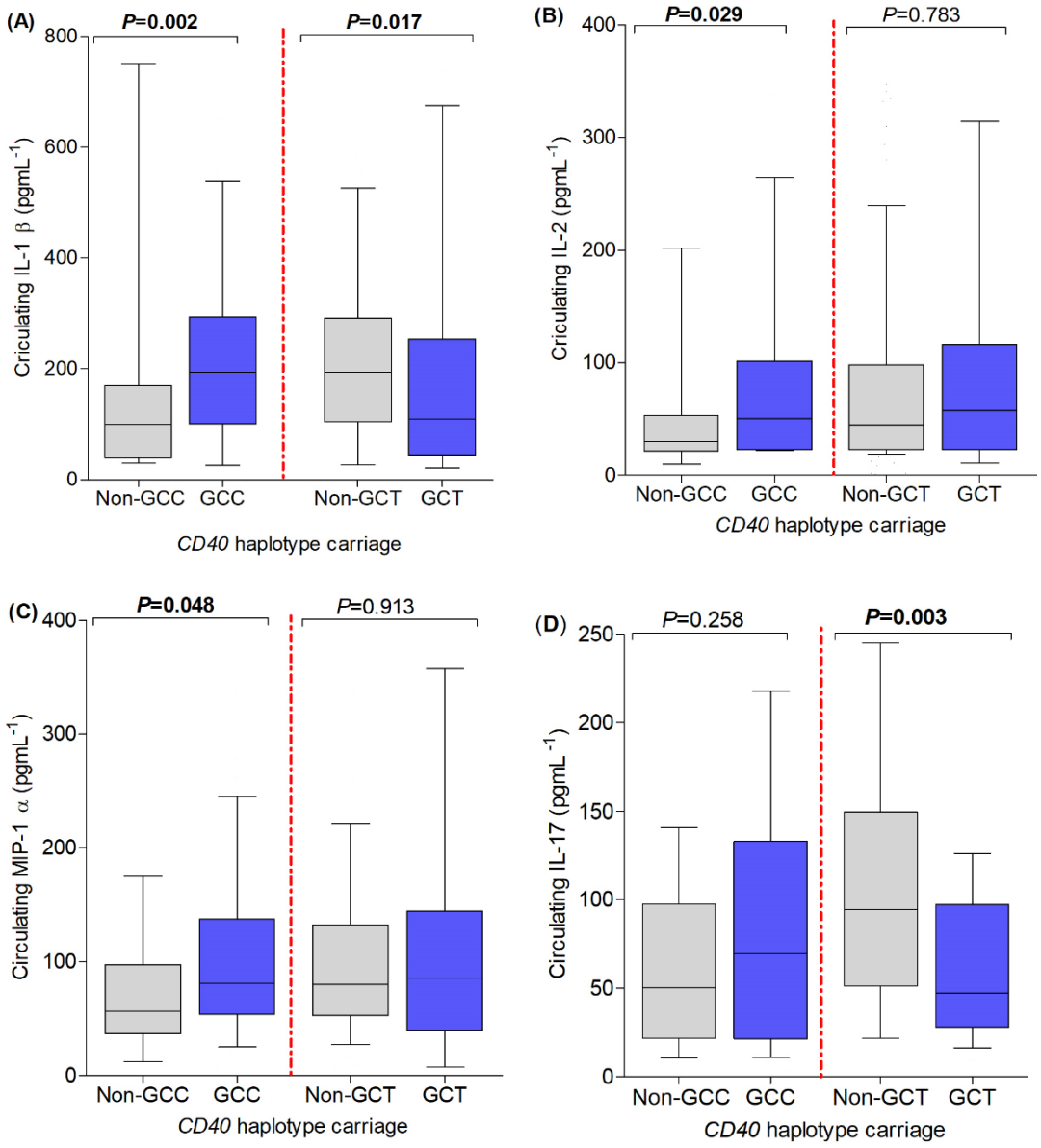

Figure 4. 3. Inflammatory mediator levels in carriage and non-carriage of haplotype:

Data are presented as box plots for haplotypes carriage and non-carriage. The boxes represent interquartile ranges; the lines through the boxes are the medians, while the whiskers show the 10th and the 90th percentiles. Levels of peripheral IL-1β, IL-2, MIP-1α, and IL-17 presented in (pgmL-1) in parasitemic children devoid of co-infections (HIV-1 and bacteraemia) (n=400) were measured using the 'Human Cytokine Twenty-Five-Plex Antibody Bead Kit'. In all the tests, comparisons between carriage and non-carriage of haplotype were performed using Mann-Whitney U test at $P \leq 0.05$.

4.3. *NFκB1 and NFκBIA* Variations and SMA

4.3.1. Distribution of genotypes in the *NFκB1* and *NFκBIA* polymorphisms within the study groups and association with SMA

To determine whether any of the genotypes were over-represented in a particular study category, the distribution of the genotypes of *NFκB1* (-8079G/A and -3297C/T) and *NFκBIA (*-826 G/A and -310G/A) and within the study groups were determined. The *NFκB1*(-8079 G/A) genotypes were distributed as follows; 30.0% GG, 48.2% GA and 21.8% AA within the non-SMA group while in the SMA group, there were 25% GG, 50.0% GA and 25.0% AA with the overall allele frequency of major G allele 0.53 and minor allele A 0.47. The allele distributions revealed consistency with HWE (χ^2=0.639, P=0.424). The distributions of the *NFκB1* (-8079 G/A) genotypes were comparable between the SMA and non-SMA (P=0.353). Further analysis of the *NFκB1*(-3297 C/T) polymorphism within the non-SMA revealed a distribution of 63.4% CC, 24.1% CT and 12.5% TT while in SMA 51.6% CC, 25.5% CT and 22.9% TT were observed. The overall major allele C frequency was 0.73 while the minor allele T had a frequency of 0.27. The TT genotype was overrepresented in the SMA group compared to non-SMA (P=0.001). The distributions of the *NFκB1* (-3297 C/T) genotypes showed deviation from HWE (χ^2=145.1, P<0.001).

Distribution of the *NFκBIA* -826 G/A genotypes in the non-SMA group were 65.8% GG, 28.8% GA and 5.4% AA while in the SMA group were 60.1% GG, 35.1% GA and 4.8% AA. The distributions of the genotypes within the study groups were comparable (P=0.242). The allele frequency of the major G allele was 0.81 while the minor A allele was 0.19. The frequencies of *NFκBIA* (-826 G/A) alleles in the whole study population revealed deviation from Hardy-Weinberg Equilibrium (χ^2=7.93 P=0.005). The *NFκBIA* (-310 G/A) genotypes

distributions in non-SMA were 83.2% GG, 14.8% GA and 2.0% AA while in the SMA were 77.7% GG, 20.2% GA and 2.1% AA, with overall major G allele frequency of 0.90 and the minor allele A frequency of 0.10. These genotype distributions did not reveal any significant differences between non-SMA and SMA (P=0.180). There was, however, a significant deviation from the HWE (χ^2=32.4 P<0.001) of the allele in the whole study population.

To determine the association between these polymorphisms and SMA (Hb< 50g/dL), bivariate logistic regression analysis in a model which involved controlling for anaemia covariates; age, sex, co-infection (HIV-1 status and bacteraemia), alpha-thalassemia, sickle cell trait (HbAS) and G6PD deficiency (Aidoo *et al.,* 2002; Ong'echa *et al.,* 2006; Perkins *et al.,* 2011; Wambua *et al.,* 2006a) was done. The analysis revealed that neither the *NFκB1*-8079 GA (OR=0.87, 95% CI=0.51-1.47, P=0.598) nor the *NFκB1*-8079 AA (OR=1.39, 95% CI=0.90-2.14, P=0.133) was associated with susceptibility to SMA using *NFκB1*-8079 GG as the reference genotype in the analysis. Further, using the *NFκB1*-3297 CC as the references, the *NFκB1*-3279CT did not show association with SMA (OR=0.65, 95% CI=0.41-1.01, P=0.058), while the *NFκB1*-3279 TT was associated with susceptibly to SMA (OR=2.13, 95% CI=1.39-3.28 P=0.001; (Table 4.5) and this was consistent with its distribution in the SMA group compared to non-SMA group. Analysis of the *NFκBIA*-826 with GG genotype as the reference revealed that both *NFκBIA*-826 AG and *NFκBIA*-826 AA genotypes were not associated with SMA (OR=1.07, 95% CI=0.49-2.23, P=0.872 and OR=0.87, 95% CI=0.40-1.83, P=0.689, respectively). Additional analysis of the *NFκBIA*-310 G/A genotypes and association with SMA using the GG as reference, similarly did not show significant association with SMA in the current study (*NFκBIA*-310 GA, OR=1.48, 95% CI=0.97-2.25, P=0.066 and *NFκBIA*-310 AA, OR=0.97, 95% CI=0.28-3.51, P=0.997). When SMA (Hb<6.0 g/dL) was used, the carriage of the mutant

genotype of the *NFκB1* -3297TT revealed association with risk to SMA (OR=1.93, 95% CI=1.09-2.38). Carriage of other genotypes did not reveal any associations with SMA. These results are summarised in Table 4.5.

Table 4. 5. Distribution of genotypes in the *NFκB1* and *NFκBIA* polymorphisms within the study groups and association with SMA

Genotypes	N (%) with genotype in group [a]			SMA (Hb<5.0 g/dL)			SMA (Hb<6.0 g/dL)		
	Non-SMA (Hb≥5.0 g/dL, n=838)	SMA (Hb<5.0 g/dL, n=188)	*P*-value[b]	OR	95% CI	*P*-value[c]	OR	95% CI	*P*-value[c]
NFκB1(-8079 G→A, rs980455)									
GG, n (%)	251 (21.8)	47 (25.0)		Ref	-	-	Ref	-	-
GA, n (%)	404 (48.2)	94 (50.0)	0.353	0.87	0.51- 1.47	0.598	0.92	0.74- 2.01	0.713
AA, n (%)	183 (30.0)	47 (25.0)		1.39	0.90- 2.14	0.133	1.01	0.82- 1.98	0.299
Y (0.47)									
NFκB1(-3297 C→T, rs747559)									
CC, n (%)	531 (63.4)	97 (51.6)		Ref	-	-	Ref	-	-
CT, n (%)	202 (24.1)	48 (25.5)	**0.001**	0.65	0.41-1.01	0.058	0.89	0.59-1.81	0.109
TT, n (%)	105 (12.5)	43 (22.9)		2.13	1.39-3.28	**0.001**	1.93	1.09-2.38	**0.039**
Y (0.27)									
NFκBIA (-826 G→A, rs2233406									
GG, n (%)	551 (65.8)	113 (60.1)		Ref			Ref		
GA, n (%)	242 (28.9)	66 (35.1)	0.242	1.07	0.49-2.23	0.872	1.51	0.32-2.62	0.909
AA, n (%)	45 (5.4)	9 (4.8)		0.87	0.40-1.83	0.689	0.87	0.540-2.01	0.829
Y (0.19)									
NFκBIA (-310 G→A, rs2233409)									
GG, n (%)	697 (83.2)	146 (77.7)		Ref			Ref		
GA, n (%)	124 (14.8)	38 (20.2)	0.180	1.48	0.97-2.25	0.066	1.17	0.97-2.25	0.104
AA, n (%)	17 (2.0)	4 (2.1)		0.97	0.28-3.51	0.997	0.89	0.39-2.28	0.909
Y (0.10)									

[a] Data are presented as n (%) of children within the study groups. Children with parasitemia were categorized on the basis of presence or absence of severe malaria anaemia SMA. [b] Statistical significance was determined by the χ^2 analysis. [c] Statistical significance was determined by logistic regression analysis controlling for confounders age, sickle cell trait, G6PD and α-thalassemia(reduced model). P-values in bold indicate statistical significance. Y; frequency of the minor allele in the whole population.

4.3.2. Distribution of the *NFκB1* and *NFκBIA* haplotypes

Since haplotypes reveal associations that may not be discernable based on individual genotypes, haplotype constructs of both *NFκB1*-8079G→A/-3297C→T and *NFκBIA*-826G→A/-310G→A were used to further determine association between these haplotypic structures and malaria outcome, controlling for confounding factors observable in the reduced model. The distributions of these haplotypes were first compared between non-SMA and SMA groups. As shown in Figure 4.4 (A), carriage *NFκB1*-8079G/-3297C (GC) haplotype was higher in the non-SMA 632 (75.4%) compared to SMA 121 (64.4%), P=0.002, while the GT haplotype was lower in non-SMA 50 (6.0%) compared to the proportions in SMA 26 (13.8%), P<0.001. *NFκB1*-8079A/-3297C (AC) haplotype was comparable between the clinical groups with non-SMA having 369 (44.0%) while SMA had 71 (37.8%), P=0.117. Further analysis showed that carriage of AT haplotype was relatively lower in the non-SMA 275 (32.8%) compared to SMA group 82 (43.6%), P=0.005. Analysis of *NFκBIA*-826G→A/-310G→A haplotypes showed comparable distributions in the proportions of the common GG haplotype in non-SMA 779 (93.0%) and SMA 176 (93.6%), P=0.748. There were in addition no proportional differences in the carriage of the GA haplotype between non-SMA 26 (3.1%) and SMA 7 (3.7%), P=0.663. Moreover, the AG haplotype were also comparable in non-SMA and SMA [(177 (21.1%) and 40 (21.2%), respectively, P=0.963]. The AA haplotype with both variant alleles where lower in non-SMA 128 (15.3%) and SMA 38 (20.2%), P=0.097 though not significantly different as shown in Figure 4.4 (B).

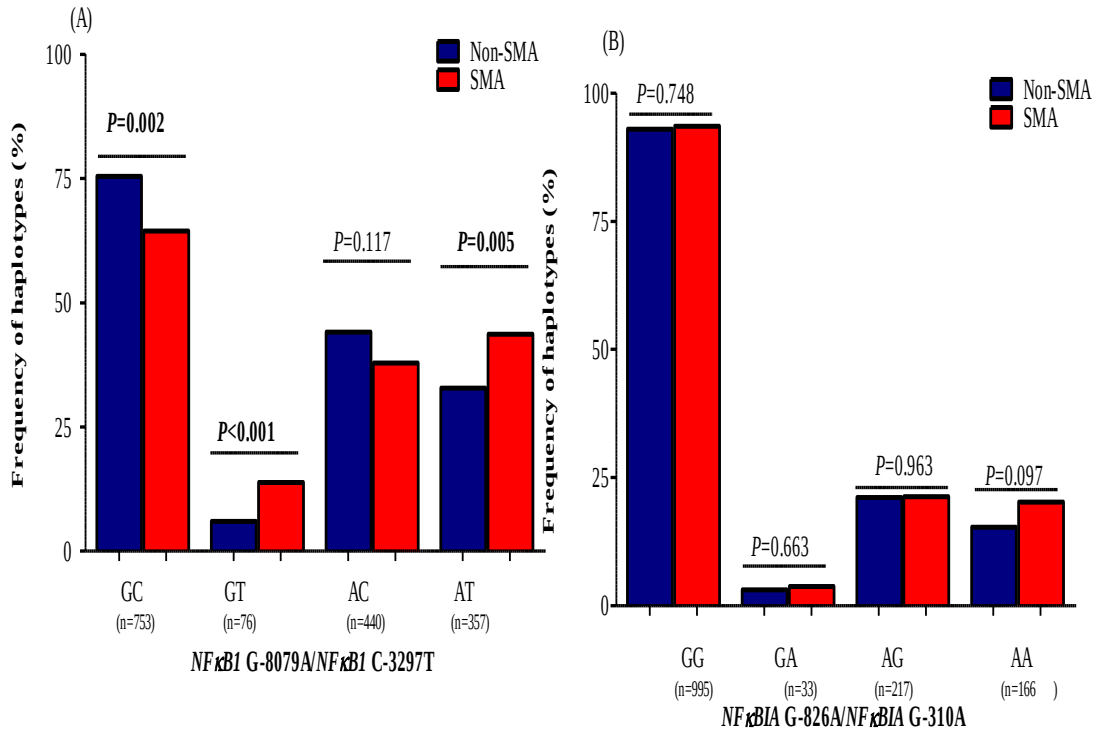

Figure 4. 4. (A and B): Distribution of *NFκB1* G-8079A/*NFκB1* C-3297T and *NFκBIA*G-826A/G-310A

Children (n=1,026) were categorized into non-SMA and SMA groups. Statistical significance was determined by χ^2 analysis. *P*-values in bold were statistically significant at $P \leq 0.05$.

