ANTIBODY RESPONSES TO *PLASMODIUM FALCIPARUM* ANTIGENS IN HIV INFECTED ADULTS IN BONDO SUB COUNTY HOSPITAL, SIAYA COUNTY, KENYA

BY

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DECLARATION

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

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DEDICATION

I dedicate this thesis to my parents Mary Odhiambo and Lucas Odhiambo and my siblings for the encouragements and moral support they provided me while pursuing this program.

ABSTRACT

Malaria and human immunodeficiency virus (HIV) are co-endemic in sub-Saharan Africa. Infection with HIV results in B cell anomalies. Antibodies are critical in protection against malaria and it is hypothesized that B cell anomalies resulting from HIV infection interfere with antibody responses contributing to severe and frequent malaria episodes. Individuals infected with HIV have elevated antibody and C-reactive protein (CRP) levels. However, it is unclear whether malaria-specific antibodies, particularly immunoglobulin M (IgM), total immunoglobulin G (IgG) and IgG subclasses levels would be increased or decreased, given the evidence of impaired B cell responses to other antigens due to HIV. Furthermore, how malaria-specific antibodies correlate with viral load (VL) and CRP levels in HIV infected individuals is unclear given their link in HIV disease progression. *Plasmodium falciparum* (*Pf*) vaccines studies have associated malaria protection with antibodies against apical membrane antigen-1 (AMA-1) and glutamate-rich protein-R0 (GLURP-R0). The current study aimed to: determine quantities and prevalence of antibody isotypes (IgG and IgM) against *Pf* antigens (AMA-1 and GLURP-R0); determine quantities and prevalence of IgG subclasses in response to *Pf* selected antigens and; measure the correlation of *Pf* specific antibody isotypes and subclasses with VL, $CD4^+$ counts and CRP levels. A comparative cross-sectional study using a sample size of 181 comprising of 52 HIV negative and 129 HIV positive adult participants seeking care at Bondo sub-County Hospital was conducted. Data from Bondo Sub-County hospital have shown an overlap in malaria and HIV infections. Antibody and CRP levels were tested using ELISA. The $CD4^+$ cell and VL counts were obtained using FACSCount and Abbott m2000 analyzer respectively. Medians and proportions of *Pf*-specific antibody levels were compared using Wilcoxon Rank-Sum and Chi-Square tests respectively. Correlations of *Pf*-specific antibodies with VL and CRP were obtained using Spearman correlation. The study found that IgM, IgG1 and IgG3 levels against both AMA-1 and GLURP-R0 were significantly high in HIV infected individuals ($P<0.0001$). Antibody responses against AMA-1 were lower in individuals having $CD4^+$ counts $\leq$200 cells/ml ($P=0.01$). Levels of IgM and IgG1 against both AMA-1 and GLURP-R0 were associated with CRP levels ($P=0.01$, 0.05, 0.02 and 0.004 respectively) and IgM and IgG1 against both antigens were associated with VL ($P=<0.001$, 0.02, 0.02 and 0.01 respectively). The data suggest that HIV infection leads to increased IgM, IgG1 and IgG3 responses, but low $CD4^+$ counts are associated with lower total IgG and IgG1 responses. These findings provide an insight into a better understanding of malaria-specific antibody responses due to HIV infection. Future studies should assess the cellular mechanisms leading to increased antibody levels in HIV-malaria co-infection.

TABLE OF CONTENTS

ABBREVIATIONS AND ACRONYMS

AU	Arbitrary Unit
AMA-1	Apical membrane antigen 1
AIDS	Acquired Immunodeficiency Syndrome
ARV	Antiretroviral
CD4	Cluster of differentiation 4
CRP	C- Reactive protein
ELISA	Enzyme-linked immunosorbent assay
KEMRI	Kenya Medical Research Institute
HIV	Human Immunodeficiency virus
Igs	Immunoglobulins
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IU	Indiana University
GLURP-R0	Glutamate-rich protein R0 (non-repetitive region)
GC	Germinal Center
MHC	Major histocompatibility complex
OD	Optical density
PBS	Phosphate buffered saline
Pf	*Plasmodium falciparum*
SGS	School of graduate studies
Tfh	T follicular helper cells
VCT	Voluntary Counseling and Testing
WHO	World Health Organization

OPERATIONAL TERMS

Pfs48/45 is a transmission-blocking target protein expressed by gametocytes of *Plasmodium falciparum* and plays a crucial role in the fertilization. It is a vaccine candidate against sexual stages of *Plasmodium falciparum.*

Apical Membrane Antigen (AMA-1) is a protein expressed in the membrane of *Plasmodium falciparum*, a protozoan parasite that causes the most deadly form of malaria in humans

Glutamate-rich protein R0, non-repetitive region (GLURP-R0) is a protein expressed in the membrane of both blood and liver stages of *Plasmodium falciparum* protozoan parasites that cause deadly malaria in humans.

Hypergammaglobulinemia is a medical condition distinguished by the increased levels of antibodies in the blood serum/plasma.

Responder is ELISA optical density greater than three SD above unexposed North American OD (AU values ≥1).

Response is a protective reaction in the body to fight against disease causing antigens especially malaria and HIV through production of antibodies.

Rosetting is a pathogenesis process in which red blood cells of malaria-infected cells bind uninfected red cells

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE
INTRODUCTION

1.1. Background Information

Malaria and human immunodeficiency virus (HIV) infections each contribute to death of millions globally and are the leading causes of death in low-income regions of Sub-Saharan Africa (Murray *et al*., 2014; WHO, 2015). There is a significant overlap in malaria and HIV epidemics in the studied populations (Abu-Raddad *et al*., 2006; Herrero *et al*., 2007). Previous findings that HIV infection is associated with high malaria prevalence raise a public health concern that these epidemics may be fueling one another, and because of this. Several studies have been done to understand the cause and implications of malaria and HIV co-infection (Abu-Raddad *et al*., 2006; González *et al*., 2012; Verhoeff *et al*., 1999; Whitworth *et al*., 2000). These studies suggest that HIV leads to the suppression of malaria targeting immune responses leaving the host susceptible to frequent episodes of malaria (González *et al*., 2012; Whitworth *et al*., 2000). However, the actual mechanism that HIV predisposes to frequent episodes of malaria has not been fully understood. Therefore, it is necessary to better understand the influence of HIV on malaria specific immune responses to inform malaria vaccine development for use in countries with a high prevalence of HIV.

B cells are key component of adaptive immunity with antibodies found to provide long-term immunity against multiple diseases upon vaccination (Crotty *et al*., 2003; Hammarlund *et al*., 2003). Their major functions of B cells include antibody production, CD4+ T cell activation and production of cytokines upon activation (LeBien and Tedder, 2008). Indeed, humoral immunity has been previously found to be crucial in protection against malaria (Sabchareon *et*

al., 1991). Studies have correlated antibodies to pre-erythrocytic and blood stage malaria antigens to protection from clinical malaria (John *et al*., 2008; Nebie *et al*., 2008) and there is research suggesting that these antibodies are sufficient to control clinical disease (Sabchareon *et al*., 1991). Furthermore, malaria specific antibody frequency has been found to rise progressively with malaria exposure (Wipasa *et al*., 2010). The degree of effective immunity against blood stage malaria parasites was established to depend on isotypes and subclasses (Ismail *et al*., 2014). Immunoglobulin M and G (IgM and IgG) have been previously reported to be protective against malaria through phagocytosis and lysis mediated by opsonization and complement respectively (Branch *et al*., 1998; Pleass *et al*., 2016). In contrast, IgM has been reported to enhance *Pf* pathogenesis by binding to the surface of *Pf*-infected erythrocytes resulting to rosette formation (Ghumra *et al*., 2008; Rowe *et al*., 2002). Structurally, Immunoglobulin G is divided into 4 different structures (subclasses) and cytophilic subclasses (IgG1 and IgG3) are reported to be more protective against malaria compared to noncytophilic subclasses (IgG2 and IgG4) (Eisenhut, 2007; Nimmerjahn and Ravetch, 2008; Stanisic *et al*., 2009).

Human immunodeficiency virus has been shown to have a major impact on B cell immunity (De Milito, 2004). Early observations on HIIV infection included hypergammaglobulinemia increase rates of autoimmune disorders and B cell malignancies (Moir and Fauci, 2009). More recently, major phenotypic abnormalities in peripheral B cells have been described including a decline in the naïve and resting memory pool paired with an expansion in activated B cells as well as atypical or “exhausted” B cells (De Milito, 2004; Moir and Fauci, 2009). Immune activation due to ongoing viral replication is hypothesized to be the genesis of B cell immunopathogenesis (Marconi *et al*., 2009)..

Within the field of malaria and HIV co-infection, prior studies examined variable quantities of malaria specific antibodies (Hasang *et al*., 2014; Mount *et al*., 2004). Preliminary data from a cohort study of individuals sampled from Bondo Kenya does not show a significant decline in malaria protection among individuals with HIV infection when compared to HIV negative individuals from Bondo sub-County (unpublished data). However, these studies only assessed the levels of total IgG which past study found to be poor predictor of malaria protection (Marsh *et al*., 1989). Also, existing data shows that in HIV-1 infected Kenyans adults, individuals living in high malaria transmission areas had sero-reactivity to a greater number of *Pf* antigens when compared with the low malaria endemic areas, suggesting that the ability of HIV infected adults to either accumulate or maintain antibody production is intact (Nnedu *et al*., 2011) Given the contrasting results on *Pf*-specific total IgG levels due to HIV, there is a need for a study to provide more insight into the malaria antibodies response in HIV positive patients. Also, IgM has been found to enhance malaria immunity and pathogenesis but no study has been conducted to compare their quantities against malaria antigens in HIV infected and uninfected population.

Immunoglobulin G subclasses are crucial as far as malaria protection malaria protection is concerned (Eisenhut, 2007; Nimmerjahn and Ravetch, 2008; Stanisic *et al*., 2009). It has been proved that HIV affects B cell populations (De Milito, 2004; Moir and Fauci, 2009) and higher total IgG1 and IgG3 titers were reported in HIV infected compared to uninfected population (Scharf *et al*., 2001). However, the high IgG1 and Ig3 titers reported in HIV positive population were total antibodies and there is a need to investigate quantities of IgG subclasses specific to malaria antigens in HIV infected and uninfected population in answering the cause of frequent

malaria cases observed in HIV infected individuals.

C-reactive protein (CRP) is a conserved plasma protein and a marker of acute response (Gewurz *et al.*, 1982) and was found to be protective against pre-erythrocytic stages of malaria (Pied *et al.*, 1989). Previous studies found that CRP is a marker of HIV progression independent of $CD4^+$ counts and viral load (Lau *et al.*, 2006)**.** In addition, cases of mortality and morbidity were reported in CRP elevated levels as a result of HIV infection (Borges *et al*., 2014; Drain *et al.*, 2007; Kuller *et al*., 2008)**.** Given the information on probable immunosuppression due to high CRP levels in HIV infected individuals, it is not known whether antibody responses against malaria associate with CRP levels in HIV infected individuals.

Studies have established that B cell immunopathogenesises correlated both with HIV viremia as well as $CD4^+$ count (De Milito, 2004; Moir *et al*., 2001; Moir and Fauci, 2009). Surprisingly, no correlation was reported between *Pf*-specific antibodies and $CD4^+$ counts (Nnedu *et al*., 2011). Other studies suggest that the breath of reactivity to malaria antigens may be slightly diminished in the setting of HIV infection but no relationship was also seen between malaria-specific antibodies with $CD4^+$ T cell counts or viral load (Subramaniam *et al*., 2015). Although no relationship was depicted between malaria-specific antibodies and viral load or $CD4^+$ counts in HIV population, previous studies only tested levels of total IgG. There is a need to correlate the levels of all antibodies found to be protective against malaria (IgM, total IgG and IgG subclasses) with viral load and $CD4^+$ counts.

Studies on T cell derangements in HIV may lend some insights into how HIV may affect antibody level and functionality. It has been known that the generation of antibody responses

require help from T cells (Claman *et al.*, 1966). This help was found to occur during germinal center (GC) reaction where T cells orchestrate the process of somatic hypermutation, class switch recombination, and selection of B cells, ultimately leading to the generation of high affinity memory B cells and plasma cells (Kelsoe, 1995; Liu *et al.*, 1996; MacLennan, 1994). In the germinal center, B cells effectively compete for stimulation by a group of $CD4^+$ T cells called T follicular helper cells (Tfh). The highest affinity B cells more efficiently engulf and present on MHC class II molecules T cell epitopes for the antigen targeted by the B cell receptor (Crotty, 2015). With a higher degree of Major histocompatibility complex (MHC) class II presentation, high affinity B cells are able to more efficiently engage Tfh cells with the corresponding T cell receptor, receiving signaling critical to their survival and differentiation (Crotty, 2015). Numerous studies have found Tfh abnormalities cells as a result of HIV infection (Cubas *et al.*, 2013; Lindqvist *et al.*, 2012) including impaired Tfh function and accumulation of these cells in lymphoid tissue. Based on these studies, it is likely that an alteration in the number or function of Tfh cells in HIV infected individuals may affect B cell development hence impacting on the process of affinity maturation and isotype switching. Based on the HIV impacts on Tfh cells, this study therefore hypothesized that there are differences in quantity of antibodies produced by HIV infected and uninfected individuals. This was assessed by testing immunoglobulin isotype (total IgG and IgM) and subclasses (IgG1-4) levels in response to selected *Pf* synthetic antigens (AMA-1 and GLURP-R0) and their association with $CD4^+$ counts, viral load and CRP levels.

