ACQUIRED *Plasmodium falciparum*-SPECIFIC ANTIBODY RESPONSES ARE ASSOCIATED WITH EFFICACY TO ARTEMISININ-BASED COMBINATION THERAPY (ACT) IN THE TREATMENT OF UNCOMPLICATED MALARIA IN KOMBEWA, WESTERN, KENYA

BY

OYUGI GEOFFREY ODHIAMBO

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN APPLIED PARASITOLOGY AND VECTOR BIOLOGY

DEPARTMENT OF ZOOLOGY

MASENO UNIVERSITY

©2018

DECLARATION

I declare that this thesis is my own original work and has not been presented in any institution of higher learning for the award of a degree certificate. All referenced sources are duly quoted and any semblance observed is coincidental.

GEOFFREY ODHIAMBO OYUGI

PG/MSC/00088/2012

Name--Signature--------------------Date--------------------

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

Dr. Daniel Ochiel, Ph.D.

Department of Zoology. Maseno University.

Signature ……………………………………………….. Date……..

Prof. Walter G.Z.O. Jura, Ph.D.

Department of Zoology. Maseno University.

Signature…………………………………………………Date……..

ACKNOWLEDGEMENTS

Without the support, guidance and patience of the following people this thesis would not be a reality. It is to them I owe my deepest gratitude;

All things are made possible by the blessings of the Almighty God, in whom we live and have our being. I thank Jehovah God for His divine favors over my life to enable me reach this far.

I would like to express my sincere gratitude and appreciation to my Supervisor, Dr. Daniel Ochiel, who has remained devoted from the start to completion of this course. He organized for funding through CNHR, formulated and supervised laboratory assays. His encouragements and timely counsels will forever remain pillars in my pursuit for academic excellence. I am also indebted to Prof. Walter G.Z.O. Jura, Maseno University, who agreed to serve as my supervisor, despite his many academic and administrative commitments. His wisdom, knowledge and commitment, have been a beckon of encouragement and enthusiasm over the entire period.

My special thanks also go to the entire teaching and non-teaching staff of Zoology Department, Maseno University,who directly or indirectly contributed to the success of this work.

I thank Dr. John Waitumbi, Walter Reed Project/KEMRI, for giving me the opportunity to work in his laboratory as a graduate student. He provided a conducive working environment, supervised laboratory assays and thesis write up. I am grateful for his valuable scientific advice, constructive criticism, encouragements and unwavering commitment throughout the study. I have learnt a lot from him and acknowledge his commitment in ensuring that this work was done well.

This thesis owes its existence to the help, support and inspiration of Walter Reed Laboratory personnel who selflessly introduced and trained me on laboratory techniques. Special thanks to Beth Mutai, Moureen Maraka,Joram Siangla,Nancy Nyakoe, George Awinda and

Clement Masakhwe. I wish to appreciate my fellow graduate colleagues whose co-operation and insights contributed to this work. I am also grateful to the administrators and the IT team for their continuous support and assistance during the time of this work.

My deep-felt gratitude goes to my family for their unflagging love and support throughout my life; this thesis would simply have been impossible without them. I am indebted to my Father and Mother, Boaz Oyugi and Jane Oyugi, for their financial, emotional and social support throughout my life as a student. Special thanks go to my sisters and brothers for standing with me during the entire period. My deepest appreciation goes to my loving wife, Jacky Jeff, for her constant support, encouragements and presence. She has remained to be a pillar in my life and a source of inspiration. My bundles of joy have been my twin daughters, Favor and Gift, together with baby Jayden. They kept me laughing even when things were tough.

DEDICATION

This thesis is dedicated to my loving parents, Mr. Boaz Oyugi and Mrs. Jane Oyugi, for their support and care throughout my life. Special dedication also goes to my wife, together with my children, Pretty, Gift and Jayden, for standing by me during this period.

ABSTRACT

Significant advancement achieved in the chemoprophylaxis and chemotherapy of malaria has been pivotal for eventual reduction of malaria prevalence. However, a major setback has been the emergence of resistance to antimalarial drugs. Towards the goal of curbing emergence of resistance, artemisinin-based combination therapies (ACT), is now adopted by many countries as the first-line treatment for malaria. The recently reported cases of resistance to ACT in South East Asia (SEA), have raised concerns on future of ACT. Therefore, an urgent need exists to fully understand the molecular determinants of ACT efficacy. This study aims to determine the baseline magnitude and breadth of antibody responses and also to associate the antibody responses to efficacy of ACT. We hypothesize that in Kombewa-western Kenya, a holoendemic region, pre-existing antibody responses to specific *P. falciparum* antigens is a predictive correlate of ACT efficacy in patients with uncomplicated malaria. There has been reported delay in parasite clearance following artemisinin treatment in Kenyan and resurgence of parasite prevalence and malaria vector in Kombewa recently. This sub-study was conducted as part of the larger two-arm (Artesunate-Mefloquine (ASMQ) and Artemether Lumefantrine (AL) randomized, open-label trial. Baseline sera from 82 patients (AL=40,ASMQ=42) enrolled in the arms of the trial were analyzed for total IgG against erythrocytic (Apical membrane Antigen-1 (HB3and 3D7 strains), Merozoite surface protein -1(3D7 and FVO strains) and pre-erythrocytic stage (Liver Stage Antigen , Cell-traversal for Ookinetes and sporozoites and Circumsporozoite protein) *P. falciparum* antigens using Luminex. Since ACT efficacy can be assessed based on parasite clearance rate, patients were grouped into fast clearers and faster clearers, using parasite clearance half-life ($PC_{1/2}$).The threshold was fixed at the 25th percentile which was 2.02 hours. Variables were compared using Mann-whitney U test, χ^2 test and z test as appropriate to examine associations between immunological endpoints and clinical endpoints. Patients generally had high prevalence of IgG antibodies (91-100%) and those >5 years of age had significantly higher titers of anti-AMA1HB3-specific antibodies (p=0.0316) than those <5 years. Faster clearers had levels of AMA1HB3-specific antibodies significantly higher (p=0.0065) than fast clearers. This association was significant even when ASMQ subjects were analyzed (p=0.0073). However, in AL arm anti-LSA-1 antibodies were significantly higher in faster clearers. This finding suggests that specific IgG antibodies againstAMA1-HB3 and LSA-1, observed in this setting, could be predictive of ACT efficacy and could therefore be used in surveillance for resistance (delayed clearance). This result should be confirmed in a large-scale study.

TABLES OF CONTENTS

DECLARATION i
ACKNOWLEDGEMENTS ii
DEDICATION iv
ABSTRACT v
TABLES OF CONTENTS vi
LIST OF ABBREVIATIONS AND ACRONYMS viii
LIST OF TABLES x
LIST OF FIGURES xi
LIST OF APPENDICES xii
CHAPTER ONE : INTRODUCTION 1
1.1 Background 1
1.2 Problem statement 7
1.3 Justification of the study 8
1.4 Significance of the study 9
1.5 Study objectives 9
1.5.1 General objective 9
1.5.2 Specific objectives 9
1.5.3 Research questions 9
CHAPTER TWO : LITERATURE REVIEW 11
2.1 Malaria etiology, prevalence and burden of disease 11
2.2 Malaria symptoms 13
2.2.1 Uncomplicated malaria 13
2.3 Immunity against malaria parasite 15
2.4 Treatment options for malaria 19
2.5 Determinants of antimalarial efficacy 22
CHAPTER THREE : MATERIALS AND METHODS 24
3.1 Study area 24
3.2 Study design 26
3.3 Sample size 26
3.4 Sampling procedure 27

3.5 Study populations 27
Inclusion and exclusion criteria 27
3.6.1 Inclusion criteria 27
3.6.2 Exclusion criteria 28
3.7 Data collection 28
3.8 Data management and analysis 33
CHAPTER FOUR : RESULTS 35
4.1 Patients characteristics in the ACT efficacy trial 35
4.2 Magnitude of Malaria-specific antibody responses in study participants 36
4.3. Breadth of antibody responses 40
4.4. Magnitude of antibody responses and Parasite clearance rate 41
CHAPTER FIVE : DISCUSSION 45
5.1 Magnitude of Malaria specific antibody responses 45
5.2 Breadth of Malaria specific antibody responses 46
5.3 Association between Malaria specific antibody responses and treatment outcome 47
CHAPTER SIX : SUMMARY,CONCLUSIONS AND RECOMMENDATIONS 49
6.1 Summary of study findings 49
6.2 Overall conclusions and implications 49
6.3 Recommendations for the study 50
6.4 Recommendations for future research 50
REFERENCES 52
APPENDICES 69
Appendix I. Ethical approval document. 69
Appendix II. Study procedure for adults. 70
Appendix III. Study procedures for children 71
Appendix IV. Bead calculation templates. 73
Appendix V. Plate map 77

LIST OF ABBREVIATIONS AND ACRONYMS

ACT Artemisinin-based combination therapy
AL Artemether plus lumefantrine combination
AQ Amodiaquine
AS Artesunate
AS+AQ Artesunate plus amodiaquine combination
AS+MQ Artesunate plus mefloquine combination
AS+SP Artesunate plus sulfadoxine-pyrimethamine combination
BW Body weight
CQ Chloroquine
DHA+PPQ Dihydroartemisinin plus piperaquine combination
GRADE Grading of Recommendations Assessment, Development and Evaluation.
HIV/AIDS Human immunodeficiency virus/ acquired immunodeficiency syndrome
IC50 Concentration providing 50% inhibition
CSP Circumsporozoite protein
Pf CelTOS *Plasmodium falciparum* cell-traversal for Ookinetes and sporozoites protein
Pf AMA-1 3D7 *Plasmodium falciparum* Apical membrane Antigen-1-3D7
Pf AMA-1-HB3: *Plasmodium falciparum* Apical membrane Antigen-1-HB3
MSP1-42 3D7: Merozoite surface protein 1-42 -3D7
MSP1-42 FVO: Merozoite surface protein 1-42-FVO
LSA Liver Stage Antigen
MIC Minimum inhibitory concentration
MQ Mefloquine
PQ Primaquine
SP Sulfadoxine-pyrimethamine
WHO World Health Organization
GMEP Global Malaria Eradication Programme.
DDT Dichlorodiphenyltrichloroethane
SEA South East Asia.
MFI Median fluorescence intensity.
SAT Saturation array technology.
$PC_{1/2}$ Parasite clearance half-life.
ACPF Adequate clinical and parasitological failure.
TF Treatment failure .
CF Clinical failure.
CRC Clinical research Centre.
GLURP Glutamate rich protein.
KEMRI Kenya Medical Research Institution
WRP Walter Reed project.
HDSS Health and demographics surveillance systems.

ARDS	Acute respiratory distress syndrome.
Irbc	Infected red blood cell.
VSA	Variant surface antigens.
SNP	Single nucleotide polymorphism.
NO	Nitric oxide.
ICAM-1	Intercellular Adhesion Molecule 1.
BSA	Bovine serum albumin.
PBS	Phosphate-buffered saline
EBA-175RII	Erythrocyte-Binding Antigen (Region II of the 175-kDa)

LIST OF TABLES

Table 1. Pre-erythrocytic and erythrocytic antigens used. 31
Table 2. Baseline characteristics of patients enrolled in the two arms of the study. 36
Table 3. Table showing Magnitude and prevalence of antibody responses for both study arms. 39
Table 4. Breadth of antibody reactivity for all patients enrolled in the study and for different age categories. 42
Table 5. The magnitude of antibodies responses between faster clearers and fast clearers in all patients in the study. 43
Table 6. Magnitude of antibodies responses between fast clearers and faster clearers among ASMQ patients. 44
Table 7. Magnitude of antibody responses between fast clearers and faster clearers among patients receiving AL. 44
Table 8. A comparison between breadth of antibody reactivity and parasite clearance rate. 45

LIST OF FIGURES

Figure 1. Malaria parasite life cycle. 11
Figure 2. Map of the study area HDSS 26
Figure 3. Antibody reactivity against malaria specific antigens of patients in the study. 38
Figure 4. The IgG antibody titers for all antigens categorized by age. 40
Figure 5. Antibody titers for all antigens by gender. 41

LIST OF APPENDICES

Appendix 1. Ethical approval document. 69
Appendix 2. Study procedure for adults. 70
Appendix 3. Study procedures for children 71
Appendix 4. Bead calculation templates. 73
Appendix 5. Plate map 77

CHAPTER ONE

INTRODUCTION

1.1 Background

There are a total of 104 countries and territories in which malaria is presently considered endemic. Globally, an estimated 3.4 billion people are at risk of malaria (WHO, 2013). WHO estimates that 207 million cases and 627,000 deaths of malaria occurred, globally, in 2012.Most cases (80%) and deaths (90%) occurred in Africa, and most deaths (77%) were in children under 5 years of age (WHO, 2013). The major cause of mortality in children and pregnant women is due to *P falciparum*. In malaria-endemic areas, young children are particularly more susceptible to malaria. In these areas, it is estimated that a quarter of all childhood deaths are due to malaria (Snow *et al.*, 2001). However with exposure, older children and adults develop essentially complete protection from severe illness and death, although sterile immunity is probably never achieved (Aponte *et al.*, 2007). While prevention has become an increasingly important strategy for control and eradication of malaria in developing countries, effective treatment remains an integral component of malaria eradication (Tanner *et al.*, 2015). In 1955, WHO launched Global Malaria Eradication Programme (GMEP) (WHO, 1999) which was focused on two areas of interventions, namely: Chloroquine (CQ) for treatment and control and DDT for vector control. This initiative had a significant impact on, particularly, low transmission areas such as India. However, due to lack of political will, emergence of chloroquine-resistant, *Plasmodium* parasite and that of DDT-resistant *Anopheles* mosquito, the campaign was eventually stopped (WHO, 1999). The termination of the GMEP witnessed a surge in malaria cases in most parts of the world (WHO, 1999). Similarly, childhood deaths increased in Africa partly due to emergence and spread of chloroquine-resistant *Plasmodium* parasite (Snow *et al.*, 2001). CQ was then

replaced as a first line therapy by Sulfadoxine-pyrimethamine (SP) in many parts of the world. However, resistance to sulfadoxine soon developed, further limiting the available treatment options (Wongsrichanalai & Meshnick, 2008).

The spread of *P. falciparum* resistant to CQ and SP necessitated increased research and innovation in the discovery of a novel antimalarial with more emphasis on ACT(White & Pongtavornpinyo, 2003). ACT combines a derivative of the natural product called artemisinin, which is an extremely potent and fast-acting antimalarial endoperoxide, with a longer-lasting partner drug that continues to reduce the parasite biomass after a short time (Fairhurst *et al.*, 2012; WHO, 2013). ACT has the advantage of improving cure rates, reducing development of resistance and decreasing transmission of drug-resistant parasites. The total effect of artemisinin combinations (which can be simultaneous or sequential) is to reduce the chances of parasite recrudescence and resistance (WHO, 2010b).

ACT was first recommended by the WHO as first-line treatment for falciparum malaria in 2006 due to treatment failures observed with the traditional anti-malarials such as CQ and SP (Lin *et al.*, 2010). Six combinations are currently recommended they include: Artemether-lumefantrine(A/L), artesunate–amodiaquine (AS/AQ), artesunate–mefloquine (AS/MQ), artesunate–sulfadoxine–pyrimethamine A/SSP) and dihydroartemisinin–piperaquine (DHA/PPQ) and Artesunate/Pyronaridine (A/PD). ACT is now widely adopted as the first or second line of therapy for malaria in Asia and most African countries; this is mainly due to its enhanced effectiveness in parasite clearance (WHO, 2010a). Recent reports of high failure rates associated with ACT suggest the possibility of clinical artemisinin resistance along the Thai-Cambodia border (Vijaykadga *et al.*, 2006;Noedl *et al.*, 2008). Researchers realized an increase in the number of patients who were still parasitemic on day 3 which indicates a change in pattern of

parasite susceptibility to artemisinins and is probably the first stage of artemisinin resistance (Wongsrichanalai & Meshnick, 2008).

Despite the changes observed in parasite sensitivity to artemisinins, it appears that the clinical and parasitological efficacy of ACT has not yet been compromised, even in the Greater Mekong sub-region. This is possibly because of the effect of partner drug which has a longer half-life than artemisinin (Wongsrichanalai & Meshnick, 2008; Slater, *et al.*, 2016). Nonetheless the efficacy of both components of ACT could be reduced if one partner drug loses efficacy for this might jeopardize the other drug (WHO, 2010b). Other suspected foci have been identified in the Greater Mekong sub-region, but are not yet confirmed although the extent of the problem is still being investigated (Fairhurst *et al.*, 2012). Historically, malaria drug resistance has emerged from SEA (focal point) and spread globally (Wongsrichanalai & Meshnick, 2008). Hence, with the observed patterns of decreased efficacy to ACT in SEA, it indicates possible emergence of resistance. The mechanisms of ACT resistance is due to mutations of the PfK13 propeller domain and involves an unconventional mechanisms based on quiescence state leading to parasite recrudescence as soon as drug pressure is removed (Paloque *et al.*, 2016). The current status of artemisinin resistance especially insight into whether it has spread beyond the Greater Mekong sub-regions and likelihood of emergence of resistance in new foci are unclear. There is also unanswered question about which tools and methods will be most effective in addressing artemisinin resistance (WHO, 2010a). This is partly due to inability of available laboratory techniques to accurately predict artemisinin resistance (Laufer, 2009). The problem is further compounded by the lack of known molecular markers for artemisinin resistance in different settings (Lim *et al.*, 2009). ACT resistance manifests as slow parasite clearance this is an important measure of anti-malarial drug efficacy (Flegg, *et al.*, 2011). It is determined by slope

of the log-linear segment in the middle of the parasite clearance curve which has the least inter-individual variance and is the focus of therapeutic assessment (White *et al.*, 2011). Parasite clearance is however determined by several other factors including, drug pharmacokinetics and pharmacodynamics, parasite genetic characteristics, parasite load, innate host resistance mechanisms and acquired immunity to malaria (Flegg *et al.*, 2011). Acquired immunity to malaria is of great interest in the assessment of antimalarial drug resistance. This is because adults living in areas of high transmission acquire partial immunity to malaria, and are often able to clear infection without drug treatment, despite the fact that they are not immune to clinical disease (Doolan *et al.*, 2009). This means that good therapeutic responses may be seen with ineffective drugs in individuals with good immunity. However, the role of acquired immunity on efficacy of ACT drug is not fully investigated and more needs to be done in terms of functionality of antibodies and endpoints that can confer efficacy and resistance. Acquired immunity is affected by age, transmission intensity, nutritional status, co-infection with HIV and pregnancy (WHO, 2010b).Infants and children are, however, at increased great risk since their immunities are still naïve. Moreover, natural immunity to malaria is acquired with age and degree of exposure (Aponte *et al.*, 2007). Studies with immunoglobulin passive transfer have demonstrated the possible role of IgG antibodies as effectors in immune-mediated protection to malaria (Sabchareon *et al.*, 1991). IgG subclasses, IgG1 and IgG3 are thought to play a key role in protection (Courtin *et al.*, 2009; Stanisic *et al.*, 2009). Antibody subclasses may neutralize parasites directly by inhibiting parasite invasion or growth in erythrocytes or indirectly by opsonization through binding to the Fc receptor IIA (Bredius *et al.*, 1994). *P.falciparum* pre-erythrocytic and erythrocytic antigens have consistently shown that they are key targets of naturally acquired immunity to malaria. Erythrocytic antigens such as MSP-1-42 (3D7), MSP-1-

42(FVO), AMA-1 (HB3), and AMA-1 (3D7) are considered important targets of naturally acquired immunity (Chitnis et al. 2001) and antibodies against these antigens inhibit invasion of erythrocytes *in-vivo,*opsonization of merozoites for phagocytosis and antibody-dependent cellular inhibition (Coley *et al*., 2007). A number of *P.falciparum* pre-erythocytic antigens have also been studied and found to be producing malaria specific antibodies. They include CelTOS, CSP and LSA (Ferraro *et al*., 2013). CelTOS has been shown to play a pivotal role in the cell traversal of host cells by malaria parasites. It has been established that immunization with pfCelTOS resulted in potent humoral and cellular immune responses and most importantly induced sterile protection against heterologous challenges with *P.berghei*s porozoites in mice (Bergmann-Leitner *et al*., 2011). LSA and CSP have consistently shown that they offer protective immune responses, hence are used as vaccine candidates.

