PREVALENCE OF SICKLE CELL TRAIT AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY AND THEIR ASSOCIATION WITH IMMUNO-GLOBULIN-G ANTIBODY RESPONSES TO SELECTED *PLASMODIUM FALCIPARUM* ANTIGENS IN KANYAWEGI SUB-LOCATION, WESTERN KENYA

BY

FWAYA MICHAEL OCHIENG'

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MASENO UNIVERSITY

DECLARATION

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

Fwaya Michael Ochieng'

PG/MSc/067/2011

Signature..... Date..... **23.11.2018**

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

Dr. Kiprotich Chelimo

Department of Biomedical Sciences and Technology,

Maseno University,

Maseno, Kenya.

Signature..... Date..... **28.11.2018**

Prof. Walter G.Z.O. Jura

Department of Zoology,

Maseno University,

Maseno, Kenya.

Signature..... Date..... **28.11.2018**

Prof. Kazura James

Case Western Reserve University

Cleveland, USA

Signature..... Date..... **28.11.2018**

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ABSTRACT

The prevalence of glucose-6-phosphate dehydrogenase (G6PD) deficiency and sickle cell trait (SCT) may vary within very close localities in malaria endemic areas due to dynamics in malaria incidence and demographics. Immunoglobulin-G (IgG) antibodies against *Plasmodium falciparum* (Pf) circumsporozoite protein (CSP) and apical membrane antigen-1 (AMA1) confer protection against malaria. Both G6PD deficiency and SCT increase resistance to Pf malaria and it is not well understood if this protection arise as a result of altered levels of IgG responses to these Pf antigens. The objectives of this study were to: determine the prevalence of SCT and G6PD deficiency; determine the association of SCT and G6PD deficiency with anti-CSP and anti-AMA1 IgG antibody responses in residents of Kanyawegi sub-location, Kisumu county of western Kenya. In this cross-sectional study, a total of 300 individuals from malaria endemic Kanyawegi sub-location participated. Venous blood was collected for laboratory assays, malaria parasite detection and enumeration. G6PD phenotyping was done using paper chromatography while PCR, restriction enzyme digestion and gel electrophoresis was used to genotype Haemoglobin-S (HbS). IgG levels against CSP and AMA1 were determined using indirect ELISA. Chi-square was used to compare the proportions of SCT and G6PD deficiency between groups. Comparison of IgG levels between groups was done using Mann Whitney U test. Association between anti-CSP and anti-AMA1 IgG antibody responses and different HbS and G6PD deficiency phenotypes was assessed using logistical regression analysis. Results showed that G6PD deficiency and SCT prevalence were 8.3% and 14.4% respectively and low frequencies of Anti-CSP IgG antibodies were significantly associated with G6PD deficiency [p=0.020] and SCT [p=0.016] but no influence on frequency of anti-AMA1 IgG antibodies by G6PD deficiency [p=0.819] and SCT [p=0.862]. However, in children with either SCT or G6PD deficiency, the malaria negative ones had significantly higher levels of anti-CSP IgG than the malaria positive ones [p=0.040; p=0.003]. This suggests that anti-CSP IgG antibodies collaborate with SCT and G6PD deficiency in protecting against paediatric clinical malaria but not in adults. Similar studies involving other genetic disorders like thalassemia need to be done to create a clear understanding of malaria immunogenetics. Information on prevalence of SCT and G6PD deficiency is important when planning for malaria chemotherapy and research in this area. Understanding humoral responses and these genetic disorders help elucidate knowledge on anti-malarial immunity and vaccine targets since the CSP-cased vaccine under trial elicit IgG antibodies which may give different findings in individuals with these genetic disorders.

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LIST OF ABBREVIATIONS AND ACRONYMS

AMA1 -Apical Membrane Antigen 1

CR1 -Complement Receptor-1

CSP -Circumspozoite Protein

DAF -Decay Accelerating Factor

G6PD -Glucose 6 Phosphate Dehydrogenase

HbS -Haemoglobin S

Ig -Immunoglobin

iRBC -infected Red Blood Cell

IRS -Indoor Residual Spraying

ITNs -Insecticide-Treated Nets

LSA1 -Liver Stage Antigen 1

MSP -Merozoite Surface Protein

PCR -Polymerace Chain Reaction

PfEMP1 -*Plasmodium falciparum* Erythrocyte Membrane Protein-1

RFLP -Restriction Fragment Length Polymorphism

SCT -Sickle Cell Trait

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CHAPTER ONE

INTRODUCTION

1.1 Background Information

Malaria is a global health concern that accounts for over 216 million clinical cases and 445,000 deaths annually (WHO, 2017). In high malaria transmission regions of the world, there is the existence of genetic variants that cause marked variations in resistance levels to malaria attack within human population (Howes *et al.,* 2013). Mutations leading to Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency confer such individuals low risk of developing cerebral malaria, severe malaria as well as low malaria parasitemia (Uyoga *et al.*, 2015; Manjurano *et al.,* 2012; Segeja *et al.,* 2008) while those with Sickle Cell Trait (SCT) afford 60% protection against clinical malaria, severe malaria, infant mortality and severe malarial anaemia (Manjurano *et al.,* 2012; Aidoo *et al.,* 2002; Hobbs *et al.,*2002). Such protective advantages however, come at a survival cost to those with Sickle Cell Anaemia (SCA), 90% of whom succumb to health complications before the age of five years (Ware, 2013) while G6PD deficient individuals suffer neonatal jaundice (Luzzatto, 2012) and haemolytic anaemia due to use of some anti-malarial drugs such as *primaquine* and *dapsone* (Dunyo *et al.*, 2011). With such an impact on human health, periodic monitoring and mapping of the prevalence of these disorders is important in malaria endemic areas which attract a lot of anti-malaria interventions.

Malaria infection is thought to exert strong selective pressure on genes of these genetic disorders (Piel *et al.*, 2010) and influence their prevalence globally. In sub-Saharan Africa, where over 90% of global malaria cases occur (WHO, 2017) higher average prevalence of G6PD deficiency and SCT stand at 9% and 30% respectively (Carter *et al.,* 2011;Piel *et al.,* 2010) with marked inter-ethnic

variation (Leslie *et al*., 2013; Sarr *et al.,* 2006; Samilchuk *et al*., 1999; Nafa *et al.,* 1994). In Kenya, high prevalence of G6PD deficiency (13.9%) and SCT (26%) occur in the malaria endemic Lake Victoria basin of western Kenya (Hunja, 2012; Moorman *et al.,* 2003) but vary in close proximities (Mulama et al., 2014; Suchdev *et al.,* 2012*;* Aidoo *et al*., 2002) thus calling for more sub-regional investigations for a better picture of their prevalence. In Kanyawegi sub-location in the Lake Victoria basin, -the site of the current study-, prevalence of G6PD deficiency stands at 7% and 26% for SCT (Moorman *et al.,* 2003). Given that early deaths occur in SCA cases (Ware, 2013) eliminate defective haemoglobin-S (HbS) coupled with changing malaria global patterns (WHO, 2017) and this area having high entomological inoculation rate (EIR) of over 300 bites per person per year (Beier *et al.,* 1990) with reported increase in pyrethroid resistance in malaria parasite vector (Schroeder *et al.,* 2018), such changes may create new dynamics in malaria incidence and thereby alter prevalence of SCT and G6PD deficiency.

In typical malaria infection, reduced risk of clinical malaria is attributed to higher levels of immunoglobulin-G (IgG) antibodies against the *Plasmodium falciparum* (Pf) antigens (Dodoo *et al.,* 2011; Noland *et al.,* 2008). More so, combined IgG antibodies against pre-erythrocytic antigens, Circum-sporozoite (CSP), Liver-Stage Antigen 1 (LSA1) and Thrombospondin-related Adhesive Protein (TRAP) have been found to be more protective against clinical malaria in children (Ajua *et al.,* 2015; John *et al.*, 2008). Additionally, high affinity antibodies directed at erythrocytic proteins; Merozoite Surface Protein 2 (MSP2) and Apical Membrane Antigen 1 (AMA1) elicit effective malaria protection (Reddy *et al.,* 2012) usually through inhibition of merozoite invasion of erythrocytes (Hooder, 2001). Whereas these proteins are key anti-malaria vaccine candidates with anti-malaria vaccines derived from CSP, LSA1 and AMA1 recombinants respectively being in at least Phase II trials due to their protective potential (Ajua *et al.,* 2015;Crompton *et al.,* 2010), the

association of their antibodies and existing malaria-related host genetic disorders against diverse geographical background has not been fully investigated.

It has been proposed by Williams *et al.* (2005) that in individuals with malaria-associated genetic disorders qualitative and quantitative changes of Pf-antigens occurs leading to changes in levels of antibodies against them. Studies in high malaria transmission areas of Burkina Faso show no association between SCT and malaria-related IgG antibodies (Afridi *et al.,* 2012; Verra *et al.,* 2007). Whereas in Gabon, Cabrerra et al, (2005) found elevated levels of IgG antibodies against Pf-Variant Surface Antigen (Pf-VSA) in individuals with SCT a study in Mali by Miura et al.(2013) found SCT to be significantly associated with lower IgG antibody levels against Erythrocyte Binding Antigen-175 (EBA175), Merozoite surface protein-1 (MSP1), MSP2 and AMA1. The influence of G6PD deficiency on antibody responses if any has remained largely unevaluated.

An earlier study in Senegal by Sarr *et al*. (2005) found significantly lower IgG antibodies in individuals with either G6PD deficiency or SCT. This study only focused on ring-infected erythrocyte surface antigen (RESA) and MSP2 but not CSP and AMA1. In as much as Verra et al. (2007) found that SCT associated significantly with higher anti-CSP and anti-AMA1 IgG antibodies in a low malaria transmission area of Burkina Faso, these could be different in regions of high malaria transmission areas such as western Kenya. Studies in malaria endemic Kanyawegi sub-location separately offer statistics on prevalence of SCT and G6PD deficiency (Mulama *et al.,* 2014; Moorman *et al.,* 2003) or humoral responses (Noland *et al*., 2008; John *et al.,*2005; John *et al.,*2003) but no literature exists on the interplay of humoral responses and SCT and G6PD deficiency. It is not clear if the antibody imbalance shown by the studies cited arise due to SCT and G6PD deficiency. This study therefore sought to determine the prevalence of SCT and G6PD deficiency and their

association with IgG antibody responses against CSP and AMA1 Pf antigens in individuals living in malaria endemic Kanyawegi sub-location of western Kenya.

1.2 Statement of the Problem

Individuals with G6PD deficiency and SCT separately exhibit protection to clinical episodes of malaria. Prevalence of these disorders in human populations varies with malaria intensity and higher prevalence of these genetic disorders being in malaria endemic areas like western Kenya. However, sub-regional variations in the prevalence of these disorders in such areas occur and may not remain stable over time due to changing demographics and malaria incidence dynamics. It is thought that anti-malarial IgG antibody imbalances occur as a result of SCT and G6PD deficiency and this may influence immunity to malaria. Since Pf antigens like CSP and AMA1 elicit production of protective IgG antibodies, studies designed to evaluate IgG antibody responses to Pf malaria in high malaria transmission regions may delineate the influence if any, SCT and G6PD deficiency separately have on IgG antibodies levels and contribute to a better understanding of anti-malarial immunity. Furthermore anti-malaria vaccine trials targeting CSP and AMA-1 Pf antigens are being rolled out in high malaria transmission areas of western Kenya yet little is known about the influence of these genetic factors on anti-malaria IgG antibody responses. These necessitated the current investigation on prevalence of SCT and G6PD deficiency and if individually these variants are associated with levels of protective IgG antibodies to CSP and AMA1 Pf-antigens in residents of a malaria endemic area of Kanyawegi sub-location, in western Kenya.

1.3 Objectives

1.3.1 General Objective

To investigate the prevalence of SCT and G6PD deficiency and their association with IgG antibody responses to CSP and AMA1 Pf-antigens in residents of malaria endemic Kanyawegi sub-location of Kisumu West sub-County, western Kenya.

1.3.2 Specific Objectives

i. To determine the prevalence of SCT and G6PD deficiency in Kanyawegi sub-location of western Kenya.
ii. To establish the association between SCT with the frequencies and levels of anti-CSP and anti-AMA1 IgG antibodies in residents of Kanyawegi sub-location of western Kenya.
iii. To establish the association between G6PD deficiency with the frequencies and levels of anti-CSP and anti-AMA1 IgG antibodies in residents of Kanyawegi sub-location of western Kenya.

1.4 Research Question and Null Hypotheses (H_0)

i. What is the prevalence of SCT and G6PD deficiency in residents of Kanyawegi sub-location of western Kenya?
ii. SCT does not influence the frequencies and levels of anti-CSP and anti-AMA1 IgG antibodies in residents of Kanyawegi sub-location of western Kenya.
iii. G6PD deficiency does not influence the frequencies and levels of anti-CSP and anti-AMA1 IgG antibodies in residents of Kanyawegi sub-location of western Kenya

1.5 Significance of the study

Anti-malarial drugs; *primaquine* and *dapsone* intended for clearance of all Pf malaria stages cause adverse haemolysis in G6PD-deficient persons (Dunyo *et al*., 2011), while individuals with Sickle Cell Anaemia (SCA) suffer severe anaemia and most die before reaching 5 years of age (Ware, 2013). Results from this study show 8.3% prevalence of G6PD deficiency and 14.4% for SCT with only 0.7% SCA cases in Kanyawegi sub-location. Having such updated statistics on prevalence of these genetic disorders will help malaria researchers plan for proper roll out of anti-malarial interventions to avoid cases of haemolysis after malaria chemotherapy in G6PD deficient persons and help SCA cases access better healthcare earlier in life. It also provides baseline information on the prevalence of G6PD deficiency (8.3%) and SCT (14.4%) that is critical to researchers and healthcare providers in malaria endemic areas such as Kanyawegi sub-location and western Kenya which attract a lot of research with intensive anti-malarial chemotherapeutic interventions.

This study has shown that SCT and G6PD deficiency separately associate with lower frequencies of anti-CSP IgG antibodies but no association with anti-AMA1 IgG antibodies in residents of Kanyawegi sub-location. Such immuno-genetic information has implication on the design of CSP-based anti-malarial vaccines, proper characterization of the study participants in anti-malarial vaccine trial to minimize confounding effects and probably adjustments in dosages when individuals with these genetic disorders are used in the vaccinate trials and future immunisation.

1.6 Limitations of the study

i) The current study was part of a major study carried since 2010 and the sampling procedure followed could have implication on reflecting the true prevalence of SCT and G6PD deficiency in Kanyawegi sub-location.

ii) A larger sample size could have given a higher accuracy in terms of prevalence of the genetic disorders in the study population.

iii) More females were recruited in the study than males and this had implications on comparing prevalence of G6PD deficiency against gender since G6PD deficiency disorder arise from mutations on the X sex chromosome.

iv) The G6PD deficiency phenotyping method used could not distinguish between the female heterozygotes, hemizygotes and homozygotes which have implications on the G6PD deficiency status of the females.

v) Other known malaria-associated genetic disorders including thalassemia, Haemoglobin-C (HbC), pyruvate kinase deficiency were not investigated in the current study thus the cross-interaction of these disorders in immune development if any, could not be brought out and understood.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology and control of malaria

Malaria is caused by five *Plasmodium* species - *P. ovale, P.vivax, P. malariae, P. knowlesi* and *P. falciparum* – of which *Plasmodium falciparum* (Pf) is the most virulent causing serious health burden annually (WHO, 2017; Nguetse *et al*, 2016; Crompton *et al.*, 2010). This is attributed to its ability to attack erythrocytes of all stages and adhere to vascular endothelium leading to severe complications like cerebral malaria and acute renal failure (Barfod *et al.,* 2011). Its leading mosquito vectors are the females of *Anopheles gambiae, An. pharoensis* and *An. funestus* species with six species of the *An. gambiae* complex being the most effective ones in Africa where prevalence and effects of malaria are a record higher than other regions with pregnant women, infants and immune-compromised individuals being at a higher risk (WHO, 2017; Omolade, 2012; Dobaño *et al.,* 2008).

The life cycle of the malaria parasite commences when an infected female *Anopheles* mosquito injects sporozoites into a mammalian host, a large number of which migrate to the liver, invade the hepatocytes, mature into schizonts which contain merozoites that bud off the hepatocytes a week later while enclosed in merosomes that contain up to thousands of these infective stages (Crompton *et al.,* 2010). The merozoites invade the erythrocytes, feed on haemoglobin and grow into ring-shaped form, trophozoites then schizonts which later release more merozoites to continue erythrocyte invasion. Some merozoites undergo differentiation to form gametocytes which are picked up by mosquitoes when they bite. On reaching the mosquito mid-gut, fertilization takes place and the resultant motile zygotes known as ookinetes penetrate the mid-gut, embed themselves in mid-gut membrane, invade cells and multiply. Numerous tiny and elongated sporozoites escape into the haemocoel, migrate into

the salivary glands and are released into the host when the mosquito takes its next meal (Omolade, 2012). This ability to live in secondary hosts, enhance the parasite's survival and transmission.

Various attempts have been made to control and treat malaria. Insecticide treated nets (ITNs), indoor residual spraying (IRS) and use of artemisinin-based combination therapy (ACT) are being promoted for prevention and curative purposes respectively more so in malaria endemic communities and have managed a remarkable decline in global malaria incidences (WHO, 2017). However, these achievements are being faced with a number of challenges, the main one being resistance of malaria vector and parasite to chemical control due to high rate of genetic mutations in the two organisms (Schroeder *et al*., 2018; WHO, 2017). Attempts are being made at development of vaccines with *Mosquirix*™ (CSP-based anti-malarial vaccine) showing promise (Ajua *et al., 2015;* Crompton *et al.,* 2010). However, more research effort is still required to combat the disease.

Over 10.9% of Kenya's population live in western Kenya, an area with over 63% of hospital bed admissions of children being due to malaria (Kapesa *et al.,* 2018), 28% malaria parasite pool in adults (Jenkins *et al*., 2015) and malaria incidence of 20.7% amongst adolescents (Otieno, 2018). According to a survey by the Ministry of Health (MoH), children aged ten to fourteen years experience the highest prevalence (11%) of malaria in Kenya but with a declining trend (MoH, 2016). The lake region experience high malaria associated admissions of 47% (Kapesa *et al.* 2018) with low penetration of diagnostic tools in rural hospitals and low household ownership of ITNs more so in rural pregnant women and children being major challenges in malaria control (Okiro *et al.,* 2010). However, in the recent past, prevalence of malaria has declined from 38% in 2010 to 27% by 2015 in children below fourteen years and household ownership of ITNs improved to 63% (MoH, 2016). Such changing malaria dynamics and control strategies may have implications on prevalence of SCT

and G6PD deficiency as well as humoral imbalances in residents of Kanyawegi sub-location in western Kenya.