1.2. Statement of the Problem

Studies have observed that individuals with HIV who live in malaria endemic regions experience more frequent and severe episodes of malaria compared to their HIV negative counterparts. Humoral immune responses are important for protection against malaria and past studies showed that HIV impairs effective B cell immunity. Limited studies investigated that HIV does not dramatically impair the quantity of malaria antibodies in HIV infected persons living in malaria endemic regions. Furthermore, other studies showed different antibody reactivities against different malaria specific antigens in HIV infected population. However, only total IgG antibody was used in these investigations. To date, no research has been done to investigate the influence of HIV infection on the distribution of malaria-specific antibody isotypes (IgM and total IgG) and subclasses (IgG1-4) and their association with $CD4^+$ counts, HIV viral load and CRP levels.

1.3. Objectives

1.3.1. General objective

To assess antibody responses to selected *Plasmodium falciparum* antigens (AMA-1 and GLURP-R0) in HIV seropositive and HIV seronegative adults living in Bondo sub-County, Siaya County, Kenya.

1.3.2. Specific objectives

i. To determine the quantities and prevalence of total antibody isotypes (IgM and IgG) in response to AMA-1 and GLURP-R0in HIV seropositive and HIV seronegative adults.

ii. To determine quantities and prevalence of IgG subclasses (IgG1, IgG2, IgG3 and IgG4) in response to AMA-1 and GLURP-R0in HIV seropositive and HIV seronegative adults.

iii. To determine the correlation of *Pf*-specific antibody isotypes and subclasses with markers of HIV disease severity and immune activation including $CD4^+$ cell counts, HIV-1 viral load and CRP levels.

1.4. Hypotheses

i. There is no difference in the quantities and prevalence of IgM and total IgG against *Pf* antigens in HIV seropositive and seronegative patients.

ii. There is no difference in IgG subclasses quantities and prevalence against AMA-1 and GLURP-R0 in HIV seropositive and seronegative patients.

iii. There is no correlation between *Pf*-specific antibodies with $CD4^+$ counts, HIV-1 viral load and CRP levels.

1.5. Justification of the Study

Western Kenya is a region with a high rate of HIV and malaria co-infection. HIV infected individuals in malaria endemic areas experience frequent and severe malaria episodes compared to uninfected individuals. This clinical observation has been associated with an impairment of antibody responses to *Pf* antigens; however, prior studies have only compared the levels of only total IgG antibody levels against HIV infected and uninfected individuals. Different reactivities of malaria specific total IgG levels were reported as a result of HIV infection. In addition, the preliminary data obtained from Bondo found no statistically different in total IgG levels in HIV infected and uninfected individuals. The responses of IgM and IgG subclasses against *Pf*-specific antigens due to HIV infection have not been investigated. The $CD4^+$ counts, viral load and CRP

levels have been correlated with HIV progression but information regarding their association with *Pf*-specific antibodies as a result of HIV is missing.

1.6 Significance of the Study

The results from this study will enable a better understanding of how the antigen-specific response to *Pf* differs in HIV-infected against non-infected individuals in an endemic area, to enable future assessment of the cellular basis for these differences, and future studies on how these changes affect risk of malaria in HIV-infected individuals.

CHAPTER TWO

LITERATURE REVIEW

2.1. Malaria and HIV Infections

The prevalence and mortality cases of HIV reported in 2013 globally were high at 29.2 and 1.3 millions respectively (Murray *et al*., 2014). Malaria cases on the other hand were found to be on a steady rise from 1990 with the peak of 232 millions in 2004 and in 2013 cases and mortality declined to 165 millions and 855000 respectively (Murray *et al*., 2014). About 90% of malaria deaths were reported in Africa region (WHO, 2015). Malaria-HIV co-infection has been found to be the major cause of death of low-income sub-Saharan populations (WHO, 2015). Furthermore, malaria and HIV infections were found to overlap in studied populations (Abu-Raddad *et al*., 2006; Herrero *et al*., 2007) and recent investigation reported that it is HIV that that is fueling the overlap through the suppression of immune responses (González *et al*., 2012; Whitworth *et al*., 2000). Additionally, HIV infection was linked with B cell anomalies (De Milito, 2004; Moir and Fauci, 2009).However, studies assessing the total IgG specific to malaria in HIV infected individuals variable reactivities (Hasang *et al*., 2014; Mount *et al*., 2004). Given the limited and contrasting information on malaria specific antibody suppression as a result of HIV there is a need to find out the specific suppression mechanisms in antibodies especially IgM, total IgG and IgG subclasses since they are pivotal in malaria protection.

2.2. Antibody Isotypes and Subclasses

Antibodies are proteins, which circulate in the blood stream and are produced by B cells in response to antigens (Abbas *et al*., 2010). Antibodies are also called immunoglobulins (Igs) meaning immunity bestowed by gamma globin fraction (Abbas *et al*., 2010). There are five

major immunoglobulin classes namely: IgA, IgD, IgE, IgG and IgM, each composed of two pairs of heavy and light chains. All chains contain variable and constant domains and each antibody has specific functions in immunity and a defined structure (Schroeder and Cavacini, 2010). Differentiated B cells that have undergone the germinal center reactions with the help of T follicular cells produce these antibodies (Murphy *et al.*, 2012). At the germinal center, B cells undergo hypermutation at the V-region genes and this improves the affinity of B cells (Li *et al*., 2004; Odegard and Schatz, 2006). The high affinity B cells are believed to effectively engulf and present antigens to MHC class II T cells (Crotty, 2015). The first immunoglobulin secreted by activated B cells is IgM and is important in protection against pathogens including malaria (Perkins *et al*., 1991; Pleass *et al*., 2016). The other Igs classes are formed as a result of DNA rearrangement at the heavy chain under the influence of cytokines released by $CD4^+$ T cells (Stavnezer, 1996a, 1996b). Immunoglobulins have specific roles in immunity (Schroeder and Cavacini, 2010) and high affinity IgG and IgA have been linked to protection from viruses and bacterial toxins (Brandtzaeg, 2003), IgE mediates activation of accessory cells and is important in resistance to parasitic infections (Gurish *et al*., 2004). Total IgG, IgG subclasses and IgM has been found to be protective against malaria parasites (Branch *et al*., 1998; Bredius *et al*., 1994; Osier *et al*., 2008; Pleass *et al*., 2016).

Immunologically, IgG are structurally split into four subclasses, IgG1, IgG2, IgG3 and IgG4 and they have been linked with immunity against diseases including malaria (Nimmerjahn and Ravetch, 2008; Schroeder and Cavacini, 2010). It is evident that IgG subclasses play important roles in protection against malaria (Bredius *et al*., 1994) Furthermore, Immunoglobulin G subclasses were found to confer neutralization effect against HIV and IgG3 with the highest

neutralizing effect (Scharf *et al*., 2001). Given the evidence that IgG subclasses offer protection from both malaria and HIV, it is not known on how HIV affects specific malaria responses.

2.3. Prevalence of Antibody Isotypes and Subclasses in HIV Infected Individuals.

Human immunodeficiency virus was associated with severe and frequent cases of malaria (Abu-Raddad *et al*., 2006; Verhoeff *etal*., 1999). B cells perturbations were previously reported in HIV infected individuals (De Milito, 2004; Moir *et al*., 2008, 2001). It is possible that the B cell perturbations as a result of HIV are the driving force towards malaria severity. Infection with HIV is also reported to dysregulate Tfh cells, cells responsible for affinity maturation, somatic hypermutation and class switching of B cells (Liu *et al*., 1996). The resultant effect of HIV is was found to be an increase in the levels of "exhausted' or atypical memory B cells, which have increased inhibitory surface receptors and are hyporesponsive to stimulation *in vitro* (Moir *et al*., 2008). These findings were a clear indication that HIV infection would impair antibody responses.

Immunoglobulin profiling findings showed that HIV seropositive children have higher levels of Igs than HIV seronegative with the mean levels of IgA, IgM, IgD and IgG3 increasing with progression from HIV asymptomatic stage to AIDS (Lugada *et al*., 2004; Lyamuya *et al*., 1999, 1994; Mizuma *et al*., 1988). Immunoglobulin M and G levels against HIV are elevated in HIV infected compared to uninfected individuals (Mizuma *et al*., 1988). Current study concluded that antibodies produced in HIV infected individuals have a lower breath of binding with malaria antigens (Subramaniam *et al*., 2015). Despite the evidence that HIV elevates antibody levels, preliminary data from this study reported that there is no significant difference in total IgG malaria specific antibody levels in HIV infected compared to uninfected individuals

(unpublished data). Also, contrasting total IgG levels against malaria specific antigens were observed in HIV infected and uninfected individuals (Hasang *et al.*, 2014; Mount *et al.*, 2004). Based on the conflicting findings in total IgG levels against malaria specific antigens as a result of HIV infection, no conclusion can be drawn from the findings and there is a need to investigate total IgG malaria specific responses due to HIV.

Immunoglobulin M levels were reported to be increased as a result of HIV infection (Lugada *et al.*, 2004; Lyamuya *et al.*, 1999, 1994; Mizuma *et al.*, 1988). Levels of IgM was associated with malaria pathogenesis by facilitating rosette formation (Ghumra *et al.*, 2008; Rowe *et al.*, 2002) and phagocytic mediated malaria parasite clearance (Branch *et al.*, 1998; Pleass *et al.*, 2016). Assessment of antibody specific responses against malaria antigens as a result of HIV infection has been investigated on only total IgG antibody (Hasang et al., 2014; Mount et al., 2004; Nnedu et al., 2011; Subramaniam et al., 2015) despite IgM role in malaria protection and pathogenesis (Ghumra *et al.*, 2008; Rowe *et al.*, 2002). There is a need to investigate IgM specific malaria responses as a result of HIV infection.

Immunoglobulin G subclasses were investigated to provide neutralizing effect against HIV (Scharf *et al.*, 2001). Despite protective abilities of IgG1 and IgG3 against malaria and HIV, an infection with HIV was found to interfere with phagocytosis mediated by these antibodies (Keen *et al.*, 2007). Given the evidence of phagocytic interference of IgG subclass antibodies by HIV, there a need to look at the quantities of these antibodies in response to malaria in HIV infected and uninfected individuals.

2.4. Markers of HIV Immune Activation (CRP) and Disease Severity ($CD4^+$ count and viral load)

CD4 T cells are a primary target of HIV infection, (Wilen *et al.*, 2012) and in the absence of treatment, HIV infection leads to the progressive depletion of circulating and secondary lymphoid $CD4^+$ T cells (Masur *et al.*, 1989). Also, HIV was linked with the impairment of Tfh cells, important $CD4^+$ cells responsible for development and maturation of B cells (Cubas *et al.*, 2013; Lindqvist *et al.*, 2012). Previously, $CD4^+$ cells were found to be crucial in malaria protection (Stevenson and Riley, 2004) and the loss of CD4+ cells as a result of HIV has been assumed to be the main cause of frequent malaria episodes (González *et al.*, 2012).To confirm the importance of $CD4^+$ counts in malaria control, past studies found that low $CD4^+$ counts were linked with high parasiteamia levels (French *et al.*, 2001; Iroezindu *et al.*, 2012; Subramaniam *et al.*, 2015; Whitworth *et al.*, 2000). Preliminary results on this study (unpublished data) and previous findings (Nnedu *et al.*, 2011) found no association between the antibodies and the $CD4^+$ counts. These comparisons were only done on total IgG antibody and there is a need to do a comprehensive comparison using antibodies previously found to be protective against malaria in addition to total IgG.

C-reactive protein (CRP) is a phylogenetically conserved plasma protein (Black *et al.*, 2004) a prototype acute phase reactants (Gewurz *et al.*, 1982). The level of plasma CRP is a marker of inflammation due to an infection (Clyne and Olshaker, 1999). Elevated CRP levels have been reported in HIV infected individuals and its level has been linked with HIV immune progression independent of $CD4^+$ counts (Lau *et al.*, 2006). In addition, high CRP levels as a result of HIV infection were associated with mortality and morbidity (Borges *et al.*, 2014; Drain *et al.*, 2007; Kuller *et al.*, 2008). Despite these findings, no study has been conducted to

investigate the association between CRP levels detected in HIV infected individuals and *Pf*-specific antibodies.

HIV enters the body through CD4 cells and sustain viremia or copy numbers through continuous replication (Ho *et al.*, 1995; Perelson *et al.*, 1996). Past studies find that high viral loads were observed in the advanced stages of HIV infection (Ho *et al.*, 1989; Piatak *et al.*, 1993; Simmonds *et al.*, 1990) and predicts the chances of developing to AIDS (Mellors *et al.*, 1995; Saksela *et al.*, 1995). At initial stages of HIV a transient decline in viral load was reported and this was linked with immune responses (Daar *et al.*, 1991). Additionally, high HIV viral load has been associated with low $CD4^+$ counts (Moir and Fauci, 2009). Also, extrinsic apoptotic death of B cells have been reported to be induced with HIV viremia (Giri *et al.*, 2006; Herbeuval and Shearer, 2007). Despite influences HIV viral load has on the immune responses and death of B cells, no study has been done to associate the levels of *Pf*-specific antibodies and HIV viral load.