Magnitude of antibody responses has been shown to predict protection in some blood stage antigens such as AMA-1,MSP-1 and MSP-2 in a study done in Kampala-Uganda (Greenhouse *et al*., 2011). A separate study in Kombewa western Kenya has also established that high magnitude and prevalence of anti-EBA-175RII were present in the study area and that these antibodies had a high functional role in inhibiting interaction of EBA-175RII with erythrocytes (Ohas *et al*., 2004). The levels of specific antibody responses in Kombewa against the seven antigens has not been established currently. Therefore such an assessment could be more informative in monitoring ACT efficacy particularly in a high and steady transmission setting like Kombewa.

Breadth of antibody responses has been shown to be significantly associated to reduced risk of malaria infection (Rono *et al*., 2013). Patients exposed to diverse array of parasites antigens are thought to have better immunity that those exposed to few parasites antigens. This had been

confirmed further in a study done in Kilifi-Kenya in which high titers of antibodies to combination of different antigens (AMA1, MSP-2, and MSP-3) were more strongly predictive of clinical protection that single antigens known to be protective (Osier et al., 2008). This therefore shows that both breadth and magnitude provide robust predictors of immune status which could then be used to predict efficacy of ACT in a holoendemic setting such as Kombewa.

Antibody responses have been shown to be predictive to treatment outcome with different drug formulations. A study in Asia demonstrated that antibody immunity is an important predictor of slow-clearing phenotype with higher magnitude of antibody immunity associated with faster $PC_{1/2}$ (Ataide *et al.*, 2017). It had also been established that Malaria specific antibodies influences treatment outcome differently for different anti-malarial drugs and antigen targets, and had the greatest impact in treatment with the current artemisinins (O'Flaherty *et al.*, 2017). Antibodies to malaria vaccine candidates (MSP3 and GLURP) were found to be predictive of treatment efficacy in chloroquine and sulphadoxine pyrimethamine, with significantly higher titers in successfully treated groups than unsuccessful groups (Diarra *et al.*, 2012).

Given the widespread adoption of ACT as standard of care in malaria endemic regions, it is important to understand the effect of acquired host immunity on its efficacy and in the development of resistance to this combined therapeutic regimen. The understanding of the impact of immunity on treatment is key in delineating the changes noted in treatment in the wake of changes in malaria epidemiology in Kombewa western Kenya that is attributable to reduced efficacy of ITN's and insecticide resistant mosquitoes (Zhou *et al.*, 2011). The major goal of this study was, therefore, to determine whether pre-existing antibody responses to *P falciparum* infection is a predictive correlate of ACT efficacy in patients with uncomplicated malaria in

western Kenya. This information will be important in surveillance for resistance, especially in different transmission settings and effective clinical management of children with malaria.

1.2 Problem statement

Recent reports indicate that *P. falciparum* isolates which are resistant to artemisinin may be emerging especially in the South East Asian countries (Alker *et al.*, 2008). The molecular basis for artemisinin resistance in these settings remains undefined. Given the current wide spread use of artemisinin as the standard of care for malaria in most countries, concern exists that resistance to artemisinin-based therapies could severely derail malaria treatment and control strategies (Fairhurst *et al.*, 2012). Despite these concerns, the extent of resistance to artemisinin in different geographical settings, under varying malaria transmission intensities, remains poorly characterized. Currently there has been resurgence in parasite prevalence and malaria vector as in some parts of Western Kenya despite increasing use of insecticide treated nets and other interventions(Zhou et al., 2011). The suggested reasons for this has been reduced efficacy of ITN's, insecticide resistant mosquitoes and vector population species shift (Kapesa et al., 2017; Wanjala & Kweka, 2018). Moreover there has been reported delayed clearance of parasite following artemisinin treatment in Kenyan coast (Borrmann et al., 2011). Although slower parasitological clearance does not constitute direct evidence that ACT regimens are currently failing in Kenya, these altered parasitological responses are regarded as an early warning that this may occur in the future. With the changing patterns of malaria epidemiology being noted in western Kenya (Zhou *et al.*, 2011) and consequence changes in rate at which immunity is acquired, time trends in parasite clearance time are difficult to interpret. Therefore there is need to know how ACT efficacy is affected in a high endemic area such as Kombewa-Kenya.

1.3 Justification of the study

Due to concerns of developing resistance to traditional antimalarial drugs including CQ and SP, artemisinin-based combination therapy (ACT) is currently being used in most settings as the standard of care. However, recently reported infection by*P. falciparum* isolates resistant to ACT in the South East Asian countries have raised concerns on the long-term use of ACT in clinical management of malaria globally. A randomized controlled trial of dihydroartemisinin-piperaquine (DHA-PPQ) versus AL in Kenya also showed modest reduction in parasite clearance rate, suggesting reduced sensitivity to ACT (Borrmann *et al.*, 2011). A better understanding of the determinants of resistance to ACT and its efficacy is necessary to guide clinical management of malaria, especially in regions with high parasite transmission. The impetus for the proposed study derives from the reported findings of possible association between acquired immunity and malaria treatment outcomes with different drug formulations (O'Flaherty *et al.*, 2017). In this regard, prevalence and magnitude of antibodies to a number of *P. falciparum* antigens including MSP-1, MSP-3, MSP-19, GLURP are reported to be significantly increased in sera of patients with successful malaria treatment compared to those with treatment failure (Diarra *et al.*, 2012). With regards to ACT, surprisingly no studies have examined the influence of protective malaria specific immunity on the efficacy of ACT in a holoendemic area, and how such immune effector mechanisms impact on development of resistance to this widely used therapy. In this study, therefore, i shall specifically evaluate the association between acquired antibody responses in aiding and/or abating ACT efficacy. Towards the goal of elucidating this, the study aims to investigate the possible role of the magnitude and breadth of acquired *P. falciparum*–specific antibody responses as determinants of ACT efficacy in patients presenting with uncomplicated malaria in western Kenya.

1.4 Significance of the study

Data generated by this study will provide baseline information of the current situation regarding acquired *P. falciparum* antibody immunity in relation to efficacy to ACTs in Western Kenya. This, in turn, will potentially enable the identification of key immunological markers, in host that may assist in the early detection of resistance. Any significant impact of immunity on clearance times will be important to identify and apply to surveillance for resistance (delayed clearance), especially in different transmission settings.

1.5 Study objectives

1.5.1 General objective

To determine *P. falciparum*-specific antibodies as immune correlates of efficacy to Artemisinin Based Combination therapy in treatment of uncomplicated malaria.

1.5.2 Specific objectives

1. To determine the baseline magnitude of acquired antibody responses against specific malaria antigen in patients with uncomplicated malaria enrolled in ACT efficacy trial in western Kenya.
2. To determine the breadth of naturally acquired malaria specific antibodies against specific malaria antigens in patients with uncomplicated malaria enrolled in the ACT efficacy trial in western Kenya.
3. To determine the association between antibody responses (magnitude and breadth) and efficacy of ACT in patients with uncomplicated malaria

1.5.3 Research questions

1. What are baseline levels of *P. falciparum* specific antibody responses in patients enrolled in ACT trial?

2. What is the breadth of *P. falciparum* specific antibody responses in patients enrolled in ACT trial?
3. Does levels and breadth of *P. falciparum* specific antibody responses associate with ACT efficacy (i.e. Parasite clearance rate) in patients enrolled in ACT trial?

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria etiology, prevalence and burden of disease

Malaria is a mosquito-borne infectious disease of humans and other animals caused by parasitic protozoans of the genus *Plasmodium* (WHO, 2010b). Commonly, the disease is transmitted by a bite from an infected female Anopheles mosquito, which introduces the organisms from its saliva into a person's circulatory system (figure 1). Malaria causes symptoms that typically include

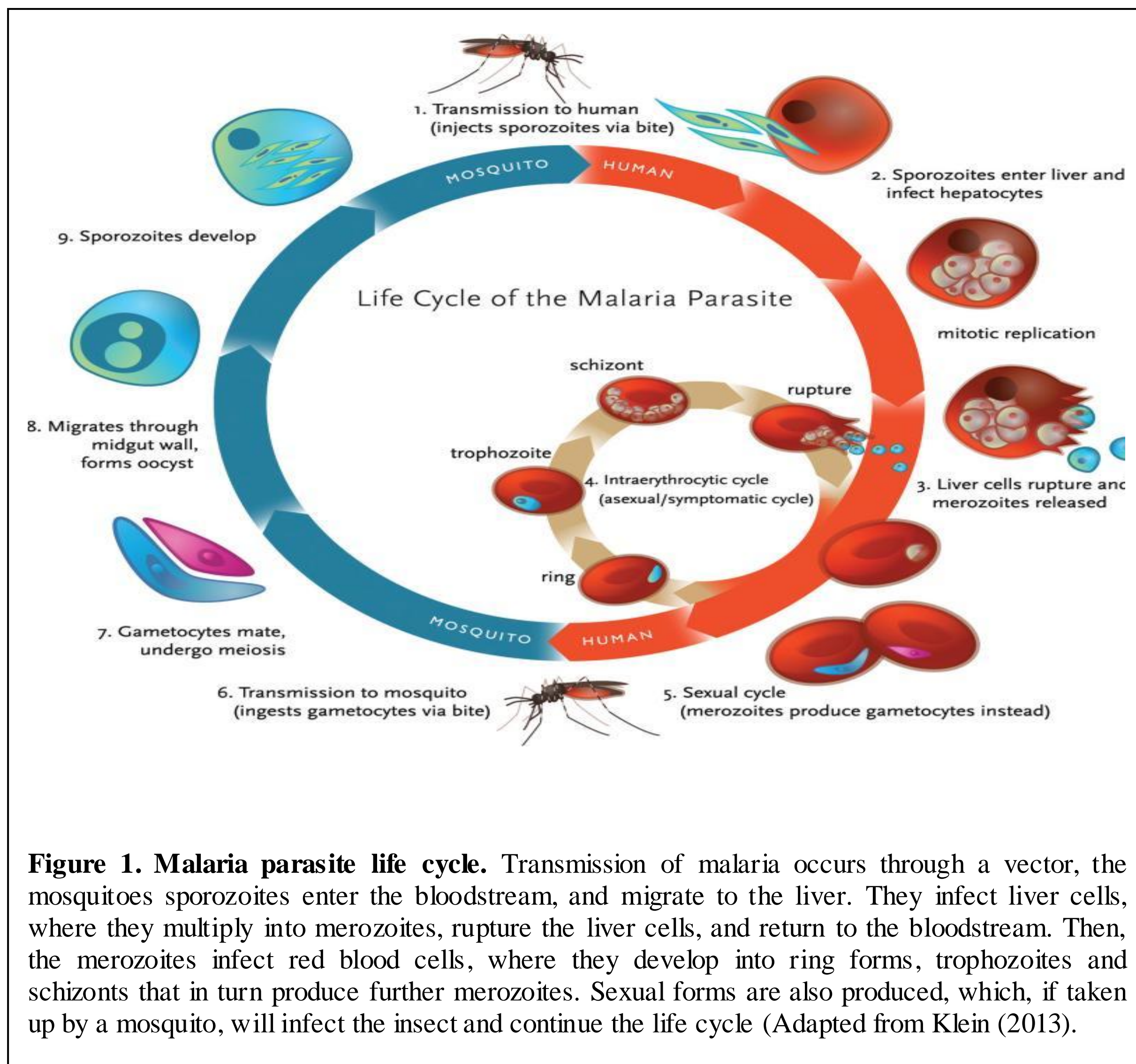

Figure 1. Malaria parasite life cycle. Transmission of malaria occurs through a vector, the mosquitoes sporozoites enter the bloodstream, and migrate to the liver. They infect liver cells, where they multiply into merozoites, rupture the liver cells, and return to the bloodstream. Then, the merozoites infect red blood cells, where they develop into ring forms, trophozoites and schizonts that in turn produce further merozoites. Sexual forms are also produced, which, if taken up by a mosquito, will infect the insect and continue the life cycle (Adapted from Klein (2013).

fever and headache, which in severe cases can progress to coma or death.

Five species of *Plasmodium* can infect and be transmitted to humans and all of these species belongs to the genus *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (Klein *et al.,* 2013). Malaria due to *P. falciparum* is the deadliest form, and it predominates in Africa (Miller et al.,2013). *P. vivax* has a wider distribution than *P. falciparum* because it is able to develop in the *Anopheles* mosquito vector at lower temperatures, and to survive at higher altitudes and in cooler climates. It also has a dormant liver stage (known as a hypnozoite) that enables it to survive during periods when anopheles mosquitoes are not present to continue transmission, such as during winter months (Hulden, 2011). The zoonotic species *P knowlensi* prevalent in Southeast Asia, causes malaria in macaques but can also cause severe infections in humans (Jongwutiwes *et al.*, 2011). Malaria is common in tropical and subtropical regions because rainfall, warm temperatures, and stagnant waters provide an environment ideal for mosquito larvae.

According to the latest estimates from WHO, there were 214 million new cases of malaria worldwide in 2015. Africa region accounted for most global cases of malaria (88%) (WHO, 2015). In 2015 there were an estimated 438,000malaria deaths worldwide most of the deaths however, occurred in African region (90%). During the same year malaria incident (new malaria cases) fell by 37% globally and by 42% in Africa.

In terms of population at risk it is children less than 5 years of age and pregnant women who are severely and disproportionately affected by the disease. For example, in 2012, malaria killed an estimated 482,000 children less than five years of age. That is 1300 children every day or one child almost every minute (WHO, 2013).

In Kenya malaria remains a major cause of morbidity and mortality with more than 70% of the population at risk of the disease according to ministry of health report 2014 (MOH, 2014).The burden is however, not homogenous but areas around lake Victoria, Western Kenya and on the coastal regions present the highest risk (MOH, 2014).

In addition to its burden in terms of morbidity and mortality, the economic effects of malaria infection can be tremendous. Households in Africa spend between $2 and $25 on malaria treatment and between $15 and $20 on prevention each month with consequent loss of resources (Onwujekwe *et al.*, 2013). In Kenya Malaria Operational Plan budget as approved by the U.S. Global Malaria Coordinator was $32.4 million in 2013 this indicates the high economic burden that malaria has on Kenyan.

2.2 Malaria symptoms

Parasite infection may result into a wide variety of symptoms, ranging from absent, very mild to severe disease and death. Consequently malaria can be classified as uncomplicated or severe (complicated) according to WHO definitions (WHO, 2010b).

2.2.1 Uncomplicated malaria

According to WHO malaria report for 2012(WHO, 2012) uncomplicated malaria is defined as symptomatic malaria without sign of severity or evidence (clinical or laboratory) of vital organ dysfunction. All symptoms and signs of uncomplicated malaria are non-specific, as shared with other febrile conditions, and can occur early or later in the course of the disease(Grobusch & Kremsner, 2005). In endemic areas, the presence of hepatosplenomegaly,thrombocytopenia and anaemia is clearly associated with malaria, particularly in children. Fever, cephalgias, fatigue, malaise, and musculoskeletal pain constitute the most frequent clinical features in malaria (National Malaria Elimination Progrmme, 2016).

2.2.2 Complicated malaria

Severe malaria occurs when infections are complicated by serious organ failures or abnormalities in the patient's blood or metabolism. The manifestations of severe malaria include: Cerebral malaria, seizures, coma, or other neurologic abnormalities, severe anemia due to hemolysis, hemoglobinuria. Other symptoms include; acute respiratory distress syndrome (ARDS), abnormalities in blood coagulation, low blood pressure, acute kidney failure, hyperparasitemia, metabolic acidosis and hypoglycemia(Trampuz *et al.*, 2003). Severe malaria is a main cause of mortality and morbidity among children under five years in Kisumu-Western Kenya (Okach, Ayisi, & Onyango, 2014). The study further established that most common presentations in a high malaria transmission like Kisumu were severe anaemia, pulmonary oedema, and cerebral anaemia. A recent study confirmed that there is an alarming number of severe forms of the disease among the school aged children at the epidemic prone setting of Western Kenya (Kapesa *et al.*, 2018). Severe malaria is a medical emergency and should be treated urgently and aggressively to avoid mortality.

Malaria is also a leading cause of anaemia in endemic areas and a major cause of complication of malaria in children (Murphy & Breman, 2001). The age distribution of severe malarial anaemia suggests that acquisition of immunity has a positive effect on haemoglobin levels. In highly endemic areas susceptibility to severe anaemia increases during the age periods when parasite density and frequency of uncomplicated episodes of malaria are highest (McElroy *et al.*, 2000). A study in Gabon established that malaria is a major risk factor for childhood anaemia and that the risk of severe malaria anaemia increases up to the age of three (Bouyou-Akotet *et al.*, 2009).

2.3 Immunity against malaria parasite

Infection with parasites and exposure to their complex antigens, generates a diverse immune response. However, a variety of protein antigens at different stages of life cycle, and rapid antigenic variations have been a major challenge resulting into evasion of immune responses (Kyes *et al.*, 2007). Innate, cellular and humoral Immunity have therefore been implicated in malaria immunity.

2.3.1 Innate immunity

Innate immunity to malaria is an inherent refractoriness of the host that prevents the establishment of the infection or an immediate inhibitory response against the introduction of the parasite. This is independent of previous infection and occurs naturally. Innate immune cells include: DC, monocytes, macrophages, NK cells, NK T cells and gamma-delta (CD) T cells (Stevenson & Riley, 2004). When DCs or monocytes-macro- phages encounter a pathogen and release cytokines such as IL-12, IL-15, IL-18, TNF-α and IFN-α/β, NK cell are activated; whereas IL-4, IL-10 and TGF-β suppress NK cell function. During malaria infections, and in response to IL-12, NK cells are the first to secrete IFN-γ (with γδ T cells and NK T cells responding later) and are able to directly kill *P. falciparum*-infected erythrocytes (Artavanis-Tsakonas *et al.*, 2003) .

2.3.2 Cellular immunity to malaria

Immunity develops with cumulative exposure as the person ages in malaria endemic areas (Aponte *et al.*, 2007). A study reported that cellular immune response induced by *P falciparum* infection can protect against both pre-erythrocytic and erythrocytic parasite stages (Perlmann. & Troye-Blomberg., 2002). Studies of rodent malaria (Doolan *et al.*, 2009), and studies of residents of malaria endemic areas (Bejon et al., 2007) indicate that T-cell cytokines responses

particularly IFN-γ responses, correlate with protection from blood stage infection and malaria illness (McCall & Sauerwein., 2010). IFN-γ is a macrophage-activating factor involved in early immune response (Artavanis-Tsakonas *et al.*, 2003). Previous studies have shown high levels of IFN-γ in plasma during severe malaria (Noone *et al.*, 2013) and that the cytokine is under regulation of IL-12 and IL-10. Crompton et al reported that IFN-γ reduces number of liver stage parasites, presumably through the nitric oxide (NO) pathway (Crompton *et al.*, 2014). This is involved in elimination of hepatic schizonts in affected hepatocytes in animal model (Doolan, 2011).Asexual blood stage *P falciparum* are responsible for malaria pathogenesis as they replicate in the blood (Miller *et al.*, 2013). It is thought that two distinct processes are involved in driving it: sequestration and inflammation (Kraemer & Smith., 2006).The parasite induces a systemic inflammatory response akin to bacterial sepsis (Clark *et al.*, 2004; Turner *et al.*, 2013) which may exacerbate iRBC sequestration by up regulating vascular adhesion molecules such as ICAM-1 (Hunt & Grau., 2003). IL-10 which is an anti-inflammatory cytokine inhibits the activity of Th1 cells, NK Cells and macrophages during infection (Couper *et al.*, 2008). It also protects against immunopathology by controlling inflammation during malarial infection. The ability to control excessive *P. falciparum*-induced inflammation in early life may be the key adaptation that confers protection from potentially life-threatening disease in young children who have yet to acquire protective antibodies, which are only reliably acquired after many years of *P. falciparum* exposure (Portugal *et al.*, 2013).

