THE FUNCTIONAL ASSOCIATIONS BETWEEN IL-4 –589T/C AND IL-6 -636G/C PROMOTER POLYMORPHISMS AND PAEDIATRIC SEVERE MALARIAL ANAEMIA IN A HOLOENDEMIC AREA OF WESTERN KENYA

By

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Abstract

Plasmodium falciparum, the most virulent and lethal human malaria parasite, is responsible for most malaria-related morbidity and mortality in sub-Saharan Africa. It manifests mainly as severe malarial anaemia (SMA) in children in malaria holoendemic areas such as western Kenya. So far, few studies have investigated genetic polymorphism in cytokines, which are important determinants of immune response regulation. Since previous studies have suggested that interleukin-4 (IL-4) and IL-6 production and their interactions seem to play a significant role in the pathogenesis of severe malaria, this study investigated the fuctional effects of IL-4 -589T/C and IL-6 -636G/C gene promoter single nucleotide polymorphisms (SNPs) on IL-4 and IL-6 cytokine production and their associations with SMA and high-density parasitaemia (HDP) during malaria infections. This was a cross-sectional study based at Siaya District Hospital (SDH) and a total of 618 and 602 study participants were studied for IL-4 -589T/C and IL-6 -636G/C SNPs, respectively. The study participants were grouped as aparasitaemic controls (AC), low density parasitaemia (LDP), HDP, SMA and non-severe malarial anaemia (Non-SMA). This was after the study participants had met the inclusion criteria which considered their natural exposure to *P. falciparum,* prior hospitalisation, intended relocation, being *P. falciparum* positive or negative and signing of the consent forms by parents/guardians, amongst others. Three millilitres of venous blood samples were collected (in ethylene diamine tetracetic acid (EDTA) vacutainer tubes and tubes without anticoagulants) from aparasitaemic children and malaria parasitemic children presenting at SDH and were used for clinical, immunological, and molecular evaluations, including haemoglobin (Hb) electrophoresis, genotyping, full haemogram and cytokine profile analyses. The IL-4 -589T/C and IL-6 -636G/C genotyping was carried out using a Taqman 5' allelic discrimination by real time PCR. Human Cytokine Twenty-Five Plex Antibody Bead Kit and a haematological analyzer were used to determine the cytokine levels and blood cell indices, respectively. The data were analysed by SPSS software. Kruskal Wallis test and the Mann-Whitney U tests were used for across group and pairwise comparisons, respectively. Proportionality was tested by χ^2 tests, while multivariate logistic regression analysis was used for determining the associations between the genotypes and malaria clinical outcomes, in a model

controlling for the confounders (age, gender, sickle-cell trait, bacteremia and HIV status). Results showed that IL-4 -589T/C SNP was significantly associated with increased susceptibility to HDP (OR; 1.64, 95%CI; 1.01-2.65, P=0.044); however, the IL-6 -636G/C SNP was not associated with any malaria disease outcome. In addition, the IL-4 -589T/C and IL-6 -636G/C SNPs were not functionally associated with circulating IL-4 and IL-6 levels, respectively. The significant departure from the Hardy-Weinberg equilibrium (HWE) shown by the IL-4 -589T/C and IL-6 -636G/C genotypic distributions, implicate the action of evolutionary forces on the genes, possibly through the exposure of the population to malaria. In conclusion, the variation in the IL-4 promoter region as found in this tudy seems to be conditioning the clinical outcomes of falciparum malaria and as a result, should be useful in the development of novel interventions for the control and management of severe malaria. The results of IL-6 -636G/C SNP from this study, on the other hand, has shown that the polymorphism neither conditions malaria disease outcomes nor circulating IL-6 levels in this population, thus necessitating studies on additional IL-6 SNPs.

CHAPTER ONE

INTRODUCTION

Background of the Study

ılaria remains a major human parasitic disease with significant morbidity and mortality ɔrldwide (Breman *et al.*, 2001; Crawley, 2004; Worrall *et al.*, 2004). About 40% of the world's pulation is at risk of acquiring malaria and approximately 3.2 billion people are at risk of alaria transmission (Croft, 2000; WHO, 2005). In these areas, an estimated 350-500 million inical cases occur annually, leading to at least 1 million deaths (WHO, 2005). An estimated)% of these deaths from malarial infections occur in sub-Saharan Africa (WHO, 2005). Further udies have shown that *Plasmodium falciparum* causes 95% of deaths due to malaria (Hoffman *al.*, 2002).

Plasmodium falciparum is the most virulent human parasite that causes cerebral malaria (CM) nd severe malarial anaemia (SMA), which are the two major severe disease outcomes ıssociated with mortality (WHO, 2000). Children below 5 years of age, pregnant women, and people without previous exposure to malaria appear to constitute the most important risk groups, since repeated malaria infections often retard development in children and lead to loss of productive activity among adults (WHO, 2000). It has been estimated that SMA affects between 1.42 and 5.66 million people and kills between 190,000 and 974,000 children below the age of five annually (Murphy and Breman, 2001). 50% of the gaps in the malaria burden, ranging between 0.4 and 1.7 million deaths annually, have also been attributed to SMA (Murphy and Breman, 2001). Earlier studies have indicated that deaths resulting from SMA have been under-estimated because of inaccurate diagnosis, low parasitaemia and confusion of the clinical picture with other causes of anaemia (Phillips and Pasvol, 1992).

Recently, epidemics in malaria have occurred in previously malaria free areas, mainly as a result of a decline in public health systems and drug resistance (WHO, 2000). Studies have demonstrated that between 25 and 30 million people from non-tropical countries visit areas in which malaria is endemic yearly, and between 10,000 and 30,000 become infected (Kain and Keystone, 1998; Croft, 2000). In addition, the burden of malaria disease outside Africa, especially in South Asia, appears greater than was estimated in the 1990s (Nahlen *et al.*, 2005). In endemic African countries, malaria accounts for 25-35% of all outpatient visits, 20-45% of hospital admissions and 15-35% of hospital deaths (WHO, 2005). The high malaria burden in Africa and other developing countries has been attributed to poverty, breakdown of malaria control programmes and resistance to the available pyrethroids, the constant emergence of drug-resistant parasites, climatic changes, political upheavals, limited affordability and accessibility to treatment (Craig *et al.*, 1999; Vulule *et al.*, 1999). Hence, malaria not only causes economic burden in developing countries, but with increasing global travel, it poses a threat to human health worldwide (Newton and White, 1999).

In Kenya, about 20 million people are infected by *P. falciparum* and about 3.5 million children are exposed to stable malaria transmission (Snow *et al.*, 1998) (Figure 1). Additional reports from Kenya estimated that daily, 400 and 72 children below the age of five years develop clinical malaria and die, respectively (Snow *et al.*, 1998). In malaria holoendemic areas, morbidity and mortality from *P. falciparum* has been shown to peak in children aged between 6 months and 24 months (Bloland *et al.*, 1999).

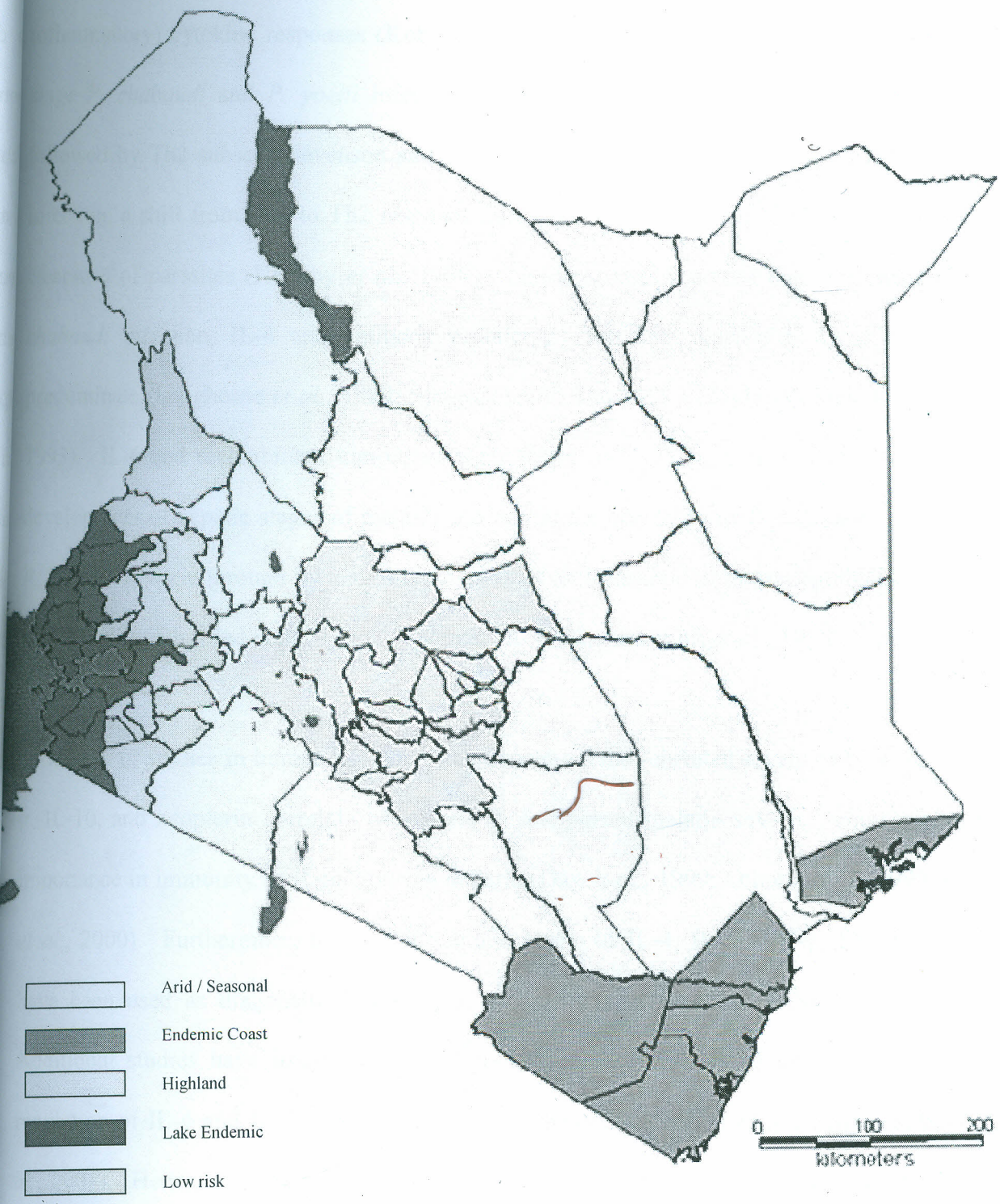


Figure 1. Map of Kenya showing Malaria Endemic Areas.

(Source. http://www.nmcp.or.ke/images/kenya.gif 1.2.3, 2003)

In murine studies, *P. yoelii* infection activates Th1 (pro-inflammatory) and Th2 (anti-inflammatory) cytokine responses (Kobayashi *et al.*, 1996). The resolution of primary blood-stage *P. chabaudi* and *P. yoelii* infections occur through sequential activation of Th1 cells followed by Th2 subset (Stevenson and Tam, 1993; Kobayashi *et al.*, 1996). In *P. chabaudi* infection, a shift from Th1 to Th2 response during peak parasitaemia has been associated with clearance of parasites (Helmby *et al.*, 1998). Earlier studies reported that following acute *P. chabaudi* infection, IL-4 and antibody producing Th2 cells, known to clear parasitaemia, predominate (Langhorne *et al.*, 1989; Stevenson and Tam, 1993; Taylor-Robinson and Phillips, 1993). IL-6 and recombinant tumour necrosis factor (r-TNF) have been shown to inhibit the development of hepatic stages of malaria and accelerate immunity to *P. chabaudi, P. yoelii* and *P. berghei* malaria through increased production of anti-plasmodial immunoglobulins (Ig) IgG1, IgG2a and IgG2b (Pied *et al.*, 1992; Vreden *et al.*, 1992; Akanmori *et al.*, 1996).