4.3.3. Association between *NFκB1* and *NFκBIA* promoters' haplotypes and SMA

Using the stringent WHO definition of SMA (Hb<5.0 g/dL), regression analysis demonstrated that carriage of *NFκB1*-8079A/-3297C GC haplotype consistent with its higher distribution in non-SMA had 40% reduced risk against SMA (OR=0.60, 95% CI=0.42-0.86, *P*=0.005). Carriage of the GT haplotype revealed an association with increased SMA susceptibly (OR=2.51, 95% CI=1.42-4.42, *P*=0.002), consistent with its higher distribution in the SMA group. The AC haplotype was not associated with SMA (OR=0.92, 95% CI: 0.64-1.33, *P*=0.651). Consistent with its high distribution in SMA group, carriage of the *NFκB1*-8079A/-3297T, AT haplotype that was higher in the SMA group, was also associated with susceptibility to SMA (OR=1.65, 95% CI=1.17-2.32, *P*=0.004), (Figure 4.5 C). When the association was run based on SMA of Hb<6.0 g/dL, the GC haplotype revealed association with protection against SMA (OR=0.70 95% CI=0.57-0.95 *P*=0.011) while the GT and the AT haplotypes still had risk with SMA (OR=1.75 95% CI=1.04-2.06 *P*=0.033) and (OR=1.14 95% CI=1.04-1.72 *P*=0.048), respectively. For the *NFκBIA* haplotypes, regression analysis did not reveal any association with susceptibility to SMA (Hb<5.0 g/dL) considering the common GG haplotype (OR=0.93, 95% CI=0.43-1.99, *P*=0.841). Carriage of the GA did not reveal association with SMA susceptibility (OR=1.24, 95% CI=0.44-3.82, *P*=0.682), as well as the AG haplotype (OR=0.59, 95% CI=0.34-0.98, *P*=0.043). Analysis of the carraige of AA haplotype revealed a significant association with SMA susceptibility (OR=1.62, 95% CI=1.02-2.57, *P*=0.042). In addition, using SMA (Hb<6.0 g/dL), there was only marginal association between the AA haplotype and susceptibility to SMA as shown in Figure 4.5 (D).

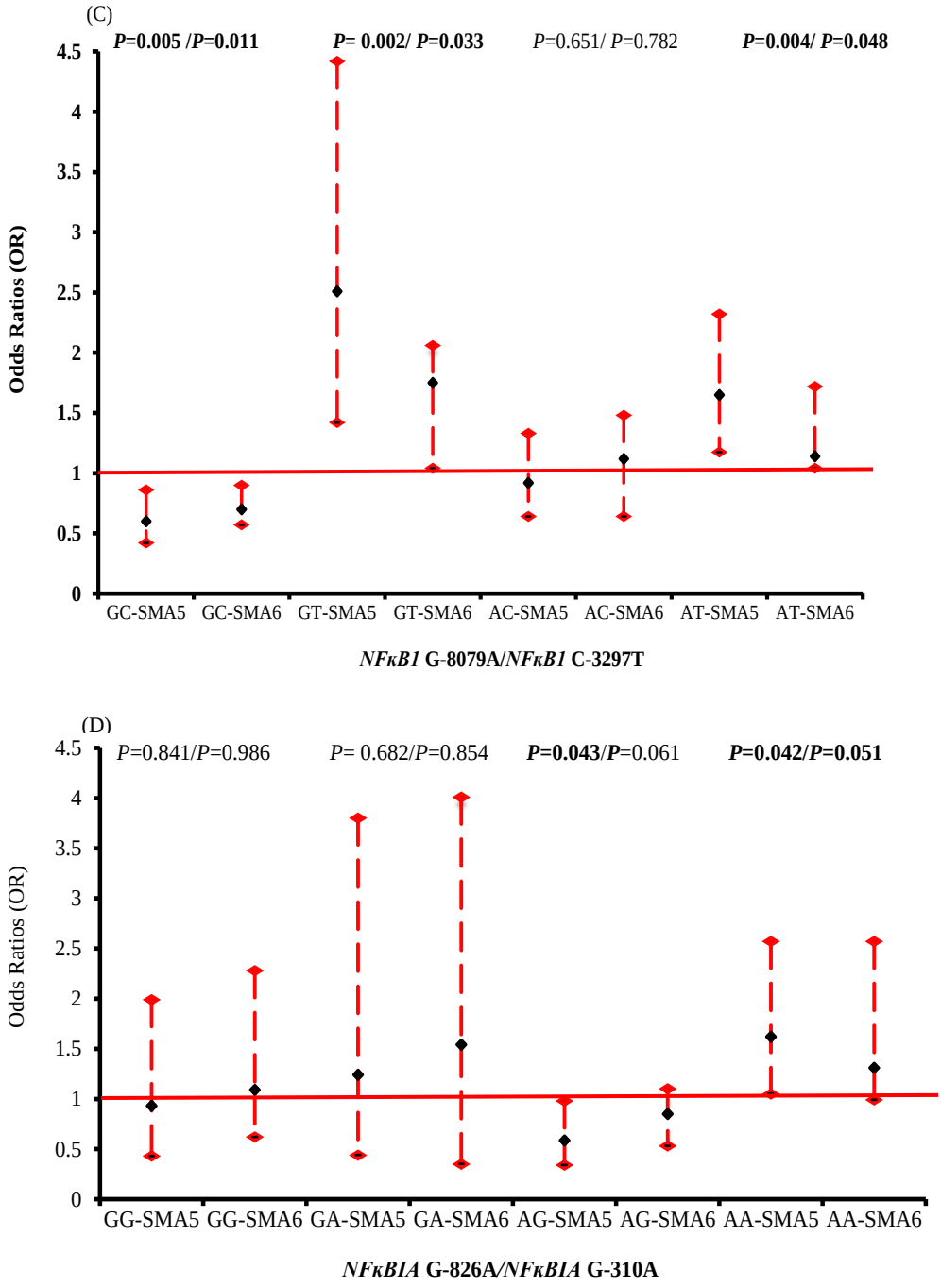

Figure 4. 5. (C and D). Associations between *NFκB1* and *NFκBIA* haplotypes and SMA (Hb<5.0 g/dL and Hb<6.0 g/dL)

Associations between *NFκBIA* haplotypes and SMA. Associations were determined in children with malaria (n=1,026). Odds ratios (ORs) and 95% confidence intervals (CIs) were determined using bivariate regression analyses, controlling for anaemia covariates; age, sex, co-infection (HIV-1 status and bacteraemia), alpha-thalassemia, sickle cell trait (HbAS) and G6PD deficiency. For each haplotype, individuals without the haplotype were used as the reference group. *P*-values in bold were statistically significant at $P \leq 0.05$. SMA5= SMA; Hb<5.0 g/dL and SMA6= Hb<6.0 g/dL.

4.3.4. Comparison of the levels of circulating IL-17 between carriage of the *NFκB1* haplotypes and correlation with Haemoglobin (Hb) levels

After determining the associations between *NFκB1* haplotypes and SMA, influence of these haplotypes associated with risk/protection on changes in the levels of circulating inflammatory mediators whose imbalances have been shown to consequently influence malaria outcome (Perkins *et al.,* 2011) were assessed. Considering the *NFκB1* polymorphisms studied in the present study, IL-17, a pleotropic cytokine was identified as the most significantly different based on the *NFκB1*-8079G→A/-3297C→T haplotypes. Analysis did not reveal differences in the levels of IL-17 in carriage of either *NFκB1*-8079A/-3297C [mean (SEM), 0.908 (0.053)] or non-AC [mean (SEM), 0.898 (0.085)], P=0.916. However, the carriage of the *NFκB1*-8079A/-3297T haplotype which was associated with susceptibility to SMA had distinctly lower levels of IL-17 [mean (SEM), 0.786 (0.063)] relative to non-*NFκB1*-8079A/-3297T [mean (SEM), 1.00 (0.062)], P=0.016. The carriage of the *NFκB1*-8079G/-3297C (GC) haplotype which had 40% reduced risk to SMA, had increased levels of IL-17, [mean (SEM), 1.03 (0.073)] compared to non-GC [mean (SEM), 0.812 (0.058)], P=0.015. In addition, IL-17 levels were also comparable in carriage of the *NFκB1*-8079G/-3297T [mean (SEM), 0.966 (0.097)] and non-GT [mean (SEM), 0.899 (0.049)], P=0.541, Figure 4.6 (A). Using Spearman rank correlation test, the correlation between circulating levels of IL-17 and haemoglobin levels were determined. The data presented here showed that there was a positive correlation between the levels of IL-17 and those of haemoglobin (ρ=0.151, P=0.027), implying that higher IL-17 levels are associated with a better outcome following malaria infection Figure 4.6 (B).

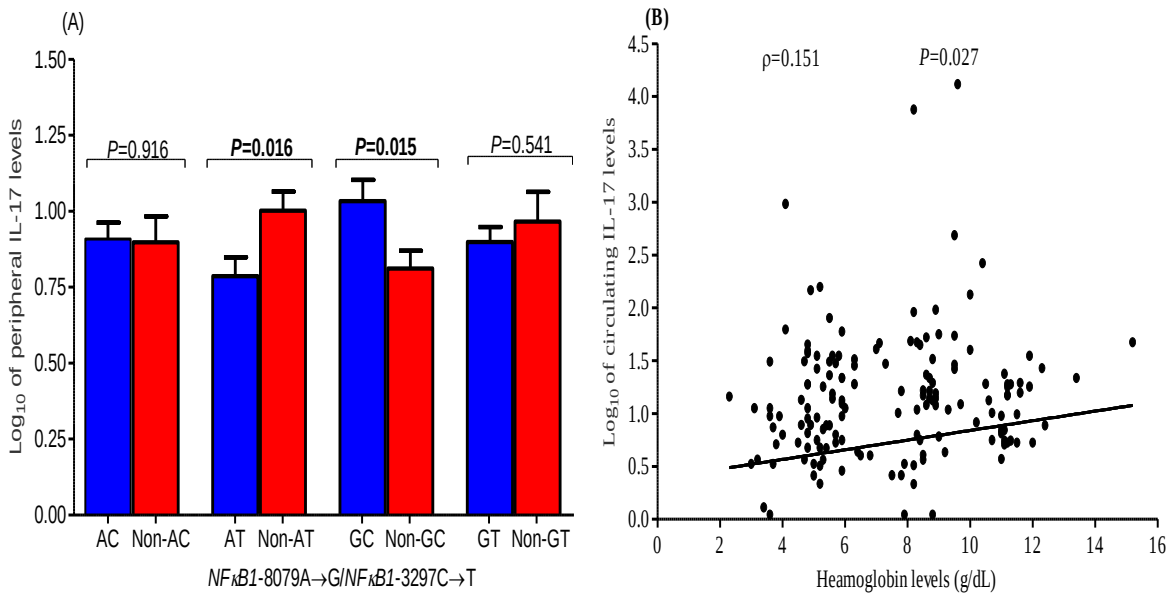

Figure 4. 6. (A and B): Comparison of the levels of peripheral IL-17 in carriage of the *NFκB1* haplotypes and its correlation between Hb levels.

(A): Comparison of the levels of circulating IL-17 in carriage of the *NFκB1* haplotypes: Data are grouped into carriage of *NFκB1*-8079G→A/*NFκB1*-3297C→T haplotypes and non-carriage for children (n=215) with *P. falciparum* malaria devoid of co-infections. Data are presented as mean with standard error of the mean [SEM] of the normalized IL-17 levels. Statistical significance determined using Student's t-test (with Welsch correction at 95% CI).

(B): Correlation between Hb levels and IL-17 levels. Correlation between normalized IL-17 peripheral levels (n=215), expressed as Log_{10}, and haemoglobin levels (Hb; g/dL) was determined by the Spearman rank correlation test. *P*-value was considered significant at $P \leq 0.05$.

4.3.5. Comparison of peripheral IL-10 and IP-10 in the carriage of *NFκBIA* haplotypes

After the finding that *NFκBIA* haplotypes are associated with SMA, effects of the haplotypes on circulating levels of IL-10 and IP-10 produced during infection was determined. The results revealed that the levels of both IL-10 and IP-10 did not differ based on the carriage or non-carriage of *NFκBIA*-826A/-310A haplotype which is associated with SMA susceptibility, [median (IQR)] for IL-10, AA haplotype [126.7 (390.0)], non-AA [229.4 (532.7), P=0.078 while for IP-10, AA haplotype [176.1, (220.0)], non-AA [210.1, (482.0), P=0.247), Figure 4.7 (A). The *NFκBIA*-826A/-310G haplotype that demonstrated an association with reduced risk of SMA had increased levels of IL-10 [255.9, (689.8)] compared to non-AG [192.7 (444.8), P=0.050]. Similarly, IP-10 levels were also elevated in the carriage of AG haplotype [268.9 (578.0)] relative to non-AG haplotype carriage [192.1 (348.0)], P=0.016, Figure 4.7 (B). The levels of IL-10 and IP-10 were also comparable in the carriage of *NFκBIA*-826G/-310A and non-carriage of this haplotype; IL-10 for GA [194.1 (553.3) and non-GA [219.5 (521.2)], P=0.311, as well as for IP-10 for GA [142.5 (350.7)] and non-GA [214.3 (411.0)], P=0.123, Figure 4.7 (C). Further, the carriage of *NFκBIA*-826G/-310G haplotype did not show any differences in the circulating levels of IL-10 and IP-10 compared to non-carriage; for IL-10 GG [220.1(521.1) and non-GG [202.0 (546.6), P=0.606, whereas for IP-10 GG [210.7 (406.1) and non-GG [121.7 (493.0), P=0.258, Figure 4.7 (D). Finally, whether the levels of IL-10 and IP-10 significantly differed between children with SMA and those with non-SMA was determined. Analysis revealed that the levels of both IL-10 and IP-10 were significantly elevated in the children with non-severe malaria anaemia; IL-10 for SMA was [145.6 (273.8) and non-SMA [433.5 (552.0)], P=0.048; and for IP-10 SMA [138.1 (184.0) while that of non-SMA was [240.0(538.1)], P=0.025, respectively as shown in (Figure 4.7 E).