2.3.3 Humoral immunity

Humoral immunity is the aspect of immunity that is mediated by macromolecules found in extracellular fluid such as secreted antibodies, complement proteins and antimicrobial peptides. Antibodies play a pivotal role in malaria immunity. The most compelling evidence for protection

against malaria arose from experiments in the 1960s where passive transfer of sera from semi-immune adults was used to treat children with clinical *P. falciparum* malaria (Cohen *et al.*, 1961). Most recent studies on antibodies and malaria have looked at the relationship between an array of plasmodium antigens and antibodies in an attempt to come up with vaccine candidates. (Good *et al.*, 2005; Vekemans & Ballou., 2008). Protective antibodies are thought to target primarily merozoite surface antigens, erythrocyte invasion ligands and variant surface antigensexpressed *by P. falciparum* infected erythrocytes (IEs) (Bull & Marsh, 2002; Good, 2004). Currently substantial evidence suggests that antibodies against merozoite antigens play an important protective role in malaria immunity and several merozoite antigens are leading vaccine candidates (Braga *et al.*, 2002). Antibodies against merozoite antigens are also thought to function *in vivo* by inhibitionof merozoite invasion of erythrocytes, opsonization of merozoites for phagocytosis, and antibody-dependent cellular inhibition(Osier *et al.*, 2014). During intra-erythrocytic development, *P. falciparum* expresses highly variant antigens on the erythrocyte surface,known as variant surface antigens (VSAs) (Beeson & Brown, 2002). *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is an example of such an antigen and is thought to be the most important target of antibodies among the variant surface antigens. This antigen plays an important role in cytoadherence and antigenic variation(Pasternak & Dzikowski, 2009).The potential to clear parasitemia depends upon IgG subclass,specifically immunoglobins IgG1 and/or IgG3(Aucan *et al.*, 2000).This is because they differ in their structures and mediate different immune effector functions (Nimmerjahn & Ravetch, 2008). IgG1 and IgG3 are the predominant subclasses produced in response to merozoite antigens (Polley *et al.*, 2006; Nebie *et al.*, 2008). IgG1 and IgG3 are cytophilic and T cell dependent, having high affinity for Fc receptors making them to bind on phagocytic cells, thereby activating effector mechanisms.

While IgG2 and IgG4 bind with lower affinity(Garraud *et al.*, 2003). For reasons that are not well understood, different merozoite antigens induce different relative levels of IgG1 and IgG3(Metzger *et al.*, 2003). It is unclear whether individuals have a bias toward producing a specific subclass regardless of the antigen or if instead the IgG subclass response is generated independently for each antigen and how this relates to protective immunity. Cytophilic IgGs therefore activate effector cell and are considered protective while non-cytophilicIgGs against the same epitope may block the protective effect of the cytophilic ones. This hypothesis has been supported by several studies. High levels of cytophilic IgG3 subclass have been associated with reduced parasitemia and protection against mild and severe malaria (Giha *et al.*, 2010; Roussilhon *et al.*, 2007). Surprisingly, high levels of IgG2 could be correlated with protection in individuals carrying the H131variant (human receptor for IgG) of monocytes Fc γ RIIareceptor which efficiently binds to IgG2.In contrast high levels of non-cytophilic IgG4 antibodies have been associated with susceptibility to malaria (Aucan *et al.*, 2001). While factors determining subclass responses to antigens are not clearly defined, antigen properties, host age, cumulative exposure, and genetic determinants have been linked with the nature of subclass responses (Aucan *et al.*, 2001; Bouharoun-Tayoun & Druilhe, 1992). For instance in twin studies it has been shown that there is concordance in monozygotic twins than in dizygotic twins for IgG levels (KatarinaSjoberg, 1992). However Stanisic in his study concluded that IgG subclass – specific responses to merozoite antigens are significantly associated with protection from high-density parasitemia and symptomatic malaria but not against parasitization *per se* (Stanisic., *et al.*, 2009).

Breadth of the antibody response is an important predictor of clinical outcome, this is because high levels of antibodies to multiple antigens have been shown to have a strong

association with protection from infection compared to antibodies against a single antigen (Osier *et al.*, 2008b). It has also been shown that one potential mechanism for antibody related protection is the combined effects of antibodies that target different steps during the life cycle of the parasite (John *et al.*, 2005).

2.4 Treatment options for malaria

While disease prevention has become an increasingly important component of malaria control efforts in Africa, prompt treatment with effective drugs will remain the cornerstone of control for the foreseeable future (Campbell, 2008).

2.4.1 Historical perspective of malarial therapy

Malaria was once found throughout many regions of the world including Northern America and Northern Europe. It was eliminated from a North America, Europe and parts of Asia and South America during 1950s following global campaign on DDT, and the discovery in the 1940s that the synthetic drug CQ could effectively treat individuals safely and cheaply (WHO,1999). This helped spur malaria eradication efforts in the 1950s. However, the emergence of CQ resistance diminished its therapeutic efficacy and doomed initial efforts to eradicate the disease. Moreover, restrictive use of DDT further led to spread of disease. The demise of these initial eradication efforts led to a resurgence in the disease and a significant change in the ecology, as CQ-resistant parasites spread from Southeast Asia to Africa (Payne, 1987). In the ensuing years, CQ was replaced as a first-line drug by SP but resistance to SP soon emerged and spread widely (Nair *et al.*, 2003; Roper *et al.*, 2003).When the parasites became resistant to these drugs and DDT use was restricted because of environmental and health hazards, malaria returned to many areas and the number of deaths peaked at 1.8 million in 2004. Nevertheless, because of novel, more effective medicines such as ACT, improved vector control, increased funding and public

awareness, the mortality rate has recently declined by ~30% globally, suggesting that it is time to consider new malaria elimination or even eradication campaigns (Flannery *et al.*, 2013).

2.4.2 Recent successes and challenges in treatment of malaria

The first-line treatment for *P. falciparum* infections in regions where chloroquine resistant parasites are present is a combination therapy of artemisinin derivatives with partner drugs that ideally have longer half-lives than artemisinin-derivatives. Artemether- lumefantrine (Coartem) and amodiaquine-artesunate (Coarsucam) are the most widely used, whereas dihydroartemisinin-piperaquine (Euartesim) and artesunate-pyronaridine (Pyramax) are the most recently approved (Anthony *et al.*, 2012). Several reformulations with doses specific for children and pregnant women are in clinical trials (Anthony *et al.*, 2012). Artemisinin derivatives are hypothesized to interact with Fe-II species in the parasite's food vacuole, and early ring stage parasites combat this by slowing down hemoglobin digestion (Klonis *et al.*, 2011). Artemisinins are fast acting and very potent against blood-stage parasites and show activity against early sexual stages of the parasite, which is important for blocking transmission. Their major limitation is a short half-life, which is why they are partnered with longer lasting drugs. ACTs are the most effective drugs to treat *P. falciparum* malaria. Reduced sensitivity to artemisinin monotherapy, coupled with the emergence of parasite resistance to all partner drugs, threaten to place millions of patients at risk of inadequate treatment of malaria since Artemisinin is the only hope left (Fairhurst *et al.*, 2012).The first signs of artemisinin resistance came from a study conducted in 2008, in which parasite clearance times after initial artesunate monotherapy were slower in patients from the Eastern Thai-Cambodian border (84 hours) than from the Thai-Myanmar border in the West (48 hours)(Dondorp., 2009). Furthermore, the proportion of slow-clearing infections (defined as a parasite half-life of ≥6.2 h) on the Thai-Myanmar border increased from 0.6% in 2001, to 20% in

2010 approaching the rate of 42% observed in Cambodia between 2007 and 2010(Cheeseman *et al.*, 2012).This suggested resistance has spread and is now present in western Thailand as well. At present, ACT resistance phenotype has been attributed to mutations in *P.falciparum* gene (PF3D7-1343700) encoding K-13 propeller domain. This is currently the best molecular marker of artemisinin resistance (Ariey *et al.*, 2014). However, these studies that established this marker were all conducted in South East Asia (Ariey *et al.*, 2014) where malaria immunity is much lower than sub-Sahara Africa. Studies done in sub-Saharan Africa have documented low frequency non-synonymous mutations in the K13 propeller domain (Kamau *et al.*, 2015; Maiga-Ascofare & May, 2016). However, these single nucleotide polymorphisms (SNPs) were different from the Asian SNPs and were not associated with any delayed parasite clearance (Ouattara *et al.*, 2015). A current study on *P. falciparum* drug resistance markers in western Kenya established that despite confirmed resistance of SP 10% of patients still use the drug resulting in high failure rates (Hemming-Schroeder *et al.*, 2018). Moreover, there was an increase in *Pfmdr1* haplotype associated to decreased lumafantrine susceptibility which calls for monitoring of AL effectiveness and potentially implementing multiple fist-line ACTs to delay partner drug from developing resistance.

In the light of the above monitoring ACT efficacy and its association to acquired malaria specific antibody responses (magnitude and breadth) could be a useful addition to molecular typing of K13 propeller polymorphism in surveillance of artemisinin resistance in Sub-Saharan Africa. Moreover, this could help in maximizing the effectiveness of drugs or suboptimal vaccines so as to lead to significant progress.

2.5 Determinants of antimalarial efficacy

Treatment failure in malaria usually results from poor compliance, inadequate dosing, pharmacokinetic factors or resistance (WHO, 2010a). However, some infections will recrudesce when none of these factors operates, this indicates that *in vivo* response to antimalarial treatment is due to many other factors other than the intrinsic susceptibility of *P. falciparum* to drugs (Travassos & Laufer, 2009). Parasite load, innate host resistance to malaria and acquired immunity to malaria have been shown to influence treatment outcome (Francis *et al.*, 2006).

2.5.1 Acquired immunity as a determinant of antimalarial efficacy

This is a key factor because it determines the host-parasite interactions and hence treatment outcome. Adults living in areas of high transmission acquire partial immunity to infection. While children in high transmission regions and everyone in low transmission regions by contrast, lack this protective immunity (Doolan et al., 2009). Infection frequently lead to symptomatic disease among these non-immune individuals (Grimwade *et al.*, 2004).Whereas semi-immune individuals can often clear infection with *P falciparum* without drug treatment(Bruce *et al.*, 2000; Djimdé *et al.*, 2003) despite the fact that they are not immune to parasitemia or clinical disease. Immunity has been shown to enhance effectiveness of antimalarial drugs. This was first observed when patients with neurosyphilis received malaria therapy i.e injection of malaria parasite to induce fever. Treatment of malaria infection was more effective if its start was delayed until the patient had experienced several paroxysms of fever and presumably had started mounting an immune response (Peters, 1987).This drug enhancing effect might also explain why quinine always seems to be more effective in regions of high transmissions (better immunity) than in areas of low transmission; a 3-day course of quinine was effective in sub-Saharan Africa but a 7-day course was needed in Thailand (Silachamroon *et al.*, 2002). Malaria eradication

measures such as residual insecticides and mass chemotherapy resulted in almost complete loss of antibody immunity in the Trobriand Islanders of Papua New Guinea (Desowitz *et al.*, 1966). Such populations are at risk of possible reinvasion by drug resistant strains from bordering areas. A study in Uganda suggested that declining malaria transmission resulted rapidly into decreasing host resistance in young children, and to decrease effectiveness of antimalarial drugs (Greenhouse *et al.*, 2009).With more intense efforts to control malaria, large populations might be at risk of losing immunity thereby enhancing the risk of decreasing efficacy of partly-effective treatments. Treatment efficacy improves with increasing age and malaria endemicity, suggesting that acquired immunity may play a role in determining the efficacy of anti-malarial treatments (Staedke et al., 2004;Greenhouse et al., 2010). The direct role of acquired antibody immunity in influencing malaria treatment outcome is conflicting (Flaherty et al., 2017).

Several studies have shown that treatment outcomes such as treatment failure (TF), clinical failure (CF), andadequate clinical and parasitological failure (ACPF) have correlations with responses to merozoite antigens (Aubouy *et al.*, 2007; Enevold *et al.*, 2007b). In a study in Tanzania, children were treated with SP and Amodiaquine and children with ACPR had substantially higher prevalence and concentration of IgG antibodies specific to GLURP than children with treatment failure (Enevold *et al.* (2007b) irrespective of their drug regimen. Patients infected with *P.falciparum* parasites carrying drug resistant mutations sometimes overcome infection after treatment. (Dorsey *et al.*, 2001). The ability to recover has been associated with host age (Djimdé *et al.*, 2003) and transmission intensity (Khalil *et al.*, 2005), reflecting an effect of acquired host immunity. These findings therefore suggest strongly that acquired immunity enhances the clinical efficacy of antimalarial drugs.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study area

The project was conducted in the context of a larger trial entitled "*In- vivo* and *in-vitro* efficacy of artemisinin combination therapy in Kisumu County, Western Kenya" (PI: Dr Ben Andagalu, KEMRI/WRP, Sponsor: GEIS, US Dept of Defense). The study was approved by the ethical review committee of the Kenya Medical Research Institute, Nairobi Kenya (KEMRI; approved protocol # 2518) and the institutional review board of the Walter Reed Army Institute of Research, Silver Spring, MD (WRAIR; approved protocol #1935). (See attached document in appendix I). The study was explained to all subjects and each consented before being enrolled in the study.

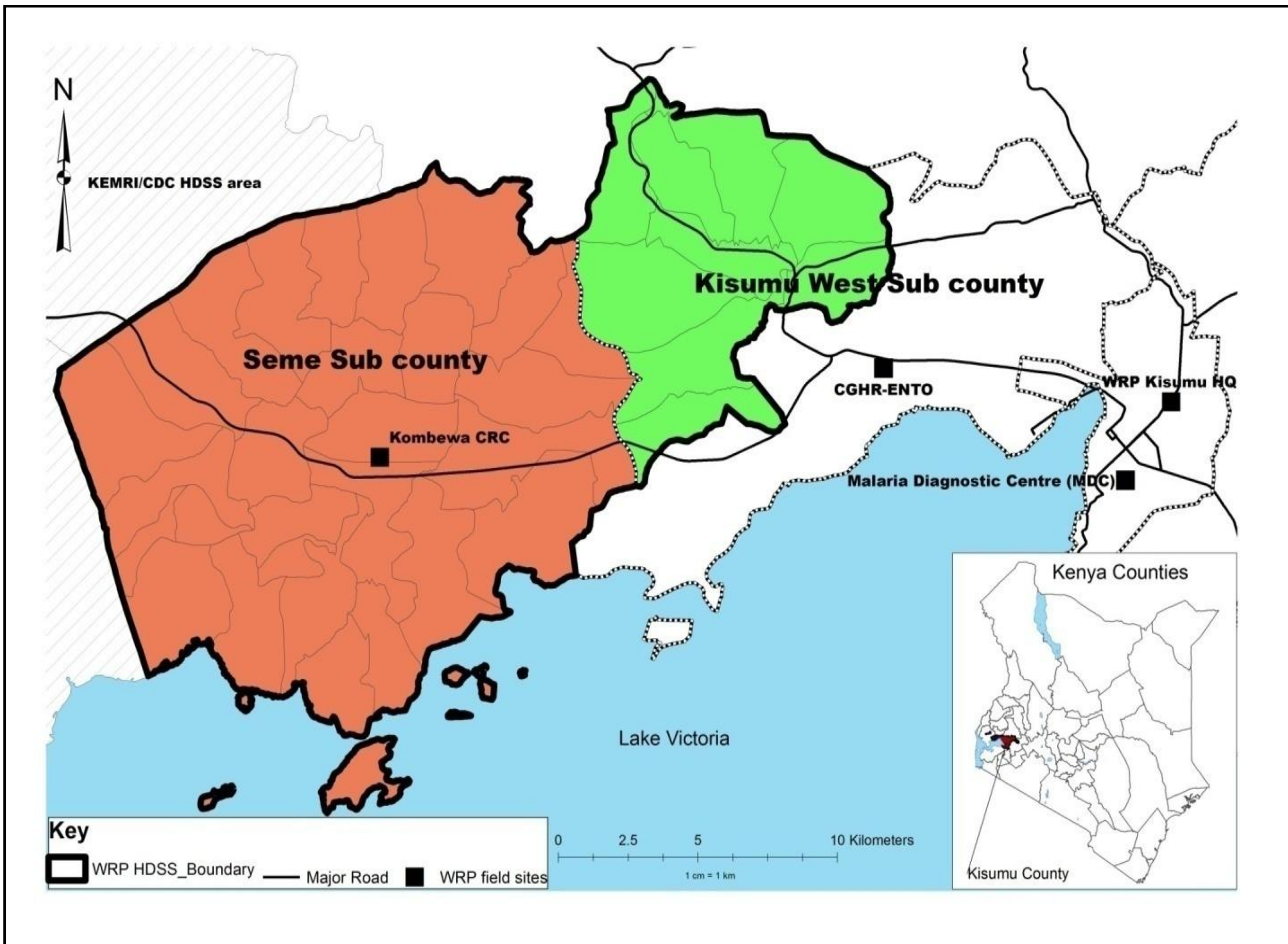

Figure 2. Map of the study area HDSS; also showing the study site (Kombewa CRC) (Wanja *et al.*, 2016)

The study was done between June 2013 and 2014 in Kombewa Division (figure 2), Kisumu county, western Kenya with a population of 23,000 people. It is located on longitude 34°45' E and latitude 0°10' S, with elevation of 150–1,250 m above sea level. Kombewa is has a rolling terrain bisected by small semi-permanent swampy streams with poor drainage with warm and dry weather. Malaria is holoendemic in this region, occurring throughout the year and with peak seasons 1-2 months after rainy season (March thru August) and (October thru December) (Idris *et al.*, 2016). Malaria transmission peaks 1-2 months after the rainy season. Mean monthly rainfall is 120.7 mm while maximum and minimum temperatures are 29.1^{0}C and 18.4^{o}C,

respectively. Malaria infections are predominantly due to *P.falciparum* accounts for 98% of malaria cases, the remaining 2% being caused by *P. Malariae* and *P.ovale*. The major malaria vectors are; *Anopheles gambiae*, *Anopheles arabiensis* and *Anopheles funestus* (National Malaria Elimination Progrmme, 2016).

3.2 Study design

The study design was a two-arm (ASMQ and AL) randomized trial nested within a larger trial entitled "*In vivo* and *in vitro* efficacy of ACT in Kisumu County, Western Kenya"

3.3 Sample size

Sample size was calculated using the below stated formulae according to (Chow *et al.*, 2003). This formula takes into account design effects and also takes care of population in both arms of the study.

$$n = (Z\alpha/2 + Z\beta)2 * [p1(1-p1) + p2(1-p2)]/(p1-p2)2$$

In this case;

$Z_{\alpha/2}$ is the critical value of the Normal distribution at α/2 (for a confidence level of 95%, α is 0.05 and the critical value is 1.96).