A number of studies in human hosts have demonstrated that elevated levels of IL-4, IL-6, TNF-α, IL-10, and neopterin correlate with acute *P. falciparum* malaria severity, emphasizing their importance in immunity to *P. falciparum* malaria (Day *et al.*, 1999; Othoro *et al.*, 1999; Biemba *et al.*, 2000). Furthermore, the production and levels of IL-4, IL-6, IFN-γ, TNF-α and IL-10 have been used as diagnostic markers for malaria disease severity (el-Nashar *et al.*, 2002). Additional studies have implicated the promoter regions of the IL-4 and IL-6 genes in the regulation of IL-4 and IL-6 plasma levels, respectively (Cartwright *et al.*, 2001; Hulkkonen *et al.*, 2001). Hence, the aim of this study was to examine the functional relationship of IL-4 –589T/C and IL-6 -636G/C promoter polymorphisms on the acquisition of parasitaemia, SMA (Hb<6 g/dL, and any density parasitaemia) and high-density parasitaemia (HDP; ≥ 10,000

parasites/μL). The functional relationship was investigated in children naturally exposed to *P. falciparum* infection in a holoendemic area of western Kenya.

1.2 Justification and Objectives of the Study

1.2.1 Justification of the study

Despite the intensive research for many decades, the knowledge on the mechanisms of protective immunity against malaria infection remains poorly understood (Hviid, 2005; Doolan and Martinez-Alier, 2006; Schmidt *et al.*, 2008). Consequently, slow progress in the development of an effective and safe vaccine against malaria has been reported (Hviid, 2005; Doolan and Martinez-Alier, 2006; Schmidt *et al.*, 2008). One method of dissecting the complex patho-physiological processes involved in severe malaria is to identify genetic traits that confer protection from severe and fatal malaria and then use these associations to investigate the role of particular mechanisms that regulate infection (Morahan *et al.*, 2002; Williams, 2006a, b).

There is evidence that cytokine gene and receptor polymorphisms are associated with varying quantities of cytokine production (Cartwright *et al.*, 2001). For example, studies have shown that variation in the promoter region of the IL-6 gene regulates IL-6 plasma levels through the multiple response elements located in the IL-6 -174G/C SNP (Keller *et al.*, 1996; Hulkkonen *et al.*, 2001). Investigations on IL-6 promoter SNP at -597 G/A, -572 G/C, -373 $A_{(n)}/T_{(n)}$, and -174 G/C, have revealed their influence on transcription through complex interactions (Terry *et al.*, 2000). Concerning IL-4 -589T/C promoter SNP, studies have shown that it enhances IL-4 production (Bijlsma *et al.*, 2002).

Previous studies have shown that elevated levels of IL-4 and IL-6 correlate with *P. falciparum* malaria severity (Othoro *et al.*, 1999; Biemba *et al.*, 2000) that appear to enhance proliferation and differentiation of B-lymphocytes (Richaud-Patin *et al.*, 1995; Premasiri *et al.*, 2005). Furthermore, TNF-α and IL-6 accelerate immunity against hepatic stages of *P. chabaudi, P. yoelii*, and *P. berghei* malaria in mice, thus demonstrating the importance of these molecules during both blood-stage and pre-erythrocytic stages of malaria (Pied *et al.*, 1992; Vreden *et al.*, 1992; Akanmori *et al.*, 1996). IL-6 has been implicated in the pathogenesis of malaria in hyper- and holoendemic areas (el-Nashar *et al.*, 2002; Gourley *et al.*, 2002). IL-4 on the other hand, appears to regulate Th1 cytokine (IFN-γ and TNF-α) production and prevents the development of iron deficiency anaemia, and therefore, could be protective against severe malaria (Grau and Lou, 1995; Nyakeriga *et al.*, 2004; Nyakeriga *et al.*, 2005). The balance between Th1 cytokines (e.g. IL-6, IL-8, IFN-γ and TNF-α) and Th2 cytokines (IL-4 and IL-10) may play a role in the clinical presentation of *P. falciparum* malaria and iron homeostasis (Kurtzhals *et al.*, 1998; Othoro *et al.*, 1999; Nyakeriga *et al.*, 2005). However, the effects of IL-4 –589T/C and IL-6 -636G/C promoter polymorphisms in regulating IL-4 and IL-6 cytokine production, and their associations with SMA and haematological complications in children from *P. falciparum* holoendemic transmission areas, such as western Kenya, has largely remained undefined. This study was designed to determine whether IL-4 –589T/C and IL-6 -636G/C SNPs, which are host genetic factors, are involved in the regulation and development of SMA and HDP in a phenotypically well-characterized population of children aged 3-36 months presenting at hospital for the first time with *P. falciparum* infection. The functional changes in IL-4 and IL-6 cytokine production in relation to these polymorphisms on malaria disease severity were also investigated.

1.2.1 The main objective of the study

To determine the associations between IL-4 -589T/C and IL-6 -636G/C SNPs and SMA

1.2.2 Specific objectives of the study

a) To determine the associations between IL-4 -589T/C and IL-6 -636G/C promoter SNPs with acquisition of parasitaemia, SMA (Hb<6 g/dL) and HDP (≥10, 000 MPS/μL) in children presenting with acute malaria.

b) To examine the functional association between IL-4 -589T/C and IL-6 -636G/C promoter genotypes and circulating IL-4 and IL-6 cytokine levels, respectively, in children presenting with acute malaria.

1.2.3 Null hypotheses

a) There is no relationship between SNPs in the IL-4 -589T/C and IL-6 -636G/C promoter with acquisition of parasitaemia, SMA and/or HDP in children with acute malaria.

b) There is no functional relationship between SNPs in the IL-4 -589T/C and IL-6 -636G/C and IL-4 and IL-6 plasma levels, respectively, in children presenting with acute malaria.

1.2.4 Assumptions

a) Relationships exist between SNPs within the IL-4 -589T/C and IL-6 -636G/C promoter regions, acquisition of parasitaemia, SMA and/or HDP in children presenting with acute malaria.

b) Polymorphisms within the IL-4 –589T/C and IL-6 -636G/C promoter affect circulating IL-4 and IL-6 production, respectively, in children presenting with acute malaria.

1.2.5 Significance of the study

The identification of the functional relationships between the IL-4 -589T/C and IL-6 -636G/C promoter polymorphisms and *P. falciparum* malaria infection will provide insight into the immuno-regulatory action of these SNPs on IL-4 and IL-6 cytokine production, SMA and/or HDP, and other malaria clinical presentations. The results of this study will enhance the understanding of immuno-genetic risk factors of severe malaria and help identify molecules that can be utilized in the development of novel interventions for the control and management of severe malaria in children living in *P. falciparum* holoendemic transmission areas, such as western Kenya.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria Prevalence

Malaria remains one of the major public health problems in the world (WHO, 2005). It arises from protozoan infection of red blood cells by *P. falciparum, P. vivax, P. ovale, or P. malariae* (Miller *et al.*, 1994; Croft, 2000). Due to its extensive geographical distribution, malaria affects most populations in sub-tropical and tropical regions (WHO, 2005). It has been estimated that 3.2 billion people from 107 countries and territories are at risk of malaria transmission, which include China, Guyana, Haiti, India, Papua New Guinea, West and East Africa (Figure 2) (Picq, 1982; WHO, 2000).

Patterns of malaria transmission and disease seem to vary between regions and within individual countries (WHO, 2005). The diversity in transmission arises from variation between the human hosts, malaria parasites, mosquito vectors, ecological conditions and socio-economic factors (WHO, 2005). In Vanuatu, for example, it has been shown that homozygous children with α-thalassemia had increased incidence to *P. vivax* and *P. falciparum* malaria, while in Thailand helminth-infected patients had increased incidence of *P. falciparum* (Maitland *et al.*, 1996; Nacher *et al.*, 2002). Pregnancy and early post-partum, on the other hand, have been associated with *P. vivax* and *P. falciparum* (Diagne *et al.*, 2000).

Legend

Areas in which malaria disappeared, been eradicated or never existed.

Areas with limited risk

Areas where malaria is endemic

Figure 2. Distribution of Malaria Endemic Areas of the World

(Source: http://www.cdc.gov/malaria/images/graphs/geodistribution.gif, 2000)

2.2 Malaria Morbidity and Mortality

Malaria kills on its own or in conjunction with other causes (Premji *et al.*, 1995; Molineaux, 1997). Deaths from *P. falciparum* mainly arise from malaria anaemia, CM, metabolic acidosis or a combination of these (Miller *et al.*, 1994). The total burden of malaria morbidity has been shown to correlate positively with transmission intensities (Marsh and Snow, 1999). The incidence of life-threatening malaria appears to decrease with the increase in transmission intensity (Molineaux, 1997). In addition, seasonal variation promotes transition from passive to active immunity (Molineaux, 1997).

Evidence of dramatic peaks and troughs of severe anaemia that are regular, predictable and useful in gauging the health and survival of young children has been reported (Owusu-Agyei *et al.*, 2002). Majority of pediatric mortalities associated with *P. falciparum* infections occur within the first 12 hours of admission (Marsh *et al.*, 1995; Allen *et al.*, 1996) in sub-Saharan Africa as compared to iron deficiency (Zucker *et al.*, 1996). Higher morbidity and mortality in patients with malaria anaemia have been demonstrated in areas with sustained infection rather than areas with fluctuating patterns of infection (Slutsker *et al.*, 1994). Additional studies have correlated severe anaemia with parasite density in areas with fluctuating pattern of infection (Lackritz *et al.*, 1992; Slutsker *et al.*, 1994; Owusu-Agyei *et al.*, 2002).

High levels of plasma lactate, creatinine, and decreased plasma bicarbonate have been reported as major predictors of death from severe malaria (Allen *et al.*, 1996). Hypoglycaemia, jaundice, impaired consciousness and respiratory distress are useful predictors of high risk of death (Marsh *et al.*, 1995; Allen *et al.*, 1996). Deaths of non-immune individuals mainly arise from CM, renal

insufficiency, pulmonary oedema, disseminated intra-vascular coagulation and acute lesions (Miller *et al.*, 1994). It has been demonstrated that high lymphocyte and monocyte counts, the overall magnitude of the cytokine responses, including TNF-α, IL-2SR, IL-6 and IL-10, and the imbalance between the pro- and anti-inflammatory responses are important determinants of mortality (Day *et al.*, 1999; Ladhani *et al.*, 2002).

The increase in malaria-specific mortality in sub-Saharan Africa has been attributed to drug resistance to *P. falciparum* infection with resultant increase in severe anaemia (Ekvall, 2003). Additionally, the presence of a highly efficient vector (*Anopheles gambiae*), a large rural population, a high degree of mobility and an inadequate health care infrastructure, appear to maintain malaria as one of the most important causes of illness and death in the sub-Saharan Africa (Bloland *et al.*, 1999).

2.3. Malaria Diagnosis, Treatment and Prevention

Previous studies have reported malaria misdiagnosis, inaccurate microscopic species identification, and incorrect and delayed treatment for falciparum malaria (Kain *et al.*, 1998). Progression of malaria from mild through complicated to severe disease appear to be as a result of missed or delayed diagnosis (Pasvol, 2005). Measurements of acute phase reactants (C-reactive protein and serum amyloid A) are valuable in the assessment of the severity of *P. falciparum* malaria and antimalarial treatment (Gillespie *et al.*, 1991). Thus, to prevent malaria-associated morbidity and mortality, improved health information on malaria, appropriate preventive measures, improved recognition of infection by physicians, rapid and accurate laboratory diagnosis, and prompt initiation of effective therapy are essential (Kain *et al.*, 1998).