2.2 Sickle Cell Trait and Malaria

2.2.1 Genetic Basis of Haemoglobin S Variant

Adult haemoglobin (HbA) is composed of two α-chains and two β-chains synthesized from translation of genes in chromosomes 16 and 11 (11p15.5) respectively (Vitor *et al.,* 2012). The haemoglobin (Hb) molecule has over 1153 variants (Silva *et al.,* 2013) and it is not clear why its genes suffer such a high number of mutations. The haemoglobin S (HbS) variant arise from an 18A>T (rs334) substitution at the second position of codon 6 of the β-globin gene (Piel *et al.,* 2010; Enevold *et al,* 2005) leading to formation of valine (GTG) instead of glutamic acid (GAG) in the β -globin chain ($\beta_{Glu}6_{Val}$) of haemoglobin (Ghansah *et al.,* 2012; Jeremiah 2006). The resultant HbS haemoglobin causes the normal bi-concave erythrocytes to sickle, be fragile, get easily trapped in the micro-vasculature and lyse under low oxygen tension leading to poor oxygen supply to tissues (Saleh-Gohari & Mohammadi, 2012).

In the homozygous state (HbSS), sickle cell anaemia (SCA) occurs, and the victim has severe pain, anaemia, splenomegaly, hepatomegaly and most die before five years of age (Sadarangani *et al.,* 2009). In Africa, SCA is a bigger health problem since malaria worsen their condition by aggravating anaemia (Williams *et al.,* 2005) such that very low prevalence of 0-1.5% are usually reported in studies (Jeremiah, 2006; Terlouw *et al.,* 2004). In the heterozygous state (HbAS), individuals have a mild condition, Sickle Cell Trait (SCT) that has a malaria-related survival value.

2.2.2 Sickle Cell Trait and Malaria Protection

In malaria endemic areas, children with SCT experience 60% protection against severe malaria anaemia (SMA), high density parasitemia (Aidoo *et al.,* 2002), and up to 90% reduction in severe malaria (SM) in early life (Manjurano *et al.,* 2012). This protection is thought to be effective within the first 2-16 months of life (Aidoo *et al.,* 2002). However, a study at the Kenyan coast found the protection level in *SCT* children being incremental from an initial 20% to almost 60% over the first ten years of life (Williams *et al.,* 2005). How this protection is achieved in an individual is not clearly understood.

It has been suggested that sickled erythrocytes release more of the toxic hydrogen peroxide (H_2O_2) and super-oxide anions (O_2^-) that retard the growth of *Pf* (Balgir, 2012). *In vitro* studies show HbS-ring-infected erythrocytes (iRBCs) suffer enhanced phagocytosis (Ayi, 2004) while *Pf* experience accelerated death when HbS- iRBCs are incubated under low oxygen concentration (Balgir, 2012; Friedman, 1978). HbS iRBCs in *SCT* children beyond 49 months show higher copy numbers of complement receptor-1 (CR_1) and CD_{55}, which protect erythrocytes against complement-mediated lysis, and are thought to be a source of protection given that children with SMA express their low copy numbers (Stoute *et al.,* 2013). Inhibition of *P. falciparum* growth in HbS iRBCs due to translocation of three micro-ribonucleic acids-RNAs (*miR-223, miR-451* and *let-7i*) into the parasite has been described (La Monte *et al.,* 2012) and it has been proposed that there is humoral role to this protection against malaria in individuals with SCT (Willliams *et al*., 2005). Taken together, these findings suggest that malaria protection in SCT individuals could be a product of many mechanisms. The current study determined the association between SCT and malaria-related IgG antibody responses that may have implications on anti-malarial immunity.

2.2.3 Distribution of Sickle Cell Trait

Mutation leading to HbS and its related disorders are a major global health concern mainly in malaria endemic areas of Mediterranean, Africa, India and Middle East (Piel *et al,* 2010). It has prevalence of 1-2% in both North and South Africa (Saleh-Gohari & Mohammadi, 2012) but with a high of 30% in sub-Saharan Africa (Cabrera *et al.,* 2005). In the low malaria transmission areas of Kenyan highlands, 3% SCT prevalence has been noted (Moormann *et al.,* 2003), 18.1% at the coast (Williams *et al.,* 2005) while in the malaria endemic Lake Victoria basin areas 26% SCT prevalence has been recorded (Moorman *et al.,* 2003) but with sub-regional variations (Suchdev *et al.*, 2012; Aidoo *et al.,* 2002). Although Kanyawegi sub-location has been shown to have an SCT prevalence of 26% (Moorman *et al*., 2003), over time, this may not remain stable due to changes in population structure, climatic conditions and malaria dynamics which may alter frequency of the HbS gene in the population. This study therefore determined the prevalence of SCT in residents of malaria endemic Kanyawegi sub-location in western Kenya.

2.3 Glucose-6-Phosphate Dehydrogenase Enzyme Deficiency

2.3.1 Glucose-6-Phosphate Dehydrogenase Enzyme and its Functions

Glucose-6-Phosphate Dehydrogenase (G6PD) enzyme is a 59Kda monomer with 515 amino acids (Moiz, 2013). It catalyses the conversion of Glucose-6-Phosphate (G-6-P) into ribulose-5-phosphate in the phosphate pentose pathway (PPP) and generates reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Moiz, 2013; Peters & Van Noorden 2009). NADPH stabilizes catalase enzyme and reduces glutathione which detoxify harmful hydrogen peroxide formed from other reactive oxygen species (ROS) (Moiz, 2013; Pamba *et al.*, 2012). Being oxygen carriers, erythrocytes are in

constant exposure to ROSs released from conversion of haemoglobin to methaemoglobin and this detoxification helps protect them from oxidative damage (Luzatto & Poggi, 2008). In the absence of G6PD enzyme, erythrocytes are deprived of NADPH, suffer oxidative damage and easily get lysed by the accumulated free radicals (Moiz, 2013). Older erythrocytes lack of nucleus and mitochondria and can not synthesize this enzyme thus suffer more damage since they can't synthesize this enzyme due to absence (Luzatto & Poggi, 2008). Infections, intake of some anti-malarial drugs and ingestion of fava beans (*Vicia faba*) that is rich in oxidation-inducing isouramil, divicine and pyrimidine aglycones (Moiz, 2013; Mehta, 1994) also induce oxidative damage to erythrocytes. Such chemicals aggravate the condition in G6PD deficient individuals who are otherwise asymptomatic, leading to acute haemolytic anaemia (AHA), haemoglobinuria and neonatal jaundice (Howes *et al.,* 2013). Severe cases like chronic non-spherocytic haemolytic anaemia (CNSHA) and splenomegaly are also experienced in the event of some G6PD genetic variants (Luzatto & Poggi, 2008). A clear understanding of what proportion of the current population have this disorder can help in timely management of its adverse effects more so in malaria endemic western Kenya where prevalence of this disorder exists at varying degrees and anti-malarial chemotherapy is intense.

2.3.2 Genetic Basis of Glucose-6-Phosphate Dehydrogenase Enzyme Deficiency

The G6PD enzyme is synthesized from translation of an 18kb long gene with 13 exons and 12 introns found in locus q28 of the telomeric region of the long arm of the X-chromosome (Peters & Van Noorden, 2009; Luzatto & Poggi, 2008). It exhibits high heterogeneity with over 400 variants arising from a few deletions and numerous single nucleotide polymorphisms (SNPs) within its introns and exons (Al-Sweedan & Awwad, 2012). Most of these variants make the G6PD unstable or alter its affinity to $NADP^+$ and G-6-P leading to its inefficiency (Peters & Van Noorden, 2009). However,

complete loss of G6PD gene due to frame shift is rare but has been linked to early foetal loss (Moiz, 2013; Luzatto & Poggi, 2008) while its over-expression is implicated in the growth of tumour in mice (Hu *et al.,* 2013). In earlier studies, Hirono and Beutler (1988) found a 376A>G substitution ($_{Asn}126_{Asp}$) in exon 5 of G6PD gene of all G6PD deficient individuals studied. This 376A>G (G6PD*A) SNP has been found to be a Class IV variant with 85% G6PD enzyme activity (Dunyo *et al.,* 2011; Vulliamy *et al.,* 1988). In later studies, 376A>G was shown to associate with variant 202G>A ($_{Val}68_{Met}$) in exon 4 to cause G6PD deficiency in 80% of G6PD deficient individuals (Hirono & Beutler, 1988). The 376A>G+202G>A haplotype which is a Class III variant with 12% G6PD enzyme activity is a common variant amongst Africans (Al-Sweedan & Awwad, 2012). However, in Middle East and Asia, 563C>T substitution ($_{Ser}188_{Phe}$) in exon 6 which leads to 3% G6PD enzyme activity is the major cause of G6PD deficiency (Al-Musawi *et al.,* 2012).

The discovery of G6PD deficiency by Hirono and Beutler (1988) in an individual who had 376A>G but lacked 202G>A proved the presence of other variants. Various studies have since confirmed there is synergistic action between 376A>G and other G6PD gene mutations to vary the deficiency and activity level of G6PD enzyme (Samilchuk *et al.*, 1999). Combination of 376A>G and 542A>T ($_{Asp}181_{Val}$) SNP found in exon 6 causes a Class II variant with less than 10% enzyme activity (Al-Sweedan & Awwad, 2012; Nafa *et al.,* 1994) while 376A>G+680G>A ($_{Arg}227_{Gln}$) result in 35% enzyme activity (Beutler *et al.,* 1992). In an isolated case of a Japanese boy, Hirono *et al.,* (2002) showed that 202G>A without 376A>G may still result in G6PD deficiency. However, such a development is yet to be encountered elsewhere.

Mutations within the G-6-P- and putative $NADP^+$ -binding regions in exon 10 of the G6PD gene leading to G6PD deficiency, have been found to cause severe conditions like Chronic non-spherocytic haemolytic anaemia (CNSHA) and splenomegaly (Beutler *et al.,* 1991). Individuals

suffering from CNSHA possess either 593G>C ($_{Arg}198_{Pro}$), 1159C>T ($_{Arg}387_{Cys}$), 1180G>C ($_{Val}394_{Leu}$), 593G>C+1057C>T and 1229G>A ($_{Gly}410_{Asp}$) (Beutler *et al.,* 1992). These Class I variants lead to less than 10% enzyme activity, are rare but lethal and usually call for multiple blood transfusion (Moiz 2013; Peters & Van Noorden 2009). Pathological contribution of G6PD deficiency could worsen with the finding of other novel pathogenic haplotypes with such individuals exhibiting clinically lower G6PD enzyme levels than those with single substitution haplotypes (Dallol *et al.,* 2012). It is therefore important to study multiple phenotypes of G6PD deficiency within a population since it may help identify more risks associated with these variants.

2.3.3 Glucose-6-Phosphate Dehydrogenase Deficiency and Malaria Protection

Individuals with G6PD enzyme deficiency afford relative protection against malaria compared to their normal counterparts. Whereas this disorder is more pronounced amongst males (Carter *et al.,* 2011; Suchdev *et al.,* 2012) the protection is afforded more by G6PD-deficient heterozygous females (Mehta, 1994) where 376A>G and 202G>A have been shown to be strongly protective against various clinical phenotypes of severe malaria (Manjurano *et al.,* 2012). Additionally, G6PD deficient heterozygous pregnant women exhibit lower risk of malaria than their normal counterparts (Mockenhaupt *et al.,* 2003). Homozygous deficient women and hemizygous deficient males are also known to be less ill of malaria than non-deficient individuals (Peters & Van Noorden, 2009).

The mechanism behind protection afforded by G6PD deficiency still remains unclear. G6PD non-deficient erythrocytes suffer 2 to 80 times greater level of parasitisation by Pf compared to the deficient ones (Balgir, 2012) and *in vitro*, ring-infected G6PD-deficient erythrocytes are more recognized and easily suffer phagocytosis by macrophages and monocytes (Luzatto & Poggi, 2008; Min-Oo & Gros 2005). The high efficiency of recognition of the infected G6PD-deficient

erythrocytes by the immune system together with oxidative damage of the erythrocyte membrane resulting in more phagocytosis is thought to help inhibit multiplication process of Pf (Mockenhaupt *et al.,* 2003). In spite of these positive attributes, treatment of malaria in G6PD deficient persons using dapsone, primaquine and sulphonamides easily lead to haemolytic anaemia (Pamba *et al,* 2012; Dunyo *et al.,* 2011). This has been a big challenge in the fight against *P. vivax* where primaquine had shown early promise in the elimination of hypnozoites (Zimmerman *et al,* 2013) and there is need for initiating pre-screening measures before the prescription of such drugs. This study determined the prevalence of G6PD deficiency in Kanyawegi sub-location which as a malaria endemic area could have a sizeable proportion of G6PD deficient individuals who may suffer haemolysis due to malaria chemotherapy silently if the prevalence of this disorder is not updated.

2.3.4 Distribution and Prevalence of G6PD Deficiency

Globally, over 400 million people have G6PD deficiency (Fortin *et al.,* 2002) especially in malaria endemic regions where the G6PD gene is thought to suffer intense selective pressure. Its absence amongst the Amerindians is thought to be as a result of their late interaction with malaria (Luzatto & Poggi, 2008). In Southern Europe, Middle East and Asia, 563C>T is the other major cause of G6PD deficiency (Al-Musawi *et al,* 2012) while in individuals of African origin, the 376A>G variant occurs at frequencies of up to 40% (Al-Sweedan & Awwad, 2012; Beutler, 1994) and together with either 202G>A, 542A>T, 680G>C or 968T>C form the main variants responsible for G6PD deficiency in Africa (Manjurano *et al.,* 2012; Carter *et al.,* 2011; Dunyo *et al.,* 2011; Nafa *et al.,* 1994).

In African populations, average G6PD deficiency prevalence stands at 10.2% (Carter *et al.,* 2011). It is largely absent in the Horn of Africa but more prevalent in sub-Saharan communities (Howes *et al.,*

2013). In Kenya 202G>A is responsible for G6PD deficiency (Shah *et al.,* 2014) whose prevalence varies with malaria incidences too but average prevalence stands at 10.8% (Carter *et al.,* 2011). Phenotypic studies show G6PD deficiency prevalence of 1.0% in low malaria transmission areas (Moormann *et al.,* 2003) but in malaria-prone western Kenya, higher prevalence of 6.8% in Nyando (Suchdev *et al.,* 2014) and 13.9% in Kombewa (Hunja, 2012) are encountered. Given that G6PD deficiency has been associated with haemolysis due to administration of some anti-malarial drugs like Dapsone (Dunyo *et al.,* 2011), malaria-endemic areas western Kenya where such drugs are likely to be used in malaria chemotherapy, need more updated sub-regional studies in order to get a better understanding of the prevalence of this disorder. This study therefore determined the prevalence of G6PD deficiency amongst the residents of malaria endemic Kanyawegi sub-location in western Kenya.

2.4 Malaria-associated Humoral Responses and Genetic factors

2.4.1 Malaria-associated Humoral Responses

Human body defense against malaria is a complex inter-play of innate, cell-mediated and humoral responses. Humoral responses involve immunoglobulins (Igs) released by B-cells targeting particular *Pf*-antigens and may act directly on the parasites and iRBCs or involve various effector cells in a synergistic defense engagement (Boudin *et al.,* 1993). The role of IgA and IgD in malaria protection is not clear but higher levels of IgE has been detected in persons with cerebral malaria (CM) and those with SM presenting with coma (Laishram *et al.,* 2012; Leoratti *et al.,* 2008). Mice with X-linked recessive B-cell deficiency lack IgM and are susceptible to *P. yoelii* attack (Leoratti *et al.,* 2008) yet IgM has been implicated in the masking of Pf-Erythrocyte Membrane Protein-1 (*Pf*EMP1)-specific IgG epitopes thereby protecting the malaria parasite from phagocytosis (Barfod *et al.,* 2011).

High IgM levels do not correlate with malaria protection in children (John *et al.,* 2005) and is thought to be the first antibody to be produced during infection (Leoratti *et al.,* 2008).

Immunoglobulin G (IgG) makes up 75% of serum antibodies and predominates in malaria protective activities (Boudin *et al.,* 1993). *In vitro,* IgG blocks the invasion of erythrocytes by the merozoites thereby interfering with parasite growth (Crompton *et al.,* 2009) and higher levels of malaria-specific IgG is detected in asymptomatic malaria cases (Leoratti *et al.,* 2008). Given that IgG levels increase with age and the risk of clinical malaria decreases with its increasing levels (Dodoo *et al.,* 2011), it seems to play a central role in naturally acquired immunity.

Repeated exposure to malaria and the complex life cycle of *Plasmodium* parasites offers the human host an advantage of interaction with a variety of Pf-proteins which help the immune system develop a pool of IgG antibodies to combat malaria infection. Distinct surface antigens are expressed on Pf at each stage of its life cycle mainly to escape immune challenge (Cowman *et al.,* 2012). Antibodies generated to target these special antigens, most of which are expressed on the parasite surface, act to modulate or alter the course of malaria infection in the host. At the sporozoite stage, a highly immunogenic CSP Pf antigen expressed stimulates CSP-specific IgG which has been shown to impair hepatic differentiation of sporozoites into merozoites in rodent malaria models (Chatterjee *et al.,* 1995) as well as offer protection in both animal models and humans (Kim *et al.,* 2011). Children with higher levels of anti-CSP IgG have fewer episodes of malaria than those with less (John *et al.,* 2003). Liver-Stage Antigens (LSA1, LSA3) which are expressed solely in infected hepatocytes are thought to play a role in hepatic schizogony and merozoite release (Kim *et al.,* 2011). Levels of these antibodies increase with age and combined high levels of IgG specific to CSP and LSA1 gave better protection to children (Noland *et al.,* 2008; John *et al.*, 2008).