Z_{β} is the critical value of the Normal distribution at β (e.g. for a power of 80%, β is 0.2 and the critical value is 0.84)

p_1 and p_2 are the expected sample proportions of the two groups from (Grigg *et al.*, 2016).

$$\mathrm{n} = \frac{(1.96 + 0.84)^2 * [0.62(1-0.62) + 0.37(1-0.37)]}{(0.62-0.37)^2}$$

$= 58.59 \cong 59$ Participants per arm.

I assume that equal group sizes are required, and we also assume that the standard deviation in each population is the same.

A total of 118 patients were enrolled in the study, 59 from each arm. From these, 96 samples were randomly selected for analysis however; complete data (Bio-data and IgG data) was obtained for 82, including 40 from the AL arm and 42 from the ASMQ arm.

3.4 Sampling procedure

Block randomization schemes with varying block size were used to come up with randomization list of treatment assignments in advance. The assignments were then placed in sequentially numbered sealed opaque envelopes. Each subsequently enrolled subject was allocated the next available envelope containing treatment assignment. This was done to reduce bias since it guaranteed that treatment assignment was not based on patient's prognostic factors.

3.5 Study populations

Patients residing within the study area, presenting with uncomplicated malaria were recruited

Inclusion and exclusion criteria

3.6.1 Inclusion criteria

1. Adults/children aged between 6 months and 65 years inclusive (minimum weight 11kg), presenting with a measured temperature of ≥37.5°C, or history of fever within 24 hours prior to presentation.

2. Mono-infection with *P.falciparum* and a baseline parasitemia of 2000-200,000 asexual parasites/ µl.
3. Ability to provide informed consent in writing. However, patients who were unable to read underwent consenting process in the presence of an impartial witness. Parental consent was required for subjects below 18 years. However, subjects aged 13-17 years were in addition to the parental consent required to provide assent.
4. Willingness of the subject or parent to the subject (in case of a child) and ability to comply with the study protocol for the duration of the study.

3.6.2 Exclusion criteria

1. Subjects were excluded if they had signs of severe malaria and severe anemia, defined as hemoglobin level below 6g/dl.
2. Mixed Plasmodium infection or mono-infection of non-*falciparum* was also excluded.
3. Patients were further excluded if subjects were unable to take oral medication and were either lactating or pregnant females.
4. History to allergy or contraindications to the study treatments was also used to exclude subjects.

3.7 Data collection

Site specific data collection tools were used to collect data and as case report forms. This included confidential forms filled by participants/parents giving pertinent details needed for the study and also medical reports given by study clinician. All these were then synchronized into one database managed by qualified study staff. These tools were completed by study teams only and no data was transferred from hospital records to study data collection document.

3.7.1 Sample collection

Venous blood samples for malaria blood films were collected at hours 0, 4, 8, 12, 18, 24 and then every 6 hours until 2 consecutive smear became negative. The blood volumes to be collected had been stated in appendices II and III on study procedures. Special precaution was taken when collecting blood from pediatric subjects. This is because volumes of blood to be drawn are more restrictive and other inherent phlebotomy difficulties may reduce the volume further down. Finally venous whole blood was collected in EDTA tubes and aliquots preserved for analysis as specified in the study protocols at USAMRU-K labs at -20^{0}C for future use. It is this stored serum samples that were used for this immunological sub-study.

3.7.2 Antigens tested

Proteins were manufactured according to current good manufacturing practice at the Walter Reed Army Institute of Research Pilot Bio production Facility (Silver Spring, MD). The seven antigens used were known vaccine candidates and they also represented both erythrocytic and pre-erythrocytic life cycle of the parasite. They are as described below;

Table 1. Pre-erythrocytic and erythrocytic antigens used.

	Antigens	Clones	Antibody function against antigens	Reference
1	LSA	3D7	Protection against sporozoite infection.	(Hillier *et al*., 2005)
2	CSP	3D7	Inhibit sporozoites invasion	(Porter *et al*., 2013)
3	CelTOS	3D7	Induce protection against sporozoites.	(Bergmann *et al*.,2011)
4	AMA-HB3	HB3	Inhibit merozoite invasion.	(Fowkes *et al*., 2010)
5	AMA-3D7	3D7	Inhibit merozoite invasion.	(Dutta *et al*., 2005)
6	MSP-1 FVO	FVO	Protection against blood stage parasites	(Lyon *et al*., 2008)
7	MSP-1- 3D7	3D7	Protection against blood stage parasite.	(Darko et al., 2005)

A table showing *P.falciparum* antigens and roles of specific IgG antibodies against them.

3.7.3 Malaria specific Antibody profiling in serum by Luminex

Sera obtained from patients at baseline (day 0) before treatment with either AL or ASMQ was screened for total IgG (i.e magnitude of response) against a representative selection of well-characterized erythrocytic and pre-erythocytic stage antigens by luminex (Kerkhof *et al*., 2015). These antigens included: AMA-1 (HB3and 3D7 strains), MSP-1-42 (3D7 and FVO strains), LSA-1,CelTOS and CSP. These antigens were selected, as previous studies have indicated that antibodies against them have been associated with protective immunity, are targets of acquired invasion inhibitory antibodies, and are vaccine candidates (Hodder, Crewther, & Anders, 2001; Persson et al., 2013). To examine breadth of reactivity sera reactivity to either 1 or multiple antigens were analyzed. Highly purified preparations of these antigens were obtained from the Walter Reed. A detailed procedure for the Luminex assay had been previously described (Ambrosino *et al*., 2010; Perraut *et al*., 2014). In brief, spectrally unique beads which had been coupled were first vortexed for 20 seconds, and sonicated for 20 seconds. Working bead mixture was prepared by diluting coupled beads in PBS-1% BSA (total volume = number of wells x 50

µl). The final concentration = 3,000 beads of each signature/50µl. See attached bead calculation template to calculate working bead mixture in appendix IV.

Sera dilution was prepared in biosafety cabinet by making aliquots of serum diluted in 1;400 with PBS-1%BSA (assay buffer) in the first well then serially diluting it from 1;400 to 1:51200. A 96 well plate (Costar, USA catalogue number 3789) was then pre-wet using 200 µl of PBS-1% BSA.50 µl of working bead mixture and 50 µl of diluted samples were aliquoted into appropriate wells (See plate map in appendix V). This were then covered with aluminum foil and incubated for 1 hour at room temperature on a titer plate shaker (Lab-line instruments, Inc, Melrose Park, IL) at 500 rpm. Supernatant was discarded by using magnetic separator (Luminex co-op. Austin, USA) and Washed four times using 200 µl of 1X PBS-0.05% Tween 20.This was followed by re-suspension of beads in 50 µl of 1X PBS- 1% BSA.

R' phycoerythrin-conjugated affinipure F (ab') 2 fragment goat anti-human IgG (H+L) (Jackson Immuno-Research Laboratories, USA Cat # 109-116-088) of concentration 500µg/ml was diluted to 2 µg/ ml in PBS-1% BSA in enough volume to add50 µl to each well.

50 µl of diluted detection antibody was added to each well then plate covered with aluminum foil and incubated for 30 minutes at room temperature on a plate shaker. Using Magnetic separator the supernatant was discarded and then washed four times with 200 µl of PBS-Tween. Finally beads were re-suspended in 100 µl of PBS1% BSA and 50 µl analyzed on the MAGPix platform (Luminex Corporation, USA). Xponent software (Luminex corporation,Texas) was used in the MAGPix setup for analysis based on the following protocols;

a). Total events: 100

b). Sample time-out: 200 sec

c). Sample volume: 50µl

d). DD Gate: 7500 to 20,000

e). Type: Magpix

Data was recorded as median fluorescence intensity (MFI) and saved in CSV format which was then exported on dedicated, password protected computers. Only authorized study personnel had access to the data. The only identifier used in this computerized database was subject's study number. This data was then kept for further analysis.

For each run, negative and positive control samples were included for analysis. Negative control was obtained from naïve human malaria sera samples (GEMINI Bio products, West Sacramento, CA) (Lot number H94MOOC).Pooled human sera samples known to be positive for the listed *P. falciparum* antigens were used as positive control (Mal-036).

The validation of the assay was done and control reference ranges established upon which the assay performance was monitored. The monitoring was done by using Levy Jennings plots in which both positive and negative control were recorded per dilution.

3.7.4 Parasite clearance rates calculation

Parasite clearance rates were calculating using Worldwide Antimalarial Resistance Network (WWARN) tool for Parasite Clearance Estimator (PCE) (WWARN ., 2009). Log transformed parasite density was plotted against time in hours to generate slope half-life. The slope half-life ($T_{1/2}$) was calculated as follows:

$$T_{1/2} = \log_e (2)/K$$

$$=0.692/K,$$ where K is the clearance rate constant.

3.8 Data management and analysis

For the larger ACT efficacy trial database was created and maintained by qualified USAMRU-K staff. Source data verification was further conducted by internal quality control staff. Moreover the study was monitored through a reciprocal monitoring scheme whereby one of the clinical research coordinator from any of the participating sites perform reciprocal monitoring at a different participating sites. Samples were de-identified and assigned numbers, code link was maintained at a separate location. All data and analysis generated both from the clinical sites and laboratory were kept on dedicated password protected computers. Only authorized study personnel had access to the data.

Statistical analysis was done using STATA version 13 (Stata Corporation, College Station, Texas) and Graph pad prism version 5. Continuous variables and proportions were compared using the two-sample Mann-Whitney,χ^2 test and z test. This was used to compare antibody concentrations or magnitude between groups (fast clearers and faster clearers) and other variables such as age, gender and parasitemia. Parasite clearance half-life ($PC_{1/2}$) was obtained from *in vivo* efficacy study where clearance half-life of the parasite infections in subset of samples in response to ASMQ and AL was obtained. Previous studies have used parasite clearance half-life values as cut-off. In this population the median $PC_{1/2}$ was 2.41hours (2.02-2.76) the 25th quartile was then used as a cut-off (2.02) (Ariey *et al.*, 2014; Bidii S. Ngalah *et al.*, 2015). Antibody cut- off per antigens were arrived at by mean plus 3 standard deviations from naïve human malaria sera samples (GEMINI Bio products, West Sacramento, CA.).To assess the breadth of responses, the threshold for high levels of antibodies was set at twice the threshold for a positive response (John *et al.*, 2005). Antibodies were analyzed in an age, gender and arm to

between one and >4antigens and proportions analyzed using Z test .Statistical significance was determined if $p \leq 0.05$.

CHAPTER FOUR

RESULTS

4.1 Patients characteristics in the ACT efficacy trial

In this ACT efficacy trial total number enrolled in AL arm and ASMQ was 82 patients as shown in Table 2. Age, gender and parasitemia at baseline were similar for both AL and ASMQ treatment groups. Of the 40 patients enrolled for AL arm, 24 were children less than 5 years of age, while 16 patients were aged 5 years and above. On the other hand, ASMQ arm had 42 patients in which 33 were children under 5 years of age, while 09 patients were aged 5 years and above. In terms of gender, 22 were males while 18 were females in the AL arm .In the ASMQ arm, 23 were males while 19 were females. Patients enrolled into AL arm had a relatively higher parasitemia at baseline compared to those in the ASMQ arm, but the difference was not statistically significant (p=0.485).

Table 2. Baseline characteristics of patients enrolled in the two arms of the study.

Variable	Study arm n (%)		
	AL (40)	ASMQ (42)	*p* value
Age (years)			0.0679
< 5	24(60%)	33(78.5%)	
5 and above	16(40%)	09(21.5%)	
Gender			0.983
Male	22(56.4)	23(54.8)	
Female	17(43.6)	19(45.4)	
Parasite density Median(IQR)	60,964.7(11,233-112,700)	26,025.8(8,001.2-93,276.5)	0.485

The study had two arms; AL (Artemether lumefantrine) and ASMQ (Artesunate mefloquine). p-values indicate the level of significance as calculated using chi- square for categorical variable and Mann Whitney U -test for continuous variables.

4.2 Magnitude of Malaria-specific antibody responses in study participants

The study determined the specific antibody responses in sera across different antigens. In this study population, between 91-100% of study participants had high responses to these antigens. Antibodies to erythrocytic antigens were generally having higher titers and prevalence than pre-erythrocytic antigens (Figure 3). IgG antibodies against AMA1-3D7 had the highest geometric mean of7889 (5705-10910) at 95% CI, while antibodies against CelTOS had the least geometric mean of 536.9 (448-642) at 95% CI (Table 3).

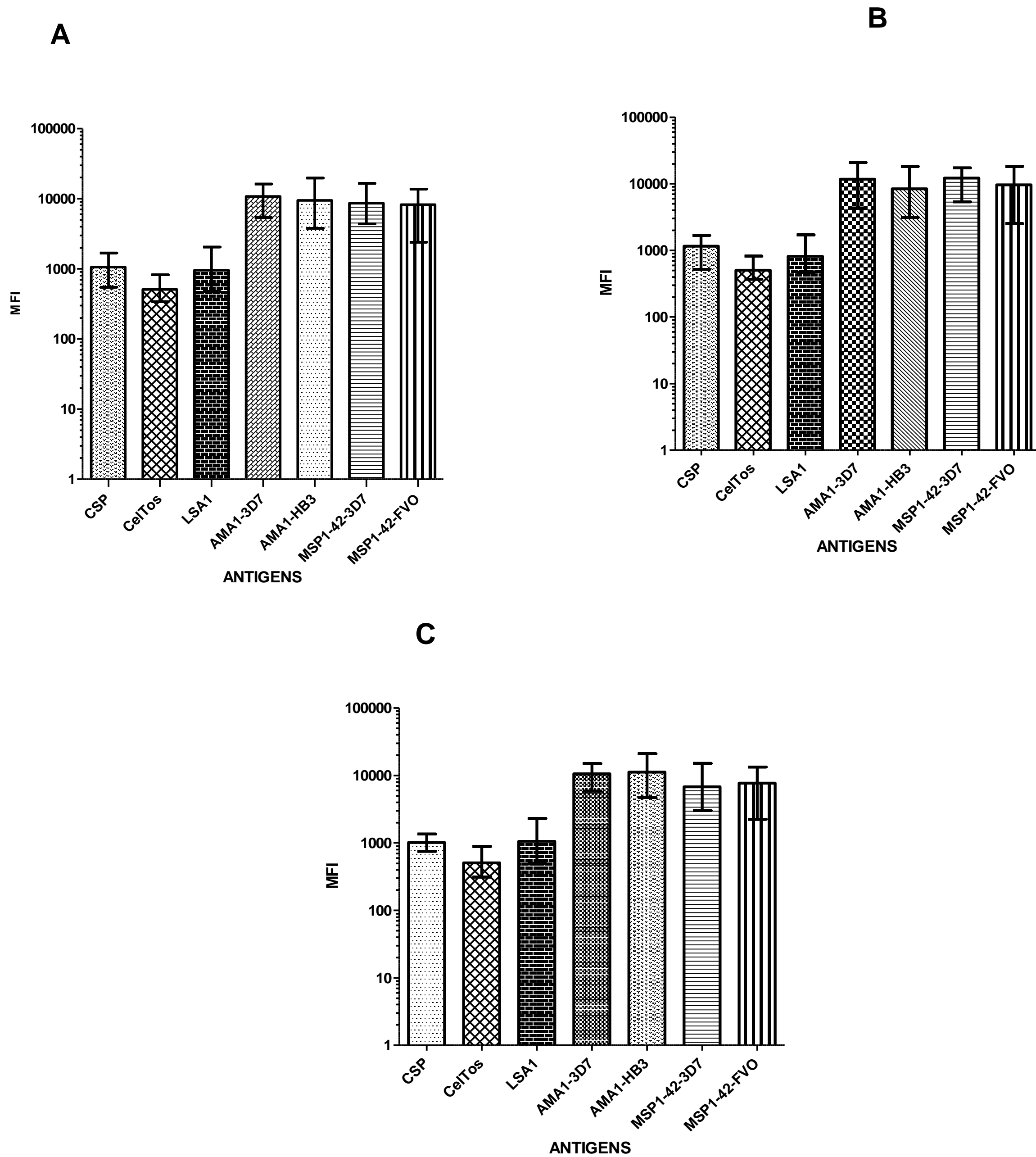

Figure 3. Antibody reactivity against malaria specific antigens of patients in the study. The magnitudes of antibody responses were quantified. Bars represent median with interquartile range.

A. Antibody reactivity of patients enrolled in both arms of the study (n=82).
B. Shows antibody reactivity of patients enrolled in ASMQ arm of the study (n=42).
C. Shows antibody reactivity of patients enrolled in AL arms of the study (n=40).

Table 3. Table showing Magnitude and prevalence of antibody responses for both study arms.

Antigen location	Pf Antigens	Geometric mean of MFI at 95%CI	N	Cut-off value	Participants above cut-off N	Prevalence (%)
Antigen reactivity of patients enrolled in both arms of the study						
Pre-erythrocytic	CSP	986.9 (808-642)	81	184.3	77	95.1
	CelTOS	536.9 (448-642)	81	196.5	74	91.6
	LSA	945.9 (758-1180)	81	160.5	78	96.3
Erythrocytic	AMA 3D7	7889 (5705-10910)	41	976.6	40	97.6
	AMA HB3	7975 (6376-9976)	81	738.3	79	97.5
	MSP 1 3D7	6979 (5341-9120)	62	156.7	62	100
	MSP 1 FVO	5706 (3932-8282)	41	172.5	40	97.6

Malaria specific antibodies magnitude and prevalence were quantified using Luminex. Pre-erythrocytic antigens (CSP, CelTOS, LSA) had relatively lower geometric mean as compared to erythrocytic antigents (AMA 3D7,AMA HB3, MSP 1 3D7 , MSP 1 FVO).The cut-off was obtained by calculating mean antibody responses (MFI) of malaria naïve patients plus two standard deviations.

4.2.1 Magnitude of antibody responses by age and gender categories

A two sample Mann-Whitney U test was used to compare differences between magnitude of antibodies titers in different age categories (≤5 years n=58 and >5 years n=23). Patients who were >5 years old had a significantly higher titers of anti- AMA1-HB3 antibodies than those ≤5 years (p=0.0316). The remaining antigens did not show any significant difference in their responses as illustrated in figure 4. The relationship between gender categories (male and female) and IgG antibody titers was, however, not found to be statistically significant (figure 5).