As a result, effective management of severe malaria has been reported to rely on rapid diagnosis, prompt administration of parenteral schizonticidal antimalarial drugs, careful fluid balance, prevention of convulsions and early recognition of complications such as hypoglycaemia, metabolic acidosis, anaemia, pulmonary oedema, renal failure, bleeding and supervening bacterial sepsis (Krishna and White, 1989).

Pre-treatment parasite densities have been shown to correlate with clinical and laboratory markers of inflammation (Gillespie *et al.*, 1991). Elevated levels of IFN-γ, TNF-α, IL-1α, IL-1β, IL-6, sIL-6R and IL-10 have been correlated with disease severity in children and adults (Grau *et al.*, 1989a; Kwiatkowski *et al.*, 1990; Baptista *et al.*, 1997). However, decreased levels of IFN-γ, IL-4, IL-6, IL-10, IL-12, IL-13 and IL-18 have been reported during schizontocidal therapy (English *et al.*, 1996; Vogetseder *et al.*, 2004; Hugosson *et al.*, 2006).

Many African children presenting with severe anaemia had neither recent symptoms nor history of malaria (Abdalla *et al.*, 1980). SMA is frequently treated with blood transfusions, which is the major vehicle for HIV transmission (Premji *et al.*, 1995). Passive transfer of antibodies from naturally immune individuals dramatically reduces parasitaemia (Bouharoun-Tayoun *et al.*, 1990; Waki, 1994; Waki *et al.*, 1995). Passive immunity has been associated with IgG2a in mice and IgG3 in humans (Groux and Gysin, 1990; Bouharoun-Tayoun and Druilhe, 1992; Waki *et al.*, 1995). Iron supplementation has been shown to increase Hb without curing anaemia in patients with inflammatory diseases (Feelders *et al.*, 1999; Verhoef *et al.*, 2002).

Cinchona alkaloids (quinine and quinidine), chloroquine, amodiaquine, sulfadoxine/pyrimethamine (SP), mefloquine, pyrimethamine/sulfadoxine/mefloquine, sesquiterpene lactones and primaquine have been used for the treatment of human malaria (Panisko and Keystone, 1990). Children treated with SP maintained clinical improvement and increased Hb concentration greater than those treated with chloroquine, indicating that chloroquine had become an ineffective therapy for clinical *P. falciparum* malaria in children (Bloland *et al.*, 1993). Administration of SP and low-dose micronutrients supplementation increased mean Hb level, thus improving childhood anaemia in malaria holoendemic areas (Ekvall *et al.*, 2000). In Kenya, artesunate and SP combination appear to be more effective against parasite clearance and gametocyte carriage than with SP alone (Obonyo *et al.*, 2003). Studies carried out in Tanzania have shown that the resistance to SP treatment against malaria arose from point mutations (Mbugi *et al.*, 2006).

Use of permethrin-impregnated bed nets and residual house-spraying have been reported to offer year-round protection against *P. falciparum* infection (Beach *et al.*, 1993; Goodman *et al.*, 2001; Mnzava *et al.*, 2001). It has been demonstrated that a combination of an effective insecticide and effective antimalarial drugs reduces malaria incidence rate by 91% (Maharaj *et al.*, 2005).

2.4 Clinical Manifestation and Pathogenesis of Malaria

2.4.1 Clinical manifestations of malaria

Malaria has been characterised by fever, headache, vomiting, tachycardia, rigors, sweating, anaemia, hepatosplenomegaly, cerebral involvement, renal failure, and shock (Croft, 2000). The occurrence of splenomegaly, hyperparasitaemia, convulsions, prostration, respiratory distress

(RD), coma, severe anaemia, hypoglycaemia, jaundice, increased blood lactate levels and a high rate of bacteraemia are common in complicated malaria (Esamai *et al.*, 1999; el-Nashar *et al.*, 2002; Chiabi *et al.*, 2004). Thrombocytopaenia, metabolic acidosis, lactic acidosis, acute phase reactants, hypotension, bilirubin, urea, sodium and albumin concentrations are indicators of poor prognosis in falciparum malaria (Imbert *et al.*, 1997; Gerardin *et al.*, 2002; Giha *et al.*, 2005); while, vomiting, fever and enlarged spleen are predictors of a *P. falciparum* parasitaemia in children and adults (Genton *et al.*, 1994).

2.4.1.1 *Cerebral malaria (CM)*

Cerebral malaria (CM) is one of the most serious complications of *P. falciparum* infection, which is accompanied by impaired consciousness (Biemba *et al.*, 2000; Wassmer *et al.*, 2003). Studies have shown that children, pregnant women and non-immune adults living in sub-Saharan Africa are more susceptible to CM (Garg, 2000; Idro *et al.*, 2005). CM is more common and severe in children in the tropics, where it is one of the most important causes of paediatric hospital admissions (van Hensbroek *et al.*, 1997; Garg, 2000; Idro *et al.*, 2005).

Clinical diagnosis of CM in African children is a collection of overlapping syndromes that act through different organ systems, which combine to cause death (Clark *et al.*, 2003). CM in African children and non-immune adults is characterized by neurological impairments, coma, sequestration of parasitized red blood cells (pRBCs) in cerebral capillaries and venules, micro-vascular obstruction by parasites, platelets, rosettes and micro-particles (Wassmer *et al.*, 2003; Wassmer *et al.*, 2004; Idro *et al.*, 2005).

The production of large quantities of TNF-α and other mediators in the host against invading parasites have been shown to be harmful to the parasite and the host (Clark and Cowden, 1992). High levels of IFN-γ, IL-1β, IL-6, IL-6R, IL-10 and TNF-α have been correlated with coma and CM (Day *et al.*, 1999; Wenisch *et al.*, 1999). Additional studies have associated high TNF-α, IL-6 and soluble IL-6 receptor (sIL-6R) levels with fatal outcome in CM patients (Kwiatkowski *et al.*, 1990; Wenisch *et al.*, 1999). However, the administration of anti-TNF-α antibody and passive immunization against mouse TNF-α to *P. berghei*-infected mice has been shown to be protective against CM (Grau *et al.*, 1987; Grau *et al.*, 1989a); while, treatment with neutralizing antibodies resolved clinical symptoms of CM faster in the experimental group than the control group (Looareesuwan *et al.*, 1999; Wenisch *et al.*, 1999).

Nitric oxide (NO) has been implicated in the pathogenesis of CM (Maneerat *et al.*, 2000). Acute induction of iNOS expression through the engagement of CD23 antigen in the brain during CM in a number of cell types enhances high NO production and iNOS expression (Maneerat *et al.*, 2000; Lopansri *et al.*, 2003; Pino *et al.*, 2004). Fatal CM has been attributed to high NO production, which appears to cause direct neurotoxicity, vasodilation and raised cerebral pressure (Weiss *et al.*, 1998).

Additional mechanisms for CM include the formation of knobs on the membrane of the pRBCs, which adhere to the endothelium and cause obstruction of cerebral micro-vessels (Aikawa *et al.*, 1990; Maneerat *et al.*, 1999; Pino *et al.*, 2004). CD36, thrombospondin, and ICAM-1 present on the membrane of endothelial cells act as receptors for the attachment of knobs of pRBCs (Aikawa *et al.*, 1990). Further studies have demonstrated that pRBCs adhere to uninfected

erythrocytes to form rosettes which also adhere to the endothelial cell surface antigen CD36 (Handunnetti *et al.*, 1992). Rosetting and cytoadherence, which involve the spontaneous binding of uninfected RBCs to pRBCs appears to hinder blood flow, thereby causing severe malaria and CM (Wahlgren *et al.*, 1992; Ramasamy, 1998).

Accumulation of platelets in the capillaries and venules of patients who died from CM suggest that platelets play a role in inducing cytoadherence of pRBC on endothelial cells (Wassmer *et al.*, 2003). Additionally, sequestrated pRBCs and reduced deformability of pRBCs have been implicated in the blocking of the capillaries in the brain, thereby depriving it of oxygen (Dondorp *et al.*, 2000). Further studies have shown that small size rosettes were protective against CM in patients with uncomplicated malaria with blood group O RBCs than those with complicated malaria from blood groups A, AB and B (Treutiger *et al.*, 1992; Wahlgren *et al.*, 1992).

2.4.1.2 *Severe malarial anaemia (SMA)*

SMA is a common, life-threatening complication of chronic *P. falciparum* malaria in infants, young children under five years of age, and pregnant women (WHO, 2005). Children with SMA appeared to have been ill for longer periods before admission and had a lower mean age compared to children with CM (Allen *et al.*, 1996; Marsh and Snow, 1999; Owusu-Agyei *et al.*, 2002). In children and adults, SMA has been defined as Hb < 5g/dL and Hb < 7.0 g/dL, respectively, with asexual forms of *P. falciparum* in peripheral blood but without specifying parasitaemia (English *et al.*, 1996; Biemba *et al.*, 2000; WHO, 2000). In western Kenya, however, SMA in children has been defined as Hb < 6.0 g/dL with any density parasitaemia (McElroy *et al.*, 2000). Further studies in western and coastal Kenya in children with severe

anaemia (Hb < 5 g/dL) has demonstrated that half of the patients had parasitaemia of less than 10,000 parasites per microliter (Zucker *et al.*, 1997; Wambua *et al.*, 2006). Consequently, anaemia in African children appears to be rarely associated with high parasitaemia, which, therefore, cannot predict the risk of death in the severely anaemic children (Wambua *et al.*, 2006).

Childhood SMA is one of the leading causes of morbidity and mortality in endemic areas of *P. falciparum* transmission, especially in holoendemic regions of sub-Saharan Africa (McElroy *et al.*, 2000; WHO, 2000; Breman *et al.*, 2001). The endemicity of *P. falciparum* has been shown to influence malarial morbidity and severity of anaemia (Mockenhaupt *et al.*, 2004). Consequently, areas with intense and sustained transmission experience more cases of severe anaemia that arises from chronic and repeated *P. falciparum* infections (Ekvall, 2003). SMA, which is more common under high transmission compared to CM, has been associated with age, fever, and sex (Allen *et al.*, 1996; Owusu-Agyei *et al.*, 2002). In malaria endemic areas of Africa, it has been estimated that between 31%-90% children and 60%-80% pregnant women suffer from SMA (Bradley-Moore *et al.*, 1985; Matteelli *et al.*, 1994; Premji *et al.*, 1995). In western Kenya, the prevalence of malaria and anaemia (Hb < 8.0g/dL) in children below five years of age has been estimated at 83% and 90%, respectively (Bloland *et al.*, 1999; McElroy *et al.*, 2000).

The pathogenesis of *P. falciparum* malarial anaemia has been shown to be complex and multi-factorial as it varies from case to case, depends on the patients' history, parasite density, degree of illness, Hb concentration and caretaker's level of education and occupation (Premji *et al.*,

1995; Issifou *et al.*, 2003; Ong'echa *et al.*, 2006). Additional investigations have indicated that malarial anaemia is influenced by nutritional factors, haemoglobinopathies, co-infections with bacteraemia and human immunodeficiency virus (HIV) (Stoltzfus *et al.*, 2000; Nussenblatt and Semba, 2002; Otieno *et al.*, 2006). Even though the mechanism underlying the causes of severe anaemia remains obscure, it has been generally accepted that it may result from haemolysis caused directly by the parasite or by dyserythropoiesis (Layez *et al.*, 2005). In low-endemic areas such as Tanzania, where the Hb of patients rarely falls below 7 g/dL of whole blood, the level of anaemia correlates with the level of *P. falciparum* parasitaemia (Kahigwa *et al.*, 2002).