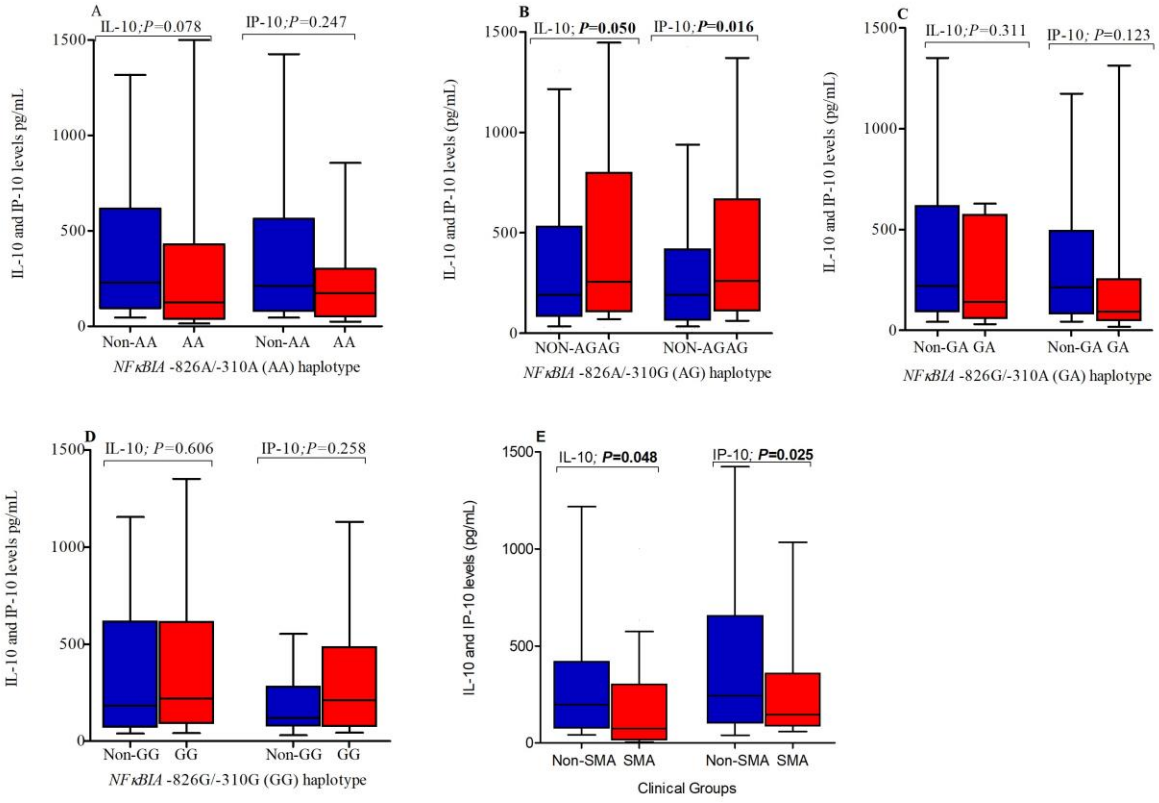

Figure 4. 7. Comparison of peripheral IL-10 and IP-10 in the carriage of *NFκBIA* haplotypes

Data are grouped into carriage of *NFκBIA* G-826A and *NFκBIA* G-310A) haplotypes and non-carriage **(A-D)** and non-SMA and SMA **(E)** for children with *P. falciparum* (n=215). Data are presented as median with interquartile range [IQR] of peripheral IL-10 and IP-10 levels. Statistical significance determined using Man Whitney U test. Significance was based at $P \leq 0.05$.

4.3.6. Distribution of *NFκB1* and *NFκBIA* genotypes combinations within the study groups and association with SMA

Using multiple variants approach (combined genotypes strategy), thirty-six (36) possible genotype combinations for the *NFκB1/NFκBIA* promoter genotypes were generated. Further analysis showed that the *NFκB1*-8079AA/*NFκBIA*-826GA combination (n=70) were higher in the SMA group [n (%); 23 (12.2%)] relative to non-SMA group [n (%); 47 (5.6%)], P=0.001. Moreover, the carriage of the *NFκB1*-3279CC/*NFκBIA*-826GG (n=405) combination consisting of the dominant alleles showed higher distribution in the non-SMA group [n (%); 345/41.2%)] compared to SMA group [n (%); 60 (31.9%)], P=0.019 while the *NFκB1*-3279TT/ *NFκBIA*-826GA combination (n=50) consisting of mutant *NFκB1*-3279TT and heterozygous *NFκBIA*-826G/A genotypes were more common in the SMA group [n (%); 23 (12.2%)] than non-SMA group [n (%); 27 (3.2%)], P<0.001. The *NFκB1*-3297CC/*NFκBIA*-310GG combination (n=517) were higher in the non-SMA group [n (%); 445 (53.1%)] relative to SMA group [n (%); 72 (38.3%)], P<0.001.

Additional analysis showed that existence of the *NFκB1*-3297TT/*NFκBIA*-310GG combination (n=118) was higher in the SMA group [n (%); 36 (19.1%)] than the non-SMA group [n (%); 82 (9.8%)], P<0.001. To determine whether these differences in distributions were associated with susceptibility to or protection against SMA, logistic regression using non-existence of each combination as the reference while controlling for confounding effects of age, sickle cell trait, G6PD and α-thalassemia in the reduced model in logistic regression was performed. Consistent with their respective distributions within the study groups, *NFκBIA*-826AG/*NFκB1*-8079AA was associated with susceptibility to SMA (OR=2.31, 95% CI: 1.30-

4.08, *P*=0.004). The *NFκB1*-3279CC/*NFκBIA*-826GG combination was associated with protection against SMA (OR=0.69, 95% CI=0.48-0.96, *P*=0.033) while the *NFκB1*-3279TT/*NFκBIA*-826G/A was associated with increased risk of SMA (OR=2.77, 95% CI=1.10-6.97, *P*=0.031). Further analysis revealed that the carriage of the *NFκB1*-3297CC/*NFκBIA*-310GG combination was associated with protection against SMA (OR=0.64, 95% CI=0.44-0.92, *P*=0.016). However, the *NFκB1*-3297TT/ *NFκBIA*-310GG combination was associated with a two-fold risk of SMA (OR=2.10, 95% CI=1.32-4.10, *P*=0.002). Using the regional definition of SMA (Hb<6.0 g/dL) the above combinations further showed association with SMA. This show the effect of combinatorial gene effects on diseases outcome in multi-factorial diseases such as malaria. These results are summarised in Table 4.6 below for combinations that were significantly different between the study groups.

Table 4. 6. Distribution of *NFκB1* and *NFκBIA* genotypes combinations within the study groups and association with SMA

Genotypes Combination	Non-SMA (Hb≥5.0 g/dL) n=838	SMA (Hb<5.0 g/dL) n=188	P-value [c]	SMA (Hb<5.0 g/dL) OR	95% CI	*P*-value[d]	SMA (Hb<6.0 g/dL) OR	95% CI	*P*-value[d]
***NFκB1*-8079G>A/*NFκBIA*-826G>A**									
AA/GA (n=70)	47 (5.6)	23 (12.2)	**0.001**	2.31	1.30-4.08	**0.004**	1.81	1.18-5.27	**0.018**
***NFκB1*-3279C>T *NFκBIA*-826G>A**									
CC/GG (n=405)	345 (41.2)	60 (31.9)	**0.019**	0.69	0.48-0.96	**0.033**	0.87	0.39-0.98	**0.046**
TT/GA (n=50)	27 (3.2)	23 (12.2)	**<0.001**	2.77	1.10-6.97	**0.031**	1.95	1.07-5.71	**0.044**
***NFκB1*-3297C>T/*NFκBIA*-310G>A**									
CC/GG (n=517)	445 (53.1)	72 (38.3)	**<0.001**	0.64	0.44-0.92	**0.016**	0.72	0.47-0.99	**0.029**
TT/GG (n=118)	82 (9.8)	36 (19.1)	**<0.001**	2.10	1.32-4.10	**0.002**	1.15	1.05-3.312	**0.024**

Data are presented as n (%) of children within the study groups with specific genotype combination. Children with parasitemia were categorized on the basis of presence or absence of severe malaria anaemia SMA (defined as Hb<5.0 g/dL, with any density parasitemia). [c]Statistical significance was determined by the χ^2 analysis. [d]Statistical was significance determined by logistic regression analysis controlling for confounding effects of age, sickle cell trait, G6PD and α-thalassemia in the reduced model. *P*-values in bold are statistically significant.

4.4. *In-vitro* Effect *PfHz* on the Levels IL-1β, IL-6 and TNF-α via the CD40 Pathway

The CD40 pathway is involved in the production of downstream molecules such as pro-inflammatory. Results from *in-vitro* stimulation of the CD40 pathway with *PfHz* demonstrate that *PfHz* caused increase in the expression levels of IL-1β (5.7-folds), TNF-α (4.6-folds) and IL-6 (6.5-folds), $P<0.05$ relative to the controls. Most importantly, it was notable that the expression levels of these pro-inflammatory cytokines peaked at 24 hours after stimulation and sharply dropped after 48 hours. In addition, when the Anti-CD40 monoclonal antibody was used either alone or in combination with *PfHz* there was further increase in the levels of the cytokines i.e. IL-1β (14.1-folds), TNF-α (12.6-folds) and IL-6 (11.0-folds), $P<0.05$ compared to the controls. However, there were no notable differences relative to the controls in the levels of these cytokines when the inhibitor (SML) was used, (Figures 4.8-4.10).

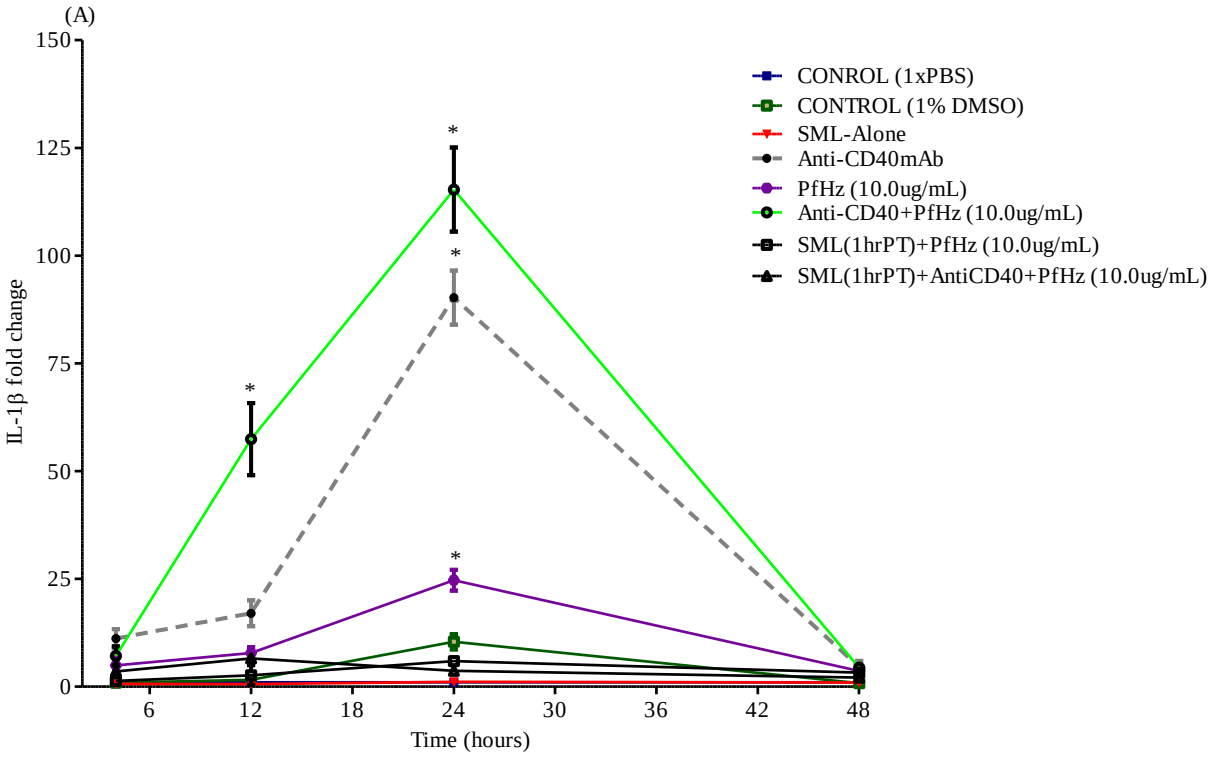

Figure 4. 8. Effects of *PfHz* treatment on IL-1β via the CD40 pathway

Quantification of expression profiles of IL-1β was performed using TaqMan® gene expression assays. Data represents average of arbitrary values for (n=2) malaria naïve individuals. β-actin was used as an endogenous control to normalization of the data by $^{\Delta\Delta}$CT method. *Represents *P*-value <0.05 with reference to the controls. Error bars are standard error of the means (SEM).

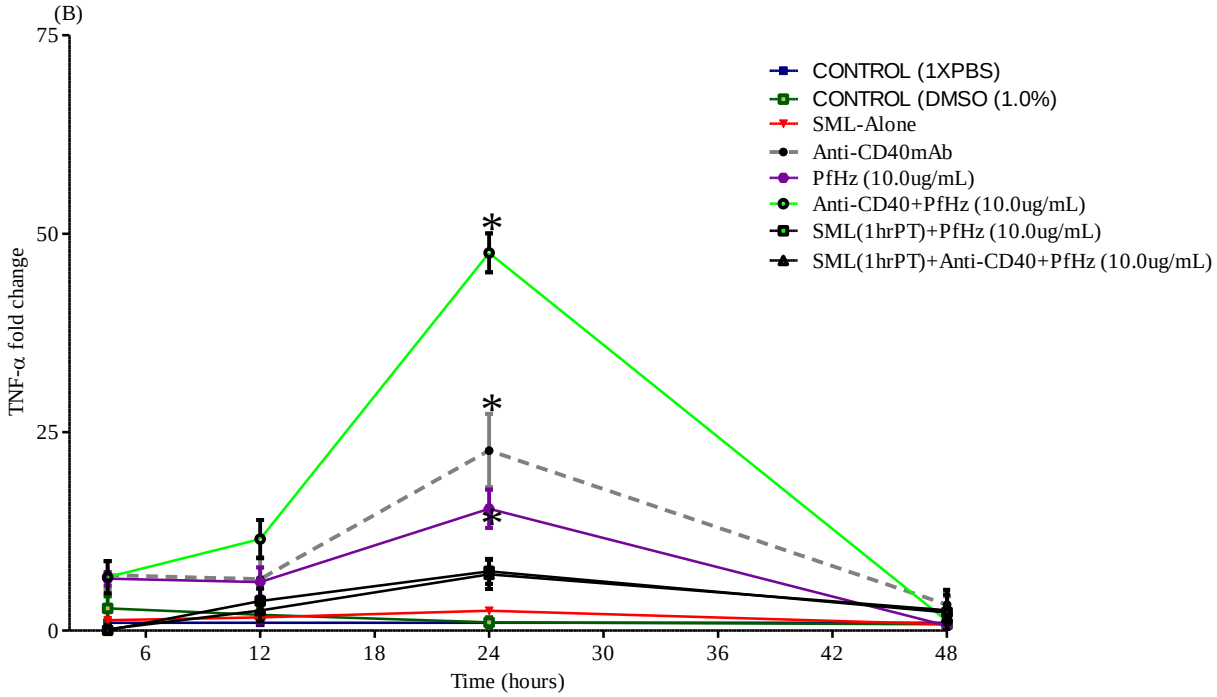

Figure 4. 9. Effects of *PfHz* treatment on TNF-α expression via the CD40 pathway

Quantification of expression profiles of TNF-α was performed using TaqMan® gene expression assays. Data represents average of arbitrary values for (n=2) malaria naïve individuals. β-actin was used as an endogenous control to normalization of the data by $^{\Delta\Delta}$CT method. *Represents *P*-value <0.05 with reference to the controls. Error bars are standard error of the means (SEM).