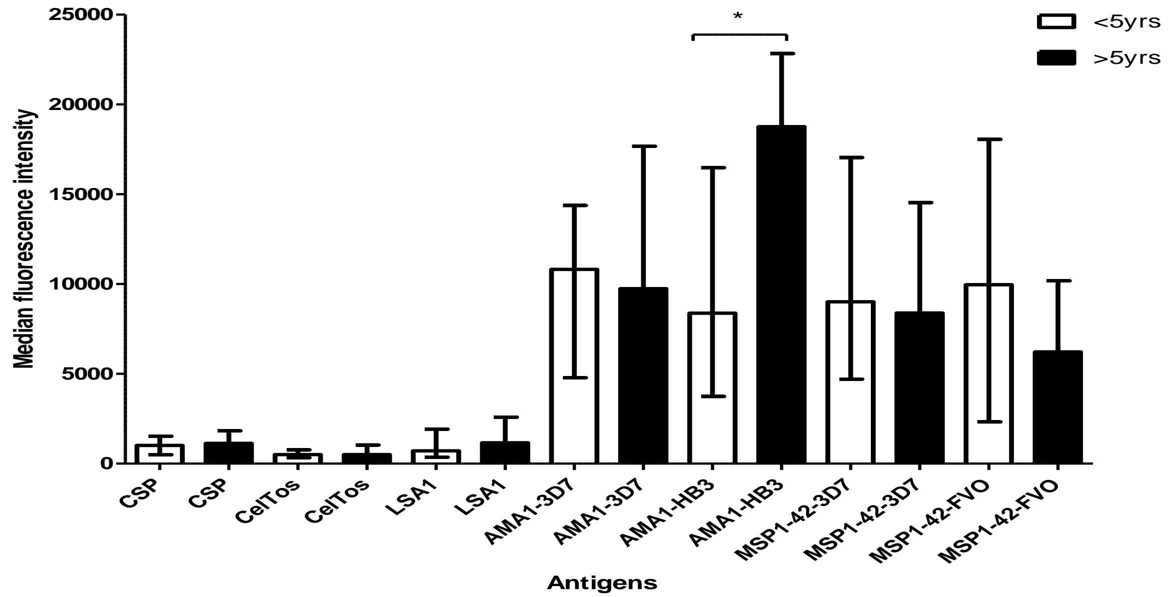

Figure 4. The IgG antibody titers for all antigens categorized by age. The antibody responses for children aged ≤5 years n=58 and >5 years n=23 were quantified. The antibody response was quantified in duplicate for each sample. Bars represent median with interquartile range. Statistical difference in antibody response between children aged ≤5 and >5 years was determined by Mann-Whitney U test (*, $p \leq 0.05$). Only AMA-HB3 had a significance difference.

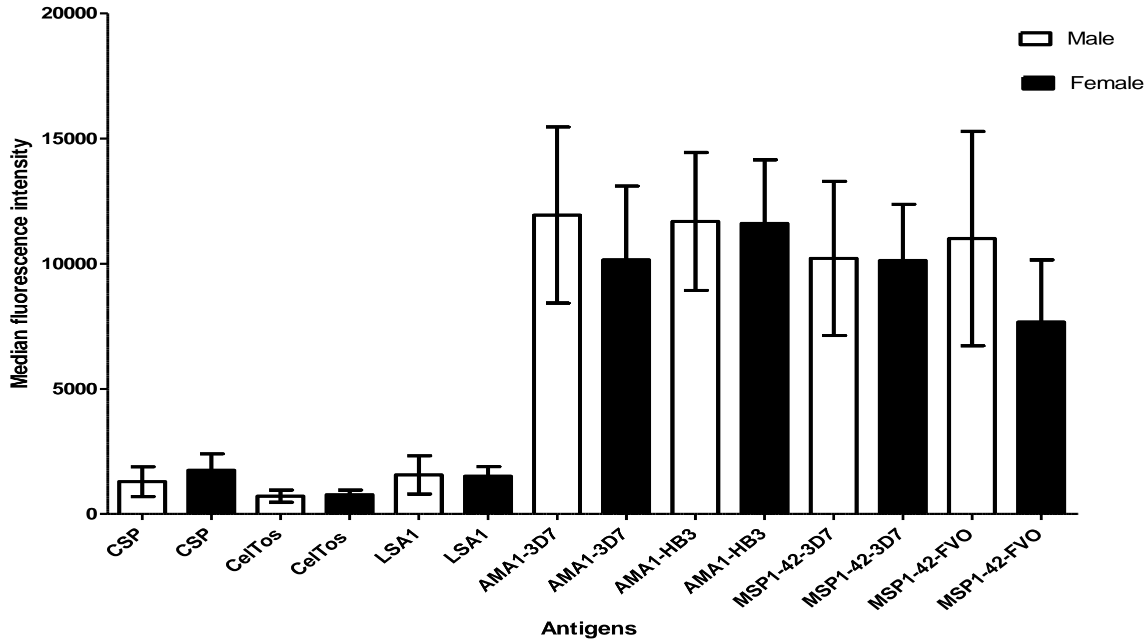

Figure 5. Antibody titers for all antigens by gender. Antibody responses per gender were quantified male (n=45) female (n=36). Bars represent median antibody titers and interquartile range. Mann Whitney U test was used to assess the significant difference within the groups and significance set at $p \leq 0.05$. No significant difference was realized.

4.3. Breadth of antibody responses

The breadth of antibody responses was defined as the number of antigens (1-7) to which an individual had antibody titers above the threshold antibody concentration as described in the analysis section. Individuals were categorized into three groups (1, 2-3 and >4) based on the number of antigens they were highly reactive to. Patients who reacted highly to only 1 antigen were 2(2.5%). While those who were reactive to 2-3 antigens were 10 (12.3%). Majority of participants i.e. 69(85.2%) had a higher breadth >4 as illustrated in table 4.

4.3.1 Breadth of antibody responses categorized by age

To assess whether age is a surrogate of IgG antibody responses, participants were categorized in two groups i.e≤5 years and >5 years. Two (2) patients responded highly to 1 antigen and both were children below 5 years. Seven (70%) individuals who were children ≤5 years had high response to 2-3 antigens while 3(30%) were subjects >5 years. Many children ≤5 years 47(68.1%) were highly reactive to >4 antigens while 7(31.9%) were those participants >5years who also were reactive to >4 antigens. Breadth of high-titers antigen-specific responses generally did not increased by age with children ≤5 years who responded highly to >4 antigens having significantly higher numbers than those >5years (p=0.0047) after analysis using Z test for proportions (Table 4).

Table 4. Breadth of antibody reactivity for all patients enrolled in the study and for different age categories.

	Breadth of antibody responses			
Number of antigens	**No of responses** N (%)	**≤5years** N (%)	**>5years** N (%)	**p-value**
1	2 (2.5)	2	0	N/A
2-3	10 (12.3)	7 (70)	3 (30)	0.2402
>4	69 (85.2)	47 (68.1)	22 (31.9)	**0.0047**

Reactivity to one or multiple antigens was assessed and subjects put into three groups and further grouped into different age categories. Level of significance was calculated using two sample tests of proportions (Z test) and significance set at p ≤ 0.05. Children ≤5 years who responded highly to >4 antigens were significantly higher in numbers than those >5years (p=0.0047).

4.4. Magnitude of antibody responses and Parasite clearance rate

IgG antibody level for all the seven antigens in both study arms were analyzed based on parasite clearance half-life as earlier described. Patients who were faster clearers had a higher titers of anti- CelTOS IgG ,anti -LSA1 IgG, anti- AMA1-3D7 IgG and anti- AMA1-HB3 IgG compared to patients who were fast clearers (Table 5). However, Only AMA1-HB3 had a statistically significant difference (p= 0.0065). The remaining three antigens (CSP, MSP1-42-3D7, MSP1-

42-FVO) had their median IgG antibody levels higher in fast clearers than in faster clearers, the difference was however not statistically significant.

Table 6 shows details of the outcome of ASMQ arm of the study. Faster clearers had a higher median antibody level in four antigens. However, only AMA1-HB3 had a statistically significant difference (p=0.0047) when Mann-Whitney U test was used to compare the difference. In the AL arm (Table 6) anti-LSA IgG antibodies had a higher median in faster clearers than in fast clearers (p=0.0396). The remaining antigens did not show any significant difference. However, faster clearers had a higher median IgG antibody than fast clearers in AMA1-HB3 antigen even though the difference was not statistically significant.

Table 5. The magnitude of antibodies responses between faster clearers and fast clearers in all patients in the study.

	Median florescent intensity		
Antigens	**Faster clearers, n=19**	**Fast clearers, n =60**	**p value**
CSP	893.6 (610.8-969.4)	1595 (1100.3-2089.7)	0.1182
CelTOS	761.1(386.7-969.6)	678.1 (578.7-943.4)	0.6443
LSA1	1617.2(964.3-2270.2)	1447.7(953.71-1941.6)	0.7193
AMA1-3D7	13583.4 (7011.1-20155.7)	9855.5 (7669.5-12041.5)	0.1404
AMA1-HB3	15842.3(11862-19822.4)	9988(7948.6-12027.6)	**0.0065**
MSP1-42-3D7	9908.2(6166.8-13649.6)	10207.6 (7956.3-12458)	0.8882
MSP1-42-FVO	6290.3(1943.8-10636.7)	10033.3 (7107.8-12958)	0.1721

Median IgG level at baseline were measured as per antigen. Values represent the median with interquartile range. The MFI was measured using Luminex. Bold represent statistically significant difference between faster clearers and fast clearers as determined by Mann Whitney U test and significance set at $p \leq 0.05$.

Table 6. Magnitude of antibodies responses between fast clearers and faster clearers among ASMQ patients.

	Median (Range)		
Antigens	**Faster clearers, n = 14**	**Fast clearers n=25**	**p value**
CSP	1078(324-1288)	1174(615-1777.5)	0.1432
CelTOS	494.7(248-843)	506(436.5-786)	0.5582
LSA1	869.5(481-1880)	716(458.5-1039.5)	0.7922
AMA1-3D7	16100(11733-23432.5)	5126(4325-5620)	0.2506
AMA1-HB3	17400.5(8380-21672.5)	4595(2447.5-10439)	**0.0047**
MSP1-42-3D7	8731(5360-17587)	13482(4940-17222.5)	0.9128
MSP1-42-FVO	9622(2525-10309)	7872.5(3178-18196)	0.7540

Median IgG level at day 0 were measured as per antigen. Value represent the median with interquartile range. The MFI was measured using Luminex. Bold represent statistically significant difference between faster clearers and fast clearers as determined by Mann Whitney U test and significance set at $p \leq 0.05$.

Table 7. Magnitude of antibody responses between fast clearers and faster clearers among patients receiving AL.

	Median (Range)		
Antigens	**Faster clearers, n=6**	**Fast clearers,n=33**	**p value**
CSP	654(395-1036)	974.5(575-1500)	0.3554
CelTOS	515(508-735)	485.25(314-912)	0.6440
LSA1	3126(1758-4540)	979.25(333-2145.5)	**0.0396**
AMA1-3D7	12791.5(4671-13328)	10501.7(6110.7-15304.2)	1.0000
AMA1-HB3	18238(16326-18770)	10120(4595-21004.5)	0.4495
MSP1-42-3D7	4665(2103-6657)	6998(3178-151115)	0.4510
MSP1-42-FVO	2515(2269-4302.5)	8668.5(3016.2-14721)	0.1489

Median IgG level at baseline were measured per antigen. Values represent the median with interquartile range. The MFI was measured using Luminex. Bold represent statistically significant difference between faster clearers and fast clearers as determined by Mann Whitney U test and significance set at $p \leq 0.05$.

4.4.1 Breath of antibody response and parasite clearance

Table 8 illustrates stratification of patients into three groups (1, 2-3 and >4) based on the number of highly reactive antigens they were responding to. Patients who reacted highly to 1 antigen were 2 in number out of which 1 was fast clearers while the remaining was faster clearer it wasn't possible to establish their p- value statistically because of insufficient data. Subjects who reacted to between 2-3 antigens were 10 out of which 2 were faster clearers while 8 were fast clearers, the difference in proportion was not significant (p=0.1037). The number of subjects that reacted to more than 4 antigens were 69 the fast and faster clearers were 52 and 17 respectively their proportion was statistically significant (p=0.0002) on analysis by Z test.

Table 8. A comparison between breadth of antibody reactivity and parasite clearance rate.

Breadth of antibody reactivity and parasite clearance rate				
Number of		**Faster clearers**	**Fast clearers**	
antigens	**N**	**N (%)**	**N (%)**	**P Value**
1	2	1 (50)	1 (50)	**N/A**
2-3	10	2 (20)	8 (80)	0.1037
>4	69	17 (24.6)	52 (75.4)	**0.0002**

Reactivity to one or multiple antigens was assessed and subjects put into three groups and further grouped into two categories bases on parasite clearance half life. Level of significance was calculated using two sample tests of proportions (Z test) and significance set at p ≤0.05. Bold value represents statistically significant difference.

CHAPTER FIVE

DISCUSSION

This study determined the baseline magnitude and breadth of acquired antibody responses against specific malaria antigens and also correlated antibody responses to ACT treatment outcome in patients with uncomplicated malaria. This was done in an attempt to provide evidence that specific malaria antibodies are predictive correlates of ACT efficacy.

5.1 Magnitude of Malaria specific antibody responses

Data from this study showed that antibody prevalence was over 90% with higher levels of IgG antibody responses for most antigens .This may suggest that these specific antigens could have been better surrogate markers of exposure or protection in this holoendemic setting. This finding is supported by a previous study which reported that the prevalence of antibodies to CSP, LSA, and MSP-2 3D7 was high among general population of Asembo location, within western Kenya, which is also malaria holoendemic setting (Zhou *et al.*, 2002). Another study had also established that IgG levels and sero-prevalence against pre- erythrocytic and erythrocytic antigens are associated with immunity against clinical malaria (Chelimo *et al.*, 2005). Kombewa, being a high transmission setting, the result may further suggest a correlation between antibodies of malaria-specific antigens and transmission intensity. A study in Gambia and Tanzania had reported higher IgG antibodies reactivity against GLURP in individuals living in high transmission area than those in low transmission area (Drakeley *et al.*, 2005).

The magnitude of antibody responses and sero-prevalence was found to be higher in erythrocytic antigens than in pre-erythrocytic antigen. This is in tandem with a study done in both unstable malaria transmission (low transmission) and stable transmission setting (high

transmission) in Kenya, which showed that in malaria holoendemic areas, antibodies against erythrocytic antigens had higher titers and prevalence than those of pre-erythrocytic antigens (Noland *et al.*, 2008).This suggests that protection in a holoendemic environment could be majorly dependent on antibodies against blood-stage antigens. A study by Greenhouse showed that stronger antibody responses to the blood-stage antigens AMA-1, MSP-1, and MSP-3, but not the pre-erythrocytic antigens CSP and LSA-1, were associated with blood- stage protection (Greenhouse *et al.*, 2011). AMA1 is known to be a target of antibodies that prevents parasites from invading red blood cells in vitro (Kennedy *et al.*, 2002; Kocken *et al.*, 2002).

The data presented in this study showed that antibody response to both clones of AMA (3D7 and HB3) had the highest titers. This suggests that AMA antibodies are highly prevalent in malaria-exposed individuals and could play a part in malaria immunity. This was also reported by Fowkes in which they established that AMA antibodies are highly prevalent and protective in malaria exposed populations and their prevalence have been noted to increase with age as naturally acquired immunity develops (Fowkes *et al.*, 2010). Antibodies against AMA-HB3 were predominantly higher in patients > 5 years compared to those ≤ 5 years. This was however in contrast with other antigens except LSA and AMA 3D7. Suggesting that antibody levels against AMA-HB3, LSA and AMA 3D7 increase with increasing age with AMA HB3 showing a stronger response. Earlier studies have further demonstrated that levels/magnitude of antibodies against some erythrocytic and pre-erythrocytic antigens increased by age (Chelimo *et al.*, 2005; John *et al.*, 2005).

5.2 Breadth of Malaria specific antibody responses

In this present study majority of study participants had a higher breadth of antibody responses (69%).This could have been as a result of a high malaria transmission setting in which the level

of antigenic diversity of *P. falciparum* populations in the area had an effect in acquisition of immunity. Several studies have shown that the antigenic and genetic repertoires of *P. falciparum* populations are wider in high-transmission areas generated by more frequent recombination events in the mosquito (Hoffmann *et al.*, 2001) .

Breadth of high-titer antigen specific responses did not increased with age in patients enrolled in the study. Those who responded highly to only one antigen (lower breadth) were all children <5 years while majority of those who responded highly to >4 antigens were also children <5 years. This was in contrast to a study done in Kilifi-Kenya in which breadth significantly increased with age (F. H. Osier *et al.*, 2008a; Rono *et al.*, 2013). The main reason for this might have been because majority of our subjects were children below 2 years of age this therefore limited our comparisons.

5.3 Association between Malaria specific antibody responses and treatment outcome

The study established that patients who were faster clearers had a higher titers of anti- CelTOS IgG ,anti -LSA1 IgG,anti- AMA1-3D7 IgG and anti- AMA1-HB3 IgG antibodies compared to patients who were fast clearers. Only AMA1-HB3 antibodies had a significant difference. This suggests a supportive role for humoral antibodies in therapeutic response to antimalarial drugs. Other studies are also in agreement that there is a stronger and more diverse antibody response that is associated with greater success in antimalarial drug treatment (Enevold *et al.*, 2007a; Mawili-Mboumba *et al.*, 2003). In the ASMQ arm of the study antibodies against AMA-HB3 was significantly higher in faster clearers than in fast clearers. This is suggesting that of the erythrocytic antigens we studied AMA-HB3 was more predictive of ACT treatment outcome than all the other remaining antigens. In other studies high levels of AMA-1 blocked sporozoite invasion of hepatocytes (John *et al.*, 2005; Silvie *et al.*, 2004).While in AL arm, LSA was

significantly higher in faster clearers than in fast clearers this was the only pre-erythrocytic antigen which was predictive of treatment outcome. This was in agreement with other studies which establishes that LSA-1 correlated with more rapid parasite clearance time (Luty *et al.*, 1998) and protection from clinical malaria.

In terms of breadth of antibody responses and parasite clearance rate, fast clearers were significantly higher than faster clearers this might have been attributed to the number of patients who were children in the study resulting in lack of adequate comparison of breadth.

CHAPTER SIX

SUMMARY OF STUDY FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of study findings

There was a high prevalence of IgG antibodies against both erythrocytic and pre-erythrocytic antigens in this setting. Moreover, erythrocytic antigens under study had a higher magnitude of IgG antibodies compared to pre-erythrocytic antigens in this study population. Antibody titers increased by age this was more evident with anti-AMA-HB3 antibodies. In addition, majority of participants showed a higher breadth of antibody responses.

Faster clearers had higher IgG antibody titers in most antigens (CelTOS, LSA, AMA-3D7 and AMA-HB3). However, AMA-HB3and LSA had a statistically significant difference.

6.2 Overall conclusions and implications

1. The magnitude of IgG antibody responses and sero-prevalence against both pre-erythrocytic and erythrocytic antigens were high ; Implying that the population could have been highly exposed to malaria.

2. The magnitude and prevalence of antibodies against erythrocytic antigens was found to be higher than antibodies against pre-erythrocytic antigens. Suggesting that in this setting (holoendemic) protection could be majorly dependent on antibodies against erythrocytic antigens.
3. There was a high breadth of responses in the study participants suggesting that the study population was malaria exposed resulting in an increased level of antigenic diversity of *P.falciparum* populations in the area.

4. Levels of anti-AMA-HB3 and LSA-1 antibodies were significantly higher in faster clearers than in fast clearers. These suggest that in the present study antibodies against the two antigens could be used as predictors of therapeutic efficacy for AL and ASMQ drugs.

6.3 Recommendations for the study

1. With further analysis AMA-HB3 and LSA could be used therefore as an important confounder in assessment of emerging ACT resistance or delayed clearance in malaria endemic populations.

6.4 Recommendations for future research

1. More comprehensive studies need to be done with a larger sample size and in different malaria transmission intensities.
2. To assess more influence of IgG antibodies on treatment outcome antibody sub-classes should be evaluated.
3. This study was limited to 7 antigens therefore further analysis should be done using other vaccine candidate antigens to evaluate the profiles of other antigens.
4. My study participants were mostly children however, better and more comprehensive antibody immune profiles could be obtained if all age groups were proportionally included.
5. Parasite clearance as a metric for drug effectiveness and resistance has limitations. Therefore, other metrics such as use of iRBC clearance rates should be explored to possibly get better immune correlates of ACT efficacy.

6. In spite of many advantages of using Luminex assay caution is necessary since if compared to ELISA it’s more robust in determining antibody titers when using serum or plasma samples.