Plasmodium falciparum infection is more common in areas where severe anaemia is a major problem, which raises the question of whether *P. falciparum* is an incidental finding or whether it is the cause of the anaemia. However, evidence from studies of antimalarial therapy demonstrates that anaemia is caused by *P. falciparum*. In an investigation that involved two study groups in Gambia, it was demonstrated that chloroquine-treated group had higher Hb than the placebo group (Van Hensbroek *et al.*, 1995). In another study in West Africa, children with moderate anaemia (Hb of 5-7.5 g/dL) who were treated with Fansidar® in a chloroquine-resistant area experienced increases in Hb relative to the group treated with chloroquine (Bloland *et al.*, 1993). These findings were consistent with the observation that in African populations, there was a rapid rise in Hb during antimalarial treatment of severely anaemic children (Abdalla *et al.*, 1980).

2.4.1.3 *Additional clinical manifestations of severe malaria*

It has been demonstrated that jaundice, hypoglycaemia and renal failure are key prognostic indicators of severe malaria (Esamai *et al.*, 1999; el-Nashar *et al.*, 2002; Chiabi *et al.*, 2004). Jaundice has been described as a yellow discoloration of the skin, sclerae of the eyes and mucous membranes. A number of studies have indicated that jaundice is a symptom of underlying pathological processes including hyperbilirubinaemia, viral hepatitis and parasitic infestations (Mishra *et al.*, 2007; Panther *et al.*, 2008; Soumare *et al.*, 2008). In malaria endemic areas, jaundice manifests in approximately 2.5% of patients with *falciparum* malaria (Anand and Puri, 2005).

Hypoglycaemia is a pathologic state that arises from a lower than normal level of glucose in the blood (<60 mg/dL). Investigations on hypoglycaemia have indicated that it is a multifactorial condition, mostly attributed to malaria, low birthweight, hyperinsulinism, hypopituitarism, prolonged fasting and treatment with quinine and quinidine (Ranque *et al.*, 2008).

Renal failure is indicated by elevated levels of serum creatinine, anaemia, haematuria, deranged acid levels and salts, including potassium, calcium and phosphate. It is a complication of *P. falciparum* malaria, which is more common in adults than children (Stone *et al.*, 1972; Cioffi *et al.*, 1986; Segasothy *et al.*, 1994). Renal failure can be diagnosed by a serum creatinine level that rises above 3 mg/dL (265 mol/L) and urinary output that is less than 400 mL in 24 hours (Mishra and Das, 2008). The possible mechanisms for renal failure include the effect of the pRBC on the microcirculation, chemical mediators, hyperbilirubinaemia, bacteria endotoxaemia

and effects of inflammation (Beutler and Cerami, 1987; Clark *et al.*, 1987; Segasothy *et al.*, 1994).

2.4.2 Factors affecting clinical manifestation of malaria

The principal mosquito vectors of malaria in Kenya are *Anophleles gambiae* s.s, *An. arabiensis,* and *An. funestus* (Beach *et al.*, 1993; Minakawa *et al.*, 1999). Malaria is caused by repeated cycles of growth of *P. falciparum, P.ovale, P. malariae* and *P. vivax* in the erythrocytes (Miller *et al.*, 1994). Infection with *P. falciparum* causes a wide spectrum of signs and symptoms, including SMA and CM (Bloland *et al.*, 1999; WHO, 2000). The clinical manifestations depend on the endemicity, age, parasite, host, geographical and social factors (Snow *et al.*, 1999; Weatherall *et al.*, 2002; Reyburn *et al.*, 2005). Additional factors that affect the clinical outcome of malaria include the asexual blood-stage of the parasite, host response to the parasitized erythrocytes, metabolites released by parasites, imbalance between pro-inflammatory and anti-inflammatory cytokines (Harpaz *et al.*, 1992; Allan *et al.*, 1995; Mackintosh *et al.*, 2004). The incidence and severity of predominant complications of severe malaria, including parasite density, fever, SMA, convulsions, CM and hypotension, correlate with seasons and the mean ages of patients (Nussenblatt *et al.*, 2001; Giha *et al.*, 2005). Age-dependent predictors of anaemia in African children are fever and parasitaemia (Premji *et al.*, 1995; Smith *et al.*, 1995).

2.4.3 Cytokines and malaria pathogenesis

A number of studies have indicated that cytokine production varied with parasite stage, parasite density, and precede the onset of clinical manifestations (Kwiatkowski *et al.*, 1990; Harpaz *et al.*, 1992; Allan *et al.*, 1995). Additional studies have shown that *P. falciparum* infected

erythrocytes rupture to release antigenic substances, malaria pigment (haemozoin), soluble antigens, and toxins, which stimulate the production of pro-inflammatory and anti-inflammatory cytokines (Pichyangkul *et al.*, 1994; Sherry *et al.*, 1995; Baptista *et al.*, 1997). Elevated levels of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1α, soluble IL-2 receptor (sIL-2R), nitric oxide (NO), IL-6, soluble tumour necrotic factor-receptor-55 (sTNF-R55) and soluble tumour necrotic factor-receptor-75 (sTNF-R75) and anti-inflammatory cytokines, including IL-10, during *P. falciparum* infection correlate with fever, parasite density, severe anaemia, jaundice, acute renal failure, CM, and hypoglycaemia (Kwiatkowski *et al.*, 1990; Shaffer *et al.*, 1991; Karunaweera *et al.*, 1992). High levels of IL-10 downregulated TNF-α (Wang *et al.*, 1994a; Wang *et al.*, 1994b; Othoro *et al.*, 1999) and decreased in patients as parasites cleared (Ho *et al.*, 1998; Gosi *et al.*, 1999). Thus, the balance between pro-inflammatory and anti-inflammatory cytokines play a major role in the clinical presentation of *P. falciparum* infection (Ho *et al.*, 1998; Kurtzhals *et al.*, 1998; Othoro *et al.*, 1999).

2.5 Cytokines and Malarial Immunity

Cytokines are a diverse group of soluble glycoproteins that modulate functional activities of cells (Feldmann *et al.*, 1998). They induce and regulate growth, differentiation, chemotaxis, activation, cytotoxicity and immunity in mature blood cells or their precussors (Troye-Blomberg, 1994; Feldmann *et al.*, 1998; Borish and Steinke, 2003). Cytokines are released by immunocompetent cells in a highly regulated fashion and may act within the same cell (autocrine), nearby (paracrine) or at distant sites (endocrine) (Seder and Paul, 1994; Delves and Roitt, 2000a, b). They are grouped as interleukins, lymphokines, monokines, interferons, chemokines and colony-stimulating factors.

Protective immunity to repeated malaria challenges appears to be dependent on immunological mechanisms, innate characteristics, genetic background of the host, Th1 and Th2 cells (Sarikabhuti *et al.*, 1988). Studies in murine models indicate that protective immunity in malaria is mediated through activation of Th1 cytokines (IL-12, IFN-γ, and TNF-α) (Sedegah *et al.*, 1994; Stevenson *et al.*, 1995; Hoffman *et al.*, 1997), which have been associated with the elimination of intracellular pathogens (Mosmann and Coffman, 1989; Mosmann and Moore, 1991). Additional studies have indicated that IL-12 initiates immune responses against blood stage of *P. chabaudi* AS and *P. berghei* XAT malaria (Mosmann and Coffman, 1989; Trinchieri, 1993; Crutcher *et al.*, 1995) through the up-regulation of Th1 cytokines (IFN-γ and TNF-α), which enhance anti-parasitic properties (Stevenson *et al.*, 1995; Yoshimoto *et al.*, 1998). Th1 cells (and macrophages) appear to enhance immunity to malaria through the production of hydrogen peroxide, reactive oxygen intermediates, reactive nitrogen intermediates, several cytokines and enzymes, which kill erythrocytic stages of malaria parasites by phagocytosis, secretion of cytotoxic factors and antibody-dependent cell-mediated cytotoxicity (ADCC) (Taylor-Robinson and Phillips, 1993; Al Yaman *et al.*, 1996; Favre *et al.*, 1999).

Antibody producing Th2 cells and IL-4 have been associated with the clearance of parasitaemia (Langhorne *et al.*, 1989; Stevenson and Tam, 1993; Taylor-Robinson and Phillips, 1993), inhibition of hepatic stages of malaria and acceleration of immunity to *P. chabaudi, P. yoelii* and *P. berghei* malaria through increased production of anti-plasmodial immunoglobulins (IgG1, IgG2a and IgG2b) (Pied *et al.*, 1992; Vreden *et al.*, 1992; Akanmori *et al.*, 1996). Enhanced activity of Th2 cells appears to resolve infection through antibody-mediated immunity (Deans

and Cohen, 1983; Taylor-Robinson and Smith, 1999) which block merozoite dispersion, inhibit cytoadherence, increase killing by ADCC and intracellular killing of erythrocytic stages (Hommel, 1996). In addition to these, immunity to malaria appears to be dependent on a balanced production of pro-inflammatory and anti-inflamatory cytokines from Th1 and Th2 cells, respectively (de Kossodo and Grau, 1993; Peyron *et al.*, 1994; Othoro *et al.*, 1999).

The pathogenesis and development of SMA has been reported as a complex interaction of multiple mechanisms (Kurtzhals *et al.*, 1998; Menendez *et al.*, 2000; Ong'echa *et al.*, 2006). However, the dysregulation of immune mediators appears to play a central role in the manifestation of SMA (Akanmori *et al.*, 2000; McElroy *et al.*, 2000; Lyke *et al.*, 2004), indicating that host genetic changes may be involved in malaria disease pathogenesis (Kurtzhals *et al.*, 1998; Akanmori *et al.*, 2000; Weatherall *et al.*, 2002). A number of studies have associated excessive Th1 and inadequate Th2 cytokine production with bone marrow suppression, dyserythropoiesis and erythrophagocytosis (Clark and Chaudhri, 1988; Anstey *et al.*, 1999; Kurtzhals *et al.*, 1998), which are linked to SMA. Further studies have shown that Th1 cytokines channel metabolically available iron to macrophages, thus limiting erythropoiesis (Abdalla, 1990; Means *et al.*, 1992; Weiss *et al.*, 1995). The Th2 cytokines, on the other hand, counteract anaemia by stimulating bone marrow function. As a result, childhood SMA has been associated directly and indirectly with Th1 and Th2 cytokine responses, respectively (Day *et al.*, 1999; Dodoo *et al.*, 2002; Ekvall, 2003). Hence, cytokines appear to play a key role in malaria outcome, iron homoeostasis and host response to parasitic infections (Taylor-Robinson and Phillips, 1993; Romagnani *et al.*, 1997; Nyakeriga *et al.*, 2005).

2.6 Genetic Variation, Cytokine Polymorphism and Malaria

A SNP is a unique nucleotide base difference between two DNA sequences (Fusari *et al.*, 2008). It has been estimated that SNPs occur on average once after every 250-1000 base pairs and account for 90% of DNA sequence variation in the human genome (Kwok and Chen, 1998; Ke *et al.*, 2004). The high density and mutational stability of SNPs make them useful DNA markers for population genetics and for mapping susceptibility genes for complex diseases (Brookes, 1999). The high frequency by which SNPs occur on the genome provides utility for trait or disease gene discovery purposes (Kruglyak, 1997).

Studies have demonstrated associations between cytokine SNPs and infections, chronic and autoimmune diseases (Bidwell *et al.*, 1999; Wood *et al.*, 2001). Cytokine promoter SNPs appear to modulate disease rather than parasitaemia (Hill *et al.*, 1991). SNPs in the promoter influence clinical outcomes by altering gene expressions, subsequent activities or quantities of gene products in the serum (Demichele *et al.*, 2003). Genetic factors affect the immune response to *P. falciparum* infection and influence the pathologic process towards SMA or CM (McGuire *et al.*, 1999; May *et al.*, 2000; Dodoo *et al.*, 2002).