2.5 Malaria Antigens

Malaria in Sub Saharan Africa is predominantly caused by *Plasmodium falciparum* parasite (Barry *et al.*, 2009). Malaria parasites have been linked with multiple genetic polymorphisms and this enable them successfully evade host immune responses causing a major obstacle in vaccine development (Escalante *et al.*, 1998; Mu *et al.*, 2007). Due to polymorphic nature of malaria parasites, previous studies concluded that future malaria vaccines should contain parasites surface proteins expressed in pre-erythrocytic, blood stage, and gametocyte stages of parasites within the host. These include: the Circumsporozoite Surface Antigen (CSP), Thrombospondin Related Adhesion Protein (TRAP), Liver Stage Antigen 1 (LSA-1), Apical Membrane Antigen 1 (AMA-1), Erythrocyte Binding Antigen 175 (EBA175), Merozoite Surface

Proteins 1-5 (MSPs 1-5), Glutamate Rich Protein (GLURP) and *Pf*s48/45 (Barry *et al*., 2009; Barry and Arnott, 2014) as shown in Figure 1 below.

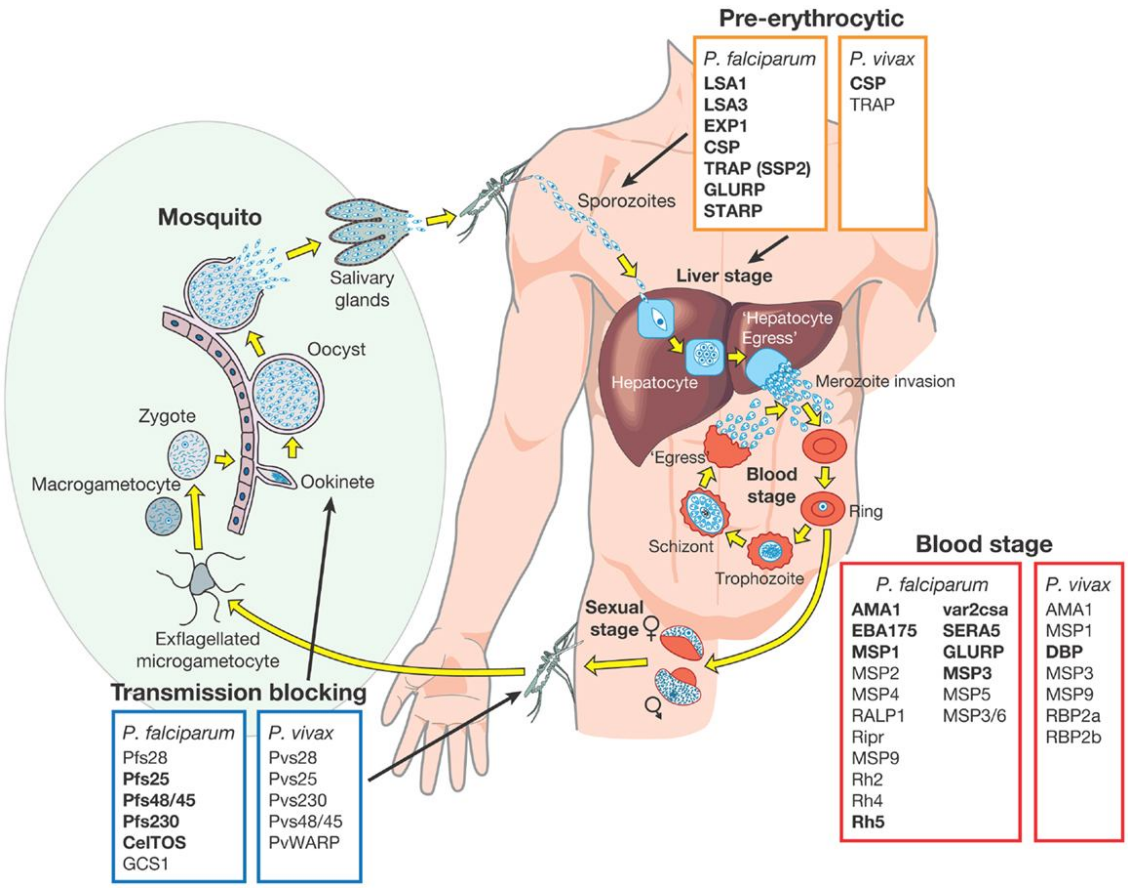

Figure 1: Malaria vaccine candidate antigens in *Plasmodium falciparum* and *Plasmodium vivax* life cycles. Adapted from (Barry and Arnott, 2014)

Selection of AMA-1 for this investigation was based on the safety and high immunogenicity findings from AMA-1 based vaccine trial conducted in Mali (Spring et al., 2009; Thera et al., 2010). Also, AMA-1 was reported to have high half life in Kenyan highland adults (Ondigo *et al*., 2014). GLURP-R0 is both pre-erythrocytic and blood stage *Pf* antigen (Barry and Arnott, 2014) and its levels have been associated with clinical malaria protection (Iriemenam *et al*., 2009). GLURP-R0 was reported to have moderate half life against total IgG

antibody in adults (Ondigo *et al.*, 2014). Also, GLURP-R0 malaria vaccine named GMZ2 is undergoing phase IIb trial test after successful phase Ia, Ib and IIa trials with high immunogenicity and safety findings (Esen *et al*., 2009; Jepsen *et al*., 2013; Mordmüller *et al*., 2010).

CHAPTER THREE
METHODOLOGY

3.1. Study Area

The study was conducted on the samples collected from the HIV voluntary counseling and testing center (VCT) in Bondo Sub County Hospital found in Bondo Sub-County. Bondo Sub-County lies between an altitude of 0° 26° to 0° 90° and from longitude 33° 58° E and 34° 35° W. The sub-County has a population of 238, 780 from the census data (2009) and Bondo Sub County Hospital is the primary health care facility in the region. HIV seropositivity was estimated to be 23.6% among adults (personal communication, Dr. Julius Oliech, sub-County health officer) and malaria transmission is holoendemic. Map of the study area is as below.

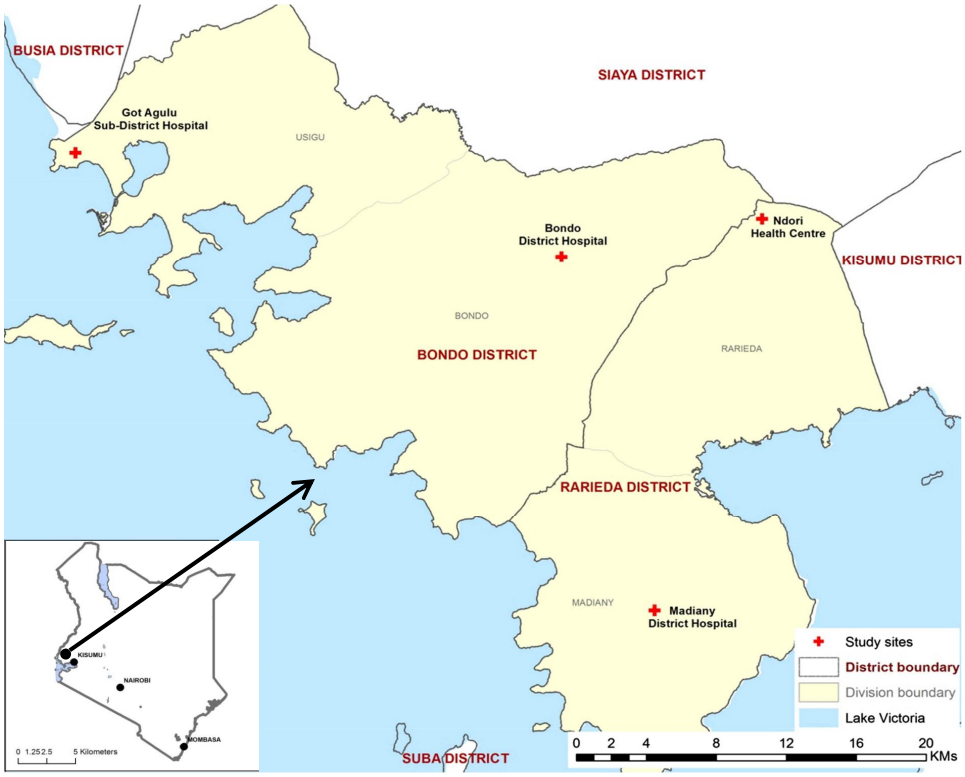

Figure 2: Map of Study Area

3.2. Study Population

Patients of 18 years of age and above undergoing HIV testing at Bondo Sub County Hospital were eligible for recruitment into the study regardless of their HIV status.

3.2.1. Inclusion criteria

1. Resident of Bondo sub-County and seeking medical health care at VCT clinic in Bondo Sub County Hospital.
2. Age of 18 years or older.
3. Negative HIV test at Bondo Sub County Hospital VCT Clinic OR newly diagnosed HIV positive at ARV Clinic.
4. Those who were able to provide informed consent and willingness to undergo study procedures.

3.2.2 Exclusion criteria

1. Those who were taking antimalarials, cotrimoxazole, corticosteroids or other immunosuppressants.
2. Fever $\geq$ 37.5°C or evidence of an acute infection. Evidence of acute infection included cough, fever, chills, and current symptoms of diarrhea, headache, abdominal pain and jaundice.
3. Chronic medical conditions including diabetes, chronic obstructive pulmonary disease, malignant or hematologic condition, inflammatory bowel disease, liver disease, the presence or treatment of which may affect immune function.
4. Those who were pregnant, as determined by urine dipstick test for pregnancy.

3.3. Study Design

This was a comparative cross sectional study to determine the effects of HIV infection on the quantity of antibodies against *Pf* antigens (AMA-1 and GLURP-R0). The study focused on individuals who were living in Bondo sub-County and were accessing medical care from Bondo sub-County Hospital.

3.4. Sample Size Determination and Sampling Techniques

Baseline data for the proposed study objectives were unavailable for calculation of power and sample size at the time of recruitment. Accordingly, a total sample size of 181 participants was used (52 HIV negative and 129 HIV positive). Assuming 80% power and α (false-positive rate) 0.05 the sample size used gave a detectable effect size of 0.21 as per the below formula (Cohen, 1992)

$$N = \left(\frac{Z_{1-\alpha/2} + Z_{1-\beta}}{ES} \right)^2$$

Where n is the sample size, α is the selected level of significance and $Z_{1-\alpha/2}$ is the value from the standard normal distribution holding 1- α/2 below it. 1- β is the selected power, $Z_{1-\beta}$ is the value from the standard normal distribution holding 1- β below it and ES is the effect size (Cohen, 1992)

$Z_{1-\alpha/2} = 1.960$

$Z_{1-\beta} = 0.84$

$$181 = \left(\frac{1.960 + 0.84}{} \right)^2$$

ES = 0.21

The sample size is 181. This is larger than the sample size of the studies examining HIV and malaria serologic reactivity as was described (Nnedu *et al.*, 2011; Subramaniam *et al.*, 2015).

All participants that were screened for HIV and were positive at the Bondo VCT center on study enrollment days were offered participation in the study (typically 2-3 days a week during the enrollment period). Every time three HIV positive people were enrolled, an enrollment was offered to HIV negative individual. These recruitment techniques were aimed at minimizing bias by study staff and ensuring proportional numbers of HIV infected and uninfected individuals were recruited.

3.5. Data Collection Procedure at the Hospital

3.5.1. Recruitment of research assistants

The study research team was composed of nurses, clinical officers, phlebotomists, and lab technologists working in Bondo hospital and attending to potential study participants. Those with the required qualifications were recruited for the job.

3.5.2. Training of research assistants

The study team was trained on all the study recruitment criteria to ensure that appropriate participants were selected. The team was trained on consenting procedures and information regarding research objectives, study procedures, inclusion and exclusion criteria, medical services, risks and benefits of the study. They were expected to adhere to the above before enrolling a participant to the study. The senior study staff from the University of Minnesota and Kenyan Medical Research Institute observed the consent process for the first week to confirm that all prospective participants obtained all of the relevant study information, were given the

opportunity to ask any questions and had the opportunity to freely decline participation. Senior study staff was available on site for the entirety of the study to answer any questions from staff or participants.

3.5.3. Clinical testing

Before or after enrollment the following tests were done:

1. HIV testing done at BondoVCT Clinic within the hospital.
2. CD4+ counting done at Bondo Sub County Hospital.
3. Malaria Rapid Diagnostic Test, conducted at Bondo Sub County Hospital.
4. Thick and thin smear prepared for malaria microscopy to all enrolled participants. Prepared slides were stained using 3% Giemsa solution and reading done as described (WHO, 2012), read by KEMRI/CDC laboratory in Kisumu. Microscopy is the goal standard for malaria parasite detection and it was meant to confirm results obtained by malaria rapid diagnostic test.
5. HIV-1 Viral load testing done at the KEMRI/CDC laboratory in Kisumu.

3.5.4. Data collection

All the data from the testing above were recorded into their respective study case report forms and were transported to laboratory alongside collected samples. All the interviews conducted by clinician to participants were recorded in clinician's case report form and this too accompanied sample to the laboratory.

3.6. Blood Collection

Approximately 40 ml venous blood was collected into sodium heparinized vacutainers by trained phlebotomists and transported to the laboratory for processing within 6 hours of collection. Plasma was obtained from the blood and stored at -20° C freezers. A drop of the blood was used for the preparation of thick and thin smear.