REFERENCES

Alker, A. P., Lim, P., Sem, R., Shah, N. K., Yi, P., Bouth, D. Y., Tsuyuoka, R., Maguire.J.D., Fandeur, T., Ariey, F., Wongsrichanalai, C., & Meshnick.S.R. (2008). PFMDR1 and *in vivo* resistance to artesunate-mefloquine in falciparum malaria on the cambodian–thai border. *American Society of Tropical Medicine and hygine, **76***(4): 641–647.

Ambrosino, E., Dumoulin, C., Orlandi-Pradines, E., Remoue, F., Toure-Baldé, A., Tall, A., Sarr, J. B., Poinsignon, A., Sokhna, C., Puget, K., Trape, J.-F., Pascual, A., Druilhe, P., Fusai, T., & Rogier, C. (2010). A multiplex assay for the simultaneous detection of antibodies against 15 Plasmodium falciparum and Anopheles gambiae saliva antigens. *Malaria Journal, **9***, 317-317. doi: 10.1186/1475-2875-9-317

Anthony, M. P., Burrows, J. N., Duparc, S., Moehrle J.J., & Wells T.N. (2012). The global pipeline of new medicines for the control and elimination of malaria. *Malaria Jounal, **11***(316): 1475-2875.

Aponte, J. J., Menendez, C., Schellenberg, D., Kahigwa, E., Mshinda, H., Vountasou, P., Tanner, M., & Alonso, P. L. (2007). Age interactions in the development of naturally acquired immunity to Plasmodium falciparum and its clinical presentation. *PLoS Med, **4***(7), e242. doi: 10.1371/journal.pmed.0040242

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A. C., Khim, N., Kim, S., Duru, V., Bouchier, C., Ma, L., Lim, P., Leang, R., Duong, S., Sreng, S., Suon, S., Chuor, C. M., Bout, D. M., Menard, S., Rogers, W. O., Genton, B., Fandeur, T., Miotto, O., Ringwald, P., Le Bras, J., Berry, A., Barale, J. C., Fairhurst, R. M., Benoit-Vical, F., Mercereau-Puijalon, O., & Menard, D. (2014). A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. *Nature, **505***(7481): 50-55. doi: 10.1038/nature12876.

Artavanis-Tsakonas, K., Eleme, K., McQueen, K. L., & Cheng, N. W., Parham,P., Davis,D.M., Riley,E.,. (2003). Activation of a subset of human NK cells upon contact with Plasmodium falciparum-infected erythrocytes. *The Journal of Immunology, **171***(10): 5396-5405.

Ataide, R., Ashley, E. a., Powell, R., Chan, J.-A., Malloy, M. J., O'Flaherty, K., … Fowkes, F. J. I. (2017). Host immunity to *Plasmodium falciparum* and the assessment of emerging artemisinin resistance in a multinational cohort. *Proceedings of the National Academy of Sciences*, 201615875. https://doi.org/10.1073/pnas.1615875114

Aubouy, A., Migot-Nabias, F., & Deloron, P. (2007). Correlations between treatment outcome and both anti-MSP119 antibody response and erythrocyte-related genetic factors in

Plasmodium falciparum malaria. *Infect Genet Evol, 7*(2):147-154. doi: 10.1016/j.meegid.2006.07.001.

Aucan, C., Traore, Y., Fumoux, F., & Rihet, P. (2001). Familial correlation of immunoglobulin G sub-class response toPlasmodium falciparum. *Infection and Immunity, **69***(2).

Aucan, C., Traoré, Y., Tall, F., Nacro, B., Traoré-Leroux, T., Fumoux, F., & Rihet, P. (2000). High Immunoglobulin G2 (IgG2) and Low IgG4 Levels Are Associated with Human Resistance to Plasmodium falciparum Malaria. *Infect Immun, **68***(3): 1252-1258.

Beeson, J. G., & Brown, G. V. (2002). Pathogenesis of Plasmodium falciparum malaria: the roles of parasite adhesion and antigenic variation. *Cell Mol Life Sci, **59***(2):258-271.

Beier, J. C., Perkins, P. V., Onyango, F. K., Gargan, T. P., Oster, C. N., Whitmire, R. E., Koech, D. K., & Roberts, C. R. (1990). Characterization of Malaria Transmission by Anopheles (Diptera: Culicidae) in Western Kenya in Preparation for Malaria Vaccine Trials. *Journal of Medical Entomology, **27***(4):570-577. doi: 10.1093/jmedent/27.4.570

Bejon, P., Mwacharo,J., Kai,O., Todryk,S., Keating,S., Lowe,B., Lang,T., Mwangi,T.W., Gilbert,S.C., Peshu,N., Marsh ,K., and Hill,A.V.S.,. (2007). The Induction and Persistence of T Cell IFN-γ Responses after Vaccination or Natural Exposure Is Suppressed by Plasmodium falciparum. *J Immunol, **179***: 4193-4201.

Bergmann-Leitner, E. S., Legler, P. M., Savranskaya, T., Ockenhouse, C. F., & Angov, E. (2011). Cellular and humoral immune effector mechanisms required for sterile protection against sporozoite challenge induced with the novel malaria vaccine candidate CelTOS. *Vaccine*, ***29***(35), 5940–5949. https://doi.org/10.1016/j.vaccine.2011.06.053

Bidii S. Ngalah, L. A. I., Agnes C. Cheruiyot, Lorna J. Chebon, Dennis W. Juma,, Peninah Muiruri, I. O., Jack Ogony, Redemptah A. Yeda, Jelagat Cheruiyot,, Emmanuel Mbuba, G. M., Angela O. Achieng, Zipporah Ng'ang'a, Ben Andagalu,, & Kamau, H. M. A. E. (2015). Analysis of Major Genome Loci Underlying Artemisinin Resistance and pfmdr1 Copy Number in pre- and post-ACTs in Western Kenya. *Scientific reports., **5***(1808).

Borrmann, S., Sasi, P., Mwai, L., & Bashraheil, M., Abdallah,A., Muriithi,S.,Fru¨ hauf,H.,Schaub,B., Pfeil,J.,Peshu,J., Hanpithakpong,W., Rippert,A.,Juma,E., Tsofa,B., Mosobo,M., Lowe,B., Osier,F.,Fegan,G., Lindega˚ rdh,N., Nzila,A., Peshu,N., Mackinnon,M.,Marsh,K.,. (2011). Declining Responsiveness ofPlasmodium

falciparumInfections to Artemisinin-Based Combination Treatmentson the Kenyan Coast. *PLoS ONE,* ***6***(11).

Bouharoun-Tayoun, H., & Druilhe, P. (1992). Plasmodium falciparum malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. *Infection and Immunity,* ***60***(4), 473–1481.

Bouyou-Akotet, M. K., Dzeing-Ella, A., Kendjo, E., Etoughe, D., Ngoungou, E. B., Planche, T., Koko, J., & Kombila, M. (2009). Impact of Plasmodium falciparum infection on the frequency of moderate to severe anaemia in children below 10 years of age in Gabon. *Malar J, 8*, 166. doi: 10.1186/1475-2875-8-166.

Braga, E. M., Barros, R. M., Reis, T. A., Fontes, C. J., Morais, C. G., Martins, M. S., & Krettli, A. U. (2002). Association of the IgG response to Plasmodium falciparum merozoite protein (C-terminal 19 kD) with clinical immunity to malaria in the Brazilian Amazon region. *Am J Trop Med Hyg,* ***66***(5), 461-466.

Bredius, R. G., Fijen, C. A., De Haas, M., Kuijper, E. J., Weening, R. S., Van de Winkel, J. G., & Out, T. A. (1994). Role of neutrophil Fc gamma RIIa (CD32) and Fc gamma RIIIb (CD16) polymorphic forms in phagocytosis of human IgG1- and IgG3-opsonized bacteria and erythrocytes. *Immunology,* ***83***(4):624-630.

Bruce, M. C., Donnelly, C. A., Packer, M., Lagog, M., Gibson, N., Narara, A., Walliker, D., Alpers, M. P., & Day, K. P. (2000). Age- and species-specific duration of infection in asymptomatic malaria infections in Papua New Guinea. *Parasitology,* ***121*** *(Pt 3)*: 247-256.

Bull, P. C., & Marsh, K. (2002). The role of antibodies to Plasmodium falciparum-infected-erythrocyte surface antigens in naturally acquired immunity to malaria. *Trends Microbiol,* ***10***(2):55-58.

Campbell, C. C. (2008). Halting the toll of malaria in Africa. *Am J Trop Med Hyg,* ***78***(6): 851-853.

Carlin, J. B., & Doyle, L. W. (2002). Sample size. *Journal of Paediatrics and Child Health,* ***38***(3): 300-304. doi: 10.1046/j.1440-1754.2002.00855.x

Cheeseman IH, Miller BA, Nair S, Nkhoma S, & Tan A, T. J., Al Saai S, Phyo AP, Moo CL, Lwin KM, McGready R, Ashley E, Imwong M, Stepniewska K, Yi P, Dondorp AM, Mayxay M, Newton PN, White NJ, Nosten F, Ferdig MT, Anderson TJ. (2012). A major genome region underlying artemisinin resistance in malaria. *Science,* ***336***(6077):79-82.

Chelimo, K., Ofulla, A. V., Narum, D. L., Kazura, J. W., Lanar, D. E., & John, C. C. (2005). Antibodies to Plasmodium falciparum antigens vary by age and antigen in children in a malaria-holoendemic area of Kenya. *Pediatr Infect Dis J,* ***24***(8): 680-684.

Chitnis, C. E. (2001). Molecular insights into receptors used by malaria parasites for erythrocyte invasion. *Curr Opin Hematol,* ***8***(2): 85-91.

Clark IA., Alleva LM., Mills AC., & Cowden WB. (2004). Pathogenesis of malaria and clinically similar conditions. *Clin Microbiol,* ***17***(3): 509-539.

Cohen, S., Mcgregor, A., & Carrington, S. (1961). Gamma-Globulin and Acquired Immunity to Human Malaria. *Nature,* ***192***: 733-737.

Coley, A. M., Gupta, A., Murphy, V. J., Bai, T., Kim, H., Foley, M., Anders, R. F., & Batchelor, A. H. (2007). Structure of the malaria antigen AMA1 in complex with a growth-inhibitory antibody. *PLoS Pathog,* ***3***(9):1308-1319. doi: 10.1371/journal.ppat.0030138.

Couper, K. N., Blount, D. G., Wilson, M. S., Hafalla, J. C., Belkaid, Y., Kamanaka, M., Flavell, R. A., de Souza, J. B., & Riley, E. M. (2008). IL-10 from CD4(+)CD25(−)Foxp3(−)CD127(−) Adaptive Regulatory T Cells Modulates Parasite Clearance and Pathology during Malaria Infection. *PLoS Pathog,* ***4***(2). doi: 10.1371/journal.ppat.1000004.

Courtin, D., Oesterholt, M., Huismans, H., Kusi, K., & Milet, J., Badaut,C.,Gaye,O., Roeffen, W., Remarque,E.J., Garcia,R.S.A., Luty,A.J.F.,. (2009). The Quantity and Quality of African Children's IgG Responses to Merozoite Surface Antigen s Reflect Protection against Plasmodium falciparum Malaria *PLoS ONE,* ***4***(10).

Crompton, P. D., Moebius, J., Portugal, S., Waisberg, M., Hart, G., Garver, L. S., Miller, L. H., Barillas-Mury, C., & Pierce, S. K. (2014). Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. *Annu Rev Immunol,* ***32***: 157-187. doi: 10.1146/annurev-immunol-032713-120220

Darko, C. A., Angov, E., Collins, W. E., Bergmann-leitner, E. S., Girouard, A. S., Hitt, S. L., … Lyon, J. A. (2005). The Clinical-Grade 42-Kilodalton Fragment of Merozoite Surface Protein 1 of Plasmodium falciparum Strain FVO Expressed in Escherichia coli Protects Aotus nancymai against Challenge with Homologous Erythrocytic-Stage Parasites, ***73***(1), 287–297. https://doi.org/10.1128/IAI.73.1.287

Darko, C. A., Angov, E., Collins, W. E., Bergmann-leitner, E. S., Girouard, A. S., Hitt, S. L., … Lyon, J. A. (2005). The Clinical-Grade 42-Kilodalton Fragment of Merozoite Surface Protein 1 of Plasmodium falciparum Strain FVO Expressed in Escherichia coli Protects Aotus nancymai against Challenge with Homologous Erythrocytic-Stage Parasites, ***73***(1), 287–297. https://doi.org/10.1128/IAI.73.1.287

Desowitz, R. S., Saave, J. J., & Stein, B. (1966). The application of the indirect haemagglutination test in recent studies on the immuno-epidemiology of human malaria and the immune response in experimental malaria. *Mil Med,* ***131***(9): 1157-1166.

Diarra, A., Nebie, I., Tiono, A., Soulama, I., Ouedraogo, A., Konate, A., … Sirima, S. B. (2012). Antibodies to malaria vaccine candidates are associated with chloroquine or sulphadoxine/pyrimethamine treatment efficacy in children in an endemic area of Burkina Faso. *Malaria Journal*, ***11***(1), 79. https://doi.org/10.1186/1475-2875-11-79

Djimdé A.A., Doumbo O.K., Traore O., Guindo A.B, & Kayentao K., D. Y., Niare-Doumbo S., Coulibaly D., Kone AK., Cissoko Y., Tekete M., Fofana B., Dicko A., Diallo DA., Wellems TE., Kwiatkowski D., Plowe CV.,. (2003). Clearance of drug-resistant parasites as a model for protective immunity in Plasmodium falciparum malaria. *Am J Trop Med Hyg,* ***69***(5), 558-563.

Dondorp AM., N. F., Yi P, Das D., Phyo AP., Tarning J., Lwin KM., Ariey F., Hanpithakpong W., Lee SJ., Ringwald P., Silamut K., Imwong M., Chotivanich K., Lim P., Herdman .T, An SS., Yeung S., Singhasivanon P., Day NP. , Lindegardh N, Socheat D, White NJ. (2009). Artemisinin resistance in Plasmodium falciparum malaria. *N Engl J Med,* ***361***(17).

Doolan, D. L. (2011). Plasmodium Immunomics. *Int J Parasitol,* ***41***(1), 3-20. doi: 10.1016/j.ijpara.2010.08.002.

Doolan, D. L., Doban, C., & Baird, J. K. (2009). Acquired Immunity to Malaria. *Clinical microbiology reviews,****22***(1).

Dorsey, G., Kamya, M. R., Singh, A., & Rosenthal, P. J. (2001). Polymorphisms in the Plasmodium falciparum pfcrt and pfmdr-1 genes and clinical response to chloroquine in Kampala, Uganda. *J Infect Dis,* ***183***(9), 1417-1420. doi: 10.1086/319865.

Drakeley, C. J., Corran, P. H., Coleman, P. G., Tongren, J. E., McDonald, S. L. R., Carneiro, I., Malima, R., Lusingu, J., Manjurano, A., Nkya, W. M. M., Lemnge, M. M., Cox, J., Reyburn, H., & Riley, E. M. (2005). Estimating medium- and long-term trends in malaria

transmission by using serological markers of malaria exposure. *Proc Natl Acad Sci U S A,* ***102***(14): 5108-5113. doi: 10.1073/pnas.0408725102.

Dutta, S., Haynes, J. D., Barbosa, A., Ware, L. A., Snavely, J. D., Moch, J. K., … Lanar, D. E. (2005). Mode of Action of Invasion-Inhibitory Antibodies Directed against Apical Membrane Antigen 1 of Plasmodium falciparum, ***73***(4), 2116–2122. https://doi.org/10.1128/IAI.73.4.2116

Enevold, A., Nkya, W. M. M. M., Theisen, M., Vestergaard, L. S., Jensen, A. T. R., Staalsoe, T., Theander, T. G., Bygbjerg, I. C., & Alifrangis, M. (2007b). Potential impact of host immunity on malaria treatment outcome in Tanzanian children infected with Plasmodium falciparum. *Malaria Journal,* ***6***:153-153. doi: 10.1186/1475-2875-6-153.

Fairhurst, R. M., Nayyar, G. M. L., & Dondorp, A. M. (2012). Artemisinin-Resistant Malaria: Research Challenges, Opportunities, and Public Health Implications. *The American Journal of Tropical Medicine and Hygiene,* ***89***(2).

Ferraro, B., Talbott, K. T., Balakrishnan, A., Cisper, N., Morrow, M. P., Hutnick, N. A., Myles, D. J., Shedlock, D. J., Obeng-Adjei, N., Yan, J., Kayatani, A. K. K., Richie, N., Cabrera, W., Shiver, R., Khan, A. S., Brown, A. S., Yang, M., Wille-Reece, U., Birkett, A. J., Sardesai, N. Y., & Weiner, D. B. (2013). Inducing Humoral and Cellular Responses to Multiple Sporozoite and Liver-Stage Malaria Antigens Using Exogenous Plasmid DNA. *Infection and Immunity,* ***81***(10): 3709-3720. doi: 10.1128/IAI.00180-13.

Flannery, E. L., Chatterjee, A. K., & Winzeler, E. A. (2013). Antimalarial Drug Discovery: Approaches and Progress towards New Medicines. *Nat Rev Microbiol,* ***11***(12), 849-862. doi: 10.1038/nrmicro3138.

Flegg, J. A., Guerin, P. J., White, N. J., & Stepniewska, K. (2011). Standardizing the measurement of parasite clearance in falciparum malaria: the parasite clearance estimator. *Malaria Journal*, ***10***(1), 339. https://doi.org/10.1186/1475-2875-10-339

Fowkes, F. J. I., Richards, J. S., Simpson, J. A., & Beeson, J. G. (2010). The relationship between anti-merozoite antibodies and incidence of Plasmodium falciparum malaria: A systematic review and meta-analysis. *PLoS Medicine*, ***7***(1). https://doi.org/10.1371/journal.pmed.1000218

Francis, D., Nsobya, S. L., Talisuna, A., Yeka, A., Kamya, M. R., Machekano, R., Dokomajilar, C., Rosenthal, P. J., & Dorsey, G. (2006). Geographic Differences in Antimalarial Drug Efficacy in Uganda Are Explained by Differences in Endemicity and Not by Known

Molecular Markers of Drug Resistance. *Journal of Infectious Diseases, 193*(7), 978-986. doi: 10.1086/500951

Garraud, O., Perraut, R., Riveau, G., & Nutman, T. B. (2003). Class and subclass selection in parasite-specific antibody responses. *Trends Parasitol, **19***(7): 300-304.

Giha, H. A., Nasr, A., Iriemenam, N. C., Balogun, H. A., Arnot, D., Theander, T. G., Troye-Blomberg, M., Berzins, K., & ElGhazali, G. (2010). Age-dependent association between IgG2 and IgG3 subclasses to Pf332-C231 antigen and protection from malaria, and induction of protective antibodies by sub-patent malaria infections, in Daraweesh. *Vaccine, **28***(7): 1732-1739. doi: 10.1016/j.vaccine.2009.12.018.

Good MF., Xu H., Wykes M., & Engwerda CR. (2005). Development and regulation of cell-mediated immune responses to the blood stages of malaria: implications for vaccine research. *Annu Rev Immunol, **23***, 69-99.

Good, M. F. (2004). Vaccine-induced immunity to malaria parasites and the need for novel strategies. *Trends in Parasitology, **21***(1): 29-34. doi: 10.1016/j.pt.2004.10.006.