It has been observed that the transition from G to C at the IL-6 -174 functionally alters gene transcription and serum levels of IL-6, with C allele variants having lower expression than G alleles (Fernandez-Real *et al.*, 2000; Terry *et al.*, 2000; Demichele *et al.*, 2003). The transcription of IL-6 is enhanced by the G allele, which has been associated with poor outcome in autoimmune and inflammatory diseases (Hulkkonen *et al.*, 2001; Humphries *et al.*, 2001; Belluco *et al.*, 2003). Further studies have associated IL-6 -174GC with higher IL-6 levels and

susceptibility to autoimmune diseases while IL-6 -174CC genotypes have been associated with lower circulating IL-6 levels (Fishman *et al.*, 1998; Jahromi *et al.*, 2000; Hulkkonen *et al.*, 2001). For IL-6 -597G/A SNP, the GG genotype has been associated with higher disease activity and disability compared with GA patients (Wilkening *et al.*, 2006). IL-6 G -636C SNP has been associated with systolic blood pressure and carotid intima-media thickness, which enhances blood pressure regulation and progression of atherosclerosis (Tanaka *et al.*, 2005). Since the IL-6 G -636C SNP has not been investigated in malaria, the current study investigated the functional association between IL-6 -636G/C and malaria disease severity in a *P. falciparum* holoendemic area of western Kenya.

Variants in the IL-4 -589T/C and high gene expression of IL-4, IL-6, IL-10 and TNF-α, have been reported to enhance the risk of developing cutaneous leishmaniasis and systemic lupus erythematosus (SLE) (Richaud-Patin *et al.*, 1995; Kamali-Sarvestani *et al.*, 2006). Recent studies in Thai adults showed that IL-4 -589T/C SNP controlled parasitaemia, modulated the balance between IL-4 and IFN-γ, thus altering the severity of malaria (Tangteerawatana *et al.*, 2007). As a result, elucidating the functional association of this polymorphism in a paediatric population naturally exposed to *P. falciparum* malaria appears critical.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Site

This study was carried out at Siaya District Hospital (SDH) in Siaya District, Nyanza Province, western Kenya (see Figure 3). The hospital is located in an area that is holoendemic for *P. falciparum* transmission with an average of 100 to 300 infective mosquito bites per annum (Beier *et al.*, 1994). SDH is located approximately 60 kilometers from Nyanza Provincial Headquarters in Kisumu District. SDH borders Busia District to the north, Kisumu District to the southeast, Vihiga and Butere-Mumias Districts to the northeast. The district covers approximately 1520 km^2 and lies between latitude 0^o 26 degrees south to 0^o 18 degrees north and a longitude of 33^o 58' east to 34^o 33' west. Siaya District experiences an equatorial climate, a temperature range between 15 to 30°C, an annual rainfall range from 800mm to 2000mm, and has an altitude of between 1140 to 1430 meters above the sea level with sloping ridges and hills with rivers that drain into Lake Victoria (Kenya, 2001). Due to these climatic conditions and topography, intense malaria transmission coincides with seasonal rainfall in April to August and November to January (Beier *et al.*, 1994).

The human population in the district is estimated to be 524,633 of which 81,304 are children less than 5 years age (Bulletin, 2003). The population growth rate stands at 0.9% (Bulletin, 2003), while infant mortality is about 176/1000 (Bulletin, 2003). Malaria is the main cause of maternal and childhood morbidity and mortality in the district (WHO, 2000). The four leading conditions accounting for the majority of inpatient morbidity at the hospital are malaria (31.6%), anaemia (10.4%), pneumonia (8.6%) and diarrhoeal diseases (5.8%) (Bulletin, 2003).

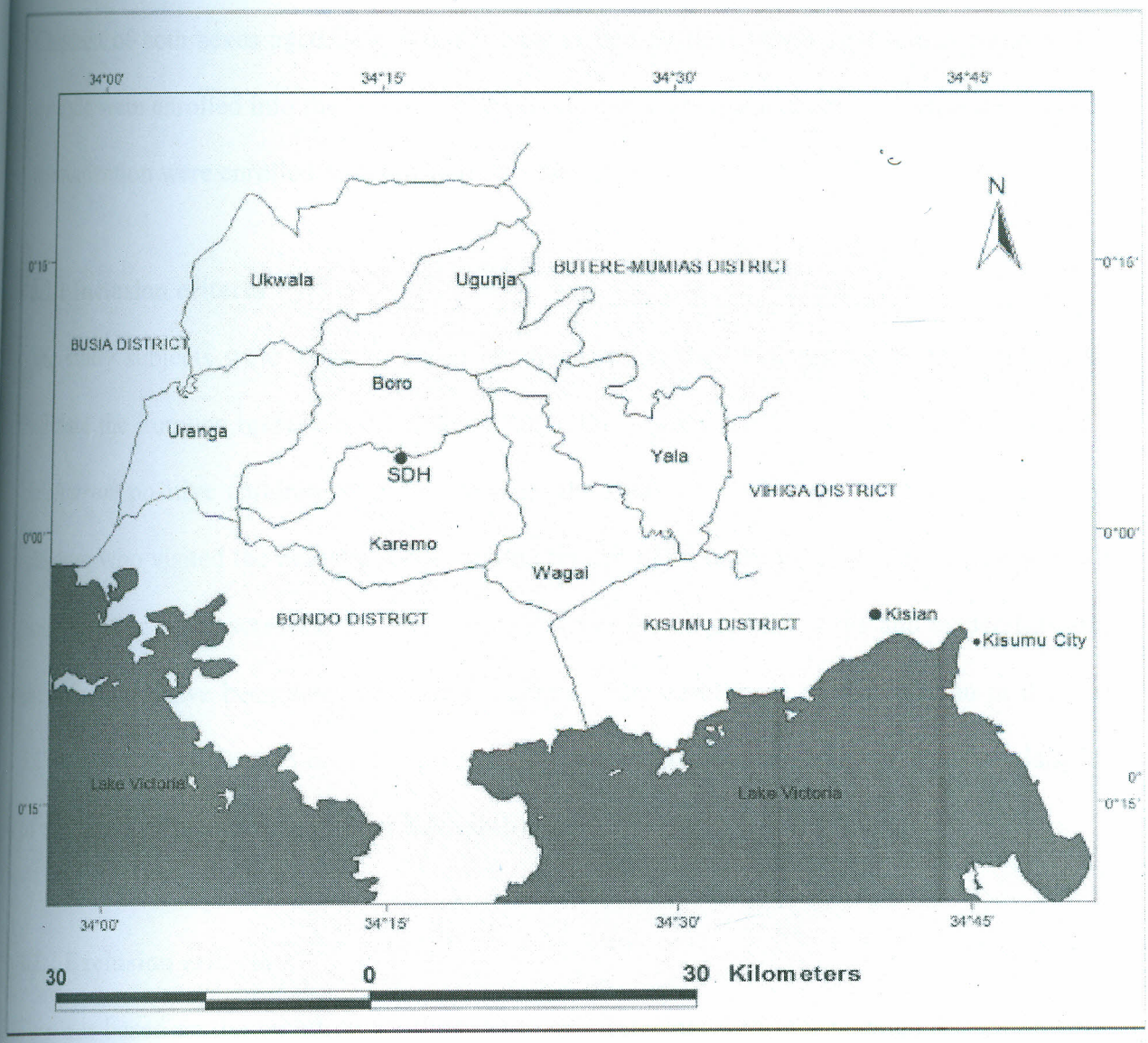


Figure 3. Map of Siaya District Showing the UNM/KEMRI Study Site (SDH)

(Map courtesy of Ong'echa *et al.,* 2006).

3.2 Study Population

Children of both sexes aged 3-36 months who visited SDH for their first documented malaria episode were enrolled into the study. Children without acute malaria who visited the hospital for immunization were enrolled as aparasitemic controls.

3.2.1 Inclusion criteria

The study subjects were selected based on the duration they had resided in the study site to validate the immune responses on children naturally exposed to *P. falciparum* infection, and *P. falciparum* positive children were enrolled into the study. Healthy, age-matched asymptomatic children who visited the maternal/child health clinic at SDH for routine immunizations were also enrolled after being screened for malaria, hookworm infection, HIV virus and bacteremia since the infections have been associated with anaemia. The enrollment of the children in the study followed written-informed consents given by the parents/guardians of the children, in a language of their choice (Dholuo, English or Kiswahili).

3.2.2 Exclusion criteria

This was based on the withdrawal or refusal to give informed consent, planned relocation within 6 months of the first hospital contact, prior hospitalisation for any reason including malaria-associated hypoglycaemia, and CM.

3.2.3 Ethical considerations

The study was approved by the ethical and scientific review committee at the Kenya Medical Research Institute (KEMRI) (See Appendix III). All parents/guardians of children received a complete explanation of the study and their participation was requested in their native language

or a language of their choice. They were given the opportunity to refuse or provide written consent before enrolment (See Appendix II). Informed consent was obtained from all the parents/guardians of children before enrolment. Participation in the study was voluntary and the participants could withdraw at any time. Access to health care was independent of participation.

All the samples collected from the study participants were code-numbered for identification and the investigators controlled access to the data. Sampling from patients and healthy controls by finger/heel prick was performed for all parasitological, immunological and genetic analysis. The sampling of blood is known to cause temporary discomfort, bruising, and pain; hence, the risks due to finger (or heel) pricks were reduced by using trained and qualified clinical staff, which carried out the process in a sterile manner to minimise risk of infection. Sterility was ensured by using betadine swabs to disinfect sites for injection. Furthermore, sterile disposable lancets were used and all sharps were stored in the appropriate biohazard sharps' containers before disposal. The sampled blood was examined for malaria parasites and anaemia; patients with malaria and anaemia received anti-malarial treatment and vitamins supplemented with iron, respectively. The aforementioned treatments as well as patient care were provided free of charge.

3.2.4 Experimental design

This was a hospital based cross-sectional study, which was part of a larger prospective longitudinal study, investigating the immunological and genetic basis of paediatric severe malaria. The study participants were categorised according to their Hb levels and presence or absence of *P. falciparum*. Heel/finger-prick blood (<100 μL) was used to determine

parasitaemia and Hb status. Screening for Hb levels was performed on heel/finger-prick blood using a HemoCue system (HemoCue AB, Angelholm, Sweden).

Based on the results of Hb and parasitaemia status, parasitaemic children were grouped as SMA (Hb<6.0g/dL) and non-SMA (Hb≥6.0g/dL). The study participants were further categorized as aparasitaemic healthy controls (AC; Hb>11 g/dL; children with malaria-negative smear for *P. falciparum* parasitaemia and free of fever or diarrhoea) for comparisons with the LDP (<10,000 malaria parasites /μL) and HDP (≥10,000 malaria parasites/μL) groups.

3.2.5 Sample Size Determination

The sample size was determined by a power and sample size calculation programme (version, 2.1.31) (Dupont and Plummer, 1990) for studies using Chi-square, Fisher's exact tests and genetic studies showing association with HDP (Awandare *et al.*, 2006). Hence, in this study, the ratio (m) of controls to cases was 114/363 = 0.314. The probability (P_0) of exposure in controls was obtained from previous studies in an adjacent study area in western Kenya which demonstrated that *P. falciparum* malaria had prevalence of 88.3% (Bloland *et al.*, 1999). The probability (P_1) of *Plasmodium falciparum* infection in malaria cases was set at 88.3%. Thus, using an α-value of 0.05 and power of 80%, the sample size for AC using power and sample size calculation programme was = 60 (Dupont and Plummer, 1990). The number of malaria cases was determined by cross-multiplication and division of 60 with the ratio (m) between controls and cases (114/363) (Awandare *et al.*, 2006), which gives 452. As a result, 452 children with acute malaria and 60 AC were needed to test the hypotheses in this study. However, a total of 111 AC and 507 parasitemic individuals were enrolled to determine the functional association

between IL-4 -589T/C and malaria disease outcomes, while 113 AC and 489 parasitemic individuals were used for IL-6 -636G/C and malaria disease outcomes, which included 10-20% additional individuals who could be lost during follow-up for the longitudinal study.