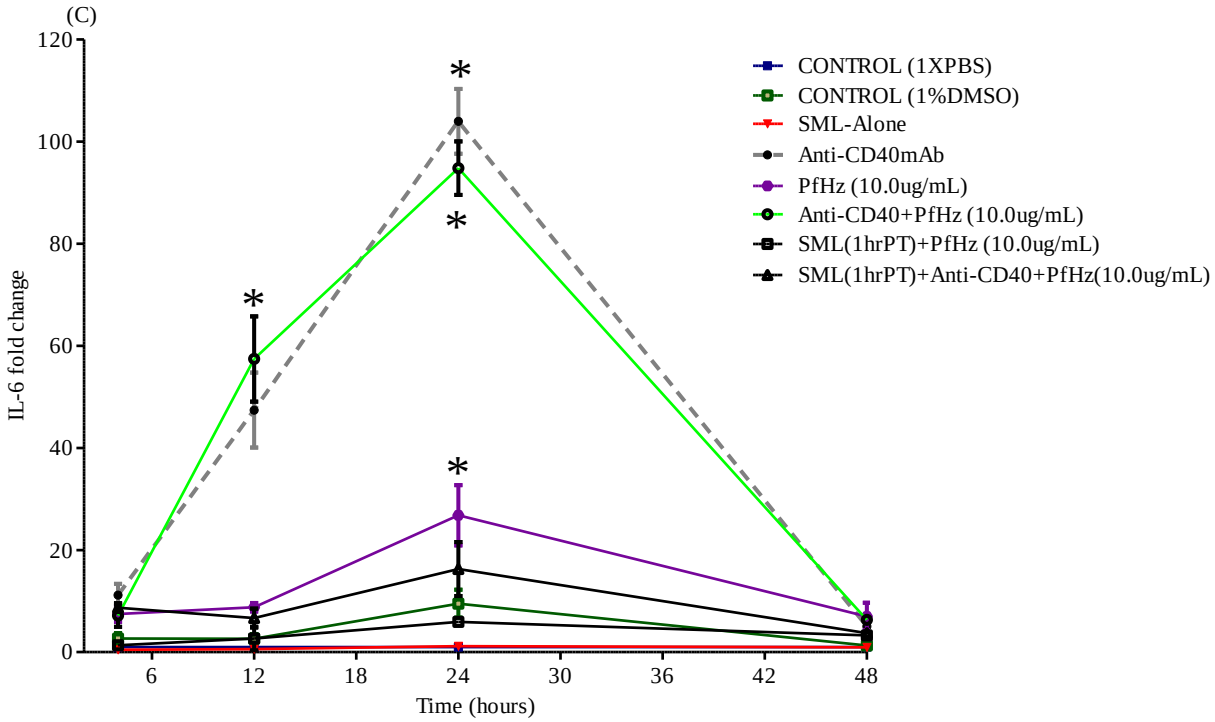

Figure 4. 10. Effects of *PfHz* treatment on IL-6 expression via the CD40 pathway

Quantification of expression profiles of IL-6 was performed using TaqMan® gene expression assays. Data represents average of arbitrary values for (n=2) malaria naïve individuals. β-actin was used as an endogenous control to normalization of the data by $^{\Delta\Delta}$CT method. *Represents *P*-value <0.05 with reference to the controls. Error bars are standard error of the means (SEM).

4.5. Interleukin 23 Receptor (*IL-23R*) Variations and SMA

4.5.1. Distribution of *IL-23R* rs1884444 G/T and *IL-23R* rs7530511 C/T genotypes in the clinical groups

Prior to determination of the association between genotypes and SMA, distributions of the *IL-23R* rs1884444G/T and *IL-23R* rs7530511C/T genotypes were determined in the clinical categories. Chi-square (χ^2) analyses showed that the distribution of the *IL-23R* rs1888444G/T and *IL-23R* rs7530511C/T genotypes were not different between the clinical groups (P=0.278 and P=0.386, respectively. *IL-23R* rs1884444G/T genotypes within the non-SMA group were 70.5% GG, 26.6% GT and 2.9% TT, while those in the SMA group were 66.0% GG, 27.8% GT and 6.2% TT. Genotypes of *IL-23R* rs1884444G/T in the non-SMA (χ^2=0.087, P=0.767) and SMA (χ^2=2.901, P=0.088) groups were consistent with Hardy-Weinberg Equilibrium (HWE). Allele frequencies of the *IL-23R* rs1884444G/T in the overall study population were 0.82 (G) and 0.18 (T), respectively. In addition, the genotypic distribution of the *IL-23R* rs1884444G/T in the overall study population was consistent with HWE (χ^2=2.21, P=0.137). The genotypic distribution of the *IL-23R* rs7530511C/T in non-SMA group was 84.5% CC, 12.6% CT, and 2.9% TT, while those in the SMA group were 79.0% CC, 17.3% CT and 3.7% TT. In both non-SMA and SMA groups, there was deviation from HWE (χ^2=12.98, P<0.001 and χ^2=6.57, P=0.010), respectively. The major and the minor allele frequency for the *IL-23R* rs7530511C/T in the overall study population was 0.89 (C) and 0.11 (T), respectively. In the overall study population, the *IL-23R* rs7530511C/T genotypic distribution showed deviation from HWE (χ^2=18.81, P<0.001), (Table 4.7).

Table 4. 7. Distribution of *IL-23R* 1884444 G/T and *IL-23R* rs7530511 C/T genotypes in the clinical groups

Genotypes	N (%) with genotype in group [a]		*P*-value [b]
	non-SMA (Hb≥6.0 g/dL) (n=207)	SMA (Hb<6.0 g/dL) (n=162)	
IL-23R rs1884444 G/T			
GG, n (%)	146 (70.5)	107 (66.0)	
GT, n (%)	55 (26.6)	45 (27.8)	0.278[b]
TT, n (%)	6 (2.9)	10 (6.2)	
X=0.18			
IL-23R rs7530511 C/T			
CC, n (%)	175 (84.5)	128 (79.0)	
CT, n (%)	26 (12.6)	28 (17.3)	0.386[b]
TT, n (%)	6 (2.9)	6 (3.7)	
X=0.11			

[a] Data are presented as n (%) of children. Children were grouped based on the modified definition of SMA (Hb<6.0 g/dL, with any density parasitemia). [b] Statistical significance determined by the χ^2 analysis. X: frequency of the variant allele.

4.5.2. Association between *IL-23R* rs1884444 G/T and *IL-23R* rs7530511 C/T genotypes and SMA

The association between individual genotypes of *IL-23R* rs1884444G/T and *IL-23R* rs7530511C/T and susceptibility to SMA was determined using logistic regression analysis, controlling for the confounding effects of age, sex, co-infection (HIV-1 status and bacteraemia), HbAS, alpha-thalassemia and G6PD deficiency (Aidoo *et al.,* 2002; Wambua *et al.,* 2006a; Wambua *et al.,* 2006b). Relative to the wild-type *IL-23R* rs1884444 (GG), no significant associations with susceptibility to SMA were observed for either the GT (OR=1.34, 95% CI=0.78-2.31, P=0.304) or TT (OR=2.02, 95% CI=0.53-7.74, P=0.286) genotypes. In addition, relative to the wild-type *IL-23R* rs7530511 (CC), neither the CT (OR=2.60, 95% CI=0.59-11.86, P=0.202) nor the TT (OR=1.66, 95% CI=0.84-3.27, P=0.142) were associated with susceptibility to SMA. Moreover, to provide a more global representation, analysis was done based on the WHO cut-off of SMA (SMA; Hb<5.0 g/dL and any density parasitemia). However, no significant association between the *IL-23R* rs1884444G/T and *IL-23R* rs7530511C/T genotypes and SMA was detectable in this study population, (Table 4.8).

Table 4. 8. Association between *IL-23R* rs1884444 G/T and *IL-23R* rs7530511 C/T genotypes and SMA

	SMA (Hb<5.0 g/dL)			SMA (Hb<6.0 g/dL)		
Genotypes	**OR**	**95% Cl**	***P*-value**	**OR**	**95% CI**	***P*-value**
	***IL-23R* rs1884444 G/T**			***IL-23R* rs1884444 G/T**		
GG, (n= 253)	Ref	-	-	Ref	-	-
GT, (n= 100)	3.69	0.89-5.13	0.174	1.34	0.78- 2.31	0.304
TT, (n= 16)	1.39	0.73-2.64	0.316	2.02	0.53- 7.74	0.286
	***IL-23R* rs7530511 C/T**			***IL-23R* rs7530511 C/T**		
CC, (n=303)	Ref	-	-	Ref	-	-
CT, (n=54)	2.02	0.43-9.42	0.372	2.60	0.59-11.86	0.202
TT, (n=12)	2.49	0.76-5.37	0.102	1.66	0.84-3.27	0.142

Children (n=369) with *P. falciparum* malaria were categorized on the basis of presence or absence of SMA. Odds ratios (OR) and 95% confidence intervals (CI) were determined using logistic regression, controlling for age, sex, co-infections (HIV-1 status and bacteraemia), sickle cell trait (HbAS), G6PD deficiency, and alpha-thalassemia. The reference groups in the logistic regression analysis were the homozygous wild-type genotypes.

4.5.3. Association between *IL-23R* rs1884444 G/T and *IL-23R* rs7530511 C/T haplotypes and SMA

Using logistic regression analysis models, controlling for the confounding effects of age, sex, co-infection (HIV-1 status and bacteraemia), HbAS, alpha-thalassemia, and G6PD deficiency (Aidoo *et al.*, 2002; Ong'echa *et al.*, 2006; Wambua *et al.*, 2006a; Wambua *et al.*, 2006b; Were *et al.*, 2011), the association between carriage of the *IL-23R* rs1884444 and rs7530511 haplotype constructs and SMA were determined. These analyses revealed that there was no association between carriage vs. non-carriage of the *IL-23R* rs1884444G/rs7530511C (GC) haplotype and SMA (OR=0.49, 95% CI=0.18-1.33, *P*=0.161). Susceptibility to SMA was also not influenced by carriage vs. non-carriage of either the GT (OR=1.04, 95% CI=0.33-3.31, *P*=0.949) or TC (OR=0.97, 95% CI=0.52-1.81, *P*=0.923) haplotypes. However, carriage of the TT haplotype was associated with a significant increase in susceptibility to SMA (OR=1.12, 95% CI=1.07-4.19, *P*=0.030). Likewise, using the WHO cut-off for SMA, only the TT haplotype was associated with the risk of SMA (OR=2.50, 95% CI=1.18-5.29, *P*=0.016, (Table 4.9).

Table 4. 9. Association between *IL-23R* rs1884444 G/T and *IL-23R* rs7530511 C/T haplotypes and SMA

	SMA (Hb<5.0 g/dL)			SMA (Hb<6.0 g/dL)		
***IL-23R* haplotypes**	**OR**	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**
GC, (n= 342)	0.34	0.13-1.17	0.107	0.49	0.18- 1.33	0.161
GT, (n= 17)	1.92	0.52-7.06	0.328	1.04	0.33- 3.31	0.949
TC, (n= 67)	1.02	0.49-2.15	0.955	0.97	0.52- 1.81	0.923
TT, (n= 56)	2.50	1.19-5.29	**0.016**	1.12	1.07- 4.19	**0.030**

Children with *P. falciparum* malaria (n=369) were grouped based on the modified definition of SMA. Odds ratios (OR) and 95% confidence intervals (CI) were determined using logistic regression model controlling for age, sex, co-infections (HIV-1 and bacteraemia) sickle cell trait (HbAS), G6PD deficiency, and alpha-thalassemia. The reference groups in the regression analysis were non-carriers of the respective haplotypes.

4.6. *FcγRIIA*-131Arg/His, *FcγRIIIA*-176F/V and *FcγRIIIB*-NA1/NA2 variations and SMA

4.6.1. Distribution of *FcγRIIA*-131Arg/His, *FcγRIIIA*-176F/V and *FcγRIIIB*-NA1/NA2 genotypes

Chi square (χ^2) analysis showed that the distributions of the FcγRIIA-131Arg/His, *FcγRIIIA*-176F/V and FcγRIIIB-NA1/NA2 genotypes were not significantly different between the clinical groups (P=0.226, P=0.162 and P=0.632, respectively). *FcγRIIA*-131Arg/His genotypes within the SMA group were 30 (26.3%) Arg/Arg, 59 (51.8%) Arg/His and 25 (21.9%) His/His. Consistency with Hardy-Weinberg Equilibrium (HWE) in the SMA group for *FcγRIIA*-131Arg/His was observed (χ^2=0.15, P=0.692). *FcγRIIA*-131Arg/His genotypes distribution in non-SMA were 39 (24.3%) Arg/Arg, 71 (44.4.0%) Arg/Hist and 50 (31.1%) His/His. Frequencies of the genotypes in non-SMA showed deviation from HWE (χ^2=4.92, P=0.027). The overall genotype distribution for the *FcγRIIA*-131Arg/His did not deviate from HWE (χ^2=0.703, P=0.402) with an overall variant allele frequency of the FcγRIIA-131Arg/His at 0.49 (Arg). The genotypic distribution of the *FcγRIIIA*-176 F/V in SMA group was 61 (53.5%) FF, 45 (39.5%) FV and 8 (7.0%) VV. The distribution of these genotypes in SMA showed consistency with HWE (χ^2=0.006, P=0.939). Within the non-SMA group, the distributions was 77 (48.1%) FF, 60 (37.5%) FV and 23 (14.4%) for VV and the genotypes showed consistency with HWE (χ^2=3.774, P=0.052). The distribution of these genotypes in overall population showed consistency with HWE (χ^2=2.510, P=0.113) and had an overall mutant allele frequency of 0.30 (V). *FcγRIIIB*-NA1/NA2 genotypes distribution in the SMA group were 6 (5.3%) NA1, 73 (64.0%) NA1/NA2 and 35 (30.7%) NA2, while in non-SMA there was 8 (5.0%) NA1, 94 (58.8%) NA1/NA2 and 58 (36.2%) NA2. The distributions of the genotypes in both SMA and

non-SMA revealed deviation from HWE normality (χ^2=15.549, P<0.001, and χ^2=14.608, P<0.001, respectively). In addition, HWE deviation was revealed by the *FcγRIIIB*-NA1/NA2 genotypes' distribution considering the whole study group (χ^2=29.74, P<0.001) with variant allele frequency of 0.36 (NA1), (Table 4.10).

Table 4. 10. Distribution of *FcγRIIA*-131Arg/His, *FcγRIIIA*-176F/V and *FcγRIIIB*-NA1/NA2 genotypes within the study groups

Genotypes	N (%) with genotype in group [a]			HWE, *P*-value (SMA+non-SMA) *
	SMA (Hb<5.0 g/dL) (n=114)	Non-SMA (Hb≥5.0 g/dL) (n=160)	*P*-value [b]	
***FcγRIIA*-131Arg/His**				
Arg/Arg, n (%)	30 (26.3)	39 (24.3)		
Arg/His n (%)	59 (51.8)	71 (44.4)	0.226[b]	0.402[b]
His/His, n (%)	25 (21.9)	50 (31.3)		
X(His)=0.48				
***FcγRIIIA*-176 F/V**				
FF, n (%)	61 (53.5)	77 (48.1)		
FV, n (%)	45 (39.5)	60 (37.5)	0.162[b]	0.113[b]
VV, n (%)	8 (7.0)	23 (14.4)		
X(V)=0.30				
***FcγRIIIB*-NA1/NA2**				
NA1/NA1	6 (5.3)	8 (5.0)		
NA1/NA2	73 (64.0)	94 (58.8)	0.632[b]	**<0.001[b]**
NA2/NA2	35 (30.7)	58 (36.2)		
X(NA1) =0.36				

[a]Data are presented as n (%) of children. Children with parasitemia were categorized on the basis of presence or absence of severe malaria anaemia SMA based (defined as Hb<5.0 g/dL, with any density parasitemia). [b] Statistical significance determined by χ^2 analysis. X; the overall minor allele frequency in the study population. *HWE; Hardy-Weinberg Equilibrium.

4.6.2 Association between *FcγRIIA*-131Arg/His, *FcγRIIIA*-176F/V and *FcγRIIIB*-NA1/NA2 and severe malaria anaemia (Hb<5.0 g/dL and Hb<6.0 g/dL)

Genetic association analysis was performed based on dominant, additive and recessive models of the *FcγR* polymorphisms. The *FcγRIIA*-131His/His dominant model did not reveal association with SMA susceptibility (OR=0.59, 95% CI=0.33-1.05, *P*=0.077). Further analysis did not reveal association between SMA using the additive (OR=1.52, 95% CI=0.72-2.93, *P*=0.298) or the recessive (OR=0.98, 95% CI=0.56-1.75, *P*=0.963) model. The dominant (OR=1.27, 95% CI=0.79-2.10, *P*=0.343) and the additive (OR=0.77, 95% CI=0.63-1.83, *P*=0.796) model of the *FcγRIIIA*-176 F/V dimorphism did not show associations with SMA. However, the recceive model of *FcγRIIIA*-176 F/V showed a trend towards protection against SMA, albeit with borderline significance (OR=0.43, 95% CI=0.18-1.02, *P*=0.056). Analysis of all the genetic models of *FcγRIIIB*-NA1/NA2 variation did not reveal any association with SMA; dominant [OR=0.76, 95% CI=0.44-1.28, *P*=0.786)], additive [OR=1.34, 95% CI=0.78-2.30, *P*=0.288] and recessive [OR=1.20, 95% CI=0.36-3.94, *P*=0.767). Using the stricter WHO definition of SMA, analysis revealed that the carriage of the recessive *FcγRIIIA*-176 V/V was associate borderline protection against SMA [OR=0.41, 95% CI=0.17-1.01, *P*=0.0.051], (Table 4.11).