Merozoites released in the blood from the liver to invade erythrocytes exhibit higher antigenic heterogeneity than other stages. Merozoite Surface Proteins (MSPs) including MSP1, MSP2 and MSP3 as well as AMA1, EBAs and *Pf*-Reticulocyte-like homologues (PfRhs) are expressed to aid erythrocyte invasion (Cowman *et al,* 2012). Children with CM expressed higher levels of IgG against conserved regions of MSP1, MSP2 and AMA1 than those with uncomplicated malaria (Dobaño *et al.,* 2008) while increased levels of IgG against MSP1 and AMA1 is associated with reduced risk of clinical malaria (Dodoo *et al.,* 2011). AMA1 is relatively conserved than MSP1 and MSP3 (Hodder *et al,* 2001), has high avidity (Ibison *et al,* 2012) and its two roles of being key in invasion of erythrocyte and hepatocytes by merozoite and sporozoite respectively (Coley *et al.,* 2007), make it a unique antigen. Further, rabbit and human anti-AMA1 IgG block merozoite invasion of erythrocytes (Hodder *et al.*, 2001) while mice immunized with its *P. chabaudi* recombinant are almost completely protected (Coley *et al.,* 2007). Taken together, CSP and AMA1 by virtue of being expressed at critical stages of the parasite's life and with good immunogenic features comprise a group of antigens that can form an effective multivalent anti-malaria vaccine. It was important therefore, to determine the association between the IgG antibody responses elicited by CSP and AMA1 against known malaria-related genetic disorders in order to help improve knowledge on malaria immuno-genetics.

2.4.2 Malaria-associated IgG Antibody Responses and SCT

Acquired immunity is thought to play a role in malaria protection experienced in SCT individuals (Williams, 2011). HbS ring-infected erythrocytes (iRBCs) undergo easy phagocytosis and exhibit higher number of autologous IgG on their surfaces as well as aggregated band 3, complement C_3c fragments and other markers of membrane damage (Ayi, 2004). It is known that cytophilic IgG works in concert with cells to help in parasite-killing responses like opsonisation, antibody-dependent

cellular inhibition and phagocytosis (Aucan *et al.,* 2000), and this could contribute to anti-malarial immunity seen in individuals with SCT. However, various studies show marked antibody imbalance in SCT cases (Miura *et al*., 2013; Sarr *et al.,* 2006). Higher total IgG against *Pf*EMP1 were recorded in SCT than Non-SCT Malian children (Cabrera *et al.,* 2005). It is not clear if similar results will be shown if CSP and AMA1 are considered in the context of higher malaria transmission areas.

A study by Miura *et al.* (2013) found lower IgG levels against AMA1 and MSP1 in SCT children but did not differ significantly when compared to their non-SCT controls. In another study, lower levels of IgG were recorded in SCT children (Sarr *et al.,* 2006). Whereas in low malaria transmission areas of Burkina Faso a higher immune response against a panel of Pf-variant surface antigens (VSAs) was seen in SCT compared to controls (Verra *et al.*, 2007), in Mali no difference in the level and range of IgG responses against a number of Pf-antigens in SCT and non-SCT cases (Tan *et al.*, 2011). The findings by later studies in Burkina Faso (Afridi *et al.,* 2012) corroborate findings by Tan *et al.* (2011). Only one study in Burkina Faso (Verra *et al*., 2007) has considered looking at anti-CSP and anti-AMA1 IgG antibody responses in SCT individuals which are leading anti-malarial vaccine candidates yet literature on such antibody responses are scanty and inconclusive more so in western Kenya where malaria is endemic and HbS disorders are common. This study therefore determined the association between SCT and levels of malaria specific IgG antibodies against CSP and AMA1 in Kanyawegi sub-location, western Kenya.

2.4.3 Malaria-associated IgG Antibody Responses and G6PD Deficiency

Ring-infected G6PD deficient red blood cells (iRBCs) bind higher amounts of autologous IgG antibodies (Mockenhaupt *et al.,* 2003) and suffer intense phagocytosis (Min-Oo & Gros, 2005). This

indicates altered immune responses due to G6PD deficiency and malaria. A study in high malaria transmission area in Senegal found G6PD deficient girls lower levels of anti-MSP2 and anti-RESA IgG antibodies compared to normal cases (Sarr *et al*., 2005) while in Tanzania there was no association between G6PD deficiency and levels of IgG antibodies against AMA1 and MSP2 in individuals with sub-microscopic Pf malaria (Shekalaghe *et al.,* 2009). It is not clear if similar results are to be found when malaria protective anti-CSP IgG antibodies are considered and a gap in knowledge exists on how G6PD deficiency associate with anti-CSP and anti-AMA1 IgG antibodies in the general population more so in western Kenya where prevalence of G6PD deficiency is high and the high malaria transmission may further have dynamics on levels of the antibodies in G6PD deficient persons. This study therefore investigated the association of between G6PD deficiency and IgG antibodies against anti-CSP and anti-AMA1 IgG in residents of malaria endemic Kanyawegi sub-location of western Kenya.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

This study recruited residents of Kanyawegi sub-location, South West Kisumu location in Kisumu West Sub-county of Kisumu County (Fig. 3.1). This area is served by Chulaimbo sub-District Hospital, a key rural health facility that offers medical training, care and collaborative research opportunities. A majority of people in this area are of the Luo ethnic group who mainly undertake fishing, small scale crop farming and livestock rearing.

It is a rural lowland area found 24 kilometers away from Kisumu City at Latitude 0^{o} $8^{'}$ North and longitude $34^{o}36^{'}$ East at 1328 meters above sea level with a population of over 3,000 people (Noland *et al*., 2008). Lake Victoria and hills on the northern parts influence its climatic conditions with temperature range being between 26-30^{o}C. Long rains are experienced between March-July and short rains within August-October. High malaria transmission occurs around the months of May-June as well as September-October with high entomological inoculation rate which exceed 300 infectious bites per person per year (Beier *et al.,* 1990). Being a malaria endemic area, it experiences increased anti-malarial interventions as well as research hence the need for updated information on prevalence of malaria-related genetic disorders. Studies done earlier indicate an SCT and G6PD prevalence of 26% and 7% respectively (Moorman *et al.,* 2003) a situation that may have changed with changing demographics and malaria dynamics.

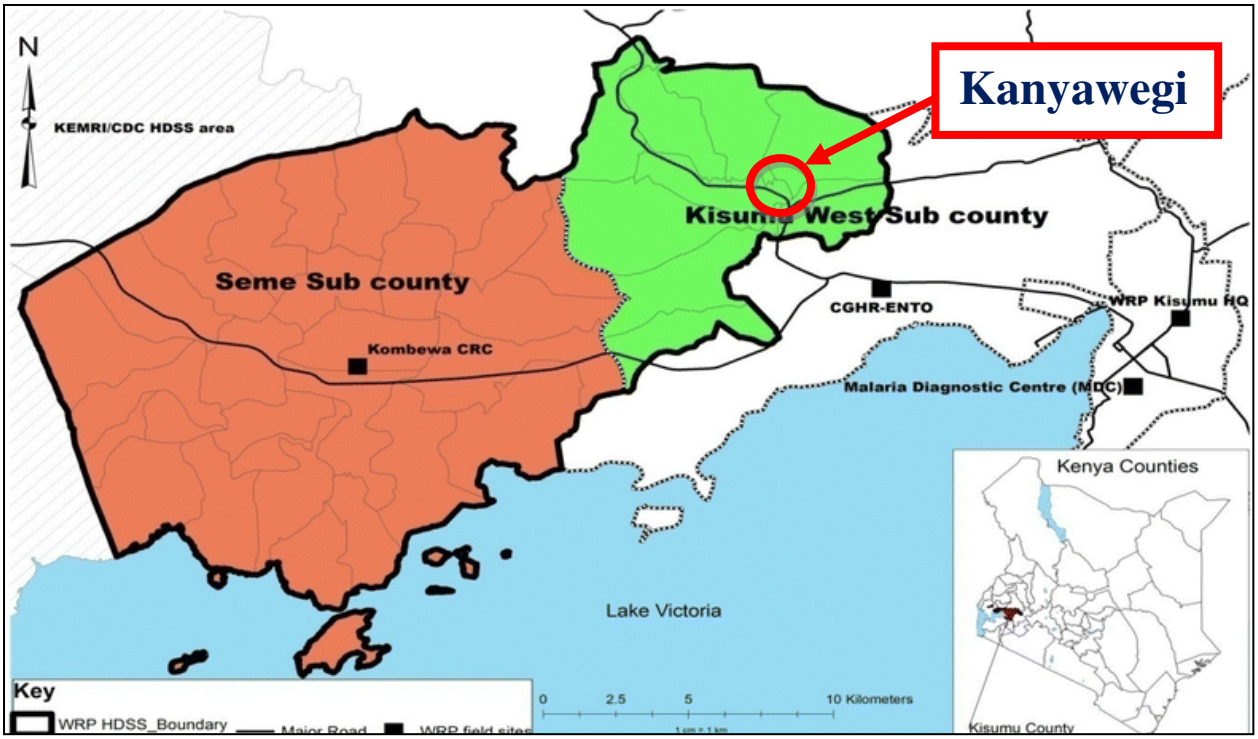

Fig 3.1: Location of Kanyawegi sub-location (0º 8' N; 34º36' E), the Study site courtesy of Wanja *et al.* (2016)

3.2 Study Design

This was a cross-sectional study on residents of Kanyawegi sub-location of western Kenya. The experimental design followed (Fig. 3.2) involved malaria parasitemia counts and G6PD-phenotyping using whole blood, Anti-CSP and anti-AMA1 IgG antibody quantification using plasma and HbS-genotyping on Deoxyribonucleic acid (DNA) samples.

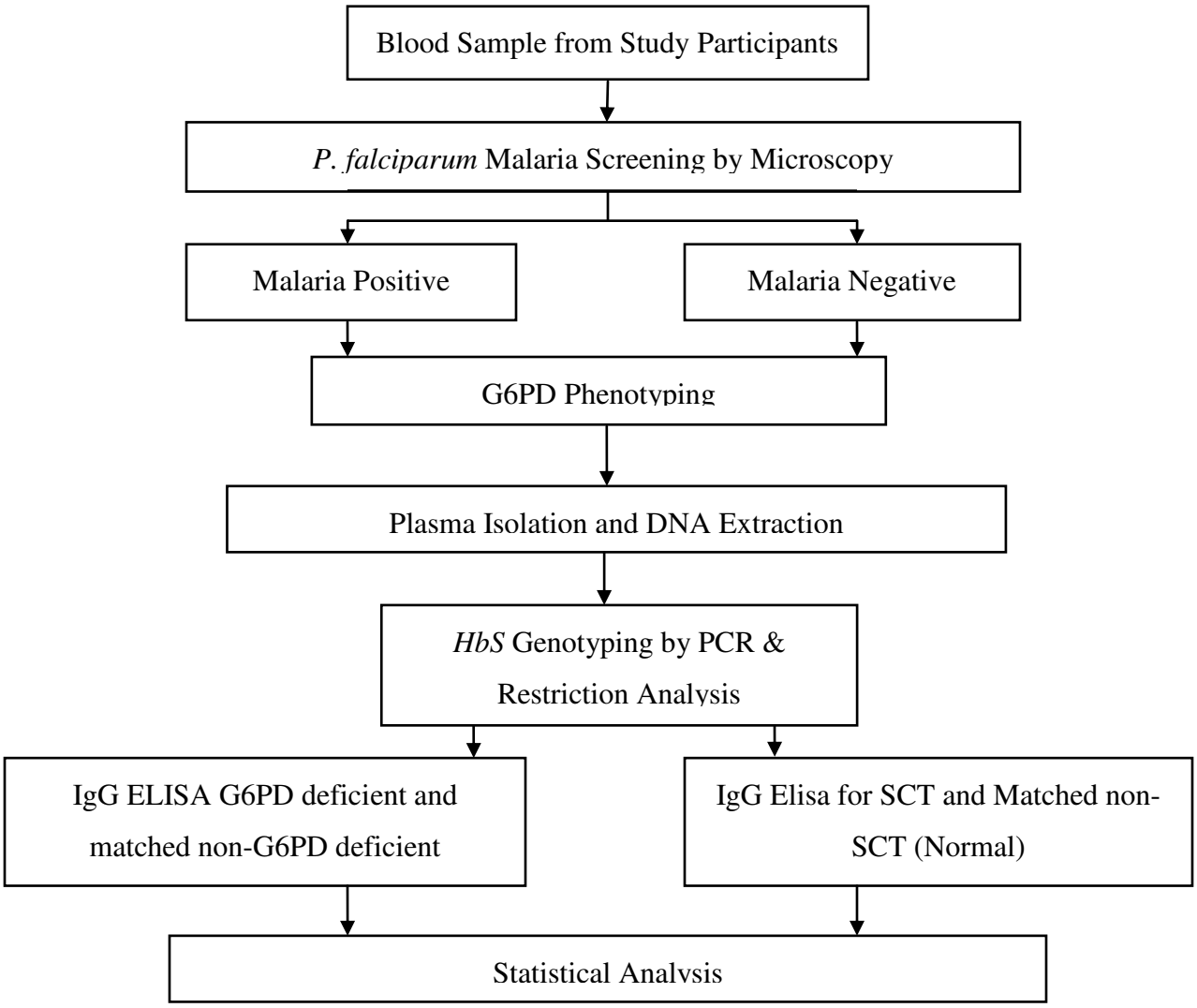

Fig 3.2: Experimental Design followed in the study

3.3 Study Population

This study was part of an ongoing study titled: "The genetic and immunological elements contributing to naturally acquired immunity to malaria during epidemiological transition in Kenya since 2010". Children (100) aged 1-10 years, of either gender from Kanyawegi sub-location, presenting with clinical symptoms of uncomplicated malaria were randomly selected from those

seeking treatment at Chulaimbo sub-District Hospital, Kisumu County. The recruited children were followed to their homes two weeks post-treatment where another 100 children of 1-10 years old and 100 adults residing in the same locality as the acute malaria cases were identified and recruited. In total, 300 individuals participated in this study.

3.3.1 Inclusion Criteria

I. For the first 100 children: Were of 1-10 years age presenting with clinical uncomplicated malaria symptoms: axillary temperature $\geq 37.9^0$C, *P. falciparum* positive of any density by blood smear, Haemoglobin levels of $\geq$ 7g/dl at recruitment, permanent residence in local areas served by Chulaimbo hospital, willingness of child's caregiver/parent to participate in blood draws, ability of parent/caregiver to give informed consent to the study.

II. For the second 100 Children, the inclusion criteria were: Age of 1-10years children with axillary temperature of $\leq 37.8^0$C and with no known malaria attacks or taking anti-malarial drugs in the last 3 months plus the other criteria as above.

III. The 100 adult participants were of: $\leq 37.8^0$C axillary temperature, $\geq$ 18years, no known malaria attacks or taking anti-malarial drugs in the previous 3 months, lived in Kanyawegi sub-location for $\geq$10 years plus.

3.3.2 Exclusion Criteria

Evidence of severe malaria like: neurologic disorder, severe respiratory stress and other chronic medical illnesses declared by participant, caregiver or parent. Additionally for adults: pregnancy and unwillingness to give informed consent.

3.4 Sample Size Estimation

Sample size for this study was determined by use of the formula by Cochran (1963) shown below. The prevalence of SCT in Kanyawegi sub-location was assumed to be at 26% based on findings of an earlier study (Moormann *et al.* (2003) but with 95% confidence level and a 5% margin of error allowed.

$$n = \frac{Z^2_{1-\alpha/2} P\,(1-P)}{d^2}$$

Where:
- $Z^2_{1-\alpha/2}$ is the standard normal quartile corresponding to $100\times (1-\alpha/_2)$
- *P* is the prevalence of *SCT* assumed to be 26%
- d is the margin of error assumed to be 5%
- n is the sample size

$$n = \frac{1.96^2 \times 0.26\,(1-0.26)}{0.05^2} = 295$$

The minimum sample size was 295 individuals. This was adjusted to 300 individuals where samples from 100 malaria positive children, 100 malaria negative children and 100 malaria negative adults were used in the study.

3.5 Laboratory Procedures

3.5.1 Blood Sample Processing

Venous blood collected in EDTA tubes was subjected to centrifugation at 10,000xg for 10mins at room temperature. Plasma was aspirated by use of pipette and dispensed into Sarstedt tubes. 1xPBS

equal to the separated plasma was added to the red blood cell pellet and mixed thoroughly. The labeled samples were stored at -80^{0}C.

3.5.2 Determination of *P. falciparum* Parasitemia Load

Detection and determination of parasitemia load for samples found *P .falciparum* positive at the hospital was ascertained at KEMRI malaria laboratory by qualified malaria microscopist. Using a pipette, a drop of blood was placed on a microscope slide and a separate glass slide used to make a thin and thick smear that was dried in the open air. The thin smear was fixed by use of 100% Methanol. The slide was then placed on a rack then flooded with 10% Giemsa stain for 20mins. The slide was tilted and the stain washed off by running water then dried in open air. Using x100 objectives and immersion oil spread on the thin and thick smears, the *P. falciparum* parasites were counted within the thick smear and quantified against 200 white blood cells (WBCs).

3.5.3 G6PD Phenotyping for G6PD Prevalence

NADPH fluorescence Spot test Sigma Kit 203-A (Sigma Aldrich, Inc., St Louis, MO, USA) was used in G6PD deficiency screening. 100µl of the reagent containing glucose-6-phosphate and NADP+ was mixed with 5µl of fresh whole blood and incubated in a warm water bath maintained at 37^{0}C. 10µl of the reaction mixture was spotted on a filter paper at the beginning (time zero), 5 and 10 minutes. The spots were then left to develop in the dark for 15minutes then visually inspected under long-wavelength (405nm) ultraviolet using ultraviolet (UV) trans-illuminator (Uvitec, Cambridge) to determine the appearance of bright fluorescence or lack of it which was proportional to the blood *G6PD* enzyme activity.