3.7. Laboratory Procedures

Collected samples were transported to the laboratory in less than 6 hours and accompanied with their respective forms. Samples were verified against the forms to avoid any error and peripheral blood mononuclear cells (PBMCs) isolated. Plasma was stored in -20°C freezer and PBMCs in liquid nitrogen tank.

3.7.1 Total IgG test

Total IgG reactivity to AMA-1 and GLURP-R0 antigens was done by standard ELISA as previously described (Chelimo *et al*., 2005; John *et al*., 2005; Kariuki *et al*., 2003). Briefly, recombinant *Pf* antigens were diluted in 1xPBS to obtain a concentration of 0.1 µg/µL. 96 well Immunol 4 (Thermo Labsystems # 3855, US) was coated with 50 µL of diluted antigens for overnight at 4°C after which they were washed three times with 1×PBS/Tween 20. The plates were blocked with 5% Blotto (non-fat dry milk) in 1xPBS for 1 hour at room temperature and washed thrice again with 1×PBS/Tween 20.Participants' samples and North American Controls (NACs) were diluted (1:100) in Blotto and 50µL added into each well and incubated for 2 hours at room temperature. North America Controls composed of control sample group haven't been exposed to malaria (negative controls for malaria). The plate was washed thrice with

1×PBS/Tween 20 and 50 µL goat anti-human IgG-Alkaline Phosphatase diluted (1:1000) in Blotto added and incubated for 1 hour at room temperature. This was followed with six washes with 1×PBS/Tween 20 and addition of substrate and incubated in dark for 30 minutes Reaction was stopped by addition of 3N NaOH and OD value read at 405 nm (Molecular Devices, Sunnyvale, CA)

3.7.2 IgM test

IgM reactivity to AMA-1 and GLURP-R0 antigens was done by standard ELISA as previously described (Chelimo *et al*., 2005; John *et al*., 2005; Kariuki *et al*., 2003). In brief, recombinant *Pf* antigens were diluted in 1xPBS to obtain a concentration of 0.1 µg/µL. Coating was done by adding 50 µL of diluted antigens into96 well Immunol 4 (Thermo Labsystems # 3855, US) for overnight at 4°C after which they were washed once with 1×PBS. The plates were blocked with 1×Seablock blocking buffer for 1 hour at room temperature and washed once again with 1×PBS. Samples and North America Controls (NACs) were diluted (1:100) in 1×Seablock buffer and added into each well and incubated for 2 hours at room temperature. North America Controls composed of control sample group haven't been exposed to malaria (negative controls for malaria). The plate was washed thrice with 1×PBS/Tween 20 and goat anti-human IgM-Alkaline Phosphatase diluted (1:4000) in 1xSeablock buffer added and incubated for 1 hour at room temperature. This was followed with six washes with 1×PBS/Tween 20 and addition of substrate and incubated in dark for 30 minutes Reaction was stopped by addition of 3N NaOH and OD value read at 405 nm (Molecular Devices, Sunnyvale, CA).

3.7.3 Antibody subclasses (1gG1-4) test

Reactivity of IgG subclasses (IgG1, IgG2, IgG3 and IgG4) againstAMA-1 and GLURP-R0 antigens were determined using standard ELISA as described (Chelimo *et al*., 2005; John *et al*., 2005).In summary, recombinant *Pf* antigens were diluted in 1xPBS to obtain a concentration of 0.1 µg/µL. Coating was done by adding 50 µL of diluted antigens into 96 well Immunol 4 (Thermo Labsystems # 3855, US) for overnight at 4°C after which they were washed twice with1×PBS/Tween 20. The plates were blocked at room temperature with blocking buffer (PBS/3% BSA) for one hour and washed twice with PBS/Tween 20. Plasma samples and NACs were diluted (1:100) in diluent buffer (PBS/1% BSA) and 50 µl/well added, sealed and incubated at 4°C overnight. North America Controls composed of control sample group have not been exposed to malaria (negative controls for malaria). Plates were washed three times with PBS/Tween 20 and secondary biotinylated antibody (mouse anti-human IgG1-4) added and incubated at room temperature for 45 minutes. The plates were washed thrice using PBS/Tween 20, Streptavidin conjugated Alkaline Phosphatase added and incubated at room temperature for 30minutes. This was followed by three times wash using PBS/Tween 20 and additional three washes with PBS only. During the last wash, Alkaline Phosphatase substrate developing solution was prepared by dissolving 1 Alkaline Phosphatase tablet in 5mL Diethanolamine Buffer and the solution allowed to completely dissolve and equilibrate to room temperature. Substrate solution of 50 µL was added in each well and allowed to develop for20 minutes in dark at room temperature. Plates were then stopped with 3N NaOH and read at 405 nm (Molecular Devices, Sunnyvale, CA).

3.7.4. CD4+ counting

3.7.4.1. Sample preparation and staining procedure

All the samples processed were arranged on a rack and labeled for sample processing. Each patient sample reagent tube tab was then labeled with the patient accession number/ the number identified the tube of blood. Each tube was then vortexed upside down for 5 seconds and then upright for 6 seconds. Each reagent tube was opened with the coloring station, the tube was then slid upright into the coloring station. When the tube was securely positioned, the lever was pulled down to core the tube. The lever was then allowed to return to its original position. Each tube was then transferred from the coloring station to the workstation, keeping each one upright. Each BD vacutainer was inverted 5-10 times to adequately mix the blood. 50 microliters of whole blood was then pipetted into each reagent while changing tips in between and discarding the tips. Each tube was capped and vortexed upright for 6 seconds. Each tube was incubated for 60-120 minutes at room temperature. The tubes were then uncapped and 50 microliters of fixative solution pipetted into each one, changing the tips in between tubes.

3.7.4.2. Running the samples on FACSCount

The FACSCount (BD Biosciences, San Jose, CA) was switched on and the reagents vortexed upright for 5 seconds. The CD4 tube was then uncapped and the cap set aside. The reagent tube was placed in the sample holder. The run button was then pressed and a message appeared informing that the test had started. When the analysis was complete, the sample holder lowered. The CD4 tube was then removed and recapped. It was then disposed as a biohazard waste. Patients' results were displayed and printed. The sample button was pressed to run the next sample.

3.7.5. Viral load testing

HIV-1 viral load was measured from dried blood spot (DBS) as described (Marconi *et al*., 2009). In brief, two blood spots previously collected on Whartman 903 filter paper for each patient's blood were punched out using a sterile puncher. The punched spots were placed into 2.0 ml of mLysis buffer provided with the Abbott sample preparation system (m2000sp) in 50 ml sealed conical tubes. The tubes were incubated at room temperature for 2 hours, with sporadic mixing. RNA was extracted from the lysate to the standard HIV-1 RNA 1.0 ml. Extraction protocol was used in accordance with the manufacturer's instructions (Abbott Molecular, Germany). Fluorescence counts were converted directly into viral load measures by the m2000real-timeanalyzer.

3.7.6. CRP level testing

CRP levels were determined by standard ELISA as previously described (Lasselin *et al*., 2012; Ryberg*et al*., 2010)using a human CRP ELISA Kit (Millipore, Cat. # CYT298) in accordance with the manufacturer's instructions. Briefly, 100µL of the diluted Standards (standard 1 diluted in 1:100 while 2 and 3 were 1:3 serial dilution from standard 1), controls (1:100) and samples (1:4000) in wash buffer were dispensed, in duplicates, into their designated wells. The plate was then incubated at room temperature (20-25^{o}C) for 30 minutes. The plate was then washed 5 times with wash solution (0.3mL per well). The plate was tapped on a stack of absorbent paper towels to remove the residual buffer. 100µL of diluted conjugate solution (anti-human CRP antibody) was added and the plate incubated at room temperature (20-25^{o}C) for 30 minutes. The plate was washed and bloated to remove residual buffer. 100µL of substrate solution was added (Care was taken not expose the micro well plate to direct sunlight by

covering it with aluminium foil). The plate was incubated for 10 minutes at room temperature (20-25^{o}C) and a blue color indicated a positive reaction. Adding 100µL of stop solution stopped the color development. The reaction mixture turned from blue to yellow. The absorbance was then determined by reading the plate at 450nm(Molecular Devices, Sunnyvale, CA). Standard curve was constructed from the standard readings and the actual plasma CRP values obtained by multiplying resultant values by 4. Results were reported as concentration of CRP (µg/mL) in samples.

3.8. Ethical Considerations

Approval to carry out this study was provided by the School of Graduate Studies (SGS) of Maseno University. Ethical approval was obtained from the Kenya Medical Research Institute (KEMRI) Ethical Review Committee in collaboration with the University of Minnesota Internal Review Boards. All participants underwent informed consent in their primary language. This included time to ask all questions regarding the purpose of the study and proposed study procedures and tests. All prospective participants were informed that participation was entirely voluntary and their willingness to participate would in no way affect the care that they received at Bondo Sub County Hospital or any other medical facility. The above testing was included in the activities described to participants during the consent process. None of the above studies altered any clinical care provided to patients an accordingly there was no ethical need to provide the above results to participants or their healthcare providers retrospectively. The study activities were conducted using a non-identifying study number for each participant. The participant's personal identifying information is kept in secured password secured database, accessible only by study personnel with training in ethical human studies research.

3.9. Data Analysis

Optical density (OD) results obtained from ELISA tests we imported into excel and arbitrary units (AU) determined. AU values were calculated by dividing the ODs of test samples by the sum of mean and 3 standard deviation (3SD) of (NACs). AU values ≥1 were considered responders or positives.

. Statistical analyses were done using STATA version 14.1 (Stata Corporation, College Station, TX) and *P*-values≤0.05 were considered significant.

Objective1: *To determine the quantities and prevalence of antibody isotypes (IgG and IgM) in response to Plasmodium falciparum antigens in seropositive and seronegative HIV patients.*

AU values for the antibody isotypesdata were compared between HIV infected and uninfected groups. To do this, the overall AU and responders AU values/levels were compared using Wilcoxon Rank-sum test. Proportions of responders to malaria antigens in HIV infected and uninfected participants were compared using Chi-square.

Objective2: *To determine quantities and prevalence of immunoglobulin G subclasses (IgG1, IgG2, IgG3 and IgG4) in response to Pf antigens*

AU values for the antibody subclasses data were compared in HIV infected and uninfected groups. To do this, the overall AU and responders AU values/levels were compared using Wilcoxon Rank-sum test. Proportions of responders to malaria antigens between HIV infected and uninfected participants were compared using Chi-square.

Objective3*: To determine the correlation of Pf-specific antibody isotypes and subclasses with markers of HIV disease severity and immune activation including CD4$^+$ cell counts, HIV-1 viral load and CRP levels.*

CD4$^+$ counts, CRP and viral load data were imported into STATA program. CD4$^+$ counts of HIV infected participants were categorized into two groups; CD4$^+$ counts ≤200 cells/mL and counts >200 cells/mL by using 1 and 0 codes respectively. Categorization of CD4$^+$ counts into counts≤/> 200 cells/mL groups were based on the counts previously recommended for ARV drug treatments initiation (Gupta *et al.*, 2006). Malaria-specific antibodies in the CD4$^+$ counts categories were compared using Wilcoxon Rank-sum test. The association of *Pf*-specific antibodies with CRP levels and viral loads were obtained by Spearman correlation test.

CHAPTER FOUR
RESULTS

4.1 Clinical Characteristics of Study Participants

This study recruited 190 participants (52 HIV negative and 138 HIV positive) living in Bondo sub-County and obtaining medical care from Bondo sub-County hospital. Malari*a*-specific antibody levels were detected on only 181 (52 HIV negative and 129 HIV positive). Only Age and CRP levels were found to be different when HIV infected and uninfected individuals were compared (P=0.03 and <0.001 respectively). Grouping of HIV clinical stages was done as per WHO recommendation in appendix V. Table 1 below is the demographic characteristics of study participants.

Table 1: Participants' demographic characteristics

	HIV negative	***HIV positive***	***p value***
Number of Participants	52	138	
Age, y, mean (sd)	28.4 (11.5)	32.2 (10.3)	**0.03**
Female sex, n (%)	25 (48.1)	84 (60.9)	0.11
Malaria Positive, n (%)	3 (5.8)	11 (8.0)	0.6
Bed net use, n (%)	43 (84.3)	115 (84.6)	0.97
CRP, g/dL, median (IQR)	0.52 (0.27, 1.15)	4.72 (0.87, 26.12)	**<0.001**
CD4 count (cells/mL), mean, (sd)		328.6 (228.5)	
Participants with CD4 <200, n (%)*		42 (30.7)	
WHOHIV Clinical Stage, n (%)			
Stage 1		36 (26.1)	
Stage 2		55 (39.9)	
Stage 3		44 (31.9)	
Stage 4		3 (2.2)	
HIV-1 Viral load (copies/mL), Median (IQR)		50370 (14,546-198,155)	

Study participants' clinical characteristics. P-value comparing HIV-negative to HIV-positive participants from t-test for means (age and $CD4^+$ counts), chi-square for proportions (female sex, malaria positive, $CD4^+$ counts<200 and WHO HIV clinical Stage), and Wilcoxon-rank Sum test for medians (CRP levels). IQR=Interquartile range: 25^{th} percentile-75^{th} percentile.