Table 4. 11. Association between *FcγRIIA*-131Arg/His, *FcγRIIIA*-176F/V, *FcγRIIIB*-NA1/NA2 and severe malaria anaemia

			SMA (Hb<5.0 g/dL)			SMA (Hb<6.0 g/dL)		
***FcγR* genotypes**	**SMA**	**Non-SMA**						
***FcγRIIA*-131Arg/His**			**OR**	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**
Dominant, (His/His, n=75)	25	50	0.67	0.44-1.01	0.064	0.59	0.33-1.05	0.077
Additive, (Arg/His, n=130)	59	71	1.03	0.61-2.73	0.432	1.52	0.72-2.93	0.298
Recessive, (Arg/Arg, n=69)	30	39	1.12	0.67-1.65	0.907	0.98	0.56-1.75	0.963
***FcγRIIIA*-176 F/V**								
Dominant, (F/F, n=138)	61	77	1.75	0.62-2.09	0.543	1.27	0.79-2.10	0.343
Additive, (F/V, n=105)	45	60	0.64	0.59-1.65	0.780	0.77	0.63-1.83	0.796
Recessive, (V/V, n=31)	8	23	0.41	0.17-1.01	**0.051**	0.43	0.18-1.02	**0.056**
***FcγRIIIB*-NA1/NA2**								
Dominant, (NA2/NA2, n=93)	35	58	0.86	0.31-1.20	0.832	0.76	0.44-1.28	0.786
Additive (NA1/NA2, n=167)	73	94	1.41	0.78-2.47	0.345	1.34	0.78-2.30	0.288
Recessive, (NA1/NA1, n=14)	6	8	1.60	0.48-3.31	0.885	1.20	0.36-3.94	0.767

Children with acute malaria (n=274) were grouped based on SMA (defined as Hb<6.0 g/dL, or Hb<5.0 g/dL) with any density parasitemia). Odds ratios (OR) and 95% confidence intervals (CI) were determined using bivariate logistic regression controlling for age, gender, co-infections (HIV-1 and bacteraemia) sickle cell trait (HbAS) and G6PD deficiency. The reference groups in the logistic regression analysis were the absence of the respective models for each genotype. n= the number of participants with the respective genotype. *P*-values were considered significant at $P \leq 0.05$.

4.6.3. *FcγRIIA*-131/*FcγRIIIA*-176/*FcγRIIIB* haplotypes distribution within the study groups and association with severe malaria anaemia

Prior to performing regression analysis to determine the association between the *FcγRIIA*-131His/Arg, *FcγRIIIA*-176F/V and *FcγRIIIB-NA1/NA2* haplotypes and SMA, the distribution of the carriage of the haplotypes within the study groups were compared. In total, eight haplotypes were generated after haplotype construction. Four most common haplotypes with an overall frequency >8.0% in the whole population were selected. The haplotypes were distributed as follows; *FcγRIIA*-131Arg/*FcγRIIIA*-176F/*FcγRIIIBNA2*, (0.33), *FcγRIIA*-131His/*FcγRIIIA*-176F/*FcγRIIIBNA1* (0.12), *FcγRIIA*-131His/*FcγRIIIA*-176F/*FcγRIIIBNA2* (0.15) and *FcγRIIA*-131His/*FcγRIIIA*-176V/*FcγRIIIBNA1* (0.17). Among these four common haplotypes, *FcγRIIA*-131Arg/*FcγRIIIA*-176F/FcγRIIIBNA2 haplotype was higher in children with SMA relative to non-SMA group (P=0.044). The distributions of the other three haplotypes were comparable between the SMA and non-SMA groups; *FcγRIIA*-131His/*FcγRIIIA*-176F/*FcγRIIIB*-NA1 (P=0.104), *FcγRIIA*-131His/*FcγRIIIA*-176F/*FcγRIIIB*-NA2 (P=0.269) and *FcγRIIA*-131His/*FcγRIIIA*-176V/*FcγRIIIB*-NA1 (P=0.188), Table 4.12.

Using bivariate logistic regression analysis controlling for age, sex, co-infection (HIV-1 status and bacteraemia), sickle cell trait (HbAS) and G6PD deficiency (Aidoo *et al.,* 2002; Ong'echa *et al.,* 2006; Wambua *et al.,* 2006a; Wambua *et al.,* 2006b; Were *et al.,* 2011), the association between carriages of the *FcγRIIA*-131/*FcγRIIIA*-176/*FcγRIIIB* haplotypic structures and severe malaria anaemia (SMA; Hb<6.0 g/dL and SMA; Hb<5.0 g/dL with any density parasitemia) was determined. This analysis revealed that the carriage of the *FcγRIIA*-131Arg/*FcγRIIIA*-176F/*FcγRIIIB*-NA2 haplotype was associated with increased risk of severe

malaria anaemia relative to none carriage (OR=1.70, 95% CI=1.02-2.93, *P*=0.036) and (OR=1.79, 95% CI=1.23-3.10, *P*=0.024) for SMA<6.0 g/dL and SMA<5.0 g/dL, respectively. Further regression analysis did not show any association between carriage of FcγRIIA-131His/*FcγRIIIA*-176F/*FcγRIIIB*-NA1 (OR=1.80, 95% CI=0.98-3.30, *P*=0.057), *FcγRIIA*-131His/*FcγRIIIA*-176F/*FcγRIIIB*-NA2 (OR=0.76, 95% CI=0.44-1.32, *P*=0.334) and *FcγRIIA*-131His/*FcγRIIIA*-176V/*FcγRIIIB*-NA1 (OR=0.71, 95% CI=0.41-1.25, *P*=0.234) haplotypes and SMA, (Table 4.12).

Table 4. 12. ***FcγRIIA*-131/*FcγRIIIA*-176/*FcγRIIIB-NA1/NA2*** **haplotypes distribution within the study groups and association with SMA**

***Ha*plotypes**	**Study Groups**			**SMA (Hb<5.0 g/dL)**			**SMA (Hb<6.0 g/dL)**		
	SMA n (%)	**Non-SMA n (%)**	***P*-value[a]**	**OR**	**95% CI**	***P*-value[b]**	**OR**	**95% CI**	***P*-value[b]**
131Arg/176F/NA2 (n=171)	79 (69.3)	92 (57.5)	**0.044**	1.79	1.23-3.10	**0.024**	1.70	1.02-2.93	**0.036**
131His/176F/NA1 (n=59)	30 (26.3)	29 (18.1)	0.104	1.92	0.99-2.78	0.054	1.80	0.98-3.30	0.057
131His/176F/NA2 (n=87)	32 (28.1)	55 (34.4)	0.269	0.60	0.56-1.62	0.247	0.76	0.44-1.32	0.334
131His/176V/NA1 (n=79)	28 (24.6)	51 (31.9)	0.188	0.51	0.31-1.31	0.142	0.71	0.41-1.25	0.234

Children with acute malaria (n=274) were grouped based on SMA (defined as Hb<5.0 g/dL or Hb<6.0 g/dL). Odds ratios (OR) and 95% confidence intervals (CI) were determined using bivariate logistic regression controlling for age, gender, co-infections (HIV-1 and bacteraemia) sickle cell trait (HbAS), alpha-thalassemia and G6PD deficiency. The reference groups in the regression analysis were the non-carriage of respective haplotypic structures. n; the number of participants with the respective haplotype. n (%); number (percentage) of participants with respective haplotype in each study group. [a]*P*-value determined using Chi-square (χ^2). [b]*P*-values were determined using logistics regression analysis. All *P*-values were considered statistically significant at $P \leq 0.05$.

4.6.4. Association between *FcγRIIA*-131Arg/His, *FcγRIIIA*-176F/V and *FcγRIIIB*-NA1/NA2 haplotypes and parasitemia

Since the FcγRs are important determinants in phagocytosis of parasites, the study determined if carriage of *Fc*γRs haplotypes was associated with parasitemia levels. Results revealed that carriage of *FcγRIIA*-131His/*FcγRIIIA*-176F/*FcγRIIIBNA1* haplotype [mean (SEM); 4.37 (± 0.079), n=59] relative to non-carriage [mean (SEM); 4.12 (± 0.052), n=215], *P*=0.009), was associated with higher parasitemia. Additional analysis showed that the level of parasitemia was comparable between the carriage and non-carriage of *FcγRIIA*-131Arg/*FcγRIIIA*-176F/*FcγRIIIBNA2* haplotype [mean (SEM); 4.18 (± 0.057), n=171] versus non-carriage [mean (SEM); 4.17 (± 0.074), n=103], *P*=0.976) and *FcγRIIA*-131His/*FcγRIIIA*-176F/*FcγRIIIB*-NA2 [mean (SEM); 4.23 (± 0.073), n=87] versus non-carriage [mean (SEM); 4.16 (± 0.056), n=187, *P*=0.520]. Further analysis also showed that the level of parasitemia was also comparable between those with *FcγRIIA*-131His/*FcγRIIIA*-176V/*FcγRIIIBNA1* haplotype [mean (SEM); 4.21 (± 0.079), n=79] versus those without the haplotype [mean (SEM); 4.16 (± 0.096), n=195], *P*=0.587), [Figure 4.11 (A-D)].

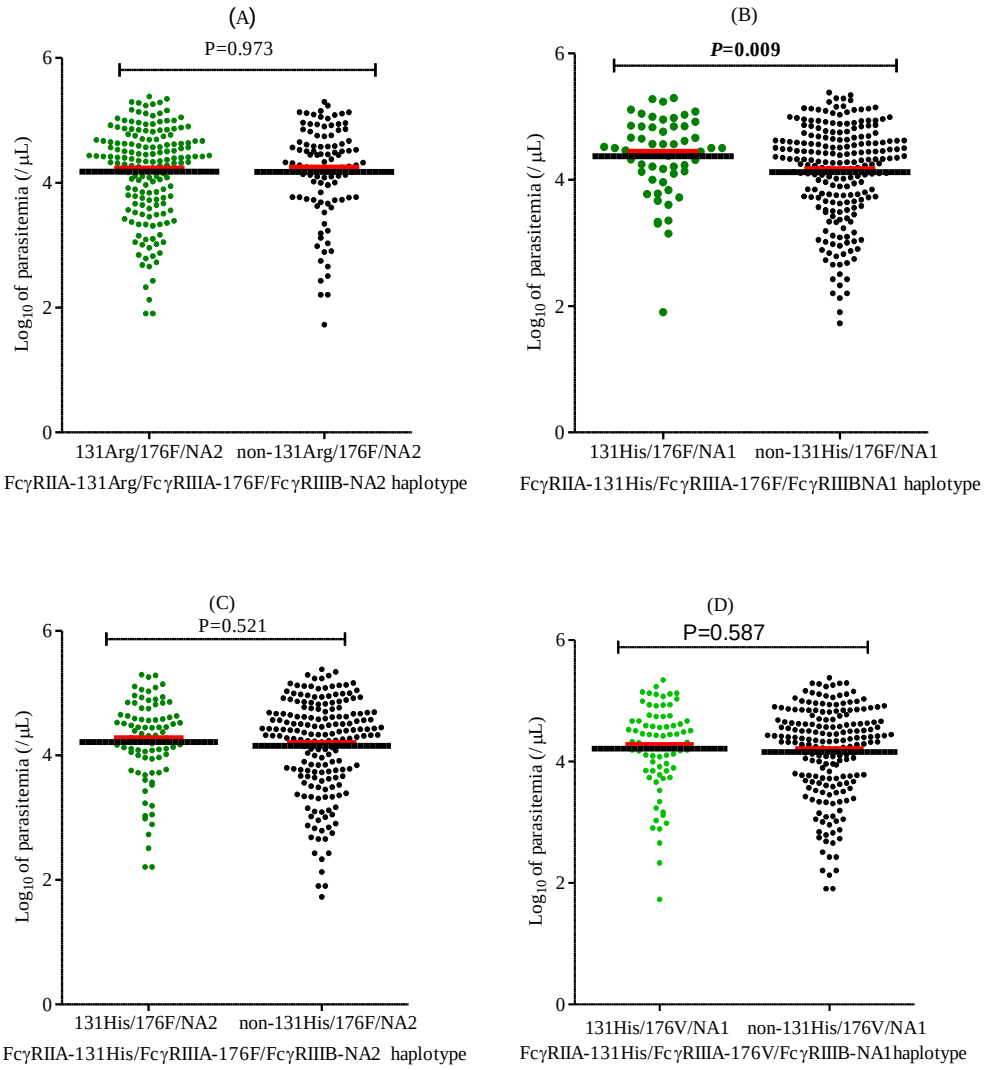

Figure 4. 11. Association between *FcγRIIA*-131Arg/His, *FcγRIIIA*-176F/V and *FcγRIIIB*-NA1/NA2 haplotypes and parasitemia levels

Data are presented as scatter plots for carriage and non-carriage of respective haplotype constructs. The thick black lines through the scatters represent mean, while the red lines above the mean line represent the standard error of the mean (SEM). The carriage of *FcγRIIA*-131His/*FcγRIIIA*-176F/*FcγRIIIB*-NA1 haplotype which was marginally associated with susceptibility to SMA had higher levels of parasitemia (*P*=0.009). Differences in parasitemia levels were determined using unpaired t-test with Welsch correction at 95% confidence interval.

CHAPTER FIVE: DISCUSSION

5.1. Introduction

The genetic mechanisms involved in susceptibility to multifactorial diseases such as SMA among immune-naïve children residing in *P. falciparum* holo-endemic areas remains partially understood. Using whole genome scans, a number of previously unknown candidate genetic markers associated with SMA have been identified. To further provide more insights into the understanding of this complex molecular mechanism, this study determined the associations between variations in cell receptors to immune molecules i.e. *CD40, IL-23R, FcγR* and their associated pathway genes i.e. *NFκB1* and *NFκBIA,* and SMA, a common clinical outcome of severe malaria in children from *P. falciparum* holo-endemic transmission areas was done. In addition, *PfHz* was used to induce the CD40 pathway and the downstream molecules measured to demonstrate the effects of *PfHz* found in children with SMA on pro-inflammatory cytokines.

5.2. Association between CD40 Promoter Polymorphism and Susceptibility to Severe Malaria Anaemia and the Alterations in Malaria Associated Pro-inflammatory Cytokines

Published literature exploring associations between *CD40* polymorphisms and malaria susceptibility are limited. In the current study, using the additive genetic model, the carriage of both the *CD40*-508GA and *CD40*-173CT in children from this region are associated with protection against SMA, a finding that is consistent with their proportionally higher distribution within the non-SMA clinical group. On the other hand, the carriage of *CD40* -508AA and -173TT mutants for these polymorphisms were associated with increased risk of SMA in both

additive and recessive model. The findings from the regression analysis were in agreement with their respective distributions, implying that existence of the mutants of *CD40* rs1800686 (-508G/A) and rs752118 (-173C/T) are associated with greater risk of SMA in this population. As such, the inheritance of the mutant alleles of these variations is likely a risk factor to SMA. Previously, a study revealed that the GT haplotype of *CD40* rs1800686 (-508G/A) and rs752118 (-173C/T) was associated with the risk of ischemic stroke in Korean population with hypertension (Cho *et al.,* 2012). In asthmatic patients, the *CD40*-508AA was found to be associated with elevated levels of IgE (Park *et al.*, 2007). Immunological studies have shown that elevated levels of IgE is pathogenic in malaria (Perlmann *et al.,* 1994; Perlmann *et al.,* 1997; Perlmann *et al.*, 2000). Production of high levels of IgE conditioned by the inheritance of *CD40*-508AA could be one possible mechanism of susceptibility in severe malaria however not investigated in the current study.