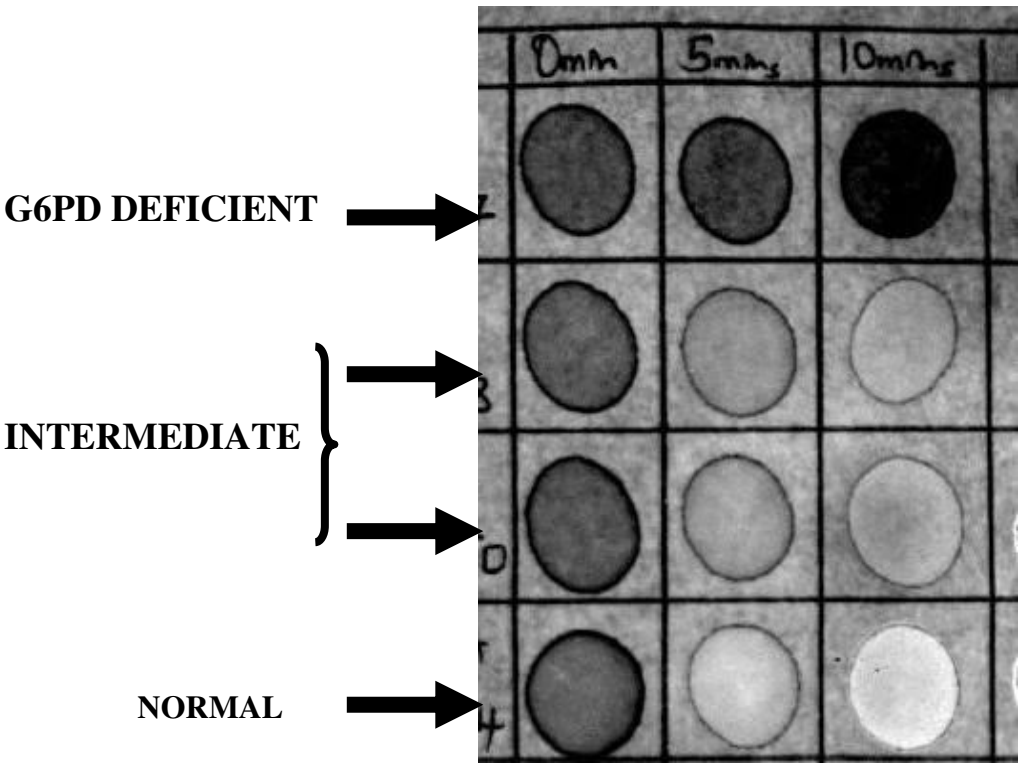

Fig 3.3:Fluorescence emissions of samples to show the different phenotypes of G6PD deficiency using the NADPH Fluorescence Spot Test. Dark spots on the right indicate Severe G6PD deficiency, moderate emission show intermediates (mild deficiency) while bright emissions on the lower right indicate normal G6PD activity

Based on the intensity and presence or absence of fluorescence emissions, the samples were scored as normal (high emission), intermediate (moderate emission), or deficient (no emission) (Segeja *et al.,* 2008) as shown in Fig 3.3 above. This procedure was carried out to identify G6PD deficient samples from the non-deficient ones.

3.5.4 DNA Extraction

DNA extraction was done as per the QIAmp® DNA Mini kit protocol. Into a sterile 1.5ml micro-centrifuge tube, 4µl of RNase A was pipetted and 200µl of anti-coagulated blood added. Incubation

at room temperature was done for 2minutes then 20µl of Proteinase K and 200µl of *Buffer AL* were pipetted into the mixture and vortexed for 10s. The reaction mixture was incubated at 56^0C for 10 minutes and 200µl of absolute ethanol added to each sample before hard vortexing to create homogeneity. About 630µl of the mixture was pipetted into the center of a DNeasy spin column sitting in a 2ml collection tube and spun at 8,000rpm for 1min in an Eppendorf centrifuge at room temperature. The flow through was discarded in 10% bleach and the DNeasy spin column placed in new collection tube. Buffer AW1 (500µl) of was added and incubation done for 5mins at room temperature then centrifuged at 8,000xg for 1min. The flow-through was discarded in 10% bleach and the DNeasy spin column placed in fresh collection tubes. Buffer AW2 (500µl) of was added and incubation done for 5mins. The columns were centrifuged at 13,200xg for 3mins. Flow-through tubes were discarded and the spin columns transferred to separately labeled 1.5ml micro-centrifuge tubes. 100µl of Buffer AE was added to each spin column then incubated for 5mins before centrifugation at 8,000xg for 1min for DNA elution. Another 100µl Buffer AE was added for a second elution step. The spin columns were discarded in 10% bleach. About 200µl DNA obtained was stored at -20^0C.

3.5.5 Sickle Cell Trait Genotyping Using PCR-RFLP

Genotyping for Sickle Cell Trait was done as per procedure described by Husain et al (1995). The PCR Mastermix was prepared by multiplying the number of reactions with the following quantities: 1.25µL of 50mM 10x PCR Buffer (500mM KCl, 100mM Tris-Cl, pH 8.3, 15mM $MgCl_2$), 1.0 µL of the 4dNTPs' Cocktail (200µM each, Thermo AB-0196), 0.1µL of the forward primer (5'-TCCTAAGCCAGTGCCAGAAG-3') and 0.1µL of reverse primer (5'-GAATTCGTCTGTTTCCCATTCTAAAC-3') (20 µM each, Fisher's Primer Manufacturing Company) (Appendix 1), 0.625µL of Red Taq Polymerase (Sigma, AB-0908/A) and 9.425µl of

Sterile PCR water. Master-mix (12.5µl) was aliquoted into each tube before addition of 0.5µL of DNA template. The following PCR thermocycling conditions were used: 95^0C for 5mins, then 95^0C for 1min, annealed at 60^0C for 1min and initial extension at 72^0C for 1min with 40 cycles from step 2 and final extension achieved at 72^0C for 3mins. The 769bp PCR amplicon was detected on a SYBR Gold (1:10000)-stained 2% agarose gel and visualized under Ultraviolet (UV) trans-illuminator (Uvitec, Cambridge).

The resultant 769bp amplicon was subjected to restriction enzyme digestion whereby for a reaction, 9.0µl of sterile PCR water, 0.5µl of CutSmart™ NEB Buffer and 0.5µl *Bsu361* (New England Bio-labs) restriction enzyme was constituted. 10µl of this restriction digest master-mix was dispensed per well together with 3.5µl of the PCR product and the reaction digestion set over a 37^0C heating block for 30mins and inactivated at 80^0C for 20mins.

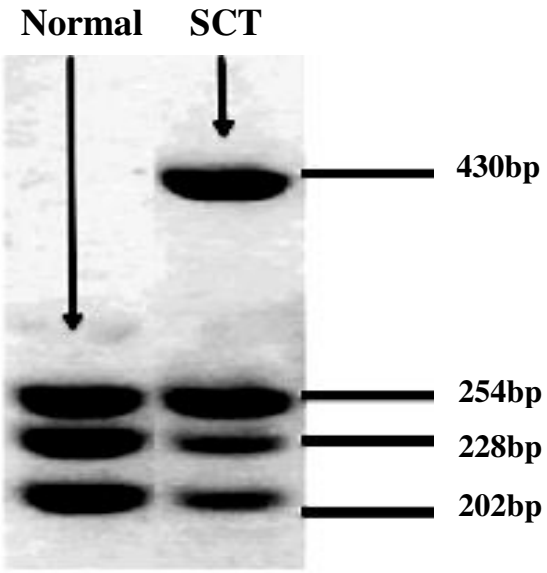

Fig.3.4: A photomicrograph of gel electrophoresis showing the identification bands for Normal (254bp, 228bp and 202bp) and Sickle Cell Trait (430bp, 254bp, 228bp and 202bp) phenotypes after digestion of fragment by *Bsu361* restriction enzyme.

The resultant restriction fragment length polymorphism (RFLP) fragments were detected by loading 5µl of digest fragment and 2µl of gel loading dye into SYBR Gold (1:10,000 dilution in 1xTBE) stained 3% agarose gel covered with 100ml 1xTBE using 170volts for 45mins. *Bsu 361* has a recognition site at codon 6 in the normal beta globin gene, and cleaved the normal amplified beta globin DNA into fragments which were visualized under Ultraviolet (UV) trans-illuminator (Uvitec, Cambridge) and used to identify the various genotypes as follows: 254, 228 and 202bp fragments for HbA/A genotype (Normal/Non-SCT), 430, 254, 228 and 202bp fragments for SCT (HbA/S genotype) while 430 and 254bp fragments for SCA (HbS/S genotype).

3.5.6 Determination of anti-CSP and anti-AMA1 IgG antibody levels

An indirect Enzyme-linked immunosorbent assay (ELISA) was used to measure IgG antibody levels against CSP and AMA1 *Pf* antigens. The CSP protein (Jackson Immuno-Research, West Grove, PA) was dissolved in 0.01 M phosphate-buffered saline (PBS) to a final concentration of 10µg/ml and 0.1µg/ml for AMA1. 50µl of antigen solution was coated on micro titer wells of Immulon-4 plates (Dynex Technologies, Chantilly, VA) and then incubated at 4°C overnight. After washing twice with 1xPBS, 0.05% Tween20 (PBST), the plates were blocked using 50µl of 5% non-fat powdered milk in PBS. 50µl sample plasma diluted 1:100 in 5% powdered milk was added to wells in duplicate and incubated for 2 hrs at room temperature. After washing thrice with PBST, 50µl of alkaline Phosphatase–conjugated goat anti-human IgG (Jackson Immuno-Research, West Grove, PA) diluted 1:2,500 in 5% powdered milk was added then incubated for 1hr. After extensive washing with PBST, 50µl *p*-nitrophenyl-phosphate was added in accordance with the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO). 50µl/well of 3N NaOH was used to stop the reaction. Optical density

(OD) was measured at 405 nm using an ELISA plate reader (Opsys MR, Dynex) and Revelation Quicklink® Software against malaria positive and negative controls.

3.6 Statistical Analyses

The variables collected and recorded included: case number, malaria status (positive or negative), body temperature, haemoglobin level, age (in years), sex, Pf parasitemia levels, optical density (OD) values for IgG levels, G6PD deficiency status (normal, moderate or deficient) and HbS (Normal and SCT). Both descriptive and inferential statistics were used to represent the analysed data.

The prevalence levels of G6PD deficiency and SCT were expressed as percentages and their frequencies analysed using Chi-square test to check for variations amongst HbS genotypes and G6PD phenotypes. This was done using Excel Spreadsheets as well as SPSS software version 12.0.

The categorical variables were coded to numerical forms. IgG antibody levels were expressed as arbitrary units (AU) calculated by dividing the OD value of each sample by the mean OD for sera of malaria negative donors (from Cleveland, Ohio who had never been exposed to malaria) plus 3 standard deviations (Noland *et al*., 2008; John *et al*., 2005).Comparisons for variation in IgG antibody levels amongst the various groups of the expected HbS genotypes and G6PD phenotypes was done by Mann Whitney U tests using Graph-pad statistical software package was used to analyze the generated data by use of.

Logistical regression analysis using SPSS software version 12.0 was carried out when determining the association between the individual HbS genotypes and G6PD phenotypes and anti-CSP and anti-AMA 1 IgG antibody levels while controlling for malaria status, gender and age as confounders. All tests were two-tailed with p-value of ≤ 0.05 considered statistically significant for each test.

3.7 Ethical Considerations

This study was part of an ongoing CWRU/KEMRI collaborative study titled 'Naturally Acquired Immunity to Malaria during the Epidemiologic Transition in Kenya' (SSC#2207/UH IRB #06-11-22/DMID #11-0036) approved by the KEMRI's Ethical Review Committee (Appendix 2). Parental consent for the 1-10 year old children was sought and the consent forms signed.

The consenting process, confidentiality and rights of the participants were guaranteed as stipulated in Appendix 3. Data integrity was achieved by ensuring the information generated from the field and assays were maintained by the Principal Investigator in the laboratory while codes were used to ensure data and participant confidentiality. The data generated was stored in excel spreadsheets under password.

CHAPTER FOUR

RESULTS

4.1 Demographic, clinical and laboratory characteristics of the study participants

A total of 300 participants were included in this study of which: one hundred (n=100) were *P. falciparum* (Pf) malaria positive children, one hundred (n=100) Pf-malaria negative children and one hundred (n=100) Pf-malaria negative adults.

Table 4.1: Demographic and clinical characteristics of the study participants.

		Malaria +ve Children	Malaria -ve Children	Malaria -ve Adults	*p*-value
No. of Participants n=300		100	100	100	
Gender n, %	Males	53,(53%)	43,(43%)	15,(15%)	
	Females	47,(47%)	57,(57%)	85,(85%)	
Age, (years)	mean	4.65	5.28	31.9	**<0.001** [a]
Haemoglobin level, g/dL		10.10	11.00	12.15	**<0.001**[a]
Body temp. (^{o}C), mean		37.96	36.5	36.3	**<0.001**[a]

Data is presented as numbers and proportions in parentheses for gender and means for age, haemoglobin level and body temperature. [a] Statistical Significance determined by Kruskal-Wallis test. Values in bold were all found to be statistically significant at $p<0.05$.

As shown in Table 4.1, malaria positive individuals had significantly lower haemoglobin level compared to the malaria negative ones ($p<0.001$). There was marked elevated axillary body

temperature in the malaria positive compared to malaria negative individuals ($p<0.001$), a clear indication of the physiological effects of Pf attack in human beings.

4.2 Prevalence of Sickle Cell Trait

As shown in Table 4.2, the prevalence of SCT in Kanyawegi sub-location stands at 14.43%. Higher prevalence of SCT (18.5%) is seen in malaria negative individuals compared to 8.0% in malaria positive ones. A significantly lower proportion of SCT occured in malaria positive individuals (18.6%) compared to 81.4% in the malaria negative individuals (children and adults combined) ($p=0.035$). Only 0.7% of the study population had SCA while 85.0% were normal.

Table 4.2: Distribution of HbS phenotypes amongst study participants in terms of age and malaria status

HbS Phenotypes	Malaria +ve Children	Malaria -ve Children	Malaria -ve Adult	Average Prevalence	*p*-value
Participants n=300	100	100	100		
Normal	91, (91.0%)	80, (80.0%)	84, (84.0%)	85.0%	
SCT	8, (8.0%)	19, (19.0%)	16, (16.0%)	14.3%	**0.035**[a]
SCA	1, (1.0%)	1, (1.0%)	0, (0.0%)	0.7%	

Data is presented as numbers and proportions in parentheses. SCT=Sickle Cell Trait; SCA=Sickle Cell Anaemia; HbS=Haemoglobin S.
[a]Statistical significance determined by χ^2 analysis with $p<0.05$ considered statistically significant.

Table 4.3: A comparison of demographic, clinical and laboratory characteristics of study participants against their SCT status.

Characteristics		Normal	SCT	*p*-value
Participants	n=298	255, (85.57%)	43, (14.43%)	**<0.001**[a]
Gender: Males	(n,%)	106, (86%)	17, (14%)	
Females	(n,%)	149, (85%)	26, (15%)	<0.810[a]
Age, years,	(median)	7.0	6.0	0.227[b]
Malaria Status	*Pf* Positive	91, (35.6%)	8, (18.6%)	
	Pf Negative	164, (64.4%)	35, (81.4%)	**0.035**[a]
Parasitemia	(median)	1487.0	775.0	0.117[b]
Hb Level, g/dL	(median)	10.8	10.9	0.721[b]
RBC $x10^{12}$/L	(median)	4.0	4.5	0.081[b]
WBC	(median)	7.1	7.1	0.434[b]
HCT	(median)	34.6	34.7	0.692[b]

Data is presented as numbers and proportions in parentheses for gender and malaria status and medians for age, parasitemia, haemoglobin level, red blood cell, white blood cells and haemocrit counts. [a] Statistical significance determined by χ^2 analysis**.** [b] Statistical significance determined by Mann Whitney U test. Values in bold were all statistically significant at $p<0.05$. Hb=haemoglobin; RBC= red blood cells; WBC=white blood cells; HCT = haemocrit; Pf= *Plasmodium falciparum.*
n=298 since the 2 cases of SCA were excluded in this comparison of SCT versus normal cases.

Table 4.3 shows individuals with SCT and normal individuals in terms of malaria status (p=0.035) and amongst participants (p=0.001) but not in relation to the considered attributes like age (p=0.227), gender (p=0.810), Haemoglobin level (p=0.721)

4.3 Prevalence of G6PD Deficiency

In respect to G6PD deficiency, individuals were categorized as either G6PD normal, moderate or deficient.

Table 4.4: Distribution of G6PD status against age and malaria status of the participants.

G6PD Phenotypes	Malaria +ve Children	Malaria -ve Children	Malaria -ve Adult	Average Prevalence	*p*-value
Participants (n=300)	100	100	100		
Normal	81, (81.0%)	76, (76.0%)	72, (72.0%)	76.3%	
Moderate	13, (13.0%)	16, (16.0%)	17, (17.0%)	15.3%	
Deficient	6, (6.0%)	8, (8.0%)	11, (11.0%)	8.3%	**0.033**[a]

Data is presented as numbers and proportions in parentheses. s[a] Statistical significance in differences in prevalence of G6PD deficiency against malaria status was determined by χ^2 analysis with $p<0.05$ considered statistically significant.

As shown in table 4.4, 8.3% of the human population at Kanyawegi have G6PD deficiency, 15.3% are moderate while 76.3% are normal. A higher G6PD deficiency prevalence of 6.3% was observed amongst the malaria negative persons compared to 2.0% in the malaria positive cases. A significantly lower proportion of G6PD deficiency (24.0%) occurred in malaria positive individuals compared to 76.0% in malaria negative ones (p=0.033).

Table 4.5: A Comparison of Clinical, Demographic and Laboratory Characteristics of Study Participants against their G6PD Status.

Characteristics		G6PD Normal	G6PD Deficient	*p*-value
Participants	n=254	229	25	**<0.001**[a]
Gender: Males	(n,%)	77, (33.6%)	11, (44.4%)	
Females	(n,%)	152, (66.4%)	14, (55.6%)	0.590[a]
Age, years,	(median)	6.5	8.0	0.464[b]
Malaria Status	*Pf* Positive (n,%)	81, (35.4%)	6, (24.0%)	
	Pf Negative(n,%)	148, (64.6%)	19, (76.0%)	**0.069**[a]
Parasitemia	(median)	848.0	780.0	0.383[b]
Hb Level, g/dL	(median)	11.2	10.9	0.783[b]
RBC $x10^{12}$/L	(median)	4.5	4.1	**0.008**[b]
WBC	(median)	7.1	6.6	0.766[b]
HCT	(median)	35.8	34.4	0.397[b]

Data is presented as numbers and proportions in parentheses for gender and malaria status and medians for age, parasitemia, haemoglobin level, red blood cell, white blood cells and haemocrit counts.[a] Statistical significance determined by $\chi 2$ analysis. [b] Statistical significance determined by Mann Whitney U test. Values in bold were all statistically significant at $p<0.05$. Hb=haemoglobin; RBC= red blood Cells; WBC=white blood cells; HCT = haemocrit

There was no significant difference in the occurrence of G6PD deficiency in terms of gender (p=0.590) and Pf parasitemia load (p=0.383) as per Table 4.5. There was however, a tendency towards association between G6PD deficiency and malaria status (p=0.069). The G6PD deficient individuals recorded insignificantly low WBC, HCT and Hb levels compared to the G6PD normal individuals (p=0.766; p=0397 and p=0.783 respectively). However, the RBC counts were significantly low (p=0.008).