4.2. Quantities and Prevalence of Antibody Isotypes in Response to *Pf* Antigens in HIV Infected and Uninfected Adults

This study compared IgM and total IgG responses to AMA-1 and GLURP-R0 in HIV infected and uninfected adults. Medians of overall AU values were compared using Wilcoxon-rank Sum test. Results indicated that there is no difference in total IgG levels against both AMA-1 and GLURP-R0 in HIV infected and uninfected individuals (P=0.99 and 0.12 respectively). On the other hand, IgM levels against both AMA-1 and GLURP-R0 were found to be significantly

higher in HIV infected as compared to uninfected individuals (*P*<0.0001) as shown in Table 2 below.

Table 2: Comparison of overall AU values of total IgG and IgM

	HIV negative (n=52)	***HIV positive (n=129)***	***P value***
IgG-AMA-1, p50 (IQR)	2.74 (1.09-5.06)	2.5 (1.37-4.13)	0.99
IgG-GLURP-R0, p50 (IQR)	0.92 (0.66-1.60)	1.04 (0.74-2.03)	0.12
IgM-AMA-1, p50 (IQR)	0.82 (0.75-0.91)	0.97(0.81-1.24)	**<0.0001**
IgM-GLURP-R0, p50 (IQR)	0.91 (0.81-1.22)	1.00 (0.81-1.29)	**<0.0001**

Total IgG and IgM responses to *Pf*-antigens. *P*-value comparing HIV-negative and HIV-positive participants using the medians of the overall AU values by Wilcoxon-rank Sum test. p50=Median, IQR=Interquartile range: 25th percentile-75th percentile. AMA-1= Apical membrane protein-1 and GLURP-R0= Glutamate-rich protein region 0

This study also investigated total IgG and IgM responders against both AMA-1 and GLURP-R0. Medians of responders antibody levels were compared using Wilcoxon-rank Sum test in HIV infected and uninfected adults. Results shown that there was no difference in the levels of total IgG that responded against AMA-1 and GLURP-R0 (*P*=0.16 and 0.78 respectively). Only IgM responders levels against AMA-1 were found to be significantly higher in HIV infected and uninfected adults (P=0.004) as shown in Table 3 below.

Table 3: Comparison of responders AU values of total IgG and IgM.

	HIV negative (N=52)	***HIV positive (N=129)***	***P value***
IgG-AMA-1, p50 (IQR), n	3.44 (2.01-5.71), 41	3.05 (1.68-4.57), 115	0.16
IgG-GLURP-R0, p50 (IQR), n	1.65 (1.28-3.15), 24	1.70 (1.23-3.47), 71	0.78
IgM-AMA-1, p50 (IQR), n	1.06 (1.02-1.13), 7	1.29 (1.13-1.57), 65	**0.004**
IgM-GLURP-R0, p50 (IQR), n	1.38 (1.14-2.19), 21	1.44 (1.16-2.00), 94	0.66

Total IgG and IgM responses to *Pf*-antigens. *P*-value comparing HIV-negative and HIV-positive participants using the medians of the responders AU values by Wilcoxon-rank Sum test. p50=Median, n=number of responders. IQR=Interquartile range: 25^{th} percentile-75^{th} percentile. AMA-1= Apical membrane protein-1 and GLURP-R0= Glutamate-rich protein region 0

The study also assessed the proportions of IgM and total IgG levels that responded to AMA-1 and GLURP-R0. Comparison of proportions was done by Chi-Square test. No significant difference was observed in the proportion of total IgG responders against both AMA-1 and GLURP-R0 (*P*=0.06 and 0.28 respectively). Proportions of IgM that responders were significantly higher against both AMA-1 and GLURP-R0 in HIV infected compared to uninfected adults (*P*<0.001) as per Table 4 below.

Table 4:Comparison of total IgG and IgM by proportions of responders

	HIV negative (N=52)	***HIV positive (N=129)***	***P value***
IgG-AMA-1, n (%)	41 (79)	115(89)	0.07
IgG-GLURP-R0, n (%)	24(46)	71(55)	0.28
IgM-AMA-1, n (%)	7(13)	65(50)	**<0.001**
IgM-GLURP-R0, n (%)	21(40)	94(73)	**<0.001**

Total IgG and IgM responses to *Pf*-antigens. *P*-value comparing the proportions of HIV-negative and HIV-positive participants total IgG and IgM that responded to *Pf*-antigens by Chi-Square test. n=number of responders. %= percentage of responders. AMA-1= Apical membrane protein-1 and GLURP-R0= Glutamate-rich protein region 0.

4.3. Quantities and Prevalence of Immunoglobulin G Subclasses (IgG1, IgG2, IgG3 and IgG4) in Response to *Pf* Antigens

Comparison of overall AU values/levels for IgG subclasses against AMA-1 and GLURP-R0 in HIV infected and uninfected adults were done using Wilcoxon-rank Sum test (Table 5). Results indicated that IgG1 and IgG3 levels against both AMA-1 and GLURP-R0 were significantly higher in HIV infected compared to uninfected participants ($P<0.0001$ for each). IgG4 levels against only GLURP-R0 was also higher in HIV infected participants compared to HIV uninfected ($P=0.03$). IgG2 levels against both AMA-1 and GLURP-R0 and IgG4 against AMA-1 were significantly not different in HIV infected compared to HIV uninfected participants ($P=0.94$, 0.72 and 0.99 respectively).

In Table 6, responders' median AU values of IgG subclasses against AMA-1 and GLURP-R0 were compared using Wilcoxon-rank Sum test. Results indicated that only IgG1 against AMA-1 and IgG3 against GLURP-R0 were statistically higher and different in HIV infected compared to HIV uninfected (*P*=0.006 and 0.0001 respectively).

Results in Table 7 compared the proportions of IgG subclasses responders' against AMA-1 and GLURP-R0. It was found that proportions of IgG1 and IgG3 that responded to AMA-1 and GLURP-R0 were significantly higher in HIV infected compared to uninfected participants (*P*<0.001).

Table 5: Comparison of overall AU values of Subclasses

	HIV negative (n=52)	***HIV positive (n=129)***	***P value***
IgG1-AMA-1, p50 (IQR)	1.10 (0.94-1.60)	1.68 (1.26-2.23)	**<0.0001**
IgG1-GLURP-R0, p50 (IQR)	0.85 (0.76-1.03)	1.23 (0.95-1.85)	**<0.0001**
IgG2-AMA-1, p50 (IQR)	0.76 (0.72-0.87)	0.77 (0.70-0.89)	0.94
IgG2-GLURP-R0, p50 (IQR)	0.81 (0.75-0.96)	0.80 (0.73-0.98)	0.72
IgG3-AMA-1, p50 (IQR)	1.11 (0.71-2.36)	2.45 (1.4-5.55)	**<0.0001**
IgG3-GLURP-R0, p50 (IQR)	1.40 (1.06-3.22)	2.76 (1.84-5.57)	**<0.0001**
IgG4-AMA-1, p50 (IQR)	1.01 (0.89-1.12)	1.00 (0.90-1.15)	0.99
IgG4-GLURP-R0, p50 (IQR)	0.69 (0.66-0.77)	0.74 (0.69-0.82)	**0.03**

IgG subclasses (IgG1-4) responses to *Pf*-antigens. *P*-value comparing HIV-negative and HIV-positive participants using the medians of the overall AU values by Wilcoxon-rank Sum test.

p50=Median, IQR=Interquartile range: 25th percentile-75th percentile. AMA-1= Apical membrane protein-1 and GLURP-R0= Glutamate-rich protein region 0.

Table 6: Comparison of responders AU values of IgG Subclasses

	HIV negative (N=52)	***HIV positive (N=129)***	***P value***
IgG1-AMA-1, p50 (IQR), n	1.43 (1.18-1.82), 31	1.77 (1.36-2.26), 117	**0.006**
IgG1-GLURP-R0, p50 (IQR), n	1.35 (1.07-2.32), 14	1.54 (1.22-2.10), 90	0.38
IgG2-AMA-1, p50 (IQR), n	1.24 (1.17-1.29), 6	1.18 (1.07-1.44), 24	0.45
IgG2-GLURP-R0, p50 (IQR), n	1.28 (1.14-1.70), 12	1.14 (1.08-1.76), 30	0.50
IgG3-AMA-1, p50 (IQR), n	2.16 (1.50-5.31), 28	2.99 (1.70-6.06), 114	0.33
IgG3-GLURP-R0, p50 (IQR), n	1.56 (1.17-3.89), 42	2.91 (1.93-5.78), 124	**0.0001**
IgG4-AMA-1, p50 (IQR), n	1.12 (1.04-1.32), 27	1.17 (1.06-1.31), 63	0.57
IgG4-GLURP-R0, p50 (IQR), n	1.11 (1.11-1.11), 1	1.17 (1.16-1.18), 2	0.22

IgG subclasses responses to *Pf*-antigens. *P*-value comparing HIV-negative and HIV-positive participants using the medians of the responders AU values by Wilcoxon-rank Sum test. p50=Median, n=number of responders. IQR=Interquartile range: 25th percentile-75th percentile. AMA-1= Apical membrane protein-1 and GLURP-R0= Glutamate-rich protein region 0.

Table 7: Comparison of IgG subclasses by proportions of responders

	HIV negative (N=52)	***HIV positive (N=129)***	***P value***
IgG1-AMA-1, n (%)	31(60)	117(90)	**<0.001**
IgG1-GLURP-R0, n (%)	14(27)	90(70)	**<0.001**
IgG2-AMA-1, n (%)	6(12)	24(19)	0.25
IgG2-GLURP-R0, n (%)	12(23)	30(23)	0.98
IgG3-AMA-1, n (%)	28(54)	114(88)	**<0.001**
IgG3-GLURP-R0, n (%)	42(81)	124(96)	**<0.001**
IgG4-AMA-1, n (%)	27(52)	62(49)	0.71
Ig4-GLURP-R0, n (%)	1(2)	2(2)	0.86

IgG subclasses responses to *Pf*-antigens. *P*-value comparing the proportions of HIV-negative and HIV-positive participants IgG subclasses that responded to *Pf*-antigens by Chi-Square test. n=number of responders. %= percentage of responders. AMA-1= Apical membrane protein-1 and GLURP-R0= Glutamate-rich protein region 0.

4.4. Correlation of *Pf*-specific Antibody Isotypes and Subclasses with Markers of HIV Disease severity and Immune Activation ($CD4^+$ Cell Counts, HIV-1 Viral Load and Plasma CRP Levels).

Results in Table 8 and 9 shown comparison of antibody levels against AMA-1 and GLURP-R0 by $CD4^+$ counts (CD4 Counts>200, CD4 Counts≤200) in HIV infected participants. Only total IgG against AMA-1 for overall AU values and IgG1 against AMA-1 for responders AU values were significantly lower in CD4 Counts≤200 compared to CD4 counts>200 (P=0.01).

Table 8: Comparison of overall AU values by $CD4^+$ count categories

	CD4 Counts>200, n=88	***CD4 Counts≤200, n=40***	***P-value***
AMA-1 IgG, p50 (IQR)	3.18 (1.67-4.58)	1.95 (1.15-3.61)	**0.01**
GLURP-R0 IgG, p50 (IQR)	1.05 (0.75-1.82)	1.02 (0.70-2.35)	0.92
AMA-1 IgM, p50 (IQR)	0.98 (0.82-1.25)	1.01 (0.78-1.35)	0.83
GLURP-R0 IgM, p50 (IQR)	1.24 (0.99-1.80)	1.15 (0.97-1.74)	0.69
AMA-1 IgG1, p50 (IQR)	1.81 (1.26-2.28)	1.45 (1.25-1.85)	0.06
GLURP-R0 IgG1, p50 (IQR)	1.29 (0.94-1.84)	1.22 (0.97-1.86)	0.97
AMA-1 IgG2, p50 (IQR)	0.76 (0.69-0.89)	0.77 (0.71-0.98)	0.36
GLURP-R0 IgG2, p50 (IQR)	0.82 (0.74-0.99)	0.79 (0.73-0.97)	0.65
AMA-1 IgG3, p50 (IQR)	2.85 (1.41-5.64)	2.44 (1.38-5.39)	0.91
GLURP-R0 IgG3, p50 (IQR)	2.89 (1.79-5.78)	2.75 (2.00-4.86)	0.84
AMA-1 IgG4, p50 (IQR)	0.99 (0.88-1.11)	1.03 (0.93-1.27)	0.08
GLURP-R0 IgG4, p50 (IQR)	0.75 (0.70-0.82)	0.73 (0.68-0.76)	0.11

Antibody responses to *Pf*-antigens by $CD4^+$ counts. *P*-value comparing HIV-negative and HIV-positive participants using the medians of the overall AU values by Wilcoxon-rank Sum test. p50=Median, IQR=Interquartile range: 25^{th} percentile-75^{th} percentile. AMA-1= Apical membrane protein-1 and GLURP-R0= Glutamate-rich protein region 0.