Greenhouse, B., Ho, B., Hubbard, A., Njama-Meya, D., Narum, D. L., Lanar, D. E., … John, C. C. (2011). Antibodies to Plasmodium falciparum antigens predict a higher risk of malaria but protection from symptoms once parasitemic. *Journal of Infectious Diseases*, ***204***(1), 19–26. https://doi.org/10.1093/infdis/jir223

Greenhouse, B., Slater, M., Njama-Meya, D., Nzarubara, B., & Maiteki-Sebuguzi, C. M., Clark,T.D., Staedke,S.G.,Kamya,M.R.,Hubbard, A.,Rosenthal,P.J., and Dorsey,G.,. (2009). Decreasing Efficacy of Antimalarial Combination Therapy inUganda Explained by Decreasing Host Immunity Rather thanIncreasing Drug Resistance. *J Infect Dis., **199***(5).

Grigg, M. J., William, T., Menon, J., Dhanaraj, P., Bridget, E., Wilkes, C. S., … James, S. (2016). Europe PMC Funders Group Artesunate – mefloquine versus chloroquine for treatment of uncomplicated Plasmodium knowlesi malaria in Malaysia (ACT KNOW): an open-label , randomised controlled trial, ***16***(2), 180–188. https://doi.org/10.1016/S1473-3099(15)00415-6.Artesunate

Grimwade, K., French, N., Mbatha, D. D., Zungu, D. D., Dedicoat, M., & Gilks, C. F. (2004). HIV infection as a cofactor for severe falciparum malaria in adults living in a region of unstable malaria transmission in South Africa. *Aids, **18***(3): 547-554.

Grobusch, M. P., & Kremsner, P. G. (2005). Uncomplicated malaria. *Curr Top Microbiol Immunol, **295***, 83-104.

Hemming-Schroeder, E., Umukoro, E., Lo, E., Fung, B., Tomás-Domingo, P., Zhou, G., … Yan, G. (2018). Impacts of antimalarial drugs on plasmodium falciparum drug resistance markers, Western Kenya, 2003-2015. *American Journal of Tropical Medicine and Hygiene*, ***98***(3), 692–699. https://doi.org/10.4269/ajtmh.17-0763

Hillier et al. (2005). Process development and analysis of liver-stage antigen 1, a preerythrocyte-stage protein-based vaccine for Plasmodium falciparum. *Infection and Immunity*, ***73***(4), 2109–2115. https://doi.org/10.1128/IAI.73.4.2109-2115.2005

Hodder, A. N., Crewther, P. E., & Anders, R. F. (2001). Specificity of the Protective Antibody Response to Apical Membrane Antigen 1. *Society*, ***69***(5), 3286–3294. https://doi.org/10.1128/IAI.69.5.3286

Hoffmann, E. H., da Silveira, L. A., Tonhosolo, R., Pereira, F. J., Ribeiro, W. L., Tonon, A. P., Kawamoto, F., & Ferreira, M. U. (2001). Geographical patterns of allelic diversity in the Plasmodium falciparum malaria-vaccine candidate, merozoite surface protein-2. *Ann Trop Med Parasitol, **95***(2): 117-132. doi: 1080/00034980120045833.

Hulden, L. (2011). Activation of the hypnozoite: a part of Plasmodium vivax life cycle and survival. *Malar J, **10***: 90. doi: 10.1186/1475-2875-10-90.

Hunt NH., & Grau GE. (2003). Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. *Trends Immunol, **24***(9).

Idris, Z. M., Chan, C. W., Kongere, J., Gitaka, J., Logedi, J., Omar, A., … Kaneko, A. (2016). High and Heterogeneous Prevalence of Asymptomatic and Sub-microscopic Malaria Infections on Islands in Lake Victoria, Kenya. *Scientific Reports*, *6*(November), 1–13. https://doi.org/10.1038/srep36958

John, C. C., Moormann, A. M., Pregibon, D. C., Sumba, P. O., McHugh, M. M., Narum, D. L., Lanar, D. E., Schluchter, M. D., & Kazura, J. W. (2005). Correlation of high levels of antibodies to multiple pre-erythrocytic Plasmodium falciparum antigens and protection from infection. *Am J Trop Med Hyg, **73***(1): 222-228.

Jongwutiwes, S., Buppan, P., Kosuvin, R., Seethamchai, S., Pattanawong, U., Sirichaisinthop, J., & Putaporntip, C. (2011). Plasmodium knowlesi Malaria in Humans and Macaques, Thailand. *Emerg Infect Dis, **17***(10): 1799-1806. doi: 10.3201/eid1710.110349.

Kamau, E., Campino, S., Amenga-Etego, L., Drury, E., Ishengoma, D., Johnson, K., Mumba, D., Kekre, M., Yavo, W., Mead, D., Bouyou-Akotet, M., Apinjoh, T., Golassa, L., Randrianarivelojosia, M., Andagalu, B., Maiga-Ascofare, O., Amambua-Ngwa, A., Tindana, P., Ghansah, A., MacInnis, B., Kwiatkowski, D., & Djimde, A. A. (2015). K13-propeller polymorphisms in Plasmodium falciparum parasites from sub-Saharan Africa. *J Infect Dis,* ***211***(8): 1352-1355. doi: 10.1093/infdis/jiu608.

Kapesa, A., Kweka, E. J., Atieli, H., Afrane, Y. A., Kamugisha, E., Lee, M., … Yan, G. (2018). The current malaria morbidity and mortality in different transmission settings in Western Kenya. https://doi.org/10.1371/journal.pone.0202031

Kapesa, A., Kweka, E. J., Atieli, H., Kamugisha, E., Zhou, G., Githeko, A. K., & Yan, G. (2017). Why some sites are responding better to anti-malarial interventions? A case study from western Kenya. *Malaria Journal*, ***16***(1), 1–14. https://doi.org/10.1186/s12936-017-2145-9

KatarinaSjoberg, J., Lucie Raharimalalat, AkLarsson, Olle Olerupt, Nuahnt. Marbiah§, Marita Troye-blomberg, and Peter Perlmanno,Carlota Doban˜. (1992). Immunology Genetic regulation of human anti-malarial antibodies in twins. *ProcNati.Acad.Sci.USA.,* ***89***: 2101-2104.

Keh, C. E., Jha, A. R., Nzarubara, B., Lanar, D. E., Dutta, S., Theisen, M., Rosenthal, P. J., Dorsey, G., Nixon, D. F., & Greenhouse, B. (2012). Associations between Antibodies to a Panel of Plasmodium falciparum Specific Antigens and Response to Sub-Optimal Antimalarial Therapy in Kampala, Uganda. *PLoS ONE, 7*(12): e52571. doi: 10.1371/journal.pone.0052571.

Kennedy, M. C., Wang, J., Zhang, Y., Miles, A. P., Chitsaz, F., Saul, A., Long, C. A., Miller, L. H., & Stowers, A. W. (2002). In vitro studies with recombinant Plasmodium falciparum apical membrane antigen 1 (AMA1): production and activity of an AMA1 vaccine and generation of a multiallelic response. *Infect Immun,* ***70***(12): 6948-6960.

Kerkhof, K., Canier, L., Kim, S., Heng, S., Sochantha, T., Sovannaroth, S., Vigan-Womas, I., Coosemans, M., Sluydts, V., Ménard, D., & Durnez, L. (2015). Implementation and application of a multiplex assay to detect malaria-specific antibodies: a promising tool for assessing malaria transmission in Southeast Asian pre-elimination areas. *Malaria Journal,* ***14***, 338. doi: 10.1186/s12936-015-0868-z.

Khalil, I. F., Alifrangis, M., Tarimo, D. S., Staalso, T., Satti, G. M., Theander, T. G., Ronn, A. M., & Bygbjerg, I. C. (2005). The roles of the pfcrt 76T and pfmdr1 86Y mutations,

immunity and the initial level of parasitaemia, in predicting the outcome of chloroquine treatment in two areas with different transmission intensities. *Ann Trop Med Parasitol, **99***(5): 441-448. doi: 10.1179/136485905x46441.

Klein, E. (2013). Antimalarial drug resistance: a review of the biology and strategies to delay emergence and spread. *Int J Antimicrob Agents, **41***(4): 311-317. doi: 10.1016/j.ijantimicag.2012.12.007

Klonis N., Crespo-Ortiz M.P., Bottova I., & Abu-Bakar N., K. S., Rosenthal PJ., Tilley L.,. (2011). Artemisinin activity against Plasmodium falciparum requires hemoglobin uptake and digestion. *Proc Natl Acad Sci U S A, **108***(28).

Kraemer SM., & Smith JD. (2006). A family affair: var genes, PfEMP1 binding, and malaria disease. *Curr Opin Microbiol, **9***(4): 374-380.

Kyes, S. A., Kraemer, S. M., & Smith, J. D. (2007). Antigenic Variation in Plasmodium falciparum: Gene Organization and Regulation of the var Multigene Family. *Eukaryot Cell, **6***(9): 1511–1520.

Laufer, M. K. (2009). Monitoring Antimalarial Drug Efficacy: Current Challenges. *Curr Infect Dis Rep, **11***(1): 59-65.

Lim, P., Alker, A. P., Khim, N., Shah, N. K., Incardona, S., Doung, S., Yi, P., Bouth, D. M., Bouchier, C., Puijalon, O. M., Meshnick, S. R., Wongsrichanalai, C., Fandeur, T., Le Bras, J., Ringwald, P., & Ariey, F. (2009). Pfmdr1 copy number and arteminisin derivatives combination therapy failure in falciparum malaria in Cambodia. *Malar J, **8***, 11. doi: 10.1186/1475-2875-8-11.

Lin, J. T., Juliano, J. J., & Wongsrichanalai, C. (2010). Drug-Resistant Malaria: The Era of ACT. *Current infectious disease reports, **12***(3): 165-173. doi: 10.1007/s11908-010-0099-y.

Luty, A. J., Lell, B., Schmidt-Ott, R., Lehman, L. G., Luckner, D., Greve, B., Matousek, P., Herbich, K., Schmid, D., Ulbert, S., Migot-Nabias, F., Dubois, B., Deloron, P., & Kremsner, P. G. (1998). Parasite antigen-specific interleukin-10 and antibody reponses predict accelerated parasite clearance in Plasmodium falciparum malaria. *Eur Cytokine Netw, **9***(4): 639-646.

Lyon, J. A., Angov, E., Fay, M. P., Sullivan, J. A. S., Girourd, A. S., Robinson, S. J., … Barnwell, J. W. (2008). Protection induced by plasmodium falciparum MSP142is strain-specific, antigen and adjuvant dependent, and correlates with antibody responses. *PLoS*

ONE, *3*(7), 1–11. https://doi.org/10.1371/journal.pone.0002830

Maiga-Ascofare, O., & May, J. (2016). Is the A578S Single-Nucleotide Polymorphism in K13-propeller a Marker of Emerging Resistance to Artemisinin Among Plasmodium falciparum in Africa? *J Infect Dis,* ***213***(1): 165-166. doi: 10.1093/infdis/jiv414.

Mawili-Mboumba, D. P., Borrmann, S., Cavanagh, D. R., McBride, J. S., Matsiegui, P. B., Missinou, M. A., Kremsner, P. G., & Ntoumi, F. (2003). Antibody responses to Plasmodium falciparum merozoite surface protein-1 and efficacy of amodiaquine in Gabonese children with P. falciparum malaria. *J Infect Dis,* ***187***(7): 1137-1141. doi: 10.1086/368414.

McCall .B.M., & Sauerwein.R. (2010). Interferon-γ—central mediator of protective immune responses against the pre-erythrocytic and blood stage of malaria. *JLB.*

McElroy, P. D., ter Kuile, F. O., Lal, A. A., Bloland, P. B., Hawley, W. A., Oloo, A. J., Monto, A. S., Meshnick, S. R., & Nahlen, B. L. (2000). Effect of Plasmodium falciparum parasitemia density on hemoglobin concentrations among full-term, normal birth weight children in western Kenya, IV. The Asembo Bay Cohort Project. *Am J Trop Med Hyg,* ***62***(4): 504-512.

Methodology for the WWARN Parasite Clearance Estimator We have developed a simple calculator that provides measures of parasite clearance from serial parasite count data. Here we present the model, terminology and methods. (2009), 1–19.

Metzger, W. G., Okenu, D. M. N., Cavanagh, D. R., Robinson, J. V., Bojang, K. A., Weiss, H. A., McBride, J. S., Greenwood, B. M., & Conway, D. J. (2003). Serum IgG3 to the Plasmodium falciparum merozoite surface protein 2 is strongly associated with a reduced prospective risk of malaria. *Parasite Immunology,* ***25***(6): 307-312. doi: 10.1046/j.1365-3024.2003.00636.x.

Miller LH., Ackerman HC., Su XZ., & Wellems TE. (2013). Malaria biology and disease pathogenesis: insights for new treatments. *Nature Medicine,* ***19***(2): 156-167.

MOH. (2014). Ministry of health annual report 2014.

Murphy, S. C., & Breman, J. G. (2001). Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy. *Am J Trop Med Hyg,* ***64***(1-2 Suppl): 57-67.

Nair, S., Williams, J. T., Brockman, A., Paiphun, L., Mayxay, M., Newton, P. N., Guthmann, J. P., Smithuis, F. M., Hien, T. T., White, N. J., Nosten, F., & Anderson, T. J. (2003). A selective sweep driven by pyrimethamine treatment in southeast asian malaria parasites. *Mol Biol Evol, **20***(9): 1526-1536. doi: 10.1093/molbev/msg162.

National Malaria Elimination Progrmme, & Federal Ministry of Health. (2016). Malaria Indicator Survey 2015, 1–190.

Ndenga, B., Githeko, A., Omukunda, E., Munyekenye, G., Atieli, H., Wamai, P., Mbogo, C., Minakawa, N., Zhou, G., & Yan, G. (2006). Population dynamics of malaria vectors in western Kenya highlands. *J Med Entomol, **43***(2): 200-206.

Nebie, I., Diarra, A., Ouedraogo, A., & Soulama, I., Bougouma,E.C., Tiono,A.B., Konate,A., Chilengi,R., Theisen,M., Dodoo,D.,Remarque,E., Bosomprah,S., Milligan,P., and Sirima,S.B.,. (2008). Humoral Responses to Plasmodium falciparum Blood-Stage Antigensand Association with Incidence of Clinical Malaria in ChildrenLiving in an Area of Seasonal Malaria Transmission inBurkina Faso, West Africa. *Infection and Immunity, **76***(2).

Nimmerjahn, F., & Ravetch, J. V. (2008). Fc[gamma] receptors as regulators of immune responses. *Nat Rev Immunol, **8***(1): 34-47.

Noedl, H., Se, Y., Schaecher, K., Smith B.L., Socheat, D., & Fukuda MM. (2008). Evidence of artemisinin-resistant malaria in western Cambodia. *N Engl J Med, **359***(24): 2619-2620.

Noland, G. S., Hendel-Paterson, B., Min, X. M., Moormann, A. M., Vulule, J. M., Narum, D. L., Lanar, D. E., Kazura, J. W., & John, C. C. (2008). Low prevalence of antibodies to preerythrocytic but not blood-stage Plasmodium falciparum antigens in an area of unstable malaria transmission compared to prevalence in an area of stable malaria transmission. *Infect Immun, **76***(12): 5721-5728. doi: 10.1128/iai.00591-08.

Noone, C., Parkinson, M., Dowling, D. J., Aldridge, A., Kirwan, P., Molloy, S. F., Asaolu, S. O., Holland, C., & O'Neill, S. M. (2013). Plasma cytokines, chemokines and cellular immune responses in pre-school Nigerian children infected with Plasmodium falciparum. *Malaria Journal, **12***: 5-5. doi: 10.1186/1475-2875-12-5.

O'Flaherty, K., Maguire, J., Simpson, J. A., & Fowkes, F. J. I. (2017). Immunity as a predictor of anti-malarial treatment failure: a systematic review. *Malaria Journal*, ***16***(1), 158. https://doi.org/10.1186/s12936-017-1815-y

Ohas, E. A., Adams, J. H., Waitumbi, J. N., Orago, A. S. S., Barbosa, A., Lanar, D. E., & Stoute, J. A. (2004). Measurement of Antibody Levels against Region II of the Erythrocyte-Binding Antigen 175 of Plasmodium falciparum in an Area of Malaria Holoendemicity in Western Kenya. *Infection and Immunity*, ***72***(2), 735–741. https://doi.org/10.1128/IAI.72.2.735-741.2004

Okach, D. O., Ayisi, J. G., & Onyango, R. (2014). Severe malaria in western Kenya : Analysis of hospital records to determine the influence of transmission level on clinical presentation, ***2***(9), 73–78.

Onwujekwe, O., Uguru, N., Etiaba, E., Chikezie, I., Uzochukwu, B., & Adjagba, A. (2013). The Economic Burden of Malaria on Households and the Health System in Enugu State Southeast Nigeria. *PLoS ONE,* ***8***(11). doi: 10.1371/journal.pone.0078362.

Osier, F. H. A., Fegan, G., Polley, S. D., Murungi, L., Verra, F., Tetteh, K. K. A., … Marsh, K. (2008). Breadth and magnitude of antibody responses to multiple Plasmodium falciparum merozoite antigens are associated with protection from clinical malaria. *Infection and Immunity*, ***76***(5), 2240–2248. https://doi.org/10.1128/IAI.01585-07

Osier, F. H., Feng, G., Boyle, M. J., Langer, C., Zhou, J., Richards, J. S., McCallum, F. J., Reiling, L., Jaworowski, A., Anders, R. F., Marsh, K., & Beeson, J. G. (2014). Opsonic phagocytosis of Plasmodium falciparummerozoites: mechanism in human immunity and a correlate of protection against malaria. *BMC Medicine,* ***12***(1), 1-15. doi: 10.1186/1741-7015-12-108.

Ouattara, A., Kone, A., Adams, M., Fofana, B., Maiga, A. W., Hampton, S., Coulibaly, D., Thera, M. A., Diallo, N., Dara, A., Sagara, I., Gil, J. P., Bjorkman, A., Takala-Harrison, S., Doumbo, O. K., Plowe, C. V., & Djimde, A. A. (2015). Polymorphisms in the K13-propeller gene in artemisinin-susceptible Plasmodium falciparum parasites from Bougoula-Hameau and Bandiagara, Mali. *Am J Trop Med Hyg,* ***92***(6): 1202-1206. doi: 10.4269/ajtmh.14-0605.

Paloque, L., Ramadani, A. P., Mercereau-Puijalon, O., Augereau, J.-M., & Benoit-Vical, F. (2016). Plasmodium falciparum: multifaceted resistance to artemisinins. *Malaria Journal,* ***15***(1): 1-12. doi: 10.1186/s12936-016-1206-9.

Pasternak, N. D., & Dzikowski, R. (2009). PfEMP1: an antigen that plays a key role in the pathogenicity and immune evasion of the malaria parasite Plasmodium falciparum. *Int J Biochem Cell Biol,* ***41***(7): 1463-1466. doi: 10.1016/j.biocel.2008.12.012.

Payne, D. (1987). Spread of chloroquine resistance in Plasmodium falciparum. *Parasitol Today, **3***(8): 241-246.

Perlmann.P., & Troye-Blomberg.M. (2002). Malaria and the Immune System in Humans. *Malaria Immunology., **80***, 229–242.

Perraut, R., Richard, V., Varela, M.-L., Trape, J.-F., Guillotte, M., Tall, A., Toure, A., Sokhna, C., Vigan-Womas, I., & Mercereau-Puijalon, O. (2014). Comparative analysis of IgG responses to Plasmodium falciparum MSP1p19 and PF13-DBL1α1 using ELISA and a magnetic bead-based duplex assay (MAGPIX®-Luminex) in a Senegalese meso-endemic community. *Malaria Journal, **13***, 410. doi: 10.1186/1475-2875-13-410.