4.4 Sickle Cell Trait status and anti-*P. falciparum* Antigen IgG antibody levels

4.4.1 Sickle Cell Trait status and Anti-CSP IgG antibody levels

A comparison of anti-CSP IgG levels between the SCT (n=41) and Non-SCT (n=93) in Figure 4.1 as well as in SCT cases against their malaria status. Individuals with SCT showed similar anti-CSP IgG levels [median; 1.10 Arbitrary units (AU)] with the normal cases [median; 1.04AU] (p=0.940).

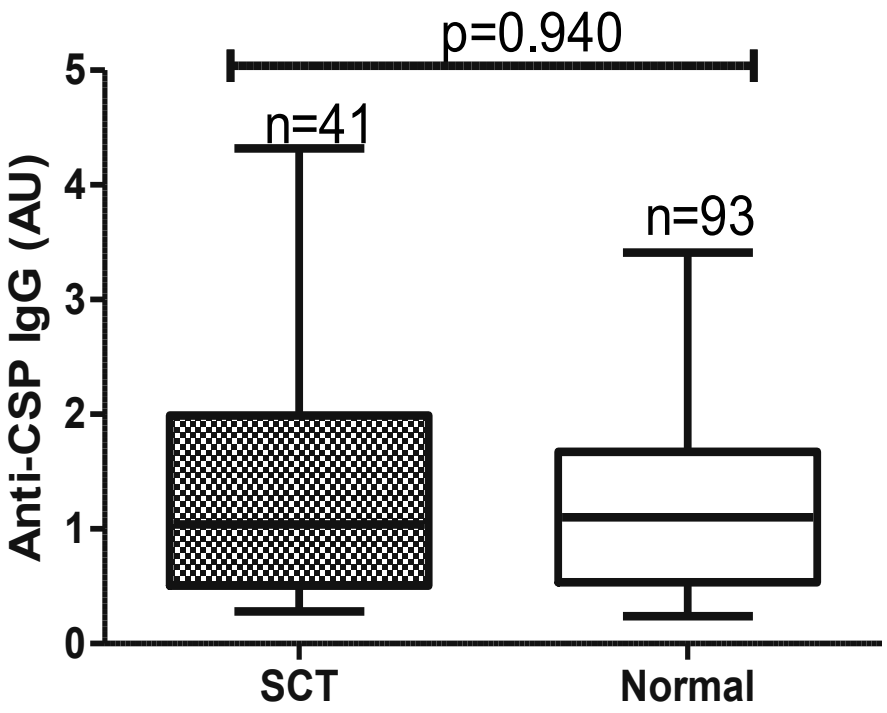

Figure 4.1: Comparison of Anti-CSP IgG levels between SCT and Normal for all individuals. Data are represented in box-plots. The boxes represent interquartile range; the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-CSP IgG antibody level comparisons between those with SCT and the Non-SCT was done using Mann-Whitney U test at $p \leq 0.05$.

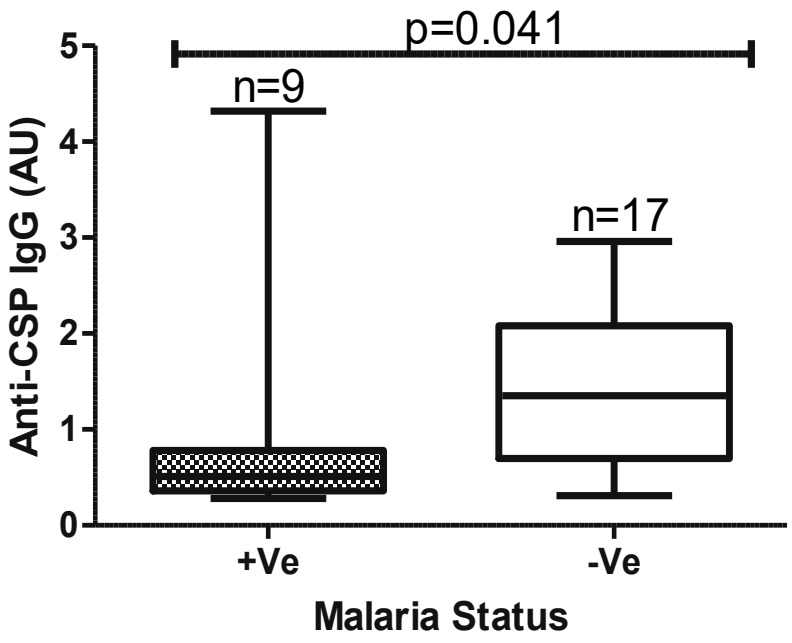

Figure 4.2: Comparison of anti-CSP IgG levels in Children with SCT against their Malaria Status. Data are represented in box-plots. The boxes represent interquartile range (IQR); the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-CSP IgG antibody level comparisons between the malaria negative SCT and the malaria positive SCT was done using Mann-Whitney U test at $p \leq 0.05$.

In Comparing anti-CSP IgG levels in children with SCT against malaria status as per Figure 4.2, malaria positive children (n=9) were found to have a significantly lower median anti-CSP IgG antibody level of 0.51 AU compared to the malaria negative ones (n=17) with median anti-CSP IgG level of 1.35AU (p=0.041).

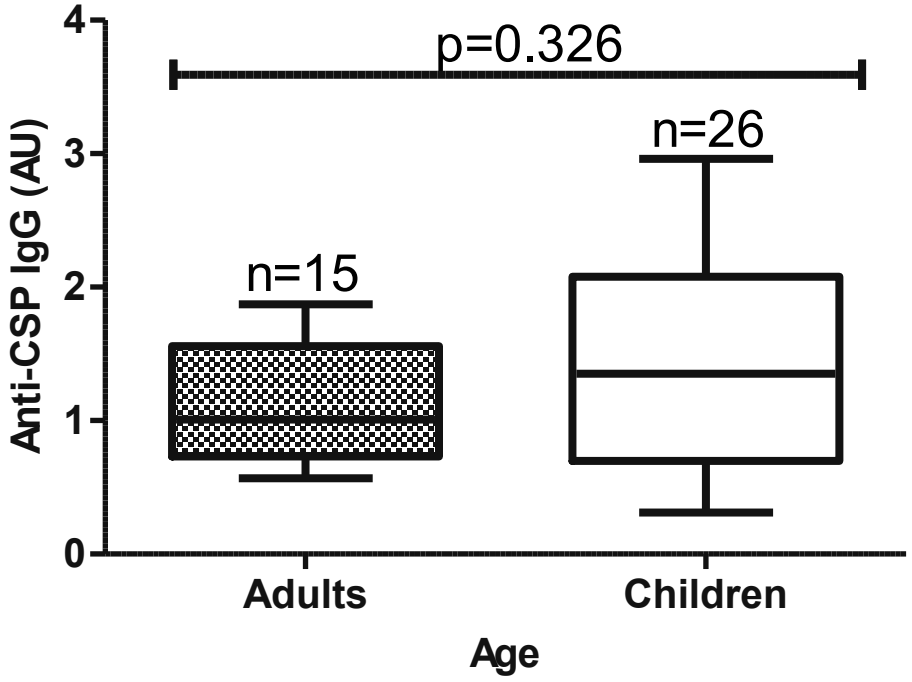

Figure 4.3: Comparison of anti-CSP IgG levels in SCT Adults and SCT children. Data are represented in box-plots. The boxes represent interquartile range (IQR); the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-CSP IgG antibody level comparisons between the adult SCT and the children SCT was done using Mann-Whitney U test at $p \leq 0.05$.

As per results in Figure 4.3 above, malaria negative SCT children (n=26) had higher median anti-CSP IgG antibody level of 1.35AU compared to 1.01AU in the SCT malaria negative adults (n=15) in children than the adults who post 1.083AU. However, at p=0.326, this difference in anti-CSP IgG antibody level as per age was not statistically significant.

4.4.2 Sickle Cell Trait status and Anti-AMA1 IgG levels

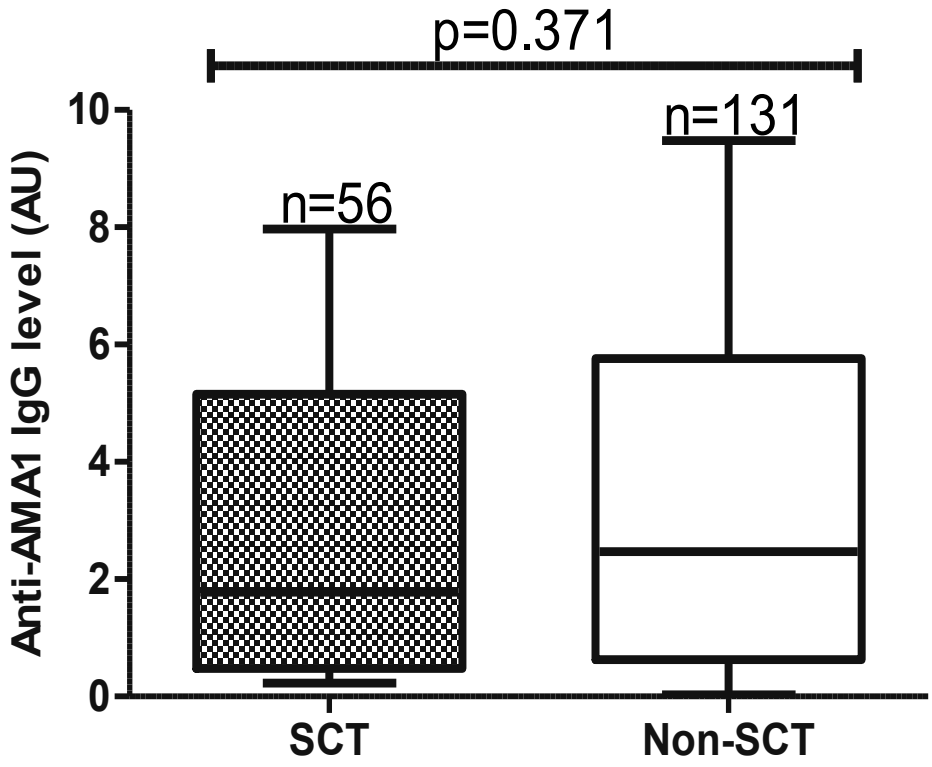

Figure 4.4: Comparison of anti-AMA1 IgG levels against SCT Status of all individuals. Data are represented in box-plots. The boxes represent interquartile range (IQR); the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-AMA1 IgG antibody level comparisons between the SCT and the Non-SCT was done using Mann-Whitney U test at $p \leq 0.05$.

Higher median anti-AMA1 IgG antibody level of 2.47AU was observed in non-SCT individuals compared to 1.78AU of those with SCT as per Figure 4.4. This difference in anti-AMA1 IgG antibody levels amongst these two groups was however not statistically significant (p=0.371).

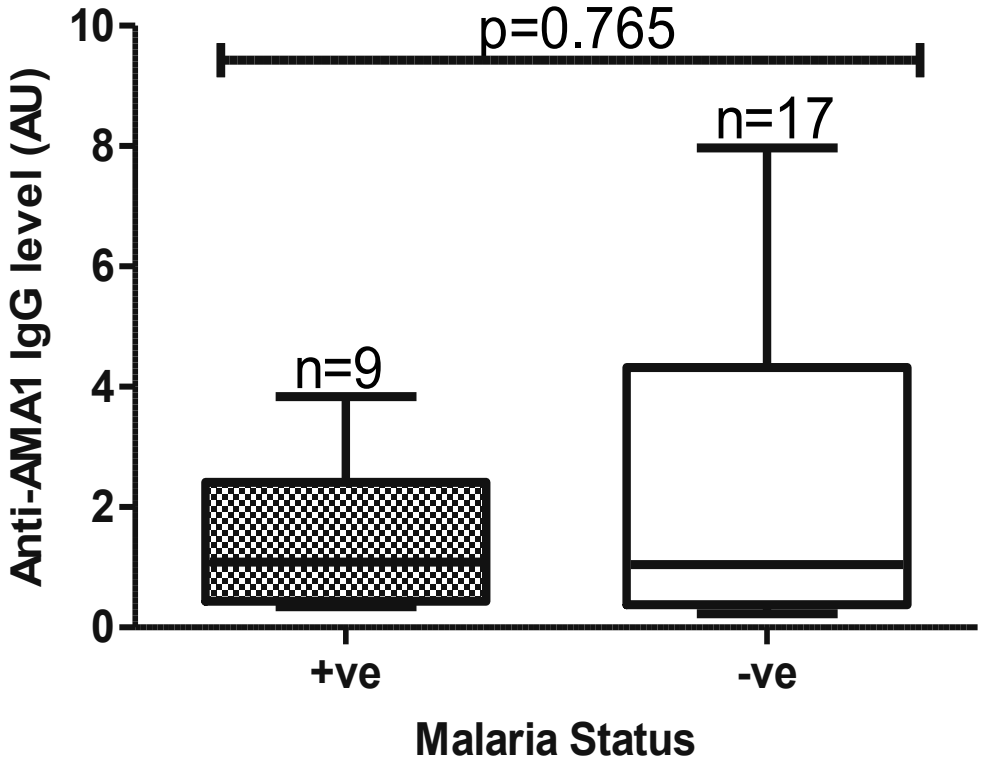

Figure 4.5: Comparison of anti-AMA1 IgG levels against Malaria Status in SCT Children. Data are represented in box-plots. The boxes represent interquartile range (IQR); the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-AMA1 IgG antibody level comparisons between the SCT malaria positive and malaria negative SCT children was done using Mann-Whitney U test at $p \leq 0.05$.

As shown in Figure 4.5, SCT malaria positive children showed similar median levels of 1.09AU anti-AMA1 IgG antibody level compared to SCT malaria negative children who had 1.05AU (p=0.765).

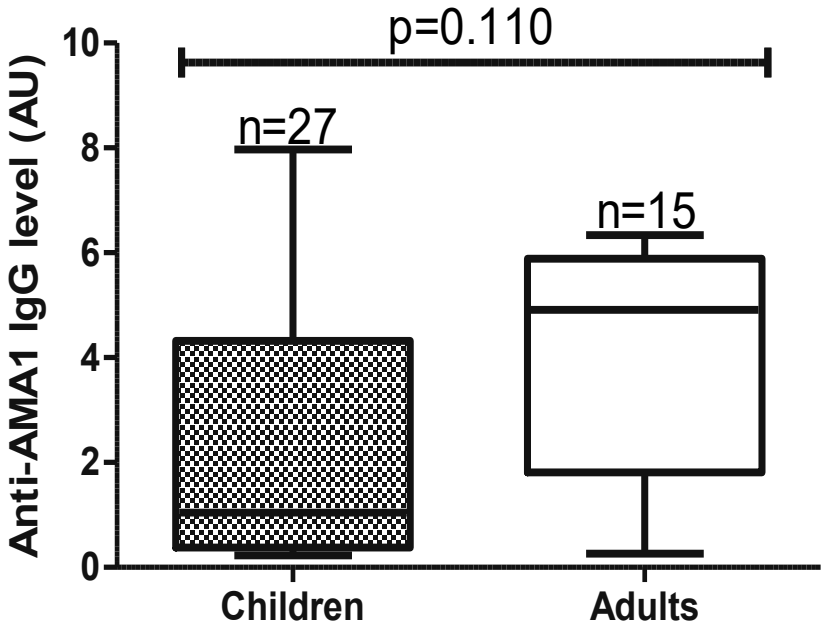

Figure 4.6: Comparison of anti-AMA1 IgG levels in SCT cases against Age. Data are represented in box-plots. The boxes represent interquartile range (IQR); the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-AMA1 IgG antibody level comparisons between the SCT malaria positive and malaria negative SCT children was done using Mann-Whitney U test at $p \leq 0.05$.

Malaria negative SCT adults express higher levels of anti-AMA1 IgG antibody levels than malaria negative SCT children (Figure 4.6). They posted a median anti-AMA1 IgG antibody levels of 4.91AU and 1.05AU respectively, however the difference in the antibody levels amongst the two groups was not statistically significant (p=0.110)

4.5 Glucose-6-Phosphate Dehydrogenase Deficiency and anti-Pf Antigen IgG levels

4.5.1 Glucose 6 Phosphate Dehydrogenase Deficiency and anti-CSP IgG Antibody levels

The level of Anti-CSP IgG antibodies was measured against gender

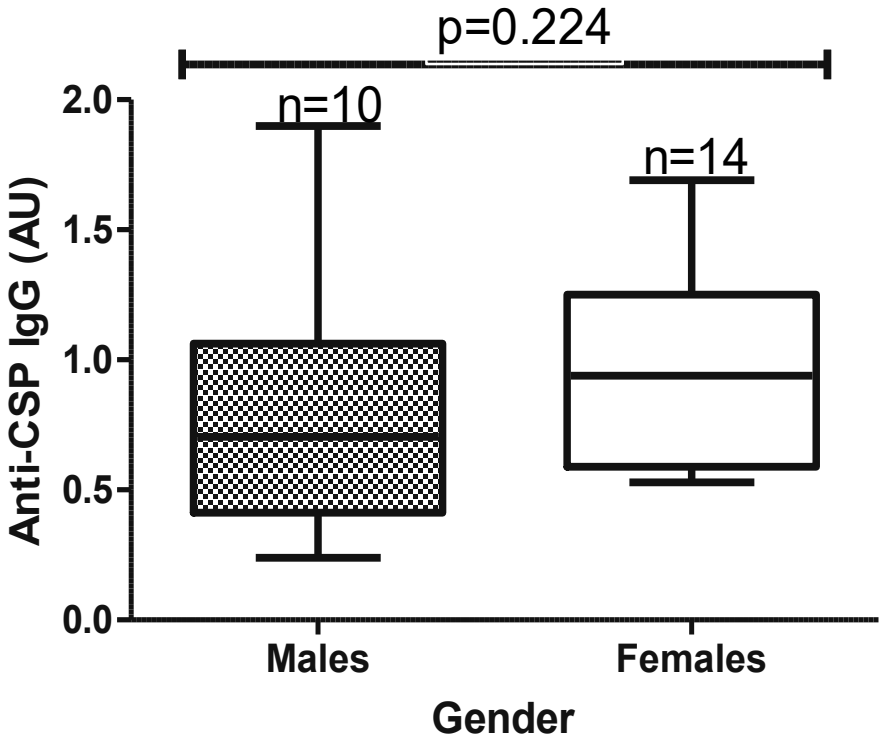

Figure 4.7: Comparison of anti-CSP IgG levels in G6PD deficient cases against gender. Data are represented in box-plots. The boxes represent interquartile range (IQR); the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-CSP IgG antibody level comparisons between the G6PD Deficient cases amongst the gender was done using Mann-Whitney U test at $p \leq 0.05$.