Table 9: Comparison of responders AU values by $CD4^+$ count categories

	CD4 Counts>200, N=88	***CD4 Counts≤200, N=40***	***P-value***
AMA-1 IgG, p50 (IQR) n	3.23 (1.72-4.61), 83	2.19 (1.40-3.74), 32	0.12
GLURP-R0 IgG, p50 (IQR) n	1.68 (1.22-3.28), 49	2.22 (1.42-3.59), 21	0.33
AMA-1 IgM, p50 (IQR) n	1.25 (1.13-1.57) 43	1.34 (1.26-1.70) 21	0.47
GLURP-R0 IgM, p50 (IQR) n	1.42 (1.19-2.00), 65	1.43 (1.14-2.13), 28	0.96
AMA-1 IgG1, p50 (IQR) n	1.84 (1.48-2.32), 79	1.53 (1.26-1.89) 37	**0.01**
GLURP-R0 IgG1, p50 (IQR) n	1.46 (1.23-2.10), 62	1.59 (1.22-2.35), 27	0.81
AMA-1 IgG2, p50 (IQR) n	1.15 (1.10-1.27), 14	1.40 (1.05-1.55) 10	0.17
GLURP-R0 IgG2, p50 (IQR) n	1.18 (1.08-1.76), 21	1.13 (1.12-1.204), 9	0.87
AMA-1 IgG3, p50 (IQR) n	3.08 (1.70-6.18), 77	2.64 (1.74-5.56), 36	0.74
GLURP-R0 IgG3, p50 (IQR) n	2.91 (1.84-5.92) 86	2.97 (2.23-5.35), 37	0.77
AMA-1 IgG4, p50 (IQR) n	1.13 (1.06-1.29), 38	1.24 (1.07-1.39), 24	0.29
GLURP-R0 IgG4, p50 (IQR) n	1.17 (1.16-1.18), 2	-0*	-0*

Antibody responses to *Pf*-antigens by $CD4^+$ counts. *P*-value comparing HIV-negative and HIV-positive participants using the medians of the responders AU values by Wilcoxon-rank Sum test. p50=Median, n=number of responders. IQR=Interquartile range: 25^{th} percentile-75^{th} percentile. AMA-1= Apical membrane protein-1 and GLURP-R0= Glutamate-rich protein region 0.

Results in Table 10 and 11 showed correlation results of overall and responders' *Pf*-specific antibodies and viral load as obtained by Spearman correlation. Overall IgM and IgG1 levels against AMA-1 and GLURP-R0 were found to weakly correlate with viral load (*P*=<0.001, 0.02, 0.02 and 0.01 respectively) as in table 10. In table 11, only IgM levels that responded to AMA-1 were found to weakly correlate with viral load (*P*=0.04).

Table 10: Correlation of overall AU values with viral load

n=128	***ρ(rho)***	***P-value***
AMA-1IgM	0.31	**<0.001**
GLURP-R0IgM	0.21	**0.02**
AMA-1IgG1	0.21	**0.02**
GLURP-R0IgG1	0.24	**0.01**
AMA-1IgG3	0.05	0.57
GLURP-R0IgG3	0.14	0.13
AMA-1IgG4	0.09	0.30
GLURP-R0IgG4	0.09	0.33

Correlation of *Pf*-specific antibodies and viral load. *ρ(rho)* values associating HIV- positive participants overall *Pf*-specific antibody levels with viral load by Spearman correlation test. n=number of HIV-positive used for correlation analysis.AMA-1= Apical membrane protein-1 and GLURP-R0= Glutamate-rich protein region 0.

Table 11: Correlation of responders AU values with viral load

N=128	***ρ(rho)***	***P-value***
AMA-1IgM, n=65	0.25	**0.04**
GLURP-R0IgM, n=94	0.10	0.32
AMA-1IgG1, n=116	0.13	0.17
GLURP-R0IgG1, n=89	0.16	0.13
AMA-1IgG3, n=114	0.04	0.66
GLURP-R0IgG3, n=123	0.13	0.16
AMA-1IgG4, n=63	0.08	0.52
GLURP-R0IgG4, n=2	-1.00*	0*

Correlation of *Pf*-specific antibodies and viral load. *ρ(rho*) values associating HIV- positive participants responders-specific antibody levels with viral load by Spearman correlation test. N=number of HIV-positive used for correlation analysis. n=number of responders. AMA-1= Apical membrane protein-1 and GLURP-R0= Glutamate-rich protein region 0.

Results in Table 12 and 13 show correlation results for overall and responders'-specific antibodies against AMA-1 and GLURP-R0 with CRP obtained by Spearman correlation. Overall IgM and IgG1 levels against AMA-1 and GLURP-R0 and IgG3 against AMA-1 were found to weakly correlate with viral load (P=0.01, 0.05, 0.02, 0.004 and 0.05 respectively) as in Table 12. In Table 13, only IgG1 levels that responded to AMA-1 were found to weakly correlate with CRP (P=0.04).

Table 12: Correlation of overall AU values with CRP by HIV status

	HIV Positive, n=129			***HIV negative, n=52***
	ρ(rho)	***P-value***	***ρ(rho)***	***P-value***
AMA-1IgM	0.22	**0.01**	0.13	0.36
GLURP-R0IgM	0.18	**0.05**	0.12	0.39
AMA-1IgG1	0.20	**0.02**	-0.19	0.18
GLURP-R0IgG1	0.25	**0.004**	0.03	0.85
AMA-1IgG3	0.17	**0.05**	-0.10	0.50
GLURP-R0IgG3	0.16	0.07	0.04	0.79
AMA-1IgG4	0.08	0.36	0.09	0.52
GLURP-R0IgG4	0.01	0.89	-0.12	0.38

Correlation of *Pf*-specific antibodies with C-reactive protein (CRP). *ρ(rho*) values associating HIV- positive and negative participants overall *Pf*-specific antibody levels with CRP levels by Spearman correlation test. AMA-1= Apical membrane protein-1 and GLURP-R0= Glutamate-rich protein region 0.

Table 13: Correlation of responders AU values with CRP by HIV status

	HIV Positive			HIV negative	
	ρ(rho)	***P-value***		***ρ(rho)***	***P-value***
AMA-1IgM, n=65	0.21	0.09	AMA-1IgM, n=7	-0.14	0.76
GLURP-R0IgM, n=94	0.17	0.11	GLURP-R0IgM, n=21	0.22	0.34
AMA-1IgG1, n=117	0.19	**0.04**	AMA-1IgG1, n=31	0.05	0.80
GLURP-R0IgG1, n=90	0.14	0.19	GLURP-R0IgG1, n=14	0.29	0.32
AMA-1IgG3, n=114	0.14	0.15	AMA-1IgG3, n=28	-0.13	0.50
GLURP-R0IgG3, n=124	0.12	0.17	GLURP-R0IgG3, n=42	-0.05	0.77
AMA-1IgG4, n=63	-0.22	0.08	AMA-1IgG4, n=27	0.11	0.57
GLURP-R0IgG4, n=2	-1.00	-	GLURP-R0IgG4, n=0	-	-

Correlation of *Pf*-specific antibodies with C-reactive protein (CRP). *ρ(rho*) values associating HIV- positive and negative participants responders-specific antibody levels with CRP levels by Spearman correlation test. n=number of responders. AMA-1= Apical membrane protein-1 and GLURP-R0= Glutamate-rich protein region 0.

CHAPTER FIVE

DISCUSSION

5.1 The Quantities and Prevalence of Antibody Isotype in Response to *Pf* Antigens in HIV Infected and Uninfected Adults.

Previously, immunoglobulin levels against specific malaria antigens have been linked with the reduction of malaria incidence (Dodoo *et al.*, 2008) confirming the earlier finding that Immunoglobulin G confers adaptive immunity against malaria (Cohen *et al.*, 1961). The first objective of this study was to determine if HIV infections was associated with a change in the prevalence and level of IgG and IgM isotypes targeting key malaria antigens, AMA1 and GLURP-R0.

This study found that HIV infected individuals have a higher titer of IgM targeting two key *Pf* antigens, AMA-1 and GLURP-R0, when compared to uninfected individuals ($P<0.0001$). When, comparing the proportions of individuals that are IgM "responders" (ELISA optical density greater than three SD above unexposed North American OD), there was also a significantly higher proportion of HIV infected individuals who were IgM responders to both AMA-1 and GLURP-R0 when compared to HIV uninfected individuals (*P* value <0.001). The results of the current study were consistent with non-malaria immunoglobulin profile findings obtained from HIV infected individuals (Lugada *et al.*, 2004; Lyamuya *et al.*, 1994; Mizuma *et al.*, 1988). In each of these studies, HIV was found to result in higher IgM levels compared to uninfected individuals. The increased levels of *Pf*-specific IgM in HIV infection are a clear indication that HIV alters malaria specific antibody responses.

The mechanism behind increased malaria antigen specific IgM is unclear. It is possible that in the setting of CD4 lymphopenia, the increase in IgM reflects memory B cells generated in

a germinal center independent pathway. These GC independent B cells are preferentially unswitched B cells in mouse models (Taylor *et al.*, 2012). Another consideration are HIV mediated derangements in the function of Tfh cells, the T cell subset that helps in the affinity maturation and switching of B cells in the germinal center reaction (Cubas *et al.*, 2013; Lindqvist *et al*., 2012).

In relation to *Pf* infection and severity, it is not clear whether increased IgM targeting malaria antigens is protective in the setting of HIV or if it leads to increased malarial disease. Several studies have demonstrated that IgM antibodies are important in the protection against numerous infections (Ehrenstein and Notley, 2010). In malaria specifically, IgM provides protection through opsonization and activation of the classical complement pathway, which mediates neutralization of malaria parasite and phagocytosis (Ehrenstein and Notley, 2010; Kinyanjui *et al*., 2003; Stanisic *et al*., 2015). Immunoglobulin M has been found to enhance malaria pathogenesis as well as immunity (Pleass *et al*., 2016). Pathogenesis of malaria is due to the fact that IgM promotes the formation of rosette, hence enabling the adherence between infected and uninfected red blood cells (Clough *et al*., 1998; Pleass *et al*., 2016). Studies on HIV and malaria co-infection have found out that HIV leads to frequent episodes and increased severity of malaria (González *et al*., 2012; Whitworth *et al*., 2000). Immunoglobulin M was linked with malaria pathogenesis and this increase in *Pf*-specific IgM levels in HIV infected participants may be a mechanism used by HIV to cause severe and frequent malaria episodes.

Another aspect that was also investigated was the response of total IgG against *Pf*-specific antigens. Previous studies demonstrated that levels of total IgG against specific malaria antigens correlate with reduction of malaria incidence (Dodoo *et al.*, 2008) confirming the early

observations that IgG confers immunity against clinical malaria (Cohen *et al*., 1961). Apical membrane antigen1 and GLURP-R0 are among a group of antigens which have been correlated with protection against clinical malaria in field studies and are being evaluated as targets for future malaria vaccine candidates (Barry and Arnott, 2014; Schwartz *et al*., 2012). This study found no significantly different total IgG levels against AMA-1 and GLURP-R0 in HIV infected compared to HIV uninfected individuals (P=0.99 and 0.12 respectively). These results were consistent with preliminary findings for the same study conducted in Bondo that found no difference in the levels of total IgG against nine of ten tested *Pf* antigens in HIV infected and uninfected participants (unpublished data). In light of this, the clinical significance of total IgG levels in HIV infected individuals is unclear the results may imply that total IgG may not play any role in malaria pathogenesis or total IgG antibodies equally protects HIV infected and uninfected individuals.

5.2 Quantities and Prevalence of Immunoglobulin G Subclasses (IgG1, IgG2, IgG3 and IgG4) in Response to *Pf* Antigens.

It was found that IgG1 and IgG3 levels were higher in HIV infected compared to uninfected individuals for both AMA-1 and GLURP-R0 (P<0.0001Previous studies found that IgG3 mediates protection against malaria more than IgG1 (Jafarshad *et al*., 2007; Tebo *et al*., 2001). Also, IgG3 was found to be better for protection against HIV-1 and this is because its flexible hinge region compared to IgG1 (Scharf *et al*., 2001). The levels of IgG1 and IgG3 were found to be high in malaria protected individuals(Bouharoun-Tayoun *et al*., 1995; Bouharoun-Tayoun and Druilhe, 1992). Despite the protection offered by IgG3 towards malaria, its high levels in HIV infected individuals were linked with B cell perturbations (Béniguel *et al*., 2004; Tomaras and

Haynes, 2009). The mechanism behind the raised levels of IgG1 and IgG3 in this study warrants further investigation as it is clear from these findings that HIV doesn't hinder class switching of antibodies. Also, it is not known whether increased levels of IgG1, and IgG3 antibodies against malaria antigens in HIV infected individuals are functionally equivalent to those produced by the HIV uninfected host. For example, they may not have the same affinity for malaria antigens due to HIV mediated dysregulation of the germinal center and Tfh cells given the evidence that HIV infection interferes with phagocytosis mediated by opsonization of IgG1 and IgG3 (Keen *et al*., 2007).

Immunoglobulin G2 and IgG4, also called non-cytophilic on the other hand were found to predominate in children and malaria unprotected adults (Bouharoun-Tayoun and Druilhe, 1992; Ferreira *et al*., 1998; Wahlgren *et al*., 1983). The study also found low reactivity of IgG2 and IgG4 (non-cytophilic antibodies) against AMA-1 and GLURP-R0 in both HIV infected and uninfected participants. The current IgG2 and IgG4 findings are in agreement with the previous findings where low *Pf*-specific IgG2 and IgG4 were reported (Stanisic *et al*., 2009). Despite low levels, IgG4 specific to GLURP-R0 was found to be higher in HIV infected compared to HIV uninfected participants (P=0.03). Looking into immune function and switching, IgG4 is believed to be switched with the help of IL-10 linking this antibody to immunosuppressive roles (Aalberse *et al*., 2009). Elevated IgG4 levels in HIV infected individuals may denote inhibitory mechanisms used by HIV to suppress host immune responses to malaria.