Persson, K. E. M., Fowkes, F. J. I., McCallum, F. J., Gicheru, N., Reiling, L., Richards, J. S., … Beeson, J. G. (2013). Erythrocyte-Binding Antigens of Plasmodium falciparum Are Targets of Human Inhibitory Antibodies and Function To Evade Naturally Acquired Immunity. *The Journal of Immunology*, ***191***(2), 785–794. https://doi.org/10.4049/jimmunol.1300444

Peters, W. (1987). *Chemotherapy and drug resistance in malaria*. London; Orlando: Academic Press.

Polley, Spencer, D., Conway, David, J., Cavanagh, David, R., McBride, Jana, S., Lowe, Brett, S., Williams, Thomas, N., Mwangi, Tabitha, W., Marsh, & Kevin. (2006). High levels of serum antibodies to merozoite surface protein 2 of Plasmodium falciparum are associated with reduced risk of clinical malaria in coastal Kenya: Elsevier Ltd.

Porter, M. D., Nicki, J., Pool, C. D., Debot, M., Illam, R. M., Brando, C., … Bennett, J. W. (2013). falciparum Circumsporozoite Protein as a Model for Vaccine Down- Selection in Mice Using Sterile Protection as an Endpoint, ***20***(6), 803–810. https://doi.org/10.1128/CVI.00066-13

Portugal S., Pierce S.K., & Crompton. P.D. (2013). Young lives lost as B cells falter: what we are learning about antibody responses in malaria. *Jounal of immunology, **190***(7), 4039-4046.

Rono, J., Osier, F. H. A., Olsson, D., Montgomery, S., Mhoja, L., Rooth, I., … Färnert, A. (2013). Breadth of anti-merozoite antibody responses is associated with the genetic diversity of asymptomatic plasmodium falciparum infections and protection against clinical malaria. *Clinical Infectious Diseases*, ***57***(10), 1409–1416. https://doi.org/10.1093/cid/cit556

Roper, C., Pearce, R., Bredenkamp, B., Gumede, J., Drakeley, C., Mosha, F., Chandramohan, D., & Sharp, B. (2003). Antifolate antimalarial resistance in southeast Africa: a population-based analysis. *Lancet,* ***361***(9364): 1174-1181. doi: 10.1016/s0140-6736(03)12951-0.

Roussilhon, C., Oeuvray, C., Müller-Graf, C., Tall, A., Rogier, C., Trape, J.-F., Theisen, M., Balde, A., Pérignon, J.-L., & Druilhe, P. (2007). Long-Term Clinical Protection from Falciparum Malaria Is Strongly Associated with IgG3 Antibodies to Merozoite Surface Protein 3. *PLoS Med,* ***4***(11), e320. doi: 10.1371/journal.pmed.0040320.

Silachamroon, U., Krudsood, S., Phophak, N., & Looareesuwan, S. (2002). Management of malaria in Thailand. *The Korean Journal of Parasitology,* ***40***(1): 1-7. doi: 10.3347/kjp.2002.40.1.1.

Silvie, O., Franetich, J.-F., Charrin, S., Mueller, M. S., Siau, A., Bodescot, M., Rubinstein, E., Hannoun, L., Charoenvit, Y., Kocken, C. H., Thomas, A. W., van Gemert, G.-J., Sauerwein, R. W., Blackman, M. J., Anders, R. F., Pluschke, G., & Mazier, D. (2004). A Role for Apical Membrane Antigen 1 during Invasion of Hepatocytes by Plasmodium falciparum Sporozoites. *Journal of Biological Chemistry,* ***279***(10): 9490-9496. doi: 10.1074/jbc.M311331200.

Slater, H. C., Griffin, J. T., Ghani, A. C., & Okell, L. C. (2016). Assessing the potential impact of artemisinin and partner drug resistance in sub-Saharan Africa. *Malaria Journal,* ***15***, 10. doi: 10.1186/s12936-015-1075-7.

Snow, R. W., Marsh, K., & Trape, J. F. (2001). The past, present and future of childhood malaria mortality in Africa. *Trends Parasitol,* ***17***(12): 593-597.

Staedke, S. G., Sendagire, H., Lamola, S., Kamya, M. R., Dorsey, G., & Rosenthal, P. J. (2004). Relationship between age, molecular markers, and response to sulphadoxine-pyrimethamine treatment in Kampala, Uganda. *Tropical Medicine and International Health*, ***9***(5), 624–629. https://doi.org/10.1111/j.1365-3156.2004.01239.x

Stanisic, D., I.,, Richard, J. S., Gilson, B. R., Murphy, V. J., Anders,R.F., Michon,P., King,C.L., Schoepflin,S., Paul Danielle,P. I., Richards,J.S., McCallum,F.J., , & Beeson, G. J., Muellum,I.,. (2009). Immunoglobulin G Subclass-Specific,Responses against Plasmodium falciparum merozoite antigens are associated with control of parasitemia and protetion from symptomatic illness. *Infection and Immunity,* ***77***(3).

Stevenson, M. M., & Riley, E. M. (2004). Innate immunity to malaria. *Nature Reviews Immunology, **4***: 169-180.

Tanner, M., Greenwood, B., Whitty, C. J. M., Ansah, E. K., Price, R. N., Dondorp, A. M., von Seidlein, L., Baird, J. K., Beeson, J. G., Fowkes, F. J. I., Hemingway, J., Marsh, K., & Osier, F. (2015). Malaria eradication and elimination: views on how to translate a vision into reality. *BMC Medicine, **13***(1): 1-22. doi: 10.1186/s12916-015-0384-6.

Trampuz, A., Jereb, M., Muzlovic, I., & Prabhu, R. M. (2003). Clinical review: Severe malaria. *Critical Care, **7***(4), 315-323.

Travassos, M. A., & Laufer, M. K. (2009). Resistance to antimalarial drugs: molecular, pharmacological and clinical considerations. *Pediatric research, **65***(5 Pt 2), 64R-70R. doi: 10.1203/PDR.0b013e3181a0977e.

Turner L., Lavstsen T., Berger SS., & Wang CW., P. J., Avril M., Brazier AJ., Freeth J., Jespersen JS., Nielsen MA., Magistrado P., Lusingu J., Smith JD., Higgins MK., Theander TG.,. (2013). Severe malaria is associated with parasite binding to endothelial protein C receptor. *Nature, **498***(7455): 502-505.

Vekemans J., & Ballou WR. (2008). Plasmodium falciparum malaria vaccines in development. *Expert Rev Vaccines, **7***(2): 223-240.

Vijaykadga S., Rojanawatsirivej C., Cholpol S., Phoungmanee D., Nakavej A., & Wongsrichanalai C. (2006). In vivo sensitivity monitoring of mefloquine monotherapy and artesunate-mefloquine combinations for the treatment of uncomplicated falciparum malaria in Thailand in 2003. *Trop Med Int Health., **11***(2): 211-219.

Wanja, E. W., Kuya, N., Moranga, C., Hickman, M., Johnson, J. D., Moseti, C., Anova, L., Ogutu, B., & Ohrt, C. (2016). Field evaluation of diagnostic performance of malaria rapid diagnostic tests in western Kenya. *Malaria Journal, **15***(1): 456. doi: 10.1186/s12936-016-1508-y.

White, N. (2011). The parasite clearance curve. *Malaria Journal, **10***(1): 1-8. doi: 10.1186/1475-2875-10-278.

White, N. J., & Pongtavornpinyo, W. (2003). The de novo selection of drug-resistant malariaparasites. *Proc. R. Soc. Lond. B, **270***: 545–554.

WHO. (1999). World Health Report. *World Health Organization 1999*.

WHO. (2010a). Global report on antimalarialdrug efficacy and drug resistance:2000–2010. *World Health Organization 2010*.

WHO. (2010b). Guide for treatment of malaria 2010. *World Health Organization 2010*, 194.

WHO. (2012). World malaria report 2012. *World health Organization 2012*.

WHO. (2013). World malaria report 2013. *WHO GLOBAL MALARIA PROGRAMME*.

WHO. (2015). World malaria report 2015. *world Health Organization 2015*.

Wongsrichanalai, C., & Meshnick, S. R. (2008). Declining Artesunate-Me fl oquine Ef fi cacy against Falciparum Malaria on the Cambodia – Thailand Border, ***14***(5), 2002–2005. https://doi.org/10.3201/eid1405.071601

Zhou, G., Afrane, Y. A., Vardo-Zalik, A. M., Atieli, H., Zhong, D., Wamae, P., … Yan, G. (2011). Changing patterns of malaria epidemiology between 2002 and 2010 in western kenya: The fall and rise of malaria. *PLoS ONE*, ***6***(5). https://doi.org/10.1371/journal.pone.0020318

Zhou, Z., Xiao, L., Branch, O. H., Kariuki, S., Nahlen, B. L., & Lal, A. A. (2002). Antibody responses to repetitive epitopes of the circumsporozoite protein, liver stage antigen-1, and merozoite surface protein-2 in infants residing in a Plasmodium falciparum-hyperendemic area of western Kenya. XIII. Asembo Bay Cohort Project. *Am J Trop Med Hyg*, ***66***(1): 7-12.

APPENDICES

Appendix I. Ethical approval document.

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 **March 20, 2013**

TO: **DR. BEN ANDAGALU,**
PRINCIPAL INVESTIGATOR

THRO': **DR. RASHID JUMA,**
THE DIRECTOR, CCR,
NAIROBI

Forwarded 26/3/2013

Dear Sir,

RE: **SSC PROTOCOL NO. 2518 (*INITIAL SUBMISSION*): IN VIVO AND IN VITRO EFFICACY OF ARTEMISININ COMBINATION THERAPY IN KISUMU COUNTY, WESTERN KENYA.**

This is to inform you that during the 213th meeting of the KEMRI/ERC held on 19th March 2013, the above referenced study was reviewed.

The Committee notes that the above referenced study seeks to assess the degree of artemisinin resistance in subjects presenting with uncomplicated *P. Falciparum* malaria.

The Committee concluded that due consideration has been given to the ethical issues that may arise from the conduct of the study and granted approval for implementation effective **19th March 2013**.

Please note that authorization to conduct this study will automatically expire on **18 March 2014**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **05 February 2014**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the SSC and ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study

Sincerely,

EAB

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

Appendix II. Study procedure for adults.

Study	Priori	Assay	D0	D	D	D3	D	D	D1	D2	D2	D3	D4	R
Eligibility			-1											
Informed			-1											
Demographic			-1											
Pregnancy			-1											
Physical			X	X	X	X	X	X	X	X	X	X	X	X
Adverse			X	X	X	X	X	X	X	X	X	X	X	X
Vital Signs			-1,	X	X	X	X	X	X	X	X	X	X	X
Concomitant			X	X	X	X	X	X	X	X	X	X	X	X
Urine sample			X											
Artesunate			4	4	4									
Mefloquine						15	1							
Artemether-			X	X	X									
Blood	1	0.1ml	-1,	24	48	72,	Δ	X	X	X	X	X	X	X
PCR for	1	1ml	-1											X
Real-time	1	0.8ml	0,	24	48	72,	Δ	X	X	X	X	X	X	
Hematology[4]	1	1ml	X	X	X	X		X	X	X	X	X	X	X
Pharmacokin	2	2ml	X			X			X					X
In vitro	2	3ml	X											X
Cryopreservat	3	2ml	X											X
Storage in	3	1ml	X											X
Microsatellite	3	2ml	X											X
Reference	3	3ml	X											X
Serology	2	5ml	X						X				X	X
PBMCs[5]	3	1ml	X											X
Blood	3	4ml	X											
Cytokine	3	2ml	X											
Biochemistry	1	1ml	X			X								
Bioassay	2	2ml	X											X
Total blood			**34.**	**4.**	**4.**	**7.6**	**-**	**1.**	**8.9**	**1.9**	**1.9**	**1.9**	**6.9**	**23.**

[1] Numbers show hour of blood sampling for microscopy, -1 is pre-enrollment; “X” indicates one sampling per day, “Δ” indicates that sample will be collected q6 until two consecutive samples are negative by microscopy

[2] Complete physical exam on Day 0, Targeted physical on all subsequent days

[3] Biochemistry tests include glucose, creatinine, AST, ALT.

[4] Hematology tests include Hb, hematocrit and platelets

[5] PBMCs = Peripheral blood mononuclear cells

[6] RC = recrudescence within the 42 days of follow-up

[7] Total blood volume collected for the entire study = 74.8mL. For participants with recrudescence, total volumes will be 97.9 mL.

*Depending on randomization scheme. 1st MQ dose is supervised, 2nd dose is unsupervised. ** - if needed.

Appendix III. Study procedures for children

Study	Priori	Assay	D0	D	D	D3	D	D	D1	D2	D2	D3	D4	R
Eligibility			-1											
Informed			-1											
Demographic			-1											
Pregnancy			-1											
Physical			X	X	X	X	X	X	X	X	X	X	X	X
Adverse			X	X	X	X	X	X	X	X	X	X	X	X
Vital Signs			-1,	X	X	X	X	X	X	X	X	X	X	X
Concomitant			X	X	X	X	X	X	X	X	X	X	X	X
Artesunate* (			4	4	4									
Mefloquine* (						15	1							
Artemether-			X	X	X									
Urine sample			X											
Blood	1	0.1ml	-1,	24	48	72,	Δ	X	X	X	X	X	X	X
PCR for	1	1ml	-1											X
Real-time	1	0.4ml	0,	24	48	72,	Δ	X	X	X	X	X	X	
Hematology[4]	1	0.4ml	X	X	X	X		X	X	X	X	X	X	X
Pharmacokin	2	2ml	X			X			X					X
In vitro	2	2ml	X											X
Cryopreservat	3	1ml	X											X
Microsatellite	2	2ml	X											X
Reference	3	2 ml	X											X
Serology	2	2ml	X						X				X	X

PBMCs[5]	3	0.5ml	X											X
Blood	3	2ml	X											
Cytokine	3	1ml	X											
Biochemistry	1	1ml	X			X								
Bioassays	2	2ml	X											X
Total blood			**21.**	**2.**	**2.**	**5.4**	**-**	**0.**	**4.9**	**0.9**	**0.9**	**0.9**	**2.9**	**15**

[1] Numbers show hour of blood sampling for microscopy, -1 is pre-enrollment; "X" indicates one sampling per day; "Δ" indicates that sample will be collected 6 until two consecutive samples are negative by microscopy.

[2] Complete physical exam on Day 0, Targeted physical on all subsequent days

[3] Biochemistry tests include glucose, creatinine, AST, ALT.

[4] Hematology tests include Hb, hematocrit and platelets

[5] PBMCs = Peripheral blood mononuclear cells

[6] RC = recrudescence within the 42 days of follow-up

[7] Total blood volume collected for the entire study = 43.1mL. For participants with recrudescence, total volumes will be 58.1mL

*Depending on randomization scheme. 1st MQ dose is supervised, 2nd dose is unsupervised. ** if indicated

Appendix IV. Bead calculation templates.

This template calculates the volume of each bead lot to add to a multiplex mix to make a final concentration of 3,000beads/50 µl for each lot. Adding 50µl of the bead mixture to each well will therefore equal 3,000 beads from each bead lot, in accordance with the Luminex SOP

-Take the number of wells used for each bead mixture and add 10% (refer to plate map for number of wells needed). Ex. 18 wells = 20 wells

-Enter adjusted number of wells used into **table i**

-Enter the # of beads needed from **table 1** in the numerator position within the "bead volume used" columns in **table ii**

-Check to make sure that the beads/micro liter concentrations are correct for each bead lot in a given bead mixture

-Label aependorf tube for each bead mixture. Add appropriate ammounts of 1XPBS, 1% BSA (see table ii, column O)

-Check to make sure the total bead mixture volume in **table ii** equals the total bead mixture volume in **table i**

-Vortex coupled bead lots for 20 seconds. Sonicate for 20 seconds. Add appropriate volume of coupled microspheres to corresponding bead mixture. See **table iii**

Mix number	Wells used (adjusted +10%)	# Beads needed per signature	Total Bead Mixture Volume (µl)
1	20	60000	1000
2	20	60000	1000
3	20	60000	1000
4	20	60000	1000

5	20	60000	1000
6	20	60000	1000

Table ii

	Bead Lots					Bead Set #1		Bead Set #2		Bead Set #3		Bead Set #4		Bead Set #5			
						1	1	2	2	3	3	4	4	5	5		
Mix Number	Bead Set 1	Bead Set 2	Bead Set 3	Bead Set 4	Bead Set 5	beads/micro	Bead Volume used (µl)	beads/micro	Bead Volume used (µl)	beads/micro	Bead Volume used (µl)	beads/micro	Bead Volume used (µl)	beads/micro	Bead Volume used (µl)	1XPBS, 1%BSA (µl)	Total Bead Mixture Volume (µl)
1	PfCelTOS - Mag030	PfCSP - Mag025	MSP 1-42 (3D7) - Mag051	AMA-1 (3D7)- Mag039	Pf LSA-1 - Mag034	5375	11.16	5300	**11.3**	4750	**12.6**	3875	**15.5**	5800	**10.3**	**939.1**	1000
2	Pf MSP1-42 (FVO) - Mag061	Pf AMA-1 (HB3) - Mag044				4400	13.64	4875	**12.3**		**#DIV/0!**		**#DIV/0!**		**#DIV/0!**	**974.1**	1000
3							#DIV/0!		**#DIV/0!**		**#DIV/0!**		**#DIV/0!**		**#DIV/0!**	**#DIV/0!**	1000
4							#DIV/0!		**#DIV/0!**		**#DIV/0!**		**#DIV/0!**		**#DIV/0!**	**#DIV/0!**	1000
5							#DIV/0!		**#DIV/0!**		**#DIV/0!**		**#DIV/0!**		**#DIV/0!**	**#DIV/0!**	1000
6							#DIV/0!		**#DIV/0!**		**#DIV/0!**		**#DIV/0!**		**#DIV/0!**	**#DIV/0!**	1000

Table iii			
Mix number	**Bead Lot**	**Bead Volume Used (µl)**	**1X PBS, 1%BSA (µl)**
1	PfCelTOS - Mag030	11.2	939.1
	PfCSP - Mag025	11.3	
	MSP1-42 (3D7) - Mag051	12.6	
	AMA-1 (3D7)- Mag039	15.5	
	Pf LSA-1 - Mag034	10.3	
2	Pf MSP1-42 (FVO)- Mag061	13.6	974.1
	Pf AMA-1 (HB3) - Mag044	12.3	
	0	#DIV/0!	
	0	#DIV/0!	
	0	#DIV/0!	

Appendix V. Plate map

USA MEDICAL RESEARCH UNIT

KENYA MEDICAL RESEARCH INSTITUTE
Jomo Kenyatta Highway
Kisumu, Kenya

P.O. Box 54, 40100 Kisumu, Kenya Tel: +254 717174935 E-mail: kisumu.admin@usamru-k.org

Indirect immunoassay using XMAP technology

Protocol ______________________Date_ _ / _ _ _ / _ _ _ _MAGPIX #_________Tracking No._______
Purpose____

A.PLATE MAP

	Negative cnt mix1	Negative cnt mix2	Positive cnt mix 1	Positive cnt mix 2	Sample 1mix 1	Sample 1mix 2	Sample 2mix 1	Sample 2mix 2	Sample 3mix 1	Sample 3mix 2	Sample 4mix 1	Sample 4mix2	
A													1:400
B													1;800
C													1;1600
D													1;3200
E													1;6400
F													1:12800
G													1:25600
H													1:51200

N/B.
1. A plate could only accommodate four samples and two controls with mix 1 and mix 2.
2. We had 8 dilutions for each sample and controls.