Comparison of the anti-CSP IgG level as per gender showed a higher median anti-CSP IgG antibody level of 0.94AU in females compared to 0.705AU in males as shown in Figure 4.7. However, this difference was not statistically significant (p=0.224).

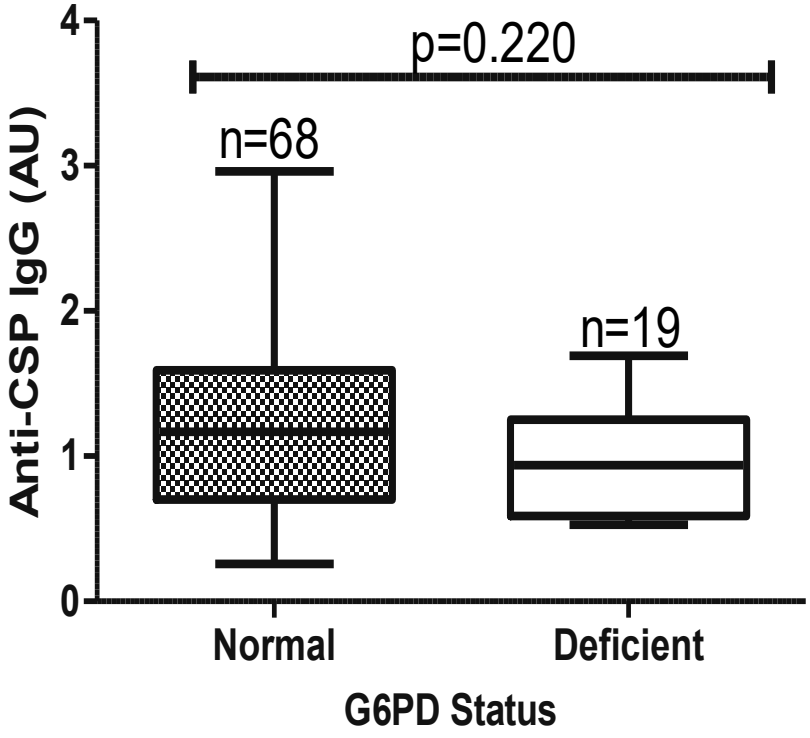

Figure 4.8: Comparison of anti-CSP IgG levels in females against G6PD deficiency status. Data are represented in box-plots. The boxes represent interquartile range (IQR); the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-CSP IgG antibody level comparisons in females against their G6PD deficiency status was done using Mann-Whitney U test at $p \leq 0.05$. The results indicate no significant differences in the levels of anti-CSP IgG antibody amongst the two groups (p=0.220).

A comparison of levels of anti-CSP IgG antibody in females showed no significant difference between those who are G6PD deficient and those who are G6PD normal as shown in Figure 4.8 (p=0.220). This is in spite of the median anti-CSP IgG antibody levels being higher in G6PD normal (1.17AU) than in the deficient females (0.94AU).

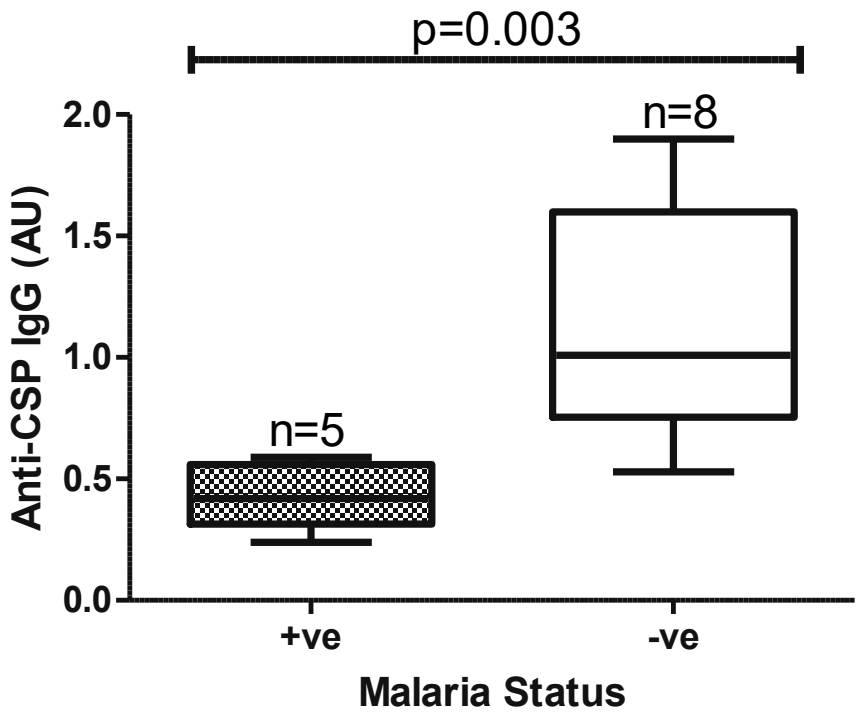

Figure 4.9: Comparison of anti-CSP IgG levels in G6PD deficient children against Malaria Status. Data are represented in box-plots. The boxes represent interquartile range (IQR); the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-CSP IgG antibody level comparisons in G6PD deficiency children against their malaria status was done using Mann-Whitney U test at $p \leq 0.05$.

When the levels of anti-CSP IgG antibody levels were compared amongst G6PD deficient children as shown in Figure 4.9, those without malaria exhibit a significantly higher median anti-CSP IgG antibody level of 1.010AU compared to those with acute malaria who score 0.42AU (p=0.003).

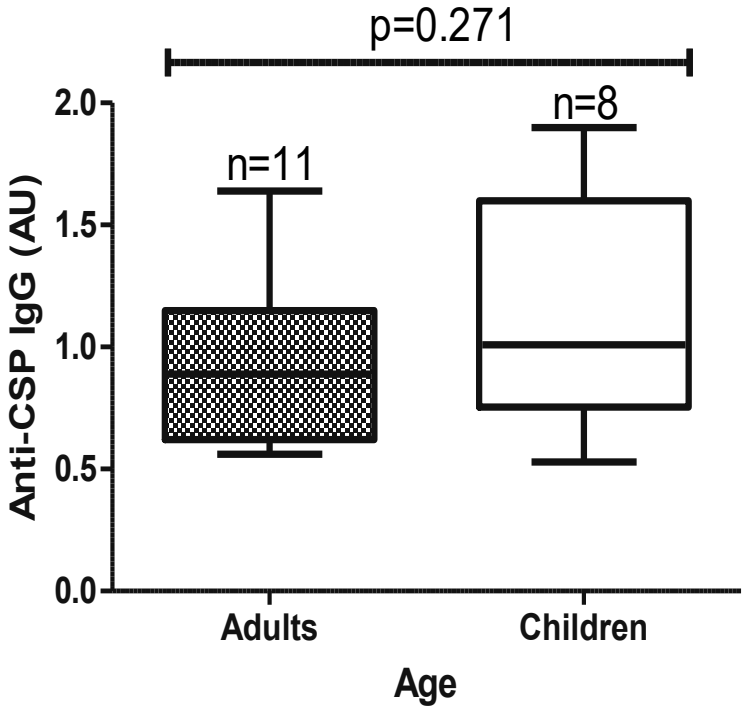

Figure 4.10: Comparison of anti-CSP IgG levels in malaria negative G6PD deficient cases against age. Data are represented in box-plots. The boxes represent interquartile range (IQR); the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-CSP IgG antibody level comparisons in malaria negative G6PD deficient cases against age was done using Mann-Whitney U test at $p \leq 0.05$.

By taking age into consideration, malaria negative G6PD deficient children showed a median anti-CSP IgG antibody of 1.01AU which is higher compared to malaria negative G6PD deficient adults who exhibited a median IgG antibody of 0.89AU. However, this variation in antibody levels was not significant (p=0.271) as shown in Figure 4.10.

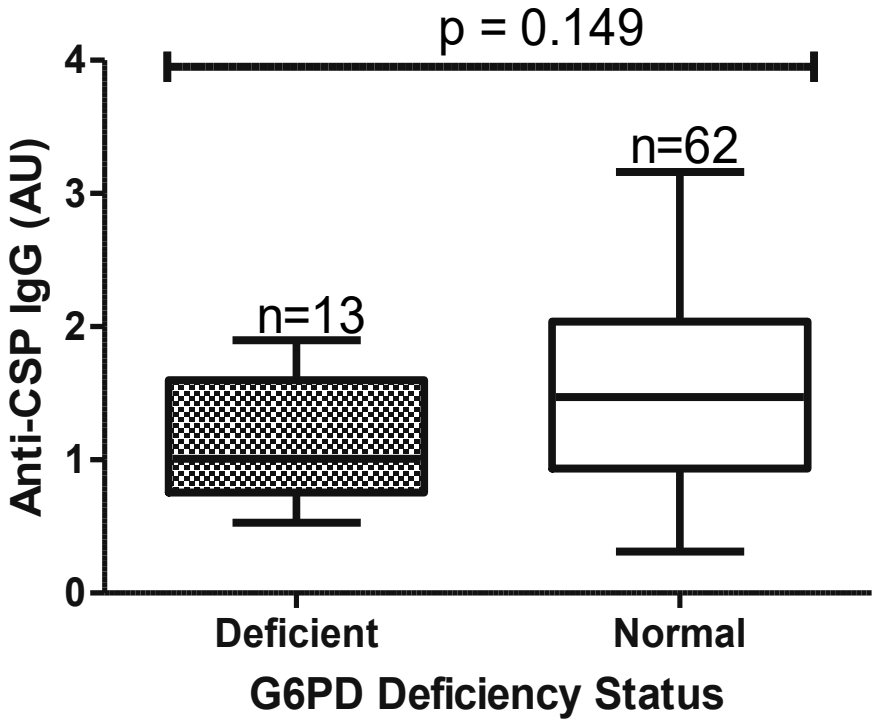

Figure 4.11: Comparison of anti-CSP IgG levels in malaria negative children against G6PD deficiency status. Data are represented in box-plots. The boxes represent interquartile range (IQR); the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-CSP IgG antibody level comparisons in malaria negative children against their G6PD deficiency status was done using Mann-Whitney U test at $p \leq 0.05$.

Although G6PD normal malaria negative children expressed higher median anti-CSP IgG antibody levels (1.47AU) compared to their G6PD-deficient counterparts (1.01AU) as shown in Figure 4.11, that difference was not statistically significant (p=0.149).

4.5.2 Glucose 6 Phosphate Dehydrogenase deficiency and anti-AMA1 IgG Antibody levels

The levels of anti-AMA1 IgG antibodies were compared in G6PD deficient cases between males and females (Fig. 4.12)

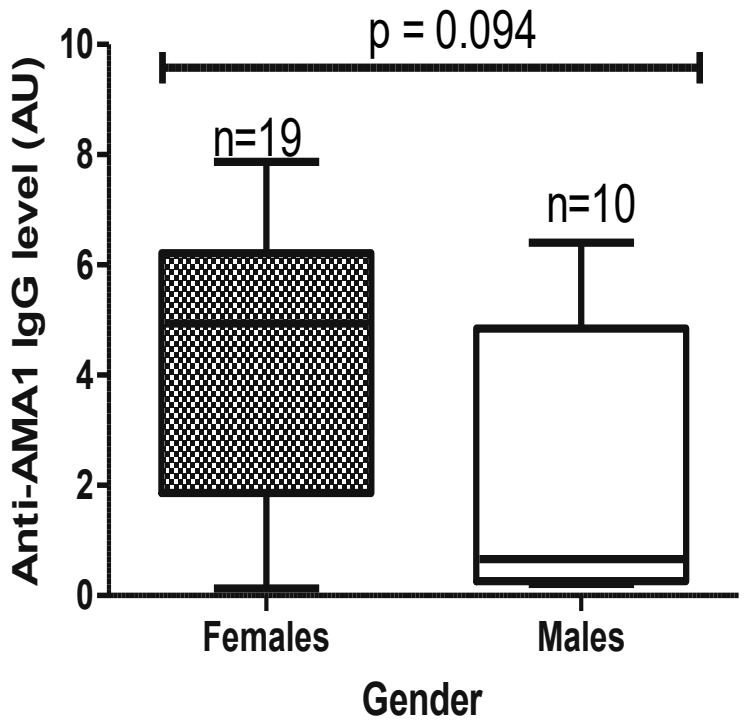

Figure 4.12: Comparison of Anti-AMA1 IgG Levels in G6PD deficient cases against gender. Data are represented in box-plots. The boxes represent interquartile range (IQR); the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-AMA1 IgG antibody level comparisons in G6PD deficient cases against gender was done using Mann-Whitney U test at $p \leq 0.05$.

According to results shown in Figure 4.12, G6PD deficient females express higher median anti-AMA1 IgG antibody levels of 4.94AU compared to G6PD deficient males who have 0.66AU, however, this difference was insignificant (p=0.094).

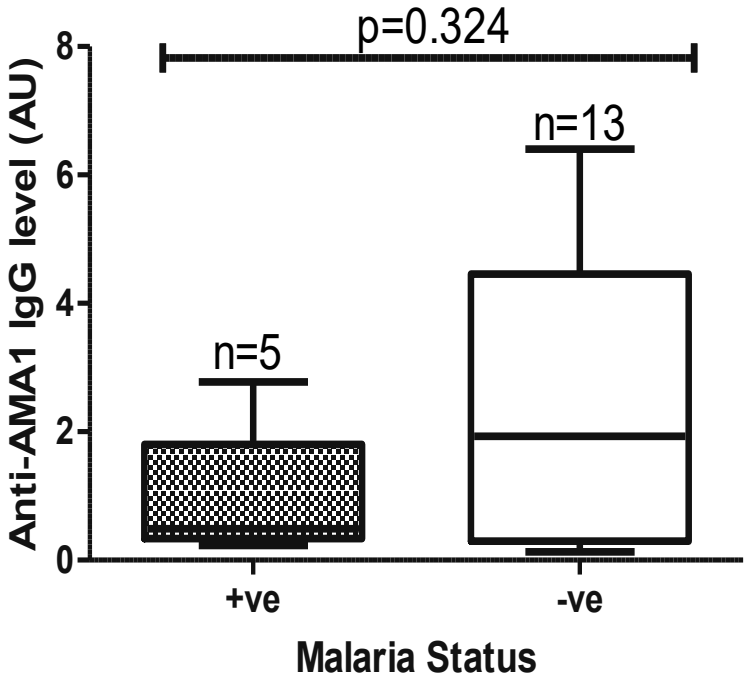

Figure 4.13: Comparison of Anti-AMA1 IgG Levels in G6PD deficient children against malaria status. Data are represented in box-plots. The boxes represent interquartile range (IQR); the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-AMA1 IgG antibody level comparisons in G6PD deficient children against malaria status was done using Mann-Whitney U test at $p \leq 0.05$.

Results in Figure 4.13 show G6PD deficient malaria negative children expressed a higher median anti-AMA1 IgG antibody level (1.93AU) compared to their malaria positive counterparts (0.490AU). This difference in their median antibody levels is however, statistically insignificant (p=0.324).

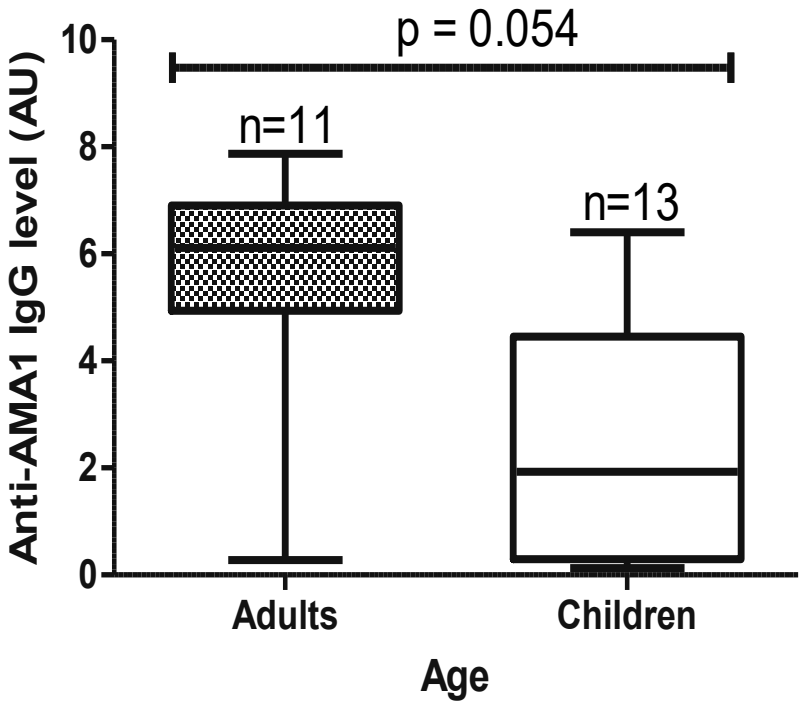

Figure 4.14: Comparison of Anti-AMA1 IgG Levels in malaria negative G6PD deficient cases against age. Data are represented in box-plots. The boxes represent interquartile range (IQR); the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-AMA1 IgG antibody level comparisons in malaria negative G6PD deficient cases against age was done using Mann-Whitney U test at $p \leq 0.05$.

Malaria negative G6PD deficient adults exhibit significantly higher median anti-AMA1 IgG antibody levels (6.11AU) compared to malaria negative G6PD deficient children (1.93AU). As shown in Figure 4.14, this difference in antibody levels was statistically significant (p=0.054).

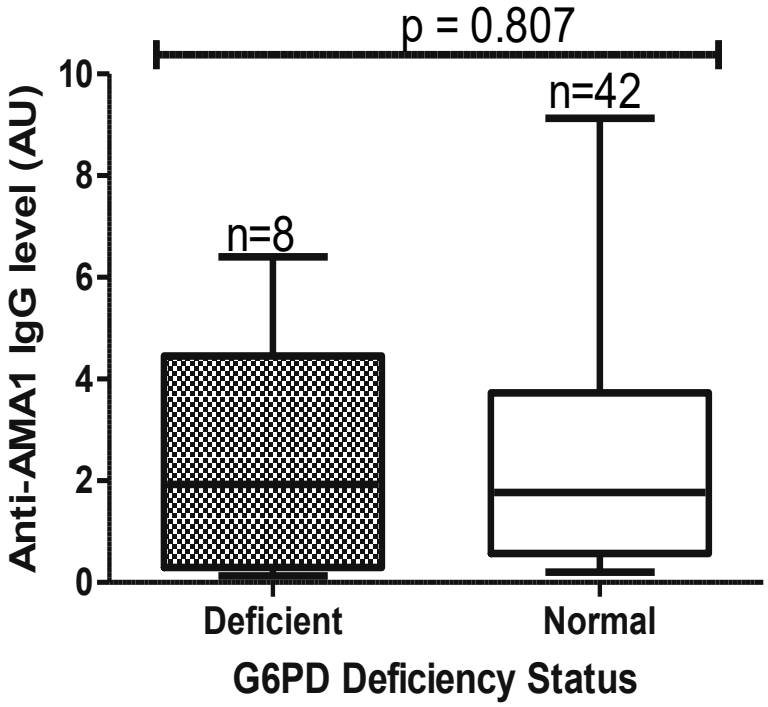

Figure 4.15: Comparison of Anti-AMA1 IgG Levels in malaria negative children against G6PD status. Data are represented in box-plots. The boxes represent interquartile range (IQR); the line through boxes is the median while the whiskers show the 10th and the 90th percentiles. Anti-AMA1 IgG antibody level comparisons in malaria negative children against their G6PD deficiency status was done using Mann-Whitney U test at $p \leq 0.05$.