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5.3 The Correlation of *Pf*-specific Antibody Isotypes and Subclasses with Markers of HIV Disease Severity and Immune Activation ($CD4^+$ Cell counts, HIV-1 Viral Load and Plasma CRP Levels).

Previously studies have shown that $CD4^+$ cells function in the development and maturation of memory B and plasma cells (Claman *et al*., 1966; Kelsoe, 1995; Liu *et al*., 1996; MacLennan, 1994). However, it has been shown that HIV affects Tfh numbers and localization, the principle $CD4^+$ T cell that help in the process of B cell development (Cubas *et al*., 2013; Lindqvist *et al*., 2012) and this may interfere with antibody responses against malaria. In addition, previous studies reported that $CD4^+$ cells and antibodies are important in malaria control (Stevenson and Riley, 2004).

In this study, CD4+ counts were categorized into two groups ($CD4^+$ counts>200 cells/mL and $CD4^+$ counts $\leq$ 200 cells/mL) and *Pf*-specific antibodies detected compared. Significant difference in the levels in the two $CD4^+$ groups were observed in total IgG and IgG1 against AMA-1 antigen (*P*=0.01) for overall and responders antibody levels respectively and lower in $CD4^+$ counts$\leq$ 200 cells/mL. Levels of these antibodies were lower in patients with $CD4^+$ counts $\leq$200 cells/mL compared to those with $CD4^+$ counts>200 cells/mL. Though the other *Pf*-specific antibodies did not show statistically significant difference in the $CD4^+$ counts categories, majority of them showed reduction in response against *Pf*-specific antibodies in $CD4^+$ counts $\leq$200 cells/mL suggesting that suppression of antibody responses in HIV infected individuals may therefore depend on $CD4^+$ counts

The lower responses of total IgG and IgG1 against AMA-1, a key blood stage antigen, in low $CD4^+$ counts ($CD4^+$ $\leq$200 cells/mL) obtained from this current study may account for the high malaria parasitemia levels previously observed in low $CD4^+$ counts amongst HIV infected

participants (French *et al*., 2001; Iroezindu *et al*., 2012; Laufer *et al*., 2006; Subramaniam *et al*., 2015; Whitworth *et al*., 2000). Studies demonstrating a decreased breadth in overall antibodies responses to malaria antigens may explain this variability when examining selected antigens (Subramaniam *et al*., 2015). In the absence of defined correlates of protection for clinical malaria it is difficult to interpret the significance of each individual serologic result.

Also investigated in the current study was the association of *Pf*-specific detected antibodies with HIV-1 viral load. Past studies hypothesized that continuous viral replication is responsible for immune activation leading to B cell immunopathogenesis observed in HIV infected individuals (De Milito, 2004; Moir and Fauci, 2009; Sodora and Silvestri, 2008). Reportedly, high viral load has been associated with low $CD4^+$ cell counts and increased immature B cells (Moir and Fauci, 2009). In addition, similar studies reported that extrinsic apoptotic B cell death was induced by HIV viremia (Giri *et al*., 2006; Herbeuval and Shearer, 2007). From the current findings, reported that there were positive associations in IgM and IgG1 levels to specific *Pf* antigens with the viral load. These suggest that the levels of the antibodies (IgM and IgG1) are influenced by HIV viral load. There is an evidence that high viral load leads to an increased immature B cells (Moir and Fauci, 2009) though the association between levels of immature B cells and antibody production warrants further investigation.

Lastly, this objective also investigated the association of *Pf*-specific antibodies with the CRP levels. The study found that IgM and IgG1 levels against AMA-1 and GLURP-R0, and IgG3 against AMA-1 were weakly correlated with CRP. The level of CRP in the plasma/serum denotes an inflammatory response as a result of an infection (Clyne and Olshaker, 1999). Elevated levels of CRP in HIV infected compared to HIV uninfected participants from the

current study were clear indication that HIV influences body's inflammatory response. Previous studies found that CRP is a marker of HIV progression independent of $CD4^+$ counts and viral load (Lau *et al*., 2006) and a number of morbidity and mortality cases have been reported as a result of HIV elevated CRP levels (Borges *et al*., 2014; Drain *et al*., 2007; Feldman *et al*., 2003; Kuller *et al*., 2008). The association between high CRP levels with high viral loads and low $CD4^+$ counts (Lau *et al*., 2006) suggest that CRP plays a role in immunosuppression. Despite the findings that IgM, IgG1 and IgG3 specific to malaria were correlated with CRP levels in HIV infected individuals, further studies should find out whether the antibodies that correlated with CRP levels are immunosuppressed.

CHAPTER SIX

SUMMARY OF THE FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of Study Findings

This study compared IgM and total IgG responses to AMA-1 and GLURP-R0 in HIV infected and uninfected adults. Results indicated that there is no difference in total IgG levels in HIV infected and uninfected individuals. On the other hand, IgM levels were found to be significantly higher in HIV infected compared to uninfected individuals. Immunoglobulin G subclasses results obtained show that only IgG1 and IgG3 levels against both AMA-1 and GLURP-R0 were higher in HIV infected compared to uninfected participants. Comparison of *Pf*-specific by $CD4^+$ counts (CD4 Counts>200, CD4 Counts≤200 cells/mL) showed that only total IgG and IgG1 were different. Correlation results show that IgM and IgG1 levels against AMA-1 and GLURP-R0 were found to associate with viral load. Also, IgM and IgG1 levels against AMA-1 and GLURP-R0 and IgG3 against AMA-1 were found to associate with CRP.

6.2: Conclusions

i. Infection with HIV leads to elevation in the levels of IgM but not total IgG against AMA-1 and GLURP-R0 antigens.

ii. HIV infection leads to an increase in the levels of IgG1 and IgG3 but not IgG2 levels against both AMA-1 and GLURP-R0 antigens. Also, HIV elevates IgG4 levels against GLURP-R0 only.

iii. $CD4^+$ counts ≤200 is associated with lower total IgG and IgG1 levels against AMA-1 antigen and IgM and IgG1 levels against AMA-1 and GLURP-R0 are weakly correlated with CRP levels and VL.

6.3: Recommendations From This Study

i. More studies should be done using multiple malaria antigens and not only AMA-1 and GLURP-R0 to find out if malaria-specific IgM levels are increased in HIV infected individuals.

ii. More studies should be done using multiple malaria antigens and not only AMA-1 and GLURP-R0 to investigate if malaria-specific IgG1, IgG3 and IgG4 levels are elevated in individuals infected with HIV.

iii. HIV infected individuals with $CD4^{+}$<200 should be given malaria prophylaxis

6.4: Recommendations For Future Studies

i. Future studies should assess mechanisms through which HIV increases the levels of IgM, IgG1 and IgG3 antibodies and determine how the increased levels correlate with malaria risk in HIV infected populations

ii. Future studies should investigate whether HIV increases the overall levels of IgM, IgG1 and IgG3 antibodies and not just the ones specific to *Pf* antigens.

iii. Future studies should look at functional responses and find out whether the increased *Pf*-specific antibody levels observed in IgM, IgG1, and IgG3 improve or impair their function.

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APPENDICES

APPENDIX I: ETHICAL APPROVAL

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 **August 5, 2015**

TO: **ANNE E. P. FROSCH,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. STEPHEN MUNGA,**
THE DIRECTOR, CGHR,
KISUMU

Dear Madam,

RE: **SSC PROTOCOL No. 2161 (*REQUEST FOR ANNUAL RENEWAL*): THE EFFECT OF HIV INFECTION ON MALARIA SPECIFIC T AND B CELL RESPONSES**

Thank you for the continuing review report for the period **June 2014** to **June 2015.**

This is to inform that during the 241st C meeting of the KEMRI/Scientific and Ethics Review Unit (SERU) held on **30th July, 2015**, the Committee **conducted the annual review and approved** the above referenced application for another year.

This approval is valid from **August 19, 2015** through to **August 18, 2016**. Please note that authorization to conduct this study will automatically expire on **August 18, 2016**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to SERU by **July 7, 2016.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to SERU for review prior to initiation.

You may continue with the study.

Yours faithfully,

FOR

PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

APPENDIX II: STUDY APPROVAL

MASENO UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: PG/MSC/00039/2013

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 15th February, 2016

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR ONYANGO E. ODHIAMBO— PG/MSC/00039/2013

The above named is registered in the Master of Science in Medical Microbiology Programme of the School of Public Health and Community Development, Maseno University. This is to confirm that his research proposal titled "Antibody Responses to Plasmodium Falciparum Antigens among HIV Patients in Bondo Sub County Hospital, Western Kenya" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

MASENO UNIVERSITY 18 FEB 2016

Prof. P.O. Owuor
DEAN, SCHOOL OF GRADUATE STUDIES

Maseno University *ISO 9001:2008 Certified*

APPENDIX III: CONSENT FORM

Appendix I: Consent Form, English Study number __ -__ __ - __ __ __ __ - __ __ - __

UMN/KEMRI Consent for Human Investigational Studies
Study Title: HIV and Malaria Interactions Study
Principal Investigator: Anne E.P. Frosch

Purpose: This is a malaria research study funded by the American Society of Tropical Medicine and Hygiene that examines how HIV infection affects people's protective response to malaria infection. Dr. Anne Frosch from University of Minnesota (UMN) in the USA and her colleagues at the Kenya Medical Research Institute (KEMRI) are asking your permission to participate in this research study. Your participation will involve collection of blood samples from you. We will use this blood to examine how your body protects against or responds to malaria infection. People eligible for this research study include individuals 18 year of age or older who are being seen at the HIV testing center at Bondo district hospital. Both HIV negative and HIV positive individuals are eligible.

Procedure: If you agree to participate, a clinical officer at Bondo district hospital will ask you a few questions about how you are feeling today and what medications you are taking. If you are eligible for the study, we will take 3 tablespoons of blood (40mL). We will use a sterile needle and tube to collect the blood after cleaning it with an alcohol swab. With this blood, we will check if you have malaria infection and for pregnancy (in women). We will also ask you to provide a stool sample to test for other infections that may make you sick. With your permission, we will provide these results to you and your doctor and give you the treatments as per the guidelines of the ministry of health Kenya. Some of the blood will be transported to the UMN/KEMRI laboratory in Kisumu. Tests done in the laboratory will tell us how well your body is protecting itself against malaria (i.e. cytokine and antibody responses. If you have HIV, we will also check your CD4 count which is a measure of the strength of your immune system and your viral load, which is a measure of how active the HIV is in your body right now. We will also store some blood for further testing on how your body fights malaria. There are no expenses involved in participating in the study. Your participation can end if you decide to end it or if the study decides to end it. We anticipate the participation of ~150 people in Bondo in this study. You will be informed promptly if information becomes available that may be relevant to your willingness to participate.

With the exception of malaria or parasitic stool infection diagnosed in the study, the study team will not provide your healthcare relating to other acute or chronic illnesses including HIV. This will be done by the local government health facility, Bondo Health Center, which provides care for these conditions according to the national guidelines.

Risks and Benefits
There are minimal risks involved in this study. The primary risk of blood collection is mild pain at the site, which lasts a few seconds. Less common reactions include bleeding, persistent pain, bruising and infection. All of these are uncommon events that have occurred in very few adults previously studied by this research group.

UMN/KEMRI Consent for Human Investigational Studies
Study Title: HIV and Malaria Interactions Study
Principal Investigator: Anne E.P. Frosch

As part of this study, you may be diagnosed with a parasitic infection such as malaria or an intestinal worm infection. If you are found to have an infection, you will be treated by the clinic and the study will cover payment of any clinic treatment expenses. Overall, we believe this is a benefit for participants because these infections can cause negative health consequences even without symptoms. However, it is possible that you will have side effects to the medicine used to treat these infections.

Finally, the results of this study will also benefit your community by helping us understand whether people with HIV need special care to treat and prevent malaria.

Compensation
You will receive Ksh800 for participation in this study. This amount will defray the cost of travel to and from the Bondo District Hospital.

Confidentiality
All information that we collect will be kept confidential, including your participation in the study, your answers to all questions and the results of tests we conduct on your blood. The results of the studies using your blood will be assigned a study number to preserve confidentiality. The principal investigator and relevant key personnel will keep a database linking your personal identifiers to the study number. Only study personnel and study monitors, auditors and institutional review boards will be allowed access to the medical information collected in this study. With your permission, we will share the results of your blood and stool tests with your doctor to help with your clinical care. Your doctor or nurse is also obligated to keep this information confidential.

Summary of your rights as a participant in a research work.
Your participation in this research is voluntary. Refusing to participate will not involve any penalty and you will receive normal care. If you decide to enroll in this study, you can withdraw yourself anytime you want without giving a reason. If information generated in the study is published or presented, your identity will not be revealed. If you experience physical injury or illness as a result of participating in this research study or you have any other concerns about your participation in the study, contact Dr. Anne Frosch (principal investigator) or her designate, Dr. George Ayodo. They can be reached at The Centre of Global Health and Child Development-Kenya, P.O.Box 130, 40100, Kisumu, or at 0737 773 914. The center is located on the northeastern shores of Lake Victoria, along the Kisumu-Busia road, about 11 km away from Kisumu.