Further analysis of the carriage of the *CD40*-1CT+TT relative to the dominant *CD40* -1CC genotype revealed association with increased susceptibility to SMA. Moreover, this study for the first time demonstrates the associations between heterozygous *CD40*-1CT or homozygous mutant *CD40*-1TT genotypes and susceptibility to SMA. *CD40*-1C/T polymorphism is located in the Kozak sequence of the 5'-untranslated region (5'-UTR) of *CD40*. The observation that the heterozygous *CD40*-1CT was associated with SMA susceptibility implies the crucial role it plays in conditioning susceptibility to SMA in children resident of *P. falciparum* holo-endemic areas. This is also consistent with its higher proportions in the SMA group. It is scientifically plausible to hypothesize that the T allele profoundly affects the Kozak sequence responsible for protein translation initiation process (Tomer *et al.,* 2002) and significantly decreases B-cell surface expression of CD40 in individuals with the CT and TT relative to CC genotype (Jacobson *et al.,*

2005). In addition, since the T allele reduce surface expression of CD40 on B-cells, its carriage in CT and TT may result into reduced secondary signals from activated T-cells required for B-cells to mount an efficacious humoral response such as immunoglobulin isotype switching and antibody secretion (Chatzigeorgiou *et al.,* 2009; Garraud *et al.,* 2002). Even though this concept was not investigated in this study, it could be a possible molecular mechanism that is involved in susceptibility to SMA in children with the carriage of the CT and TT. Furthermore, a study in the Brazilian Amazon population demonstrated that the T allele is associated with reduced IgG against *P. vivax* Duffy binding protein (*Pv*DBP) (Cassiano *et al.,* 2016). However, it remains to be determined whether this is the case with IgGs against *P. falciparum* specific antigens.

Since haplotype use is more hel*pf*ul in the elucidation of significant associations not discernable by use of individual alleles (D'Amelio *et al.,* 2012), it is of genetic value to employ haplotypic strategies to evaluate and reveal their associations with SMA. On the basis of the current selected SNPs, carriage of *CD40* (-508G/-173C/-1C) GCC haplotype consisting of all the major alleles was associated with significant protection against SMA while the carriage of the GCT consisting of the mutant allele of -1C/T was 5-folds associated with SMA risk. According to these findings, the protective effects of GCC haplotype could be attributed to the wild type alleles of the *CD40*-508G/A, -173C/T, and -1C/T and most common in the non-SMA group. One plausible explanation to this is that *P. falciparum* infectious burden and pressure experienced by residents of this region could possibly result to natural selection and adaptation leading to accumulated higher frequency of protective alleles. The finding that the carriage of the GCT haplotype was associated with increased susceptibility to SMA was rather surprising considering that existence of the wild type allele of the *CD40* -508G/A and -173C/T were observed to be higher in non-SMA and their presence did not seem to have any diluting effects

on the T allele of the -1C/T. The strong effect of T allele of –1C/T may be due to the fact that it exist at the initiation point of translation (Tomer *et al.,* 2002) and that it hinders mRNA-ribosome complex stabilization thereby decreasing surface expression of CD40 on immune cells, a mechanism that has been demonstrated in other inflammatory diseases (Shuang *et al.,* 2011; Wagner *et al.,* 2015). Of importance is that the *CD40*-1TT genotype has been shown to be associated with higher levels of soluble CD40 (sCD40) in patients with systemic lupus erythematous (SLE) a chronic autoimmune disease (Chen *et al.,* 2015; Wu *et al.,* 2016). sCD40 exhibits immunosuppressive effects since it reduces T-cell activation and immunoglobulin production (Esposito *et al.,* 2012; van Kooten *et al.,* 1994) which could possibly result to SMA.

Studies have revealed that the balances in inflammatory mediators levels is required in reducing or enhancing malaria severity (Lyke *et al.,* 2004; Ong'echa *et al.,* 2008; Oyegue-Liabagui *et al.,* 2017; Perkins *et al.,* 2000; Were *et al.,* 2009) and that these balances are mediated by genetic variations in their genes or genes in their signalling pathways (Ong'echa *et al.,* 2008; Ouma *et al.,* 2008a; Ouma *et al.,* 2010; Phawong *et al.,* 2010). From a panel of inflammatory mediators, the hypothesis that the carriage of GCC and the GCT haplotypes may directly/indirectly condition SMA via influencing the levels of the inflammatory mediators during *P. falciparum* malaria was investigated. The carriage of the 'protective' GCC haplotype was associated with higher levels of IL-1β, IL-2 and MIP1-α relative to the non-carriage. However, the carriage of the 'susceptible' haplotype GCT, had significantly reduced levels of peripheral IL-1β and IL-17. Even though the mechanism(s) by which these *CD40* polymorphisms mediated differential levels in IL-1β, IL-2, MIP-1α and IL-17 would result into SMA protection, earlier studies showed that IL-2 is a critical cytokine in the regulation of bone marrow erythropoiesis, prevents development of aneamia (Chopra *et al.,* 2015) and maintenance

of hematopoietic stem cells (Giampaolo *et al.,* 2017). Moreover, IL-1β promotes hematopoietic stem cells regeneration in response to acute insults such as a *P. falciparum* infection (Pietras *et al.,* 2016) while reduced IL-17 has been shown to be associated with SMA (Davenport *et al.,* 2012) consistent with its role in promoting haematopoiesis (Jovcic *et al.,* 2001; Krstic *et al.,* 2009). While the exact role played by increased MIP-1α in malaria still remains under investigation, a previous study in Gabonese children showed that elevated MIP-1α was associated with acute malaria (Ochiel *et al.,* 2005). As depicted by this study, use of genetic variations that have profound influence on functional changes in downstream molecules is important in identification of combinatorial gene functions involved in specific disease pathways. For further evaluation of the role of CD40 in pathogenesis, it is important to determine the peripheral levels of sCD40 in homogenous populations and relating them to the genetic variations that are shown to be involved in SMA pathogenesis in current study.

5.3. Association between NFκB1 and NFκBIA Polymorphisms and Susceptibility to Severe Malaria Anaemia and Change in IL-17, IL-10 and IP-10

Knowledge of genetic modulation of the NFκB signalling pathway is crucial since there is increasing interest in identifying biomarkers for prediction of disease risks and design of novel therapies which regulate the activity of NFκB proteins. In recent past, the central roles of polymorphisms in the promoter regions of *NFκB1/NFκBIA* genes have been demonstrated by studies in inflammatory diseases such as inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE), Behcet's disease and Grave's disease (Bank *et al.,* 2014; Gao *et al.,* 2012; Hung *et al.,* 2010; Niyazoglu *et al.,* 2014). Even though inflammatory diseases have specific clinical phenotypes and their own complex aetiology different from infectious diseases, the contribution of the NFκB proteins signalling is common and central to them. These observations

raise the question that presence of genetic variation in the promoter regions of *NFκB1* and its inhibitor alpha *NFκBIA* gene could be associated with SMA susceptibility. This hypothesis was investigated by examining the associations between promoter polymorphisms in *NFκB1* and its inhibitor, *NFκBIA*.

From this population of children from *P. falciparum* holo-endemic area, this study demonstrates that the carriage of the homozygous mutant TT alleles at the *NFκB1*-3297 C/T locus is associated with a significant susceptibility to SMA implying that its inheritance could be an important susceptibility factor. However, carriage of other individual genotypes of the investigated SNPs did not reveal significant associations with SMA. In previous malaria immuno-genetic studies, it has been shown that in multifactorial diseases such as malaria, haplotype based genetic analysis reveals associations that are not observable using individual genotypes, reviewed by (Perkins *et al.,* 2011). Considering haplotypic analysis, the existence of *NFκB1*-8079A/-3297T haplotype revealed association with susceptibility to severe malaria anaemia while the carriage of the *NFκB1*-8079G/-3297C haplotype was associated with 40% protection against severe malaria anaemia. It is evident that the inheritance of both mutant alleles (A and T) of these SNPs increases susceptibility while carriage of both wild type alleles (G and C) is associated with protection against severe form of malaria in this population.

Since it has been shown by earlier studies (Baldwin, 2001; Ghosh and Karin, 2002) that inducible expression of key inflammatory mediators including cytokines, growth factors and chemokines are determined by genetic variations, the study evaluated whether this was the case in SMA. Based on the current selected *NFκB1* polymorphisms, significant differences were detectable in the peripheral levels of interleukin-17 which also contains the NFκB response element within its promoter (Shen *et al.,* 2006). Consistently, AT haplotype which was also

associated with SMA risk had reduced peripheral IL-17 levels while the GC haplotype which was associated with protection had elevated peripheral IL-17. The results demonstrate that these polymorphisms may in part influence SMA susceptibility by modulating the circulating levels of IL-17 during acute malaria infection in children from this holo-endemic transmission area. Moreover, the levels of IL-17 also revealed a positive correlation with haemoglobin levels. Collectively, results of the current studied *NFκB1* polymorphisms suggest that increases in IL-17 levels is associated with protection against SMA. This finding is also consistent with previous studies from this population which showed children with increased IL-17 were protected from severe malaria anaemia (Davenport *et al.,* 2012; Ong'echa *et al.,* 2011). Interleukin-17 links the immune system and erythropoiesis system by enhancing the proliferation of erythroid precursor cells (Jovcic *et al.,* 2007; Jovcic *et al.,* 2004; Jovcic *et al.,* 2001). Moreover, IL-17 stimulates macrophages and some epithelial cells to produce a milieu of pro-inflammatory mediators, for example, IL-1, IL-6, TNF-α, NOS-2, metalloproteases (MMP) and chemokines, hence initiating inflammatory processes observed in infectious diseases (Onishi and Gaffen, 2010). Reduced IL-17 levels have been associated with malaria anaemia in mice models infected with *P. yoelii* (Xu *et al.,* 2013).

Previously, a study involving *in-silico* systems biology approach showed that inhibitor kappa B-alpha (IκBα) is a central hub in transcriptional responses of prevalent childhood lung diseases, including respiratory syncytial virus infection (RSV), asthma and broncho-pulmonary dysplasia (Ali *et al.,* 2013). In the present study, analysis of *NFκBIA* haplotypes revealed that the carriage of *NFκBIA*-826A/-310A haplotype (consisting of both mutant A and A alleles) was associated with susceptibility to SMA, whereas the carriage of the *NFκBIA*-826A/-310G was associated with protection against SMA risk. It is important to emphasize that the presence of

both mutant alleles (A and A) of the *NFκBIA*-826A/G and *NFκBIA*-310A/G SNPs increases risk to SMA through a mechanism that still remains to be explored. However, it is possible that during infection, followed by the production of molecules such as *P. falciparum* glycosylphosphatidylinositols (GPI) (Mbengue *et al.,* 2016) and *P. falciparum* haemozoin (*PfHz*) (Awandare *et al.,* 2011), there may be aberrant pro-inflammatory stimulation leading to SMA. Further, it is also possible that there are other mutation(s) within the promoter and /or enhancers not investigated that play a role in protection/risk reported here. The observation that the *NFκBIA*-826A/-310G haplotype was associated with protection against SMA risk in the context of this study was surprising since its general distribution was similar in SMA and non-SMA. Further analysis into this finding revealed that this protective effect might have been due to both intermediate G6PD status, since it was found that those with this haplotype and had intermediate form of G6PD were higher in the non-SMA (11.4%) compared to SMA (4.8%), P=0.004. This shows the strong impact of the carriage of G6PD heterozygous/hemizygous trait protective against severe malaria (Ruwende *et al.,* 1995). This demonstrate somewhat the genetic influence of different genes in conditioning of disease outcomes.

The carriage of *NFκBIA*-826A/-310G haplotype relative to non-carriage had increased levels of peripheral IL-10 and IP-10. It has been documented that severe malaria pathology is due to an interplay of host-related factors and parasite determined factors during infection (Mazier *et al.,* 2000; Perkins *et al.,* 2011). As a systemic inflammatory disease, literature on cytokine levels and their correlations in severe malaria are panoptic (Davenport *et al.,* 2012; Hunt and Grau, 2003; Lyke *et al.,* 2004; Mirghani *et al.,* 2011; Perkins *et al.,* 2011). In the current study, the carriage of the *NFκBIA*-826A/-310G haplotype, which was associated with protection against SMA in this population, presented with increased levels of both IL-10 and IP-

10. Consistently, IL-10 and IP-10 levels were elevated in children with non-severe malaria. Therefore, both increased levels of IL-10 and IP-10, in the context of these haplotypes, protect against severe malaria anaemia. While interpreting these findings, it is important to consider the disease definition and population stratification, since other studies have documented contradictory results on the levels of these cytokines and relationship with malaria severity. For instance, levels of IL-10 have been shown to be elevated in severe disease compared to uncomplicated malaria in Sudanese children as well as Malian children (Lyke *et al.,* 2004; Mirghani *et al.,* 2011). The role of IP-10 has been observed in a number of studies involving *P. falciparum* malaria. Serum levels of IP-10 have been shown to be significantly higher in children with cerebral malaria (CM) compared to children with SMA in Ghanaian children (Armah *et al.,* 2007). In another study in India, IP-10 levels were outstandingly elevated as disease severity increases, with highest levels among CM mortality cases (Jain *et al.,* 2008). Despite the fact that elevated amounts of IL-17, IP-10 and IL-10 were shown to confer protection against SMA in the context of this study, a previous study depicted elevated levels as predictors of multiple organ dysfunctions (MOD) in Indian patients with *P. falciparum* severe malaria (Herbert *et al.,* 2015). However, consistent with the current study, a previous study in this population also revealed that elevation in the levels of both IL-10 and IP-10 is protective against SMA (Ong'echa *et al.,* 2011). These observations can explain the complexity in the nature of networked roles of inflammatory mediators based on specific disease description and host genetic factors. The interpretation of the levels of pro- and anti-inflammatory mediators in the context of *P. falciparum* malaria should consider the fact that parasite genetic factors also in part impact on host cytokine and chemokine responses (Pattaradilokrat *et al.,* 2014).

Since combining multiple variants from the same or different genes in a molecular pathway may result in a greater predictive power of disease risk (Munde *et al.,* 2012; Song *et al.,* 2011a), this study evaluated the combined effect of *NFκB1/NFκBIA* genotypes on susceptibility to SMA. The current study showed that the carriage of *NFκB1*-8079AA/*NFκBIA*-826AG, *NFκB1*-3297TT/*NFκBIA*-826AG and *NFκB1*-3297TT/*NFκBIA*-310GG genotype combinations were associated with the risk of SMA. It is worth noting that the carriage of AA genotype of the *NFκB1*-8097 could be an important susceptibility factor in malaria. This is possible because the A allele leads to binding of glucocorticoid receptor-beta (GR-β) which leads to attenuation of expression of *NFκB1* which contains one of its responsive elements (Cato and Wade, 1996; De Bosscher *et al.,* 2003; Messeguer *et al.,* 2002). Further, the carriage of the TT genotype of *NFκB1*-3297 C/T variation is important in determining the risk of SMA in this paediatric population since its carriage in combination with either the *NFκBIA*-826 AG (heterozygous) or *NFκBIA*-310GG (wild type) is associated with increased risk to SMA. This may be explained by the finding that the T allele is responsible for the binding of the orphan nuclear receptor; chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1) which leads to transcriptional repression of many genes involved in inflammatory processes (Leng *et al.,* 1996; Smirnov *et al.,* 2000). As result of this repression, there could be aberrant production of cytokines observed during *P. falciparum* malaria**.** In the current study, presence of genotypes combination of the *NFκBIA*-826GG/*NFκB1*-3297CC and the *NFκBIA*-310GG/*NFκB1*-3297CC showed protection against severe malaria anaemia. This protective effect could be due to the *NFκB1*-3297CC since its existence with any of the *NFκBIA* wild type confers protection. These observations underscore the use of genetic combinations in genome wide association studies (de Bakker *et al.,* 2005; Pe'er *et al.,* 2006). Even though this strategy has not been applied in genetic association

studies in infectious diseases, it could be important to further explore its usefulness in future to enhance identification of genetic susceptibility factors especially for genes in a common molecular pathway.