No significant difference was seen in the medians of anti-AMA1 IgG antibody levels between malaria negative G6PD deficient (1.93AU) and normal children (1.77AU) (p=0.807) as shown in Figure 4.15.

4.6 Glucose 6 Phosphate Dehydrogenase Deficiency Status, Sickle Cell Trait and Frequency of IgG Antibodies

Prior to determining the association between G6PD deficiency, SCT and IgG antibodies against CSP and AMA1 *Pf*-antigens, frequency of IgG antibody against the *Pf*-antigens was determined. Sample plasma with Optic Density (OD) values greater than 0.36OD for CSP and 0.45OD for AMA1 were categorized as being positive or have "High" IgG antibody levels while those with ODs below cut-off were categorized as being negative or have "Low" IgG antibody levels against the specified *Pf*-antigens (Noland *et al*., 2008; John *et al*., 2005).

4.6.1 Frequency of IgG antibodies against G6PD Status

Anti-CSP and anti-AMA1 IgG antibody frequencies were compared to the genetic disorders as shown in the table below

Table 4.6: Comparison of anti-CSP and anti-AMA1 IgG antibody frequencies against G6PD status.

Antibody	Antibody Level	G6PD deficient (n, %)	G6PD Normal (n, %)	*p*-value
Anti-CSP IgG	Low	17, (58.62)	48, (42.48)	
	High	12, (41.38)	65, (57.52)	**0.042**[a]
Anti-AMA1 IgG	Low	9, (31.04)	36, (32.73)	
	High	20, (68.96)	74, (67.27)	0.852[a]

The frequencies of the anti-CSP and anti-AMA1 IgG antibodies were determined. [a] Statistical significance determined by χ^2 analysis with $p<0.05$ considered statistically significant. Significantly lower frequency of anti-CSP.

According to results shown in Table 4.6, G6PD normal individuals had a significantly higher frequency of anti-CSP IgG antibodies than the G6PD deficient individuals (p=0.042) but no significant difference was seen when anti-AMA1 IgG antibodies were compared (p=0.852).

4.6.2 Frequency of IgG antibodies against Sickle Cell Trait Status

According to results shown in Table 4.7, Sickle Cell Trait (SCT) had no significant influence on the frequency of anti-CSP IgG (p=0.202) and anti-AMA1 IgG (p=0.589) antibodies.

Table 4.7: Comparison of anti-CSP and anti-AMA1 IgG antibody frequencies against Sickle Cell Trait status

Antibody	Antibody Level	SCT (n, %)	Normal (n, %)	*p*-value
Anti-CSP IgG	Low	28, (50.91)	57, (48.31)	0.202
	High	27, (49.09)	61, (51.69)	
Anti-AMA1 IgG	Low	21, (37.50)	42, (32.06)	0.589
	High	35, (62.50)	89, (67.94)	

The frequencies of the anti-CSP and anti-AMA1 IgG antibodies were determined. Statistical significance was determined by χ^2 analysis with $p<0.05$ considered statistically significant

4.7 Association between Glucose 6 Phosphate Dehydrogenase, Sickle Cell Trait and IgG antibodies

4.7.1 Association between anti-CSP IgG antibodies, G6PD and SCT

To determine the association between low frequency of anti-CSP IgG antibodies and the two genetic disorders, binary logistic regression analysis was done controlling for age, malaria status, G6PD deficiency and Sickle Cell Trait (SCT) status.

Table 4.8: Association between anti-CSP IgG antibody frequencies, G6PD, SCT and malaria status

Genetic Disorder	OR	95% CI	*p*-value
G6PD Deficiency	0.458	0.237-0.885	**0.020**
SCT	0.432	0.218-0.856	**0.016**
Malaria	0.050	0.013-0.185	**<0.001**
Age	0.980	0.956-1.004	0.106
Females	0.952	0.480-1.887	0.887

Study participants were classified based on the presence or absence of SCT as well as G6PD deficiency and Malaria Status. Odds Ratios (OR) and 95% confidence interval (CI) were determined using binary logistic regression controlling for age and malaria. The reference groups in the logistic regression analysis were those individuals without either of the genetic disorders, males and malaria negative

As shown in Table 4.8, lower frequencies of anti-CSP IgG antibodies had significant association with G6PD deficiency (p=0.020), SCT (p=0.016) and malaria (p<0.001) but not with age (p=0.106) or gender (0.887).

4.7.2 Association between anti-AMA1 IgG antibodies, G6PD deficiency and SCT

The association between anti-AMA1 IgG antibody frequency and the two genetic disorders was done using binary logistic regression analysis while controlling for age (John *et al*., 2005), malaria status (Dodoo *et al.,* 2011; John *et al*., 2008; John *et al*., 2003) G6PD deficiency and Sickle Cell Trait (SCT) status (Miura *et al*., 2013; Verra *et al*., 2007; Sarr *et al*., 2006).

Table 4.9: Association between anti-AMA1 IgG antibody frequencies G6PD, SCT and malaria status.

Attribute	OR	95% CI	*p*-value
G6PD Deficiency	0.924	0.473-1.808	0.819
SCT	1.062	0.539-2.093	0.862
Malaria	0.621	0.265-1.459	0.275
Age	1.090	1.044-1.142	**<0.001**
Gender	1.092	0.554-2.154	0.799

Study participants were classified based on the presence or absence of SCT as well G6PD deficiency and Malaria Status. Odds Ratios (OR) and 95% confidence interval (CI) were determined using binary logistic regression controlling for age and malaria. The reference group in the logistic regression analysis were those individuals without either of the genetic disorders.

Table 4.9 shows lack of significant association in low frequencies of anti-AMA1 IgG antibodies when Sickle Cell Trait (p=0.862) and G6PD deficiency (p=0.819), malaria (p=0.275), gender (p=0.799) are taken into consideration. However, there was a significant association between low frequencies of anti-AMA1 IgG antibodies and age (p<0.001).

CHAPTER FIVE

DISCUSSION

To determine the prevalence of malaria-related genetic disorders and their association with anti-CSP and anti-AMA1 IgG antibody responses, a cross-sectional analysis of prevalence of G6PD deficiency and Sickle cell trait (SCT) was done amongst residents of a malaria endemic area of Kanyawegi. The current study revealed an SCT prevalence of 14.4% while G6PD deficiency stood at 8.3%. In terms of humoral responses, G6PD deficiency associated significantly with lower frequencies of anti-CSP IgG antibodies but not with anti-AMA1 IgG antibodies. The levels and frequencies of anti-AMA 1 IgG antibody showed no significant variation amongst individuals with SCT and those who lacked them while a significant variation was seen when the frequencies of anti-CSP IgG antibodies were taken into account.

Malaria parasite attack leads to a number of physiological changes in the human host that bring about clinical symptoms. In a typical trend, malaria positive individuals showed higher axillary body temperature with lower haemoglobin level compared to malaria negative participant which is attributed to the high splenic clearance of iRBCs. Earlier studies (Miura *et al*., 2013; Cabrera *et al*., 2005; Aidoo *et al*., 2002) showed that SCT and G6PD deficiency confer relative protection to malaria attack. The current study also found a similar result since low incidences of clinical malaria and insignificantly low Pf parasitemia was reported in individuals with these genetic disorders but with no significant differences in age and haemoglobin level with those who lacked these genetic disorders. The observed reduced incidence and clinical effects of Pf malaria on SCT and G6PD deficient individuals is thought to be as a result of enhanced phagocytosis (Luzatto & Poggi, 2008; Min Oo & Gros, 2005; Ayi, 2004; Mockenhaupt, 2003), retarded

parasite growth in sickled iRBCs and G6PD deficient iRBCs (Balgir, 2012) and humoral influence (Williams, 2011).

Most erythrocyte-based human genetic disorders thought to confer relative protection to malaria attack occur in human populations present in malaria endemic areas of the world (Fowkes *et al*., 2008). Kanyawegi sub-location, which was the site for the current study is found in western Kenya with over 45% of malaria incidence reported amongst children (Kapesa *et al*., 2018) and a high adult prevalence of the malaria parasite standing at 28% (Jenkins *et al*., 2015). Additionally, it has very high Entomological Inoculation Rate (EIR) of over 300 infectious bites per person per year (Beier *et al.,* 1994; Beier *et al.,* 1990). Earlier studies in the lake Victoria basin sites have reported higher prevalence of these genetic disorders compared to low malaria transmission areas (Suchdev *et al*., 2014; Tsang *et al*., 2014; Hunja 2012; Moorman *et al*., 2003).

In the current study, SCT prevalence stood at 14.4% that is lower compared to 27.9% (John *et al.,* 2005) and 26.5% (Mulama *et al.,* 2014) recorded earlier for the same study site. However, these two recent studies didn't set out to investigate the prevalence of SCT *per se* in the current study site and the fewer (77) study participants as used by John et al. (2005) did not effectively reflected the correct diversity of the genetic mutations in an entire population of the study site. Whereas Mulama et al. (2014) used a larger sample size (547), the inclusion criteria in their study chose only those that lacked endemic Burkitts Lymphoma (eBL) and it is therefore possible that random sampling as followed in this current study might not have been undertaken in a way that would be representative of the entire population. An early study by Moorman et al. (2003) showed prevalence of SCT being 26% but it is unlikely for the prevalence to remain the same for over ten years. The drop in prevalence of SCT as seen in the current study may be indicative of influence of changing demographics due to human immigration and emigration as

well as early deaths of SCA cases that clear the HbS gene from the population. Earlier studies done in the surrounding areas of Nyando and Asembo bay with almost similar malaria incidence and entomological inoculation rates as the current study site (Oloo et al., 1996; Beier *et al*., 1994; Githeko *et al*., 1993; Beier *et al*., 1990) showed an SCT prevalence of 17% and 17.4% respectively (Tsang *et al*., 2014; Suchdev *et al*., 2014; Hobbs *et al*., 2002; Aidoo *et al*., 2002). This higher prevalence of SCT compared to Kanyawegi may be indicative of variations resulting from underlying interplay of malaria incidences, topography, demographics and anti-malaria interventions on the spread of this genetic disorder in human populations.

Sickle cell anaemia (SCA) which is a serious health concern in malaria endemic areas of the world was only seen in 0.7% of the study participants. It was only observed in children and not adults. This is consistent with findings from other studies in western Kenya where below 2.0% SCA prevalence has been reported (Tsang *et al*., 2014; Suchdev *et al*., 2014; Terlouw *et al*., 2004). The absence of SCA cases in adults and its low prevalence show that such individuals could likely have experienced malaria and other related infections that aggravate their anaemic conditions leading to early death. It is therefore possible that deaths of individuals with SCA at an early age before reaching reproductive age as a result of poor health contribute to the continued elimination of the HbS gene from the population and thereby regulate the prevalence of SCT even when population of people in an area increases over time.

The prevalence of G6PD deficiency in the current study site stood at 8.3%. This showed a slight rise from 7% observed in an investigation in the same study site done over ten years earlier (Moorman *et al*., 2003). Compared to neighbouring areas, Kanyawegi displayed a higher G6PD deficiency prevalence than Nyando with 6.8% (Tsang *et al*., 2014; Suchdev *et al*., 2014) but lower than Kombewa with 13.9% (Hunja, 2012) found in the same malaria endemic lake

Victoria basin. Given that the G6PD gene occur on the X-sex chromosome, higher occurrence of G6PD deficiency in the males was expected as seen in earlier studies (Suchdev *et al.,* 2012; Carter *et al.,* 2011; Shekalaghe *et al*., 2009). However, this study found no marked difference of G6PD deficiency prevalence against sex. Earlier studies (Leslie *et al.,* 2013) fault the G6PD deficiency phenotyping approach used since it may give 'false' normal to males and also its inability to distinguish G6PD homozygosity and heterozygosity in females. This does not deny or confirm the existence of association between sex and G6PD deficiency since the significantly higher number of females enrolled in the current study compared to males made this comparison inconclusive.

In recent times, acquired immunity has been proposed to partially mediate protection against malaria in individuals with these adaptive genotypes (Williams, 2011; Verra *et al*., 2007; Williams *et al*., 2005). In typical malaria attack, it has been shown that increase in IgG antibody levels against known antigens of pre-erythrocytic and erythrocytic Pf stages lead to decline in malaria infections (Dodoo *et al*., 2011; Noland *et al*., 2008; John *et al*., 2008; John *et al*., 2005; John *et al*., 2003). Such generalization has not been possible in studies that have factored in the adaptive genotypes as a result of conflicting findings in earlier studies (Miura *et al.,* 2013; Afridi *et al.,* 2012; Tan *et al.,* 2011; Verra *et al.,* 2007; Sarr *et al.,* 2006).

Production of IgG antibodies against Pf Circumsporozoite protein (CSP) offer protection to both animal and human models (Kim *et al.,* 2011). Generally, children with higher anti-CSP IgG levels experience fewer episodes of malaria than those with less Anti-CSP IgG antibodies (Noland *et al*., 2008; John *et al*., 2003). In the current study, SCT as well as G6PD deficiency were associated with significantly lower frequency of the anti-CSP IgG antibodies. It was expected that for anti-CSP IgG antibodies to drive protection against malaria in G6PD deficient

and SCT individuals separately, then the antibodies levels and frequency need to be higher but this was not the case in the reported findings. Interestingly, when malaria and age were factored in, malaria positive children with SCT had significantly lower levels of anti-CSP IgG antibody compared to the malaria negative children with SCT. The same result was seen in regard to G6PD deficiency status where the malaria negative G6PD deficient children showed significantly higher levels of anti-CSP IgG antibodies than the malaria positive G6PD deficient counterparts. Taken together, these findings show that in respect to CSP, it is possible that the high level of anti-CSP IgG antibodies playing in concert with these genetic disorders and other innate factors could be driving the malaria immunity in children.

Increase in IgG antibodies against blood stage Pf antigens including AMA1 have been associated with a decline in risk of malaria infection (Dodoo *et al*., 2011; Noland *et al*., 2008; John *et al*., 2008; John *et al*., 2005) since they have Pf growth inhibitory activity and prevent erythrocyte invasion (Miura *et al*., 2013). In order to afford protection against malaria, it was expected that higher frequency of these antibodies would occur in individuals with either G6PD deficiency or SCT. However, in earlier studies (Miura *et al*., 2013; Sarr *et al*., 2006), lower IgG antibodies against blood stage Pf antigens RESA, MSP2 and EBA175 have been reported in individuals with either of these genetic disorders. In the current study individuals with SCT and G6PD deficiency separately had statisticaly insignificant low frequency of anti-AMA1 IgG antibody. This was consistent with findings done elsewhere (Miura *et al*., 2013; Afridi *et al*., 2012; Tan *et al*., 2011) in study sites with high malaria transmission just like the current study site.

G6PD deficiency and SCT have separately been shown to bring down Pf parasitemia through ratardation of Pf parasite growth by sickled and G6PD deficient infected erythrocytes (iRBCs) releasing more toxic Hydrogen peroxide and super oxides (Balgir, 2012) or through splenic

clearance given that such iRBCs suffer enhanced phagocytosis (Min-Oo and Gros 2005; Mockenhaupt *et al*., 2003). This impairment of parasite growth lead to low Pf parasitemia in individuals with these genetic disorders as seen in the current study though insignificant. This may in turn influence reduced production of IgG antibodies by B cells against erythrocytic stage antigens like AMA1 as reported in earlier studies (Miura *et al*., 2013; Sarr *et al*. 2006).

Studies done in low malaria transmission areas of Tanzania and Burkina Faso found significantly high levels of anti-AMA1 IgG antibody in individuals with either G6PD deficiency and SCT compared to those who lack the genetic disorders (Dewasurendra *et al*., 2012; Verra *et al*., 2007). A study in Burkina Faso (Verra *et al*., 2007) established siginificant difference in anti-AMA1 IgG antibody levels amongst those in low but not in high malaria transmission areas. It is therefore possible that exposure to malaria early in life and repeated malaria infection in areas of high malaria transmission lead to saturation of IgG antibodies against malaria blood stage antigens over time which in turn confound attempts at understanding the influence, if any, of SCT and G6PD deficiency on malaria-related anti-AMA1 IgG antibody levels.

The current study has established that G6PD deficiency and SCT separately associate with low frequency of anti-CSP IgG antibodies but not with anti-AMA1 IgG antibodies in general population. Whereas this study confirms that there is relative protection against malaria in individuals with either SCT or G6PD deficiency it has not been able to find a link with enhanced humoral responses reported by some studies (Dewasurendra *et al*., 2012; Verra *et al.*, 2007) for the two investigated Pf antigens. In light of the finding of this study, it is possible that generally, the elevated recognition and early clearance of G6PD deficient and HbS iRBCS could lead to low parasitemia but insignificant changes in the IgG antibodies against blood stage antigens in

the general population. However, this assertion becomes different when only children with SCT or G6PD deficiency are considred in the event of malaria attack as shown in this study

In children with SCT or G6PD deficiency, those who eventually experience clinical malaria have lower levels of anti-CSP IgG antibodies which imply that amongst this group, these genetic disorders separately play in concert with Anti-CSP humoral responses to afford them protection against attack and progression of clinical malaria. This could partly explain the early assertion by findings of Williams *et al.* (2005) and Aidoo *et al.*(2002) that the protection afforded by either of these genetic disorders against malaria attack occured mainly when the individuals are below 10 years of age.