Contact information: _____ has described to you what is going to be done, the risks, hazards, and benefits involved, and can be contacted at _____.

Further information on research subject's rights is available from: The Secretary, KEMRI Ethics Review Committee (ERC), PO Box 54840-00200, Nairobi. Tel: 020-2722541 or

UMN/KEMRI Consent for Human Investigational Studies
Study Title: HIV and Malaria Interactions Study
Principal Investigator: Anne E.P. Frosch

0722205901 or 0733400003; Email address: ERC@kemri.org or the Director of Kemri, PO Box 54840, Nairobi at 020-2722541.

Signature

Signing below indicates that you have been informed about the research in which you voluntarily agree to participate; that you asked any question about the study; and that the information given to you has permitted you to make a fully informed and free decision about your participation in the study. By signing this consent form, you do not waive any legal rights, and the investigators are not relieved of any liability they may have. You can withdraw from this study any time. You will be offered a copy of this consent form and it will be provided to you if you would like to have one.

Printed name of the participant

Signature or fingerprint of Participant

If the participant is unable to read and or write, a related witness should be present during the informed consent discussion. After the written informed form is read and explained to the participant, and after they have orally consented to their participation in the study, and have either signed the consent form, the witness attests that the information in the consent form and other written information were explained to and understood by the participant.

Name of the Person Witnessing Consent (Printed)

Signature of the Person Witnessing Date /Time

----------Date---------- ----------

Signature of the Clinician obtaining consent Printed Name

---------- Date ----------

Signature of the Principal Investigator (Affirming subject eligibility for the study and informed consent has been obtained.

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UMN/KEMRI Consent for Human Investigational Studies
Study Title: HIV and Malaria Interactions Study
Principal Investigator: Anne E.P. Frosch

Genetic studies: Our bodies are made of small building blocks called cells. These contain a substance called DNA, which codes for each body's unique instructions. Some of the molecules or signals in your body that are used to fight infections are coded for by these instructions. Because we are interested in studying how your body fights malaria, we may want to test your DNA for traits important to this. These are called genetic tests. For most of these tests, the results have no known direct implication on your health. For some of these tests, we know that certain traits are associated with autoimmune diseases such. These are diseases in which sometimes your immune response, which protects your body from infection is too strong, and can be directed at your own tissues. Rheumatoid arthritis is an example of one of these disease. However, none of the tests that we will do will tell you if you will get any disease. Because of this, results from these tests will not be given back to you or put into your medical records. We will only test your DNA for traits that tell us how your body fights infection and we will request permission for any genetic studies from the Institutional Review Board (IRB) at UMN and the KEMRI Scientific Steering Committee (SSC) and Ethical Review Committee (ERC).

We would also like to be able to test your blood for malaria DNA, these are the instructions inside the malaria parasite that codes for its makeup and function. This will tell us about how the malaria causes infection and protects itself from your body's attempts to get rid of it. The results of these tests should not affect you directly because kind of malaria infection that we are examining is a temporary infection and you will have cleared the infection by the time these studies are done.

If you do not agree to have your blood stored for genetic testing, we will destroy the sample. If, at any time you wish to withdraw your agreement, please contact Dr. George Ayodo (Box 1578, Kisumu or at 0737 773 914) and we will destroy the sample. If you do not wish to have your blood stored for genetic testing, you may still participate in this study. You will still be examined for defenses against malaria. If you have malaria, we will still treat you.

If you agree to future genetic testing, circle "YES". If you do not agree, circle "NO"

YES NO

Participant Signature*_____ Date _____

Witnessed by_____ Date_____
*A participant/parent can sign, or verbally state his/her consent in the presence of a witness who will then sign.

****
****

UMN/KEMRI Consent for Human Investigational Studies
Study Title: HIV and Malaria Interactions Study
Principal Investigator: Anne E.P. Frosch

Long-term storage and future studies: It is possible that we will discover other important factors that help us understand how your body fights malaria. We would like to store the blood that is left over from the tests we describe here for up to 10 years to look for some of these other factors. We will only use your blood to examine how your body fights malaria. A portion of your sample will be sent to the University of Minnesota in the USA for testing that cannot be done in Kenya as far as this study is concern. Otherwise, your samples will be stored at the Center for Global Health Research in Kisumu.

I understand that I have the right to withdraw my agreement to use my blood for future research anytime and for any reason. If I withdraw my agreement to use my blood for future research testing, the samples will be destroyed. I may also ask that my blood not be used for certain types of testing. To do this, I may tell Dr. George Ayodo (Box 1578, Kisumu or at 0737 773 914) of my request and he will tell the study people at UMN/KEMRI. I understand that the UMN IRB and KEMRI SSC and ERC must approve any future testing not described here.

I agree for UMN/KEMRI to store my blood for future studies of factors that may protect against malaria.

If you agree to have your blood sample stored, circle "YES". If you do not agree, circle "NO"

YES NO

If you agree to have a portion of you sample shipped to the U.S., circle "YES". If you do not agree, circle "NO"

YES NO

Participant Signature*_____ Date _____

Witnessed by_____ Date_____
*A participant/parent can sign, or verbally state his/her consent in the presence of a witness who will then sign.

APPENDIX IV: ENROLMENT AND SCREENING FORM

2754222127

HIV/MALARIA STUDY
HM1 - SCREENING AND ENROLLMENT REPORT FORM

Form Number

SECTION A: IDENTIFICATION /DEMOGRAPHIC DETAILS

Field Asst ID | Participant's Initials (F.M.L.) | Enrollment Date (dd/mm/yyyy) | Bondo Hospital Medical Record Number | Patient ID | Sex

Sex: ○ Male ○ Female

SECTION B: HIV HISTORY

B.1 Date of HIV test (dd/mm/yyyy) ___ / ___ / ___

B.2 What is the HIV status of the subject (based on the testing that the Bondo Clinic conducted)?

○ Negative ⟶ if negative, proceed to Section 3

○ Positive ⟶ if positive, complete B.3, B.4 below anc complete form HM5

B.3. Has the subject ever taken medications for the treatment of HIV?

○ No ○ Yes ⟶ if yes, stop, subject not eligible (Complete Section F)

B.4 What is the WHO clinical stage of the patient? (Use WHO staging sheet to determine the stage)

○ 1

○ 2

○ 3

○ 4

SECTION C: CLINICAL HISTORY

C.1. Has the subject received an antimalarial or cotrimoxazole in the past 2 weeks including Coartem (LA), Cotrimoxazole/Bactrim, Fansidar (SP), Chloroquine (CQ), Amodiaquine (AQ), Quinine (QN) or another antimalaria?

○ No ○ Yes ⟶ if yes, mark specific medication, subject not eligible go to Section F)

- ○ LA
- ○ Cotrim/Septra/Bactrim
- ○ SP
- ○ CQ
- ○ AQ
- ○ QN
- ○ Other, Specify_____ (review with PI)

C.2. Do you have any of the following conditions (diabetes, COPD, asthma, emphysema, chronic bronchitis, cancer/leukemia, inflamatory bowel disease, Crohns disease, ulcerative colitis, liver disease, hepatitis, cirrhosis)?

○ No ○ Yes ⟶ if yes, mark specific condition, subject not eligible go to Section F)

- ○ Diabetes
- ○ Chronic obstructive pulmonary disease (Asthma, Emphysema, Chronic Bronchitis)
- ○ Cancer/Leukemia
- ○ Inflamatory bowel disease (Crohns Disease or Ulcerative Colitis)
- ○ Liver Disease (Hepatitis, Cirrhosis)
- ○ Other, Specify_____ (review with PI)

C.3. Does the patient have any of the feel ill today with cough, fever, chills, diarrhea, headache, abdominal pain or jaundice?

○ No ○ Yes ⟶ if yes, mark specific symptoms, subject not eligible go to Section F)

Mark specific symptoms

- ○ Cough
- ○ Fever or Chills
- ○ Diarrhea
- ○ Headache
- ○ Abdominal pain
- ○ Jaundice
- ○ Other, Specify_____

C.4. Did you sleep under a bed net last night?

○ No ○ Yes

SECTION D: CLINICAL EXAMINATION

D.1. Axillary temperature: ☐☐.☐ °C **if temperature is above 37.5 (99.5), subject not eligible, go section F**

D.2. Jaundice:

○ none

○ mild → if jaundice present, subject not eligible go to Section F

○ deep → if jaundice present, subject not eligible go to Section F

SECTION E: PREGNANCY TESTING, if male proceed to section F

E.1. Pregnancy test result

○ Negative

○ Positive → if positive, subject not eligible go to Section

SECTION F: STUDY ELIGIBILITY

F.1. Are eligibility criteria met?

○ Yes → if yes,proceed with blood collection

○ No → if no, go to section H

SECTION G: RDT RESULTS

G.1. RDT Results

○ Negative

○ Positive

SECTION H: CLINICAL MANAGEMENT

H.1. Did the subject require referral to clinical team

○ No

○ Yes → if yes, mark specific reason

- ○ Symptoms of acute illness
- ○ Fever
- ○ Jaundice
- ○ Positive pregnancy test
- ○ Positive RDT
- ○ Other, Specify_____

H.2. Did the subject agree to be seen by clinical team?

○ No

○ Yes → if yes, briefly describe treatment plant by clinical team

APPENDIX V: HIV CLINICAL STAGING

2219202604

HIV/MALARIA STUDY
HM5 - WHO CLINICAL STAGING OF HIV/AIDS FOR ADULTS AND ADOLESCENTS

Form Number

Field Asst ID | Participant's Initials (F.M.L.) | Enrollment Date (dd/mm/yyyy) | Bondo Hospital Medical Record Number | Patient ID | Sex: ○ Male ○ Female

Clinical Stage1

○ No ○ Yes → if yes, mark symptoms for stage 1

- ○ 1A. Asymptomatic
- ○ 1.B. Persistent generalized lymphadenopathy

Clinial Stage 2

○ No ○ Yes → if yes, mark symptoms for stage 2

- ○ 2A. Moderate unexplained weight loss (<10% of presumed or measured body weight
- ○ 2B. Recurrent respiratory infections (sinusitis, tonsillitis, otitis media, and pharyngitis)
- ○ 2C. Herpes zoster
- ○ 2D. Angular cheilitis
- ○ 2E. Recurrent oral ulceration
- ○ 2F. Papular pruritic eruptions
- ○ 2G. Seborrheic dermatitis
- ○ 2H. Fungal nail infections

Clinical Stage 3

○ No ○ Yes → if yes, mark symptoms for stage 3

- ○ 3A. Moderate unexplained weight loss (<10% of presumed or measured body weight
- ○ 3B. Unexplained chronic diarrhea for >1 month
- ○ 3C. Unexplained persistent fever for >1 month (>37.6 oC, intermittent or constant)
- ○ 3D. Persistent oral candidiais (thrush)
- ○ 3E. Oral hairy leukoplakia
- ○ 3F. Pulmonary tuberculosis (current)
- ○ 3G. Severe presumed bacteria infections (eg pneumonia, empyema, pyomyositis, bone or joint infection, menengitis, bacteremia
- ○ 3H. Acute nectorizing ulcerative stomatistis, gingivitis, or periodontitis
- ○ 31. Unexpected anemia (hemoglobin<8g/dL)
- ○ 3J. Neutropenia (neutrophils<500 cells/uL)
- ○ 3K. Chronic thrombocytopenia (platelets <50,000 cells/uL)

Clinical Stage 4

○ No ○ Yes → if yes, mark symptoms for stage 4

- ○ 4A. HIV wasting syndrome, as defined by CDC (Involuntary weight loss>10% of the baseline body weight) associated with either chronic diarrhea
- ○ 4B. Pneumocystis pneumonia
- ○ 4C. Recurrent severe bacteria pneumonia
- ○ 4D. Chronic herpes simplex infection (orolabial, genital, or anorectal site for >1 month or visceral herpse at any site
- ○ 4E. Esophageal candidiasis (or condidiasis of trachea, bronchi, or lungs)
- ○ 4F. Extrapulmonary tuberculosis
- ○ 4G. Kaposi sarcoma
- ○ 4H. Cytomegalovirus infection (retinitis or infection of other organs)
- ○ 4I. Central nervous system toxoplasmosis
- ○ 4J. HIV encephalopathy
- ○ 4K. Crytococcosis, extrapulmonary (including meningitis)
- ○ 4L. Disseminated nontuberculosis Mycobacteria infection
- ○ 4M. Progressive multifocal leukoencephalopathy
- ○ 4N. Candida of the trachea, bronchi, or lungs
- ○ 4O. Chronic crytosporidiosis (with diarrhea)
- ○ 4P. Chronic isosporiasis
- ○ 4Q. Disseminated mycosis (eg histoplasmosis, coccidioidomycosis, penicilliosis)
- ○ 4R. Recurrent nontyphoidal Salmonella bacteremia
- ○ 4S. Lymphoma (celebral or B-cell non-Hodgkin)
- ○ 4T.Invasive cervial carcinoma
- ○ 4U.Atypical disseminated leishmaniasis
- ○ 4V.Symptomatic HIV-associated nephropathy
- ○ 4W.Symptomatic HIV-associated cardiomyopathy

HM5 WHO Clinical Staging of HIV/AIDS Form_V2 31/05/2012

Date edited | Initials | Date coded | Initials | Date verified | Initials