5.4. The In-vitro Effect of PfHz on the Expression of CD40 downstream Pro-inflammatory Cytokines (IL-1β, IL-6 and TNF-α)

Studies by have demonstrated that the *PfHz* is a potent modulator of paediatric SMA pathogenesis (Awandare *et al.,* 2011; Kempaiah *et al.,* 2016; Were *et al.,* 2009). However, the actual molecular pathway through which these occur still remains to be explained. This study utilizing molecules that have been shown to inhibit the CD40-TRAF6 interaction (Zarzycka *et al.,* 2015) and the Anti-CD40 monoclonal antibody that stimulates the CD40 pathway showed that the *PfHz* is able to stimulate and increase the expression of CD40 pathway by increasing the expression levels of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) albeit in time dependent manner. These cytokines have been shown to be elevated in malaria infection (Boeuf *et al.,* 2012; Lyke *et al.,* 2004; Olivier *et al.,* 2014) and their deregulated production is involved in the pathogenesis of SMA. The results from this *in-vitro* experiment suggest involvement in part of the CD40 pathway in influencing SMA pathogenesis. Further, investigation of the effect of *PfHz* on the NFκB phosphorylation revealed an increased phosphorylation of the NFκBp65. When the NFκB complex proteins are phosphorylated, there occurs nuclear translocation that leads to transcription of target genes including those involved in the production of cytokines (Song *et al.,* 2011b). These imply that *PfHz* as it has been demonstrated previously (Kempaiah *et al.,* 2016), is a potent stimulator of NFκB pathway.

5.5. Association between the Haplotypes of Non-synonymous Mutations within IL-23R (rs1884444 G/T and IL-23R rs7530511 C/T) and Susceptibility to Severe Malaria Anaemia

In *P. falciparum* holo-endemic transmission areas, one of the most common clinical outcomes of malaria is SMA. To further provide additional information on important immune receptor genes that condition susceptibility to SMA, the role of the IL-23/IL-17 cytokine pathway was investigated by determining the genetic association between non-synonymous mutations of *IL-23R* rs188444G/T and rs7560511C/T polymorphisms and susceptibility to SMA. The study showed that individual genotypes in *IL-23R* (rs188444G/T and rs7530511C/T) were not independently associated with susceptibility to SMA. However, carriage of the *IL-23R* rs188444T/rs7560511T (TT) haplotype was associated with increased susceptibility to SMA [using both modified (Hb<6.0 g/dL) and WHO (Hb<5.0 g/dL) definition of SMA].

The protein encoded by the *IL-23R* gene located on chromosome 1 is a subunit of the receptor for IL-23 sub-unit alpha (IL-23A) which pairs with the receptor molecule IL-12β1, both of which are required for IL-23A signalling (Parham *et al.*, 2002). The IL-23R/IL-12β1 dimer binds to IL-23, which is made up of a p19 protein and IL-12p40 sub-units (Oppmann *et al.*, 2000). In addition to its expression on memory T-cells, IL-23R is present on other immune cells, including activated antigen presenting cells (APCs), natural killer cells, and monocytes, all of which are involved in host-defence against invading pathogens (Bailey *et al.*, 2007; Cheung *et al.*, 2008; Schmidt-Weber *et al.*, 2007). Genetic variation in IL-23R plays an important role in determining the efficacy of cellular immune responses (Perkins *et al.*, 2011). The current study demonstrates that genotypic variants of *IL-23R* [i.e., rs1884444 (G/T) and rs7530511 (C/T)] are individually not associated with susceptibility to SMA.

The *IL-23R* (rs188444 G/T) is located at codon 3 in exon 2 of the *IL-23R* and results in a histidine-to-glutamine substitution. This G to T change is known to be responsible for changes in the signal peptide of the *IL-23R* and results in exon skipping, alternative splicing, or receptor malformation (Kan *et al.,* 2008) resulting in alteration of the receptor-ligand binding specificity. Previous studies have demonstrated that *IL-23R* rs1884444 variation is associated with susceptibility to oesophageal and gastric cancer, schistosomiasis-associated immune reconstitution inflammatory syndrome, and inflammatory bowel disease (Chen *et al.,* 2010; Chu *et al.,* 2012; Ogola *et al.,* 2014). However, consistent with a study in Chinese adults with pulmonary tuberculosis (PTB) and drug-resistant PTB (Jiang *et al.,* 2015), this study found no association in the current investigation between rs1884444 variants and malaria disease outcomes. The *IL-23R* rs7530511 C/T polymorphism results in a replacement of Proline for Leucine at codon 310 (P310L). The rs7530511 C/T is located adjacent to the motif sequence, WQPWS, present in the membrane-trans-membrane proximal *IL-23R* domain, and is capable of altering receptor affinity (Huber *et al.,* 2008), a variation that can influence differential production of downstream molecules. Individual genotypes of rs7530511 in this population, however, did not show any independent associations with susceptibility to SMA, despite earlier observations that the rare TT genotype of the rs7530511 was associated with autoimmune conditions, such as Graves' disease (GD) (Huber *et al.,* 2008). This could be explained by the autoimmune nature of GD which is thyroid gland specific while severe *P. falciparum* malaria which affects multiple organs.

Considering the fact that haplotypes within particular genes are often capable of exposing genetic combinations which can moderate or interact to produce effects that are not observable with individual genotypes (Ouma *et al.,* 2008b; Wilson *et al.,* 2005), haplotypes of *IL-23R*

rs1884444 G/T and rs7530511 (C/T) were therefore constructed. The current study revealed that the carriage of *IL-23R* rs1884444/rs7530511 (TT) haplotype was associated with an increased risk of SMA. This observation implies that carriage of both mutant genotypes (TT) is an important genetic risk factor for developing SMA once a child becomes infected with *P. falciparum*. Although presently undetermined, one can speculate that the TT haplotype may amplify exon skipping and/or mRNA splicing, resulting in altered affinity of the receptor for IL-23 (ligand) binding (Huber *et al.*, 2008; Kan *et al.*, 2008). Since IL-23/T-helper 17 axis leads to the production of IL-17, and other pro-inflammatory mediators, the TT haplotype may potentially inhibit the generation of pro-inflammatory mediators that aid in controlling malaria infections. However, the complex interplay between successfully controlling an infection and the generation of inflammatory-derived anaemia during a malaria infection is difficult to discern at the molecular level (Chen *et al.*, 2000). IL-17 is a prototypical example of such complexity since IL-17 bridges immune and hematopoietic regulation by stimulating early stage erythroid progenitors (i.e., burst forming unit erythroid, BFU-E) (Krstic *et al.*, 2012), and in the opposite context, inhibits late stage erythroid progenitors (Bugarski *et al.*, 2006; Bugarski *et al.*, 2004; Jovcic *et al.*, 2001). Further studies are required to delineate the influence of genetic polymorphisms within *IL-23R* on differential expression and production of inflammatory mediators to unravel the molecular mechanisms through which the IL-23/T-helper 17 axis collectively influences the development of malaria disease outcomes.

5.6 Association between FcγRIIA-131Arg/His, FcγRIIIA-176F/V and FcγRIIIB-NA1/NA2 Genetic Polymorphisms and Susceptibility to Severe Malaria Anaemia

Based on the observations that Fc gamma receptors (FcγRs) are important contributory factors for infectious disease immuno-pathogenesis, the association between the *FcγRIIA-*

131Arg/His, *FcγRIIIA*-176F/V and *FcγRIIIB*-NA1/NA2 polymorphisms and paediatric severe malaria anaemia was determined. Further, the association between the carriage of the *FcγR* haplotypes and parasite levels during *P. falciparum* infection were determined. The current study demonstrated that the *FcγRIIA*-131Arg/ *FcγRIIIA*-176F/*FcγRIIIB*-NA2 haplotype was associated with an increased susceptibility to SMA, while the *FcγRIIA*-131Arg/ *FcγRIIIA*-176F/ *FcγRIIIB*-NA1 haplotype was associated with increased levels of circulating parasites during infection. However, there was no association between the individual genotypes and SMA in this paediatric population from western Kenya.

The FcγRs constitute a crucial arm of host immune defence against extracellular challenges by infectious agents through engagement of IgGs to enable innate immune effectors cells carry out phagocytosis and other downstream processes leading to immunity (Ravetch and Kinet, 1991; Salmon *et al.*, 1992). Some polymorphisms in the FcγRs have been identified as genetic determinants of susceptibility to infectious diseases (Omi *et al.,* 2002; Ouma *et al.,* 2006). The *FcγRIIA*-131Arg/His polymorphism leads to change of Histidine to Arginine at 131 located at its second extracellular immunoglobulin-like domain (van de Winkel and Capel, 1993; Warmerdam *et al.,* 1991). The *FcγRIIA*-131His/His has efficient binding to IgG_2 as opposed to FcγRIIA-131Arg/Arg. In addition, the IgG_2 and IgG_3 antibodies have been shown to confer resistance to malaria by some studies (Aucan *et al.,* 2000; Nasr *et al.*, 2008). In the current study, however, there were no associations between *FcγRIIA*-131Arg/His polymorphism and SMA. An earlier study (Schuldt *et al.,* 2010) in Ghanaian children demonstrated that *FcγRIIA*-131Arg/Arg was associated with an increased risk of severe malaria anaemia, but not cerebral malaria or any other malaria complication. Of note is the fact that a number of studies have shown contradictory results on the actual role of this variant on malaria disease severity (Cooke

et al., 2003; Nasr *et al.,* 2007). These discrepancies may be attributed to clinical definitions of malaria, different genetic backgrounds from ethnic diversity and overall sample (population) size in previous studies.

The *FcγRIIIA*-176F/V gene displays a functional allelic polymorphism that generates allotypes exhibiting different receptor properties (Ravetch and Perussia, 1989). This study revealed no association between the *FcγRIIIA*-176F/V polymorphism and susceptibility to SMA in this paediatric population. This may imply that this particular variant is not independently associated with susceptibility to SMA. The *FcγRIIIB* is a C-terminus linked glycosylphosphatidylinositol (GPI) moiety anchored receptor, exclusively expressed on neutrophils with three characterized allotypes i.e. human neutrophil antigen (HNA-1a or NA1, HNA-1b or NA2 and HNA-1c or SH) (Bux *et al.,* 1995). The NA variants, NA1 and NA2, are a product of five non-synonymous SNPs in the first IG-like domain, with an asparagine to serine switch at amino acid position 65 resulting in glycosylation and reduced affinity in the NA2 allele (Ravetch and Perussia, 1989; Salmon *et al.,* 1990). In the current study, no association between either the NA1 or NA2 allotypes or susceptibility to SMA using common genetic models i.e., dominant, additive and recessive models was observed. However, in Ghanaian children aged 1 to 12 years, the FcγRIIIB-NA2 was associated with susceptibility to clinical malaria (Adu *et al.,* 2012). In a different study of malaria patients in Thailand, the *FcγRIIIB*-NA2 allotype was associated with cerebral malaria, but not other forms of severe malaria (Omi *et al.,* 2002). Given the differences in findings from different populations and a diversity of clinical manifestations associated with malaria, the exact role of *FcγRIIIB*-NA2 in mediating outcome of malaria disease remains to be further explored.

It is important to note that FcγRs function synergistically, especially via crosslinking, resulting in phagocytosis of immunoglobulin-opsonized immune complexes or through stimulation of neutrophil granulation leading to production of reactive oxygen species (ROS) (Mitchell *et al.,* 1994; Salmon *et al.,* 1995). In the current study, haplotypic analysis revealed that carriage of the *FcγRIIA*-131Arg/*FcγRIIIA*-176F/*FcγRIIIB*-NA2 haplotype was associated with susceptibility to SMA. This is not surprising given that the haplotype had a higher frequency in the SMA group relative to the non-SMA group. Consistent with these observations, previous studies have demonstrated that the *FcγR*-131Arg/Arg is associated with low phagocytic activity and poor immune complex clearance (Warmerdam *et al.,* 1991), which may imply that its inheritance as a haplotype, together with *FcγRIIIA*-176F and the *FcγRIIIB*-NA2 allotypes, impart decreased cellular responses to IgG-mediated stimulation (Bredius *et al.,* 1994; Koene *et al.,* 1997), and subsequently, susceptibility to SMA. Although the exact mechanisms through which the *FcγRIIA*-131Arg/*FcγRIIIA*-176F/*FcγRIIIB*-NA2 haplotype result in severe malaria susceptibility were not evaluated in the current study, it is scientifically plausible to propose that carriage of the haplotype may lead to a reduced crosslinking in neutrophils, hence low phagocytic activity resulting in reduced antibody depended respiratory burst (ADRB), a mechanism by which neutrophils provide protection against clinical malaria (Joos *et al.,* 2010; Kapelski *et al.,* 2014; Witko-Sarsat *et al.,* 2000). Moreover, the *FcγRIIA*-131Arg/Arg, *FcγRIIIA*-176F/F and *FcγRIIIB*-NA2 allotypes are associated with low binding to cytophilic antibodies, which have been shown to play major roles in ADRB (Pleass *et al.,* 2003; Shi *et al.,* 2011). Taken together, the *FcγRIIA*-131Arg/*FcγRIIIA*-176F/FcγRIIIBNA2 haplotype may culminate in a reduced protective inflammatory response leading to enhanced susceptibility in children with SMA.

The finding that the *FcγRIIA*-131His/*FcγRIIIA*-176F/*FcγRIIIB*-NA1 haplotype was associated with higher parasitemia levels is fascinating given the fact that the *FcγRIIA*-131His/His and *FcγRIIIB*-NA1 allotypes in this haplotype construct are associated with effective binding to cytophillic IgGs (Garraud *et al.*, 2003), leading to clearance of opsonized parasites as opposed to the *FcγRIIIA*-176 F/F. One possible explanation for this observation could be that high levels of parasitemia in the haplotype may be associated with the diluting effect of the *FcγRIIIA*-176F allele, which has a low binding to cytophylic antibodies (Koene *et al.*, 1997), and hence reduced clearance of parasites. However, it is worth noting that *FcγRIIIA* binding of IgG is important in induction of natural killer (NK) cells stimulatory properties which results in release of pro-inflammatory mediators, such as IL-1β, interferon-γ and tumour necrosis factor-α (Lyke *et al.,* 2004) whose imbalances have been implicated in pathogenesis of clinical malaria in children.

CHAPTER SIX: SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1. Summary of Findings

This study demonstrates that using genome wide association study (GWAS) models, genetic variations in *CD40, IL-23R, FcγRs, NFκB1* and *NFκBIA* are associated with susceptibility to *P. falciparum* SMA in immune-naïve children (aged 6-36 months) from Siaya County which is a *P. falciparum* holo-endemic transmission area. In addition, this study reveals that the *PfHz* is able to stimulate the expression of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. One possible pathway through which the *PfHz* may be involved in SMA pathogenesis is via the stimulation of CD40 pathway which transduce downstream molecules leading to production of pro-inflammatory cytokines which have been shown to be adversely deregulated in during *P. falciparum* malaria infection.

6.2. Conclusions

1. The current study reveals for the first time that *CD40* rs1800686 (-508G/A), rs752118 (−173C/T) and rs1883832 (-1C/T) polymorphisms in *CD40* gene promoter are associated with susceptibility to paediatric SMA in children from this *P. falciparum* holo-endemic transmission area. Moreover, these variations have profound influence on the levels of malaria-mediated circulating pro-inflammatory cytokines (IL-1β, IL-17, MIP-1α and IL-2) that are deregulated during infection.
2. The current study demonstrates that *NFκB1*-8079G/A/-3297 C/T and *NFκBIA*-826G→A/-310G→A promoter polymorphisms are salient genetic factors that condition

susceptibility/protection to severe malaria anaemia in children from western Kenya. These polymorphisms also mediate the production of IL-10 and IP-10 on infection by *P. falciparum*, possible mechanism involved in SMA.