3.3 Methods of Data Collection

All the research activities were undertaken at SDH and KEMRI (Kisumu) in the laboratories of UNM/CGHR/KEMRI Project. The data collection for this study was carried out from December, 2006 to May, 2008. The study was divided into clinical, parasitological, laboratory and immunogenetic investigations. The following methods were employed:

3.3.1 Clinical and parasitological examinations

All the study subjects were clinically examined on admission or routine visit to SDH by study clinicians. Each study participant underwent a full clinical and parasitological examination, including screening for *P. falciparum* parasitaemia, hookworm infection, human immunodeficiency virus (HIV virus) and bacteremia status since these infections have been associated with anaemia.

For each study participant, 3mL of venous blood was obtained in EDTA Vacutainer tubes; however, tubes without anticoagulants were used for serum measurements (Becton-Dickinson, San Diego, CA). The collected blood was used as follows: 50µL dried on filter papers and used for DNA extraction (stored at -80^{0}C and later used in DNA isolation for Taqman 5' genotyping assays), 10µL for Hb electrophoresis, 20µL for full haemogram by a Coulter Counter® (Beckman

Coulter AcT diff2) and ~2.9mL of blood used for plasma separation (used for cytokine/chemokine measurements by Human 25 Plex Cytokine Assay).

3.3.2 Cytokine measurements

Plasma samples stored at -80°C were used to determine cytokine profiles. The samples were thawed and centrifuged at 14,000 rpm (Forma Scientific Centifuge, Model No. 5681, USA) for 30 seconds to remove fibrin clots and precipitates. The IL-4 and IL-6 cytokine levels were assayed using the Human Cytokine Twenty-Five-Plex Antibody Bead Kit (Biosource International; LHC0008, Camarillo, CA, USA). In this assay, plates were read on a Luminex 100 system (Luminex) and analyzed using Bio-Plex Manager software (version IS 2.3; Bio-Rad Laboratories). The lower detection limit was 4 pg/mL for IL-4 (Bead region 77) and 3 pg/mL for IL-6 (Bead region 19). A control prepared from a pool of three healthy donors was aliquoted and used between assays for standardization of results.

Briefly, the filter bottom microplate wells were pre-wetted with working wash solution and thereafter aspirated using a vaccum manifold. The plates were washed twice and incubation buffer added into each well. Analyte-specific capture antibodies that are conjugated to beads were then pippeted into each well. A standard of analyte concentration, control samples and the test plasma samples, all in duplicates, were added into the designated wells. The test plates were sealed and foiled followed by incubation in the dark at room temperature for 2 hours on an orbital sharker. Following incubation, the plate wells were aspirated and washed twice with working solution. An analyte-specific biotinylated detector antibody was added to each well and the plates incubated in the dark at room temperature for 1 hour on a sharker. Wells were

aspirated, washed twice and streptavidin-R-Phycoerythrin added to each well. The plate was incubated in the dark for 30 minutes on an orbital sharker, aspirated and then washed three times. Washing solution was aliqouted into the wells and the mixture shaken for 2-3 minutes to resuspend the beads.

The spectral properties of the capture beads and the amount of associated R-Phycoerythrin fluorescence were used to determine the analyte concentrations using a Luminex 100 instrument (Luminex Corporation Inc., Hercules, USA). Analysis of the results was performed using the Bio-plex manager software (Biorad Laboratories Inc., Hercules, USA).

3.3.3 Genotyping for polymorphisms

DNA for determining the IL-4 and IL-6 SNP genotypes was extracted from dry blood spots using the Gentra System DNA isolation kit (Gentra, Minneapolis, MN, USA). The IL-4 -589T/C and IL-6 -636G/C SNPs were genotyped using a Taqman 5' allelic discrimination Assay-By-Design method (Applied Biosystems, Foster City, CA, USA). PCR for the Taqman assays for both the IL-4 -589T/C and IL-6 -636G/C were performed in a total volume of 5 μL with the following amplification protocol: 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute. Following PCR, the genotype of each individual was assigned automatically by measuring the allelic specific fluorescence on the ABI PRISM 7900HT Sequence Detection Systems using the SDS 2.1 software for allelic discrimination (Applied Biosystems).

3.4 Data Management, Statistical Analysis and Presentation

Data entry, cleaning, validation, and analysis were performed using SPSS (version 12.0, Chicago, Illinois, USA). Across group comparisons of medians were determined using Kruskal-Wallis tests and whenever significant, pair-wise Mann-Whitney U tests were carried out. Chi-square analyses were used to examine differences between proportions. Multivariate logistic regression analyses were used to determine the associations between IL-4 -589T/C and IL-6 -636G/C with malaria disease outcomes (acquisition of *Plasmodium falciparum* parasitaemia, HDP, SMA); however, it was necessary to control for age, gender, sickle-cell trait, HIV-1 infection and bacteremia since they have been associated with differential susceptibilities to malaria disease outcomes in such populations from malaria endemic regions (Aidoo *et al.*, 2002; Bloland *et al.*, 1999; Owusu-Agyei *et al.*, 2002; Otieno *et al.*, 2006). Analyses were performed using a modified definition of SMA (Hb < 6.0 g/dL) (McElroy *et al.*, 1999), while HDP was based on a standard definition of ≥ 10,000 parasites/μL (McElroy *et al.*, 1999; Otieno *et al.*, 2006; Ouma *et al.*, 2006). Statistical significance was defined as $P \leq 0.05$. The results of this study have been presented in scientific conferences (See Appendix 1).

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic, Clinical and Parasitological Characteristics of the Study Subjects Genotyped for the IL-4 -589T/C and IL-6 -636T/C Polymorphisms

The results of the demographic, clinical and parasitological characteristics of the study subjects for the IL-4 -589T/C and and IL-6 -636T/C polymorphisms are summarised in Table 1. For IL-4 -589T/C, aparasitemic controls (AC, n=111), children with LDP (n=188) and HDP (n=319) were examined. The results showed no significant differences in the distribution of the male and female children among the three clinical groups (AC, LDP and HDP; P=0.074; Chi-square analysis; see Table 1). Similarly, age was comparable across the clinical groups (P=0.069; Kruskal Wallis test). Hb concentrations were significantly different across the three groups (P<0.0001; Kruskal Wallis test). The AC children had significantly higher Hb levels compared to children with LDP (P<0.0001; Mann-Whitney U test) or HDP (P<0.0001; Mann-Whitney U test). Children with LDP had significantly lower Hb compared to those with HDP (P<0.001; Mann-Whitney U test). The proportion of children with SMA (Hb<6g/dL) was significantly higher in the HDP group relative to the LDP group (P=0.025; Chi-square analysis).

Table 1: Demographic, Clinical, and Parasitological Characteristics of the Study Subjects Genotyped for the IL-4 -589T/C and IL-6 -636T/C Single Nucleotide Polymorphisms.

		IL-4 -589T/C				IL-6 -636G/C			
Characteristic		AC	LDP	HDP	*P*	AC	LDP	HDP	*P*
No. of subjects, n		111	188	319		113	179	310	
Gender (%)[a]	Male	50 (45)	107 (57)	153 (48)		47 (42)	100 (56)	155 (50)	
	Female	61 (55)	81 (43)	166 (52)	0.074[a]	66 (58)	79 (44)	155 (50)	0.059[a]
Age (in months)[d]		8.0 (11)	10.84 (9)	9.0 (9)	0.069[c]	8.0 (9)	10.0 (9)	9.0 (9)	**0.027**[c]
Haemoglobin (g/dL)[d]		9.8 (5.1)	6.1 (3.2)	6.9 (3.6)	**<0.0001**[c]	9.8 (5.0)	6.1 (3.1)	6.8 (3.6)	**<0.0001**[c]
Parasite density (µ/L)[d]		0	3,338 (4,986)	38,074 (56,551)	**<0.0001**[b]	0	3,338 (5,153)	37,327 (56,461)	**<0.0001**[b]
SMA, n, (%)		N/A	79 (42.5)	107 (57.5)	**0.025**[a]	N/A	72 (42.4)	109 (36.5)	0.123[a]

Legend: Aparasitemic control (AC), children for routine immunization; low-density parasitaemia (LDP, parasites/µL <10,000); high-density parasitaemia (HDP, parasites/µL ≥10,000); severe malaria anaemia (SMA, Hb < 6.0 g/dL, with any density parasitaemia). N/A (not applicable). Statistical analysis was determined by: - [a] Chi-square analysis, [b] Mann-Whitney U test and [c] Kruskal Wallis test. [d] Data are presented as median (interquartile range).

The table shows that (i) AC children in the IL-6 -636 SNP were significantly younger than their counterparts with LDP and HDP (ii) AC in both IL-4 -589T/C and IL-6 -636G/C had significantly higher Hb than either LDP or HDP and (iii) The proportion of children with SMA was significantly higher in the HDP than the LDP group for the IL-4 -589T/C SNP.

Concerning the demographic, clinical and parasitological characteristics of the study subjects of the IL-6 -636G/C SNP, AC children (n=113), children with LDP (n=179) and HDP (n=310; see Table 1) were analyzed. The results did not demonstrate significant differences in the distribution of male and female children among the clinical groups (P=0.059; Chi-square analysis). Age was significantly different across the groups (P=0.027; Kruskal Wallis test), largely because AC children were significantly younger than those with LDP (P<0.05; Mann-Whitney U test) and HDP (P<0.01; Mann-Whitney U test). Hb concentrations were significantly different across the three groups (P<0.0001; Kruskal Wallis test). AC children had significantly higher Hb levels compared to children with LDP (P<0.0001) or HDP (P<0.0001; Mann-Whitney U test). Children with LDP had significantly lower Hb levels compared to those with HDP (P<0.05; Mann-Whitney U test). The proportions of children with SMA in the LDP and HDP groups were not significantly different (P=0.123; Chi-square analysis).

4.2 Genotypic Distributions of IL-4 -589T/C and IL-6 -636G/C in the Clinical Groups

The genotypic distributions of the IL-4 -589T/C and IL-6 -636G/C are summarised in Table 2. For IL-4 -589T/C, AC (n=111) and children with acute malaria (n=507) were examined. Overall, among children examined (n=618), 63% were TT, 24% were CT and 13% were CC (Table 2), representing a significant departure from Hardy-Weinberg equilibrium (HWE) (χ^2=81.32, P<0.005; Chi-square analysis). The genotypic distribution in AC was 59% TT, 26% CT and 15% CC (Table 2). Frequencies of the T and C alleles were 0.72 and 0.28, respectively, in AC with departure from HWE (χ^2=14.17, P<0.005; Chi-square analysis). Among children with acute malaria, there were 64% TT, 23% CT and 13% CC (Table 2), yielding allele frequencies of T=0.76 and C=0.24, respectively. Likewise, there was significant evidence of departure from HWE (χ^2=66.83, P<0.005; Chi-square analysis). Overall analysis revealed that there were no significant differences in the frequency distribution of the IL-4 -589T/C polymorphism in malaria cases compared to controls (P=0.553; the Chi-square analysis; Table 2).