In this current study, the inclusion of age as well as malaria status as comparison factors in the study helped to bring out more information not seen in other studies done in the study site more so involving the two genetic disorders. Additionally, the consideration of only two Pf antigens (CSP and AMA1) helped reduce confounding results due to antibody saturation.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 SUMMARY OF FINDINGS

The prevalence of G6PD deficiency and SCT in Kanyawegi area of Kisumu West sub-county stands at 8.3% and 14.4% respectively while only 0.7% have sickle cell anaemia. Both G6PD deficiency and SCT separately associated significantly with low frequencies of anti-CSP IgG antibodies but not with anti-AMA1 IgG antibodies. In children with SCT or G6PD deficiency, those who eventually experience clinical malaria have lower levels of anti-CSP IgG antibodies.

6.2 CONCLUSIONS

I. In residents of Kanyawegi sub-location, the prevalence of G6PD deficiency stands at 8.3% while that of SCT stands at 14.4% suggesting that in the current study site there is a sizeable proportion of people having relative protection against malaria.

II. There was significant association between SCT and low frequencies of anti-CSP IgG antibodies but not with anti-AMA1 IgG antibodies in residents of Kanyawegi sub-location in Western Kenya. This shows that such a low frequency and level of anti-CSP IgG antibodies do not contribute significantly to relative malaria protection seen in individuals with SCT.

III. There was significant association between G6PD deficiency and low frequencies of anti-CSP IgG antibodies but not with anti-AMA1 IgG antibodies in residents of Kanyawegi sub-location in Western Kenya suggesting that G6PD deficiency alter the levels and

frequency of anti-CSP IgG antibodies to levels that do not significantly contribute to relative protecting against malaria in G6PD deficient individuals.

6.3 RECOMMENDATIONS FROM THE FINDINGS OF THIS STUDY

6.3.1 Recommendations for applications of the study findings

I. The prevalence of G6PD deficiency in Kanyawegi sub-location has risen from 7% to 8.3% and that of SCT has dropped to14.4% in the last fifteen years. This shows that prevalence of malaria-related genetic disorders is dynamic in malaria endemic areas and need to be evaluated periodically to have up to date data that do not jeopardize initiatives to do with malaria chemotherapy, blood transfusion and malaria research.

II. Individuals with SCT or G6PD deficiency separately have low frequency and levels of anti-CSP IgG antibodies. Thus dose adjustments of anti-malarial vaccines derivatived from Pf-CSP may be considered during vaccine trials in high malaria transmission areas..

6.3.2 Recommendations for future studies

I. The current study only considered data of clinical nature from the study participants. Other information including participants' usage of ITNs, frequency of malaria attacks and anti-malarial drugs used could have implications on malaria outcome and IgG antibody balance

II. The association of these genetic variants (SCT and G6PD deficiency) against the anti-Pf antigen IgG sub-classes need to be considered since they might bring out more immuno-

genetic information as opposed to the current study that only evaluated total IgG antibodies

III. Only two Pf antigens (CSP and AMA1) were considered in this study. A study involving an evaluation of a panel of other Pf antigens at different time points will give more immuno-genetic information not realized by this study.

IV. The association and role of the various G6PD deficiency genetic variants be investigated against IgG antibodies of a panel of known Pf antigens. Whereas this study showed lack of association between anti-AMA 1 IgG antibodies and G6PD deficiency, no study has attempted to look at this association against the various circulating G6PD deficiency genetic variants like 202G>A, 376A>G or 563C>T.

V. The G6PD deficiency phenotyping method used has limitations and does not identify the differences more so in females, thus a quantitative or genotyping method could be more helpful since lyonisation in female heterozygotes has implication on G6PD deficiency.

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APPENDIX 1

HbS GENE PRIMERS

***HbS* GENE PRIMERS**

HbS	Forward	5'-TCCTAAGCCAGTGCCAGAAG-3’
	Reverse	5'-GAATTCGTCTGTTTCCCATTCTAAAC-3’

APPENDIX 2

APPROVAL LETTER BY ETHICS COMMITTEE

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **February 8, 2013**

TO: DR. JOHN VULULE (SITE PRINCIPAL INVESTIGATOR) & PROF. JAMES KAZURA (PRINCIPAL INVESTIGATOR, CASE WESTERN UNIVERSITY)

Dear Dr. Vulule and Prof. Kazura,

RE: SSC PROTOCOL No. 2207- REVISION 4 (RE-*SUBMISSION 3)*: NATURALLY ACQUIRED IMMUNITY TO MALARIA DURING THE EPIDEMIOLOGIC TRANSMISSION IN KENYA

Reference is made to your letter dated January 24, 2013. The ERC Secretariat acknowledges receipt of the following documents on February 5, 2013;

(a) Revised Study Protocol.
(b) Consent Document for Cohort 1 Minors with Malaria – English, Kiswahili and Dholuo Versions.
(c) Consent Document for Cohort 2 Healthy Minors – English, Kiswahili and Dholuo Versions.
(d) Consent Document for Cohort 3 Healthy Adults– English, Kiswahili and Dholuo Versions.

The is to inform you that at the 211th meeting of the Ethics Review Committee held on Wednesday, 6th February 2013, the Committee determined that the issues on 18th January 2012 are adequately addressed. Consequently, the study is granted approval for implementation effective the **6th day of February 2013** for a period of one year. Please note that authorization to conduct this study will automatically expire on **February 5, 2014**.

If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to the ERC Secretariat by **December 18, 2013**. The regulations require continuing review even though the research activity may not have begun until sometime after the ERC approval.

You are required to submit any proposed changes to this study to the SSC and ERC for review and the changes should not be initiated until written approval from the ERC is received.

Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the ERC and you should advise the ERC when the study is completed or discontinued.

Work on this project may begin.

Sincerely,

DR. CHRISTINE WASUNNA,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

APPENDIX 3

CONSENT FORMS FOR THE STUDY

UNIVERSITY HOSPITALS CASE MEDICAL CENTER
KENYA MEDICAL RESEARCH INSTITUTE
CONSENT FOR INVESTIGATIONAL STUDIES

Project Title: Naturally Acquired Immunity to Malaria during the Epidemiologic Transition in Kenya
Healthy Children (ages 1 to 10 years)

Principal Investigators: James W. Kazura, M.D. (USA) and John Vulule, Ph.D. (Kenya)

Dr. James W. Kazura from Case Western Reserve University (CWRU) in the USA and Dr. John Vulule of the Kenya Medical Research Institute (KEMRI) in Kisumu and their colleagues are inviting you to enroll your child in a research study sponsored by the National Institutes of Health (NIH). This is a research study to answer specific questions about malaria. The information from this study may help people in your area. The study doctor (the person in charge of the research) or the field assistants will explain the study to you. Research studies include only people who choose to join the study.

Please take your time to decide if you want to join this study. Some people find it helpful to talk about the study with their family and friends before they make a decision. It may also be useful to talk with your doctor and other people providing your child's medical care about the study. If you have questions or want to know more about the study, you can ask them for more information.

Your child is being asked to be in this study because he or she is healthy and lives near Chulaimbo Hospital. "You" may also refer to your child. We want to compare healthy children to children who have been hospitalized for malaria.

WHY IS THIS STUDY BEING DONE?

The purpose of this study is to find out how your child's body protects itself from malaria.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

About 400 children and 100 healthy adults from Nyanza Province will take part in this study in Kenya. About 100 healthy adults will take part in this study in the United States.

WHAT IS INVOLVED IN THE STUDY?

If you agree to have your child in the study we will ask you some questions (e.g. age, village location, home, sex of child and bed net use). This is called "demographic" information. We will also ask you some questions about the health of your child (e.g. symptoms, recent past history of malaria infections, medications, and information about physical examination findings). We will also ask you for information and tests results of your child's HIV testing. We will only obtain this information from your child's health record if you agree to allow us to have this information. Your child will not be tested for HIV for this study but we will need this information from your child's health record for this study.

We will provide a brief physical examination (e.g. height, weight, temperature) and take a blood sample to see if your child is infected with malaria. We will then do a finger prick blood smear (about 250 microliters or 5-10 drops of blood) or take a sample of blood (2-10 ml or one-half to 2 teaspoons depending on size and age) from your child's arm to find out if your child is infected with malaria parasites. If we find that your child is sick we will ask you to return to the Chulaimbo Hospital or local health center for further examination and treatment if needed. Our study will provide a cost voucher for you and your child to travel to the Chulaimbo Hospital or local health center for this purpose.

We will ask you to come back to the health center every six months for two years (4 visits). At each return visit we will take blood from your child's arm vein (2-10 ml or one-half to 2 teaspoons) and do a brief physical examination. The study will provide a cost voucher for travel for you and your child for this purpose. If we find that your child is sick we will ask you to return to the Chulaimbo Hospital or local health center for further examination and treatment if needed. The study will provide a cost voucher for travel for you and your child for this purpose.

HOW LONG WILL I BE IN THE STUDY?

Your child will be in this study for two years. You will be asked to bring your child to the health center every 6 months for two years. This amounts to a total of 4 visits.

CAN I STOP BEING IN THE STUDY?

Yes. You can decide to stop at any time.

CAN I BE TERMINATED FROM THIS STUDY?

Your child's participation in this study may be stopped by the investigators if you move away from the study area during the study or if your child develops malaria.

WHAT ARE THE RISKS OF THE STUDY?

The risk of drawing blood by venipuncture or finger stick is small although some people become lightheaded after giving blood. Your child may be uncomfortable and may cry when blood is drawn. Your child may experience momentary discomfort and/or bruising. Infection, excess bleeding, clotting, or lightheadedness are also possible after drawing blood from your hand or arm,

but these events are unlikely. Your child will be watched by members of the research team and given an opportunity to rest if you feel lightheaded.

The doctors also want to use some of your blood from this study to learn about small parts of the body, too small to see with the naked eye, called "genes." Genes are in all people and are found in almost every part of the body, including the blood. These genes carry the information that passes characteristics from parents to their children. Genes may carry information about characteristics that you see, like the color of your skin or your height; they also carry information about characteristics that you cannot see. In a family, people who share the same characteristics usually share the same genes in the blood. There can be differences in genes that determine whether your blood is strong and protects you from severe malaria. We will look for potential differences in your genes that might affect your child's susceptibility to malaria and other infectious diseases.

Allowing the doctors to study the genes in child's blood will provide very important information about the chances of getting sick with malaria. We will not tell you or anyone else, the results of your child's genetic tests.

☐ I agree to allow my/my child's blood to be tested for genes that affect malaria.

☐ I do not agree to allow my/my child's blood to be tested for genes that affect malaria.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

There may be no benefit to you or your child for being in this study. By participating in this study your child will have frequent clinical follow up resulting earlier diagnosis and treatment of malaria and other common illnesses.

WHAT OTHER OPTIONS ARE THERE?

You do not have to participate in this study. Taking part in this study is voluntary. You may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled. If your child has malaria you will be referred to the local health center and be treated according to current Kenyan Ministry of Health (MOH) treatment guidelines. There is no cost for this treatment.

WHAT ABOUT CONFIDENTIALITY?

We will keep the information we collect about your child confidential. Your child's blood sample will not have any identifying information about you on it. The samples will have a code number on it. Only the principal investigators and the study data manager will have access to this code. Any test results we obtained as part of the study will be shared with the health care providers at the health center, only with your approval. This will occur even if your child leaves the study or you decide to withdraw your child at any time.

WHAT ARE THE COSTS?

There is no cost to you or your child to participate in this study. You and your child will receive no payment for taking part in this study. All laboratory tests will be paid for by the study. If you need help in paying for transportation to come to the health center for follow up visits, the study will arrange payment. If your child develops a local infection within 7 days at the site on the hand or arm from where we drew the blood, we will provide transportation to your local provincial health center and a voucher to cover the costs of medications to treat this infection. Also, if your child becomes sick you will be referred to a local health center or clinic for treatment at no cost to you.

STORAGE AND USE OF SAMPLES FOR FUTURE STUDIES

Samples of your child's blood will be stored indefinitely in a freezer at the laboratories of the Kenya Medical Research Institute (KEMRI) in Kisumu and for the study duration at the laboratories of the Center for Global Health & Diseases at Case Western Reserve University in Cleveland, Ohio (USA), and may be used indefinitely for future testing related to scientific studies not described here, including tests related to malaria and other infectious diseases. However these samples will only be used with approval from the Kenya Medical Research Institute's National Ethical Review Committee (ERC) and the Principal Investigators' primary Institutional Review Board (IRB). You will not be contacted for additional consent. You may still participate in this study if you do not consent to us using your samples for future scientific studies about diseases affecting your community. If you check "no," then your child's samples will be stripped of the identification number in the database after the completion of this study, and will not be used by the investigators after the study is completed. If you change your mind in the future, you may contact Dr. John Vulule (the study Co- PI) at 07146 06023 (cell phone) or write to him at Centre for Global Health Research (CGHR) KEMRI, Kisumu. P. O. Box 1578 - 40100, Kisumu, Kenya

UNIVERSITY HOSPITALS CASE MEDICAL CENTER
KENYA MEDICAL RESEARCH INSTITUTE
CONSENT FOR INVESTIGATIONAL STUDIES

Project Title: Naturally Acquired Immunity to Malaria during the Epidemiologic Transition in Kenya
Healthy Children (ages 1 to 10 years)

Principal Investigators: James W. Kazura, M.D. (USA) and John Vulule, Ph.D. (Kenya)

Consent for use of your blood samples for future studies (Please check one box only) ☐ yes ☐ no

Samples of your child's blood may be sent outside of Kenya to the United States (Case Western Reserve University/Cleveland) or Australia (MacFarlane Burnet Institute of Medical Research/Melbourne) for tests related to this study.

☐ I agree to allow my child's samples to be sent to the United States or Australia for laboratory testing. My child's samples will not have their name on them or other identifying information.

☐ I do <u>not</u> agree to allow my child's samples to be sent to the United States or Australia for laboratory testing for this study.

Summary of your rights as a participant in a research study

Your participation in this research study is voluntary. Refusing to participate will not alter your usual health care or involve any penalty or loss of benefits to which you are otherwise entitled. If you decide to join the study, you may withdraw at any time and for any reason without penalty or loss of benefits. If information generated from this study is published or presented, your identity will not be revealed. In the event new information becomes available that may affect the risks or benefits associated with this study or your willingness to participate in it, you will be notified so that you can decide whether or not to continue participating in the study.

If you experience physical injury or illness as a result of participating in this research study, medical care is available at the local health center or, if more severe, you will be transported to the local hospital; however, University Hospitals Case Medical Center has no plans to provide free care or compensation for lost wages.

Disclosure of your study records

Efforts will be made to keep the personal information in your research record private and confidential, but absolute confidentiality cannot be guaranteed. The University Hospitals Case Medical Center Institutional Review Board and/or the Kenya Medical Research Institute ERC may

review your study records. In addition, for treatment studies, the study sponsor and possibly foreign regulatory agencies may also review your records. If your records are reviewed your identity could become known.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

_____ has described to you what is going to be done, the risks, hazards, and benefits involved. The study co-ordinator, Dr. Peter Suma Odada, can be contacted at (254) 0733 746854 or 0720-766550 (cell phone) or by writing to him at Centre for Global Health Research (CGHR) KEMRI, Kisumu, if you have any questions. If you have any questions, concerns or complaints about the study in the future, you may also contact him later. If you change your mind in the future, you may contact Dr. John Vulule (the study Co- PI) at 07146 06023 (cell phone) or write to him at Centre for Global Health Research (CGHR) KEMRI, Kisumu. P. O. Box 1578 - 40100, Kisumu, Kenya, or The Secretary, KEMRI Ethics Review Committee, P.O. Box 54840-00200, Nairobi; Telephone Numbers: 020-2722541, 0722205901, 0733400003; Email address: erc@kemri.org.

If the researchers cannot be reached, or if you would like to talk to someone other than the researcher(s) about; concerns regarding the study; research participant's rights; research- related injury; or other human subject issues, please call the University Hospitals Case Medical Center's Research Subject Rights phone line at (1*) (216) 983-4979 or write to: The Chief Medical Officer, The Center for Clinical Research, University Hospitals Case Medical Center, 11100 Euclid Avenue, Lakeside 1400, Cleveland, Ohio, 44106-7061 .

Signature

Signing below indicates that you have been informed about the research study in which you voluntarily agree to participate; that you have asked any questions about the study that you may have; and that the information given to you has permitted you to make a fully informed and free decision about your participation in the study. By signing this consent form, you do not waive any legal rights, and the investigator(s) or sponsor(s) are not relieved of any liability they may have. A copy of this consent form will be given to you.

X	
Printed name of minor	

UNIVERSITY HOSPITALS CASE MEDICAL CENTER
KENYA MEDICAL RESEARCH INSTITUTE
CONSENT FOR INVESTIGATIONAL STUDIES

Project Title: Naturally Acquired Immunity to Malaria during the Epidemiologic Transition in Kenya
Healthy Children (ages 1 to 10 years)

Principal Investigators: James W. Kazura, M.D. (USA) and John Vulule, Ph.D. (Kenya)

X	
Signature of Parent/Legal Guardian	Date
X	
Printed name of Parent/Legal Guardian	
X	
If Legal Guardian, indicate relationship to child	

Thumbprint of Parent or Legal Guardian
(if needed)

Study personnel (only individuals designated on the checklist may obtain consent)

X	
Signature of person obtaining informed consent	Date
X	
Printed name of person obtaining informed consent	
X	
Signature of Principal or Co-Investigator	Date
X	
Printed name of Principal or Co-Investigators	

UNIVERSITY HOSPITALS CASE MEDICAL CENTER KENYA MEDICAL RESEARCH INSTITUTE CONSENT FOR INVESTIGATIONAL STUDIES
Project Title: Naturally Acquired Immunity to Malaria during the Epidemiologic Transition in Kenya ***Healthy Children (ages 1 to 10 years)***
Principal Investigators: James W. Kazura, M.D. (USA) and John Vulule, Ph.D. (Kenya)

Signature of Witness	Date
X	
Printed Name of Witness	

UNIVERSITY HOSPITALS CASE MEDICAL CENTER
KENYA MEDICAL RESEARCH INSTITUTE
CONSENT FOR INVESTIGATIONAL STUDIES

Project Title: Naturally Acquired Immunity to Malaria during the Epidemiologic Transition in Kenya
Healthy Children (ages 1 to 10 years)

Principal Investigators: James W. Kazura, M.D. (USA) and John Vulule, Ph.D. (Kenya)

Assessment of Informed Consent		
	Yes	No
Do you understand the consent form?		
Do you have any questions?		
Question:		
Does your child have to participate in this study?		
Will we take blood from your child during this study?		
Can you refuse to have your child participate in the study at any time?		
Is there any charge for being in the study?		
Will you or your child receive any money for being in the study?		
Do you know who to call if you have questions?