3. *P. falciparum* Haemozoin is a potent modulator of the pathogenesis of *P. falciparum* malaria via the CD40 and NFκB pathways due to its ability to influence the expression levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) produced during infection. This however occurs in a time dependent manner.
4. Haplotypes of the *IL-23R* alter the risk of developing SMA, an implication of the importance of study the effects of non-synonymous genetic alterations in infectious disease.
5. *FcγRs* haplotypes, but not individual genotypes are associated with malaria disease severity, demonstrating the combinatorial effects of genes on influencing phenotypes of malaria.

6.3. Recommendations from the current study

1. Carriage of the *CD40* -1T allele should be exploited as an important marker of SMA susceptibility in this populations since most children with this allele were at risk of SMA even though its frequency was low based on this study.
2. Combined genotypes analysis for genes functioning in a molecular pathway such as the *NFκB1* and its inhibitor *NFκBIA* is more useful in identification of loci that influence disease susceptibility and should be applied in genetic studies dealing with homogenous populations.

3. Molecules that inhibit the stimulatory effects of *PfHz* through the CD40 signalling pathway should be used to help reduce the pro-inflammatory imbalanced caused by *PfHz* during *P. falciparum* malaria infection.

4. The *IL-23R* non-synonymous haplotype (rs1884444T/rs7530511T; TT) should be applied as genetic susceptibility marker in paediatric SMA as they can predict disease outcome.

5. To show the predictive use of *FcγRs* in SMA, haplotype analysis should be used as single SNP analysis does not reveal clear associations.

6.4. Recommendations for future studies

1. Further immuno-genetic studies with other ethnic groups and additional variations in *CD40* including exon variations would help to decipher the actual role of *CD40* gene variations in *P. falciparum* malaria susceptibility.

2. Future studies should consider measurements of phosphorylated NFκB to determine how much is translocated to the nucleus as this will be an indicator of cellular stimulations.

3. Drug target studies should consider the development of additional molecules that inhibit CD40 pathway to help absolve the excess pro-inflammatory effects of *PfHz* observed during *P. falciparum* malaria infection.

4. Future studies aimed at longitudinally measuring immune complexes e.g. immunoglobulins over time will help to delineate the important role of *FcγR* haplotypes on susceptibility to severe malaria in paediatric populations.

5. To fully decipher the molecular and immunological contributions of *IL-23R* variations on the pathogenesis of SMA, studies should include analysis of downstream molecules of the IL-23R/IL-17 signalling pathway such as the pleotropic IL-17.

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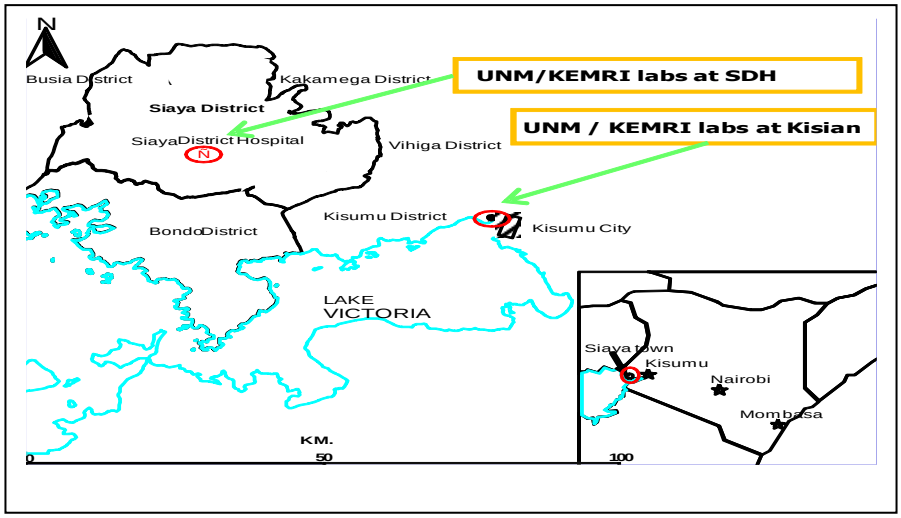
APPENDICES

Appendix 1. Map of Study Area



Appendix 2. Study Consent Forms (English Version)

Screening Form 1a: Written consent for parent/guardian for screening of child for malaria aneamia 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) to see how children fight malaria. Dr. Douglas Perkins of University of New Mexico would like your child to be screened to see if your child can 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 malaria aneamia". 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 will enroll children who are 6 to 36 months of age and follow them for 3 years for the date of enrollment. The study ends on 31 June 2011. We might learn ways to help improve treatment or prevention of malaria. Before being joining the research study your, child must have several tests to see if he or she is in the groups of children that the research study will be done in.

Screening Procedure: Since this study will only have only children with malaria and aneamia (low blood), we will have to screen your child to see if he or she has malaria and low blood. If you choose for your child to have the screening done, we will ask you about your child's health and check for malaria and low blood. To check for malaria and low blood, we will get blood (several drops) by sticking your child's heel or finger with a small needle.

Also, we know that HIV/AIDS is a problem in this area. HIV/AIDS affects the way the body fights infections. There are blood tests that can be done to learn if your child has the virus that causes AIDS. This virus is called HIV. We would like to test your child's blood for HIV exposure. The research study will enroll children that have HIV exposure and those that do not have HIV exposure. However, it important for the research study to know who has HIV exposure since it affects the way your child fights infections. If your child has HIV exposure, this means that he or she may or may not have HIV. To see if your child has HIV exposure, we must do a rapid blood test from the several drops of blood we will get by sticking your child's heel or finger with a small needle. If the rapid blood test is positive, then we must do another test to see if your child has HIV in his or her body.

If you want your child to be tested for HIV, you will have to talk to one of our HIV counselors. The counselor will give you facts about HIV. These facts will be about the HIV blood tests and how you can keep your child from getting HIV. You will receive the results of the rapid test (for HIV exposure) the same day. If your child has a positive rapid test for HIV, we will do the additional test. The additional test will tell you for certain if your child has HIV. The HIV counselor will give you the results of the additional HIV test result within two weeks when you come back to the hospital. Whether the blood test is negative or positive, you will be the only person to get the result of your child's HIV test. The results of the HIV test will be kept private to the extent allowed by law. Receiving your child's HIV test results can be stressful. If your child tests positive, this may upset you. However, knowing the tests results will benefit your child, because you will be able to act to protect your child's health. If your child's blood tests

positive for HIV, we would like you to talk with a doctor. However, if you choose, only you will get the HIV test results of your child. You do not have to speak with a doctor. We will not tell any other person the result of the test unless you ask us to do so. Testing for HIV and talking to HIV/AIDS counselors will be free of charge. If you want, we will refer your child to the Patient Support Center (PSC) at SDH so that your child can get medicine that can help fight the HIV virus. HIV can also cause low blood in your child. Professional counselors will give you facts about the problems of low blood. They will also talk with you about why it is important to go to follow-up visits and take the vitamins and drugs for malaria and HIV.

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. If your child has a problem with the finger- or heel-stick, Dr. Benjamin Esiaba, of the MOH, will treat the problem at SDH.

Benefits from the screening for malaria, low blood, and HIV are access to malaria and HIV testing and counseling for your child. 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.

Having the screening done is up to you. If your child is eligible for the research study, based on the screening results, you may choose to have your child enrolled in the research study. If you do not want your child to be in the research study, your child will still get the best possible medical care at the hospital. If you decide you want your child to be in the research study, you must discuss the research study with a member of the research team and sign the consent form for enrollment (participation).

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

Signatures:

Parent/guardian's signature: _____ Date: _____

Witness Signature: _____ Date: _____

Parent/guardian' thumbprint: _____

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

Parent/guardian's signature:_____ Date :_____

Parent/guardian's thumbprint: _____

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

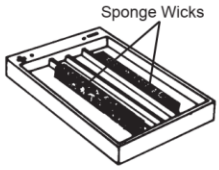
1. Remove the Titan® IV Citrate Agar Plate from the refrigerator and allow the plate to come to room temperature (15 to 30°C) while preparing the patient samples.
2. Remove the plate from the plastic bag and properly identify it by marking with a marker on the plastic support backing of the agar filled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

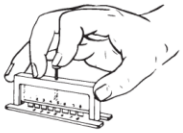
B. Preparation of Patient Sample and the Control

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

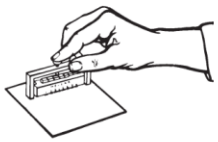
1. Pour approximately 100 mL of Citrate Buffer into each outer section of the Titan Gel Chamber.
2. Wet two sponge wicks in the buffer, and place the sponges in each outer compartment so that the top surface protrudes approximately 2 mm above the inner chamber ridges. (Care should be taken to rinse all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



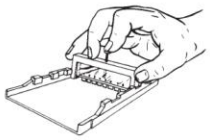


D. Sample Application

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.

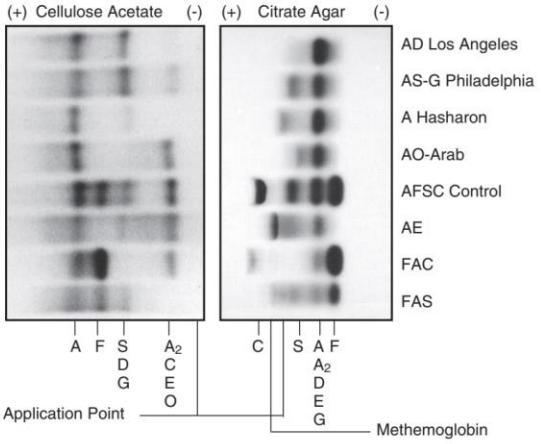


Figure 1. Electrophoretic Mobilities of Hemoglobins on Titan® III Cellulose Acetate and on Titan® IV Citrate Agar.

	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 β^{o}-Thalassemia HbA is absent.
In Sickle Cell β^{+}-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

1. Wintrobe, M.M., Clinical Hematology, 6th Edition, Lea and Febiger, Philadelphia, 1967.
2. Fairbanks, V.F., Diagnostic Medicine Nov/Dec.:53-58, 1980.
3. Schneider, R.G., et al. Laboratory Identification of the Hemoglobins, Lab Management, August, 29-43, 1981.
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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Pro. 17
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:

$$\text{Glucose-6-Phosphate (Not Fluorescent)} + \text{NADP} \xrightarrow{\text{G-6-PDH}} \text{6-Phosphogluconate (Fluorescent)} + \text{NADPH}$$

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

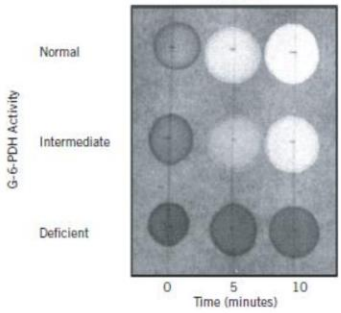
Samples with normal G-6-PDH and with G-6-PDH deficiency should be included with each group of assays to ensure reliable test performance. A sample with intermediate G-6-PDH activity could also be included. G-6-PDH Control Normal, Catalogue No. G 6888, G-6-PDH Control Intermediate, Catalogue No. G 5029, 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.

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8. Echler G: *Determination of glucose-6-phosphate dehydrogenase*. Am J Med Technol 49:259, 1983.
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**.

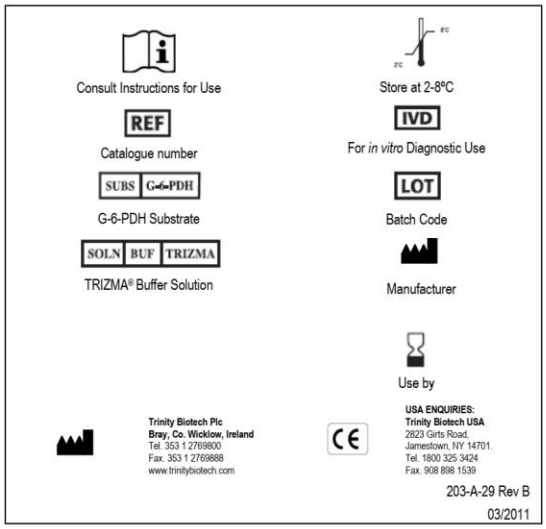
ORDERING INFORMATION

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



Appendix 5. Transcription Binding Factor Analysis

Variation	Sequence	Transcription binding factor in presence of	
		Major allele	**Minor allele**
CD40			
(rs1800686) -508 G/A	GGAAGTTGAGACGACGCG CCCACAC**[G/A]**AGGGAATT TCCTTTGAAAGAGAGCG	**(G)** G-alpha, RelA, NFκB, c-Ets	**(A)** C/EBP-β, RelA
(rs752118) -173 C/T	CAAAGAAGAAGAGCTGTC TCTGGGA**[C/T]**CATGCCTC CTCCCGTACACAGCAAG	**(C)** YY1, PPAR-α,	**(T)** XBP-1
rs1883832 (-1C/T)	GTCCTGCCGCCTGGTCTCA CCTCGC**[C/T]**ATGGTTCGT CTGCCTCTGCAGTGCG	**(C)** YY1, ENKTF, XBP-1, TBR-β1	**(T)** NONE

Appendix 6. Heamozoin Preparation Protocol

Materials

Parasitized RBCs
Falcons, Oak Ridge Tubes
1XPBS/DPBS sterile
Ultracentrifuge in Sorvall S34 rotor Combo (2-4-5)
Sonicator
Microcentrifuge tubes, sterile

Procedure

1. Collect the *Pf* infected RBCs (when parasitemia is >5%, and late trophozoites and early schizonts in predominate forms, high parasitemia is better) in 15 or 50mL falcon tubes.
2. Spin down the parasitized RBCs @ 3000rpm for 10 mins. Re-suspend the resulting pellet in 10-20mL DPBS/PBS and repeat the centrifuge @ 3000rpm for 5 mins.
3. Store the pellet (about 4ml from T-75 *pf* culture) at -20°C (If not processing on same day, thaw the sample at RT before proceeding to step 4)
4. Add a 1:1 volume of pellet: saponin soln (1% Saponin:- 1g Saponin to 100 mL of water, molecular biology reagent, from Sigma, cat 4502-1L, lot #RNBF5660). Re-suspend the pellet using a pipette. Incubate for about 20 mins. Check under haemocytometer to ensure that the RBCs are lysed.
5. Mix every 4 ml of the pRBCs to 20 ml of 1X DPBS [newly opened].
6. Transfer the batch of diluted pRBCs to OakRidge tubes (sterile)
7. Spin in ultracentrifuge Sorvall S34 rotor at 14000 rpmX20 minutes at 4°C
8. Remove the lipid membranes after the above procedure and decant supernatant
9. Resuspend the pellet in each tube in 20ml of 1X DPBS
10. Repeat spin and 1X DPBS resuspension **six** times (until the supernatant is clear). Between the spin, weigh empty sterile microfuge tubes and record.
11. Resuspend each pellet in 3 mL of 1X DPBS. Aliquot 1.5 ml of the suspension into the pre-weighed microfuge tubes, rinse the Oakridge tubes with 1mL of 1X DPBS and transfer to microfuge tube.
12. Spin at 14000 rpm for 20 minutes at 4°C in microfuge tubes. Remove supernatant and spin again for 5 minutes. Carefully, remove any remaining supernatant.
13. Allow the pellet to dry at 40°C on a dry heating block **2 days**.
14. Once the pellets have completely dried, weigh each of the micro centrifuge tubes containing dried extract. Subtract the new weight from the previous empty microfuge tube to get the weight of haemozoin.
15. Store the dried pellet at -20°C.

Appendix 7. Study Approval

15 FEB 2012

KENYA MEDICAL RESEARCH INSTITUTE

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Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
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KEMRI/RES/7/3/1 **February 13, 2012**

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

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

FORWARDED
DIRECTOR

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

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

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

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

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

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

Sincerely,

CHRISTINE WASUNNA,
FOR: SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

In Search of Better Health