Regarding IL-6 -636G/C genotypic distribution, AC (n=113) and children with acute malaria (n=489) were examined (see Table 2). In the 602 children examined, 83.7% were GG, 12.5% were CG and 3.8% were CC, representing a significant departure from Hardy-Weinberg equilibrium (HWE; χ^2=58.19, P<0.005; Chi-square analysis). The genetic distribution in AC was 77.9% GG, 15.9% CG and 6.2% CC (Table 2). Frequencies of the G and C alleles were 0.86 and 0.14, respectively, in AC with departure from HWE (χ^2=13.43, P<0.005; Chi-square analysis). Among children with acute malaria, there were 3.3% CC, 11.7% CG and 85.1% GG (Table 2), yielding allele frequencies of G=0.91 and C=0.09, respectively. Likewise, there was significant evidence of departure from HWE (χ^2=42.68, P<0.005; Chi-square analysis) in children with acute malaria. Overall analysis revealed that there were no

significant differences in the frequency distribution of the IL-6 -636G/C polymorphism in malaria cases compared to controls (P=0.138; Chi-square analysis).

Table 2. Distribution of IL-4 -589T/C and IL-6 -636G/C Genotypes in the Clinical Groups.

	Genotype	AC (*n*, %)	Malaria cases (*n*, %)	Total (*n*, %)	*P*
IL-4 -589T/C	TT	65 (59)	324 (64)	389 (63)	
	CT	29 (26)	119 (23)	148 (24)	0.553[a]
	CC	17 (15)	64 (13)	81 (13)	
Total		*n*=111	*n*= 507	*n*= 618	
Allele frequencies		*P* (T)=0.71	*P* (T)=0.76	*P* (T)=0.75	
		P (C)=0.29	*P* (C)=0.24	*P* (C)=0.25	
IL-6 -636G/C	GG	88 (77.9)	416 (85.1)	504 (83.7)	
	CG	18 (15.9)	57 (11.7)	75 (12.5)	0.138[a]
	CC	7 (6.2)	16 (3.3)	23 (3.8)	
Total		*n*=113	*n*= 489	*n*= 602	
Allele frequencies		*P* (G)=0.86	*P* (G)=0.91	*P* (G)=0.90	
		P (C)=0.14	*P* (C)=0.09	*P* (C)=0.10	

Legend: IL-4=Interleukin-4; IL-6=Interleukin-6; *P* (T) and *P* (G) are frequencies of wild alleles, while *P* (C) is the frequency of mutant allele in the population, respectively. Data are presented as proportions. Children were categorized based on the absence (aparasitaemic controls) or presence of parasitaemia (malaria cases). [a]Statistical significance was determined by the Chi-square analysis.

The table shows that the distribution of A/C and malaria cases in the population is independent of IL-4-589T/C and IL-6-636 genotypes.

4.3 Associations Between IL-4 -589T/C, IL-6 -636G/C and Malaria Disease Outcomes

The results for the associations between variation at IL-4 -589T/C, IL-6 -636G/C and malaria disease severity, determined by multivariate logistic regression analysis, are summarised in Table 3. Parasitaemia (*P. falciparum*-positive blood smear), HDP, and SMA were the primary disease outcomes. For IL-4 -589T/C, relative to homozygous T alleles, the CT (OR; 0.84, 95% CI; 0.51-1.38, *P*=0.498) and CC genotypes (OR; 0.81, 95% CI; 0.44- 1.49, *P*=0.502) were not associated with parasitaemia (Table 3). However, among parasitaemic children, relative to the homozygous T, individuals heterozygous (i.e. TC) (OR; 1.64, 95% CI; 1.01-2.65, *P*=0.044) were significantly associated with increased risk of having HDP, while presence of CC genotype (OR; 1.26, CI; 0.70-2.25, *P*=0.440) was not associated with HDP (Table 3). Additional analyses of the relationship between the IL-4 -589T/C polymorphism and SMA (Hb<6.0g/dL) showed that neither the presence of the CC (OR; 0.71, CI; 0.39-1.29, *P*=0.259) nor the CT genotypes (OR; 1.00; 95% CI; 0.64-1.57, *P*=0.986), altered the risk to the development of SMA in parasitaemic children (Table 3).

Regarding the association between variation at IL-6 -636G/C and malaria disease outcomes, relative to homozygous G alleles, the CG (OR; 0.67, 95%CI; 0.37-1.22, *P*=0.190) and CC genotypes (OR; 0.45, 95%CI; 0.17-1.15, *P*=0.095) were not associated with acquisition of *P. falciparum* parasitaemia (Table 3). Among the parasitaemic children, relative to the GG group, the CG (OR; 1.18, 95%CI; 0.64-2.16, *P*=0.593) and CC (OR; 2.41, 95%CI; 0.66-8.75, *P*=0.182) genotypes were not associated with HDP (Table 3). Additional analyses of the relationship between the IL-6 -636G/C polymorphism and SMA (Hb<6.0g/dL) showed that CC (OR; 0.80, 95% CI; 0.26-2.40, *P*=0.684) and CG (OR: 1.33, 95% CI; 0.74-2.39, *P*=0.347) groups, relative to the GG genotype, were not associated with SMA (Table 3).

Table 3. Association of IL-4 -589T/C and IL-6 -636G/C polymorphisms with malaria disease severity

SNPs	Genotype	Acquisition of parasitaemia[a]			SMA (Hb<6.0 g/dL)[b]			HDP (≥ 10,000 MPS/μL)[b]		
		OR	95%CI	*P*	OR	95%CI	*P*	OR	95%CI	*P*
IL-4- 589T/C	TT	1.00			1.00			1.00		
	TC	0.84	0.51-0.18	0.498	1.00	0.64-1.57	0.986	**1.64**	**1.01-2.65**	**0.044**
	CC	0.81	0.44-1.49	0.502	0.71	0.39-1.29	0.259	1.26	0.70-2.25	0.440
IL-6 -636G/C	GG	1.00			1.00			1.00		
	CG	0.67	0.37-1.22	0.190	1.33	0.74-2.39	0.347	1.18	0.64-2.16	0.593
	CC	0.45	0.17-1.15	0.095	0.80	0.26-2.40	0.684	2.41	0.66-8.75	0.182

Legend: Parasitaemia (*P. falciparum*-positive blood smear), HDP, and SMA were the primary disease outcomes. High-density parasitaemia (HDP≥10,000 parasites/μL); severe malaria anaemia (SMA, Hb<6.0 g/dL, with any density parasitaemia); Odds Ratios (OR) and 95% confidence interval (CI) were determined using multivariate logistic regression controlling for age, gender, HIV-1 status, bacteraemia and sickle cell trait (HbAS). IL-4 -589T/C and IL-6 -636G/C were analysed as combined groups of AC and malaria cases[a] , and [b]only malaria cases, yielding *n*=618 and *n*=507, *n*=602 and *n*=489, respectively for each polymorphism.

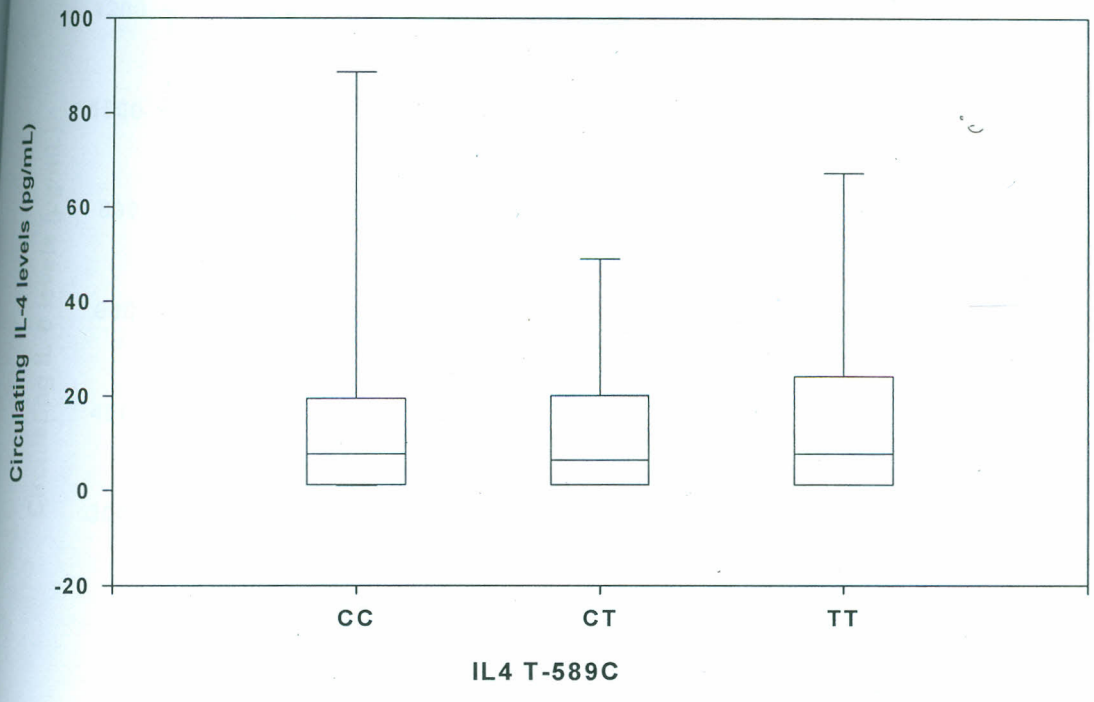
The table shows that children with IL-4 -589TC genotype is significantly associated with susceptibility to HDP.

4.4 Functional Associations Between IL-4 -589T/C and IL-6 -636G/C Variants and Circulating IL-4 and IL-6 Levels

The functional relationship between the IL-4 -589T/C polymorphism and circulating IL-4 concentrations is summarized in Figure 4. Children with acute malaria (n=198) were stratified according to IL-4 genotypes (TT, n=131; CT, n=46; CC, n=21). There was no significant differences in the levels of circulating IL-4 in the 3 genotypes (Kruskal Wallis test, P=0.841)

The functional relationship between the IL-6 -636G/C polymorphism and circulating IL-6 concentrations were examined in children with acute malaria (n=195) who were stratified according to IL-6 genotypes (GG, n=163; CG, n=27; CC, n=5) (Figure 5). There was no significant differences in the levels of circulating IL-6 in the 3 genotypes (Kruskal Wallis test, P=0.479).

Figure 4

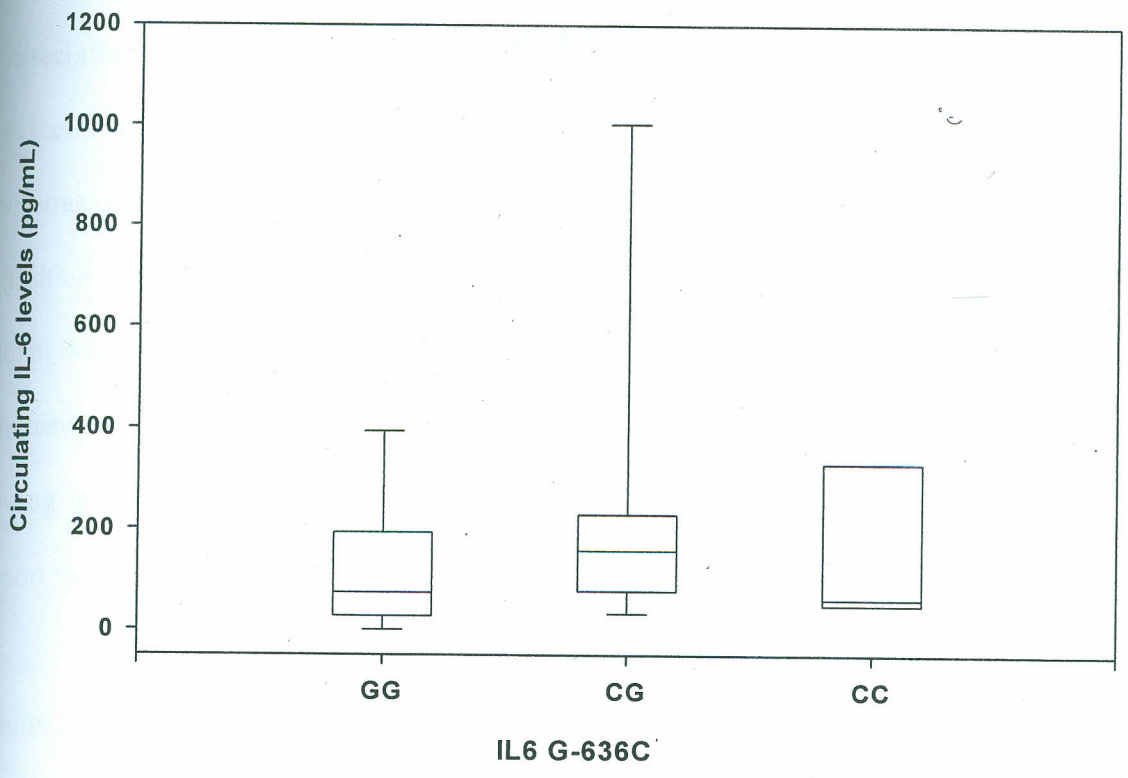


Functional Associations Between Individual IL-4 Promoter Variants and Circulating IL-4 Levels in Children with Malaria.

Circulating IL-4 levels (pg/mL) in children aged 3-36 months with malaria were measured using the Human Cytokine Twenty-Five-Plex Antibody Bead Kit and results are presented for IL-4 -589T/C (TT, n=131; CT, n=46; CC, n=21). Data are presented as box plots where the box represents the interquartile range, the line through the box is the median, and whiskers illustrate 10^{th} and 90^{th} percentiles. There were no significant associations between the IL-4 -589T/C genotypes and circulating IL-4 levels (Kruskal Wallis test, P=0.841).

The figure shows that circulating IL-4 levels are independent of IL-4 -589T/C genotypes.

Figure 5



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The figure shows that circulating IL-6 levels are independent of IL-6 -636G/C genotypes

CHAPTER FIVE

5.0 DISCUSSION

5.1 Associations Between IL-4 -589T/C and Malaria Disease Outcomes

Cross-sectional results on children aged 3-36 months presented on the IL-4 -589T/C demonstrated significant associations with HDP, but not any other malaria disease outcomes (acquisition of parasitaemia or SMA). Relative to homozygous T individuals, the heterozygous children were significantly at an increased risk of developing HDP. This finding is consistent with previous observations from the same cohort (Awandare *et al.*, 2006; Ouma *et al.*, 2006), showing that alleles that predisposes to HDP vary from those that protect from SMA, which support the notion that SMA and HDP may not be related in this cohort.

Results from this study demonstrated significant deviations from Hardy-Weinberg equillibrium in the genotypic distribution of IL-4 -589T/C. Such deviations can be attributed to evolutionary forces, possibly natural selection, that strives to fix advantageous alleles within the population. The driving forces in such endemic areas would be a continous exposure to polygenic diseases such as malaria (Kwiatkowski, 2005).

This study demonstrated significant association between IL-4 -589T/C polymorphism with malaria disease severity. Heterozygous individuals (TC) at the IL-4 -589 loci were significantly at an increased risk of developing HDP relative to homozygous T allele. Previous studies carried out in malaria endemic regions of West Africa revealed a significant association between IL-4 -590T allele and elevated anti-malarial IgG levels among the Fulani (Luoni *et al.*, 2001), while a later study carried out in the same region demonstrated a higher prevalence of *P. falciparum* infection in individuals with the same allele (Vafa *et al.*, 2007). Other studies have shown that

IL-4 through its anti-inflammtory effects has better resolution on parasites. In a study carried out in West Africa in children aged below 6 years old, there was a negative correlation between serum levels of IL-4 and parasite density, suggesting a role for IL-4 in mechanisms leading to parasite clearance (Mshana *et al.*, 1991). In addition, IL-4 in synergy with IL-10 has been demonstrated to be important for parasite clearance in later antibody-mediated phases of infection (Troye-Blomberg *et al.*, 1994). In the current study, the IL-4 -589T/C individuals who were significantly at an increased risk of developing HDP produced the lowest IL-4 levels. Hence, the increased risk to HDP could be related to their inability to mount an effective anti-parasitic and antibody-mediated response.

In the current study, IL-4 -589T/C genotypes were not associated with SMA (Hb<6.0g/dL). The lack of significant association between the IL-4 genotypes and SMA may indicate that the gene may not be functionally related with disease in this population. Previous studies carried out in West African populations in children aged between 6 months and 15 years did not find any associations between the IL-4 -589T/C polymorphism and severe malaria as defined by mixed clinical phenotypes (any density parasitaemia together with coma, prostration, repeated convulsions, Hb<5 g/dL, pulmonary oedema/respiratory distress, renal failure) (Verra *et al.*, 2004), while other studies carried out in Thai adults did not also reveal associations with malaria disease severity (defined with mixed clinical phenotype; hypoglycaemia, Hb<7 g/dL, haematocrit<20% or creatinine>30 mg/dL) (Tangteerawatana *et al.*, 2007). Thus, to delineate the relationships between the IL-4 promoter genotypes and susceptibility to SMA, additional studies have been initiated.

The genes encoding IL-4, IL-5 and IL-13 are located in the complex 5q31-33 chromosome region (Choi *et al.*, 2002; Hosseini-Farahabadi *et al.*, 2007; Tavakkol Afshari *et al.*, 2007), where in addition to the production of IL-4, they encode cytokines, growth factors and receptors, which appear to control immunity to *P. falciparum* (Gomez *et al.*, 1998; Leite-De-Moraes *et al.*, 1998; Rihet *et al.*, 1998; Luoni *et al.*, 2001). Previous studies have shown that the IL-4 gene promoter SNPs affect IL-4 transcription, production and disease progression (Scarel-Caminaga *et al.*, 2003; Nakkuntod *et al.*, 2004; Gervaziev *et al.*, 2006; Kleinrath *et al.*, 2007).

In this study, even though there was a trend in which the IL-4 -589TT and IL-4 -589CC genotypes appeared to be associated with increased IL-4 production relative to the CT genotype, this relationship was statistically not significant. These observations are inconsistent with the previous *in vitro* studies demonstrating that relative to IL-4 -589C allele, the IL-4 -589T has higher luciferase reporter activity and a different pattern of protein binding on electrophoretic mobility shift assay (EMSA) (Borish *et al.*, 1994; Rosenwasser *et al.*, 1995; Nakashima *et al.*, 2002). Such differences are not unexpected given that complex genetic interactions may exist *in vivo* relative to *in vitro* assays. Hence, further studies are warranted to delineate whether carriage of different genotypeswithin the IL-4 -589T/C affect transcription and disease severities in paediatric populations naturally exposed to *P. falciparum* malaria.

5.2 Associations Between IL-6 -636G/C and Malaria Disease Outcomes

It was hypothesized that polymorphic variability at the IL-6 -636G/C would be associated with malaria disease outcomes in paediatric population less than 3 years resident in *P. falciparum* malaria holoendemic transmission areas. Results of this study have demonstrated that relative to

homozygous G alleles, the CG and CC genotypes were not associated with acquisition of *P. falciparum* parasitaemia. Amongst parasitaemic children, the same genotypes were also not associated with HDP and SMA. Taken together, these findings show that IL-6 -636G/C polymorphism is not associated with the acquisition of parasitaemia, development of HDP and SMA in children presenting for their first time with acute malaria in this holoendemic *P. falciparum* transmission area.

Polygenic diseases such as malaria have exerted significant selective pressure on the human genome, particularly in host-immune response genes that mediate susceptibility and clinical outcomes of disease (Kwiatkowski, 2005). Allele frequencies of IL-6 -636G/C variants presented in this study are comparable to those observed in previous studies in African reference populations (Hapmap, dbSNP rs1800796). However, none of these previous studies examined frequencies of these variants in *P. falciparum* holoendemic transmission area. Genotypic distribution in this study showed a significant departure from HWE for the IL-6 -636G/C promoter variants, likely illustrating that the gene could be under selection. However, the selective forces may be uncertain or may be operating in positions in close proximity to the current promoter region, given that there were no significant associations between the variants and any of the clinical outcomes.

Previous studies carried out in the adjoining villages (Wangarot, Riwa Ojelo, and Waringa, Rarieda Division) in western Kenya demonstrated significant associations between IL-6 -174 and malaria disease severity in males aged between 12 and 35 years (Gourley *et al.*, 2002). However, no study to date has investigated the functional associations between IL-6 -636G/C

and malaria disease severity in malaria. This study presents the first finding demonstrating that this polymorphism is not associated with the acquisition of parasitaemia and development of HDP and SMA. Additional analyses have revealed that none of the genotypes was associated with protection or increased risk to SMA using both modified (Hb<6.0g/dL) (McElroy *et al.*, 1999) and WHO definition of SMA (Hb<5.0g/dL) (WHO, 2000), further demonstrating that this polymorphism may not be associated with susceptibility to SMA.

In this study, genetic variation at IL-6 -636G/C was not associated with functional changes in circulating IL-6 levels in children with malaria. Previous studies have demonstrated that the IL-6 gene is located on chromosome 7p21 and the polymorphisms within the IL-6 gene promoter affect IL-6 transcription and production (Greisenegger *et al.*, 2003; Sawczenko *et al.*, 2005; Weger *et al.*, 2005; Walston *et al.*, 2007). Further studies have demonstrated that the human IL-6 promoter contains multiple regulatory elements, which bind transcription factors belonging to the NF-kappaB (-75/-63), C/EBP (-158/-145 and -87/-76) and AP-1 (-283/-277) families (Faggioli *et al.*, 1997; Bergamini *et al.*, 1999; Faggioli *et al.*, 2004). Through such promoter regulation, IL-6 has been demonstrated to affect both innate and acquired immunity in infectious, and inflammatory and autoimmune diseases (Esfandi *et al.*, 2006; Esteve *et al.*, 2007; Kaden, 2007; Moreira *et al.*, 2007; Noto *et al.*, 2007; Sainz *et al.*, 2008); however, in the current study, the variants did not alter susceptibility to malaria disease outcomes.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study has demonstrated that parasitemic children with the IL-4 -589TC genotype were significantly more susceptible to HDP; however, the IL-4 -589T/C promoter SNP was not associated with either SMA or acquisition of *P. falciparum* parasitaemia. The IL-6 -636G/C SNP, on the other hand, was not significantly associated with acquisition of *P. falciparum* parasitaemia, HDP or SMA. In addition, the study showed that IL-4 -589T/C and IL-6 -636G/C genotypes were not functionally associated with circulating IL-4 and IL-6 levels, respectively. In summary, these findings provide an important first step for exploring the potential role of both IL-4 and IL-6 variants in malaria immunopathogenesis. This information could be useful in the designing of an effective vaccine against malaria and for identifying at risk groups that require more intense management for susceptibility to malaria.

6.2 Recommendations

Future studies should:

(a) Carry out haplotypic analyses with multiple of IL-4 and IL-6 SNPs since haplotypes are better predictors of disease severity than individual SNPs,

(b) Determine if variation in IL-4 -589T/C and IL-6 -636G/C can be used as predictors of malaria disease outcomes (morbidity and mortality) in a longitudinal study,

(c) Investigate the association between IL-4 -589T/C, IL-6 -636G/C and anti-malarial antibody levels, both cross-sectionally and longitudinally.

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