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Analysis of Humoral Immune Response to a Panel of *Plasmodium falciparum*
Blood-Stage Vaccine Candidate Antigens in Naturally Primed Populations in
Seasonal Malaria Settings in Ethiopia

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A Thesis Submitted to
The Department of Microbial, Cellular and Molecular Biology

Presented in Fulfillment of the Requirements for the Degree of Doctor of
Philosophy (Biomedical Sciences)

Addis Ababa University

Addis Ababa, Ethiopia

June 2012

ABSTRACT

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In Ethiopia, the general population is quite vulnerable to unpredictable cyclic epidemics of *Plasmodium falciparum* malaria. However, there is very little information on the anti-malaria immune profile of the population residing in the endemic regions of the country. This study was designed to investigate the nature of humoral immune response to malaria in two population groups in two endemic localities, Shewa Robit in the north and Boditi in south. In a cross-sectional study, the study participants were diagnosed for malaria infection microscopically and by the rapid diagnostic test (RDT). The sera were tested by using enzyme-linked immunosorbent assay (ELISA) for total immunoglobulin (Ig) G against *P. falciparum* blood-stage vaccine candidate antigens: apical membrane antigen 1 (AMA1), glutamate-rich protein (GLURP) R2 region, and merozoite surface protein 2 (MSP2) allelic variants (3D7 and FC27) in Shewa Robit. Total IgG against GLURP-R0, MSP3 and GMZ2 and IgG subclasses against GLURP-R0 and MSP3 were assayed in both Shewa Robit and Boditi sera. Whereas 23(8.6%) blood-smear-positive cases for *P. falciparum* were detected in Boditi, all Shewa Robit study participants had no detectable *P. falciparum* infection. At both localities total IgG prevalence and levels to GMZ2 were significantly higher than the response to the component domains

(GLURP-R0 and MSP3) indicating the induction of strong GMZ2-specific natural antibodies. There was significant difference between the median antibody level to GMZ2, GLURP-R0 and MSP3 compared to the responses to other antigens tested in Shewa Robit, indicating that GMZ2 could be a more relevant blood-stage malaria vaccine candidate antigen. Higher total IgG and subclass prevalence and levels were detected in Shewa Robit than Boditi, suggesting difference in the intensity of malaria transmission in the two localities and/or genetic differences between the two population groups in their response to blood-stage *P. falciparum* antigens. In both study sites, IgG subclass antibody levels to GLURP-R0 were significantly higher than that to MSP3 for all corresponding subclasses in most individuals, indicating the higher relative immunogenicity and protective potential of GLURP-R0 compared to MSP3. Against both GLURP-R0 and MSP3, the ratio of cytophilic to noncytophilic antibodies was >1 in the majority of the study participants, in both study sites, indicating the induction of protective antibodies against the two antigens. Analysis of age-related pattern in antibody levels against the antigens tested showed a positive association with increasing age for most antigens suggesting the role of intrinsic age-related factors in immune maturation. The age factor appears plausible as there was no evidence for increase in antibody response with increasing frequency of reported past clinical malaria. Overall, the study has shown that Ethiopian population groups residing in unstable and seasonal malaria epidemiological settings have a high prevalence and levels of long-lived antibodies that readily recognize *P. falciparum* blood-stage vaccine candidate antigens, particularly GMZ2 and its component fractions (GLURP-R0 and MSP3). Furthermore, detection of high level antibody responses in non-febrile smear-negative individuals without history of reported past malaria episodes may possibly be an indication of a low-grade, asymptomatic (submicroscopic) infections in the induction and maintenance of high level protective

immunity. Therefore, to determine the implication of submicroscopic infections in the induction and boosting of malaria immunity versus the existence of long-lived malaria-specific antibodies in the absence of boosting from submicroscopic infection, PCR confirmation of the microscopy-negative samples would be necessary.

Keywords: antigen, blood-stage vaccine, cytophilic IgG subclass, ELISA, Ethiopia, falciparum malaria, noncytophilic IgG subclass

ACKNOWLEDGEMENTS

The journey was long, tough and painful demanding great resilience. Individuals, groups of individuals, communities, institutions and countries at large had their valuable inputs to the success of this work deserving sincere recognition and credit.

It all begins with Professor Beyene Petros. His responsibility and contribution as research supervisor is understandably huge. His commitment from the very early consultation phase to conceive the research idea and proposal development up to producing the first draft was really painstaking and can hardly be detailed. Not necessarily face to face, we were always together mentally and emotionally thinking of each other worrying about the day to day progress of the project which sometimes moved and sometimes got tougher. The heavy burden of revising, trimming and finalization of the dissertation; orchestrating its public presentation and securing final approval is on him. Taking this opportunity I express my heartily respect and thanks!

My closest friend dear brother Dr. Ahmad Mustefa, Department of Chemistry, Addis Ababa University is a source of incredibly persistent comfort. Lucky enough I had the opportunity to benefit from his computational and computer skills for multiple purposes. Thanks Ahmad!! Please keep your sense of humor and helping those who are in need of your assistance.

Ato Tesfaye Getachew, Medical Faculty, Addis Ababa University was vital and an expert in tackling the tedious bureaucracy on financial matters and in providing profound support for field works. W/O Amelework Ayado's laboratory assistance was valuable. Dr Haileeyesus Adamu's friendship was lovely both at home and abroad

when we were trying to cross the rigors of graduate study together. Dr Alemayehu Worku, School of Public Health, Addis Ababa University was highly acknowledged for statistical advice.

Dr Gurja Belay, former Head of Department of Biology and the then Department Academic and Graduate Committees, especially Dr Amare Gessesse, were instrumental in getting my contentious application for enrollment to the so called In-House PhD Program passed. I had Dr Gurja's rock-solid backing during the initial phases of the study when it was most difficult to ignite and get the field work started. At this period there was simply tremendous friction producing more heat than light!

The Office of Vice President for Research and Publication and Dean of School of Graduate Studies, Addis Ababa University has provided financial support for which I am grateful. But the critical funding was made available by the German Academic Exchange Service (DAAD) and Ministry of Education, Federal Democratic Republic of Ethiopia. I am indebted to these institutions and the respective governments.

Professor Klavs Berzins, Wenner-Gren Institute, Department of Immunology, Stockholm University, Sweden and Drs. Benjamin Mordmüller, Meral Esen and Professor Peter Kremsner, Institute of Tropical Medicine, University of Tübingen, Germany had created an opportunity for me to visit the respective institutes, made the available state-of-the-art facilities and guided me during the immunological data generation processes.

Mr Anthony Ajua and Nnaemeka Iriemenam were very helpful in assisting with the lab work in Germany and Sweden respectively. Ato Tamirat Gebru and his family

socially supported me much when I was in Germany and suffering from ill health. Dr Imad Faluk, Mrs Sonja, all other staff members and the then MSc/PhD students of Institute of Tropical Medicine, University of Tübingen and Wenner-Gren Institute, Stockholm University were friendly. The excellent scientific and social environment in the two laboratories made an indelible impression on me.

Government representatives, community leaders, health personnel in the study areas and the study populations are thanked for their moving cooperation and enthusiasm. While apologizing for not exhausting the list I am duly grateful to drivers, field assistants, administrative staff, friends and colleagues collectively.

My family had been so patient during my absence from home and when I was deeply absorbed by the project work depriving them of their right of getting my assistance and close guidance.

TABLE OF CONTENTS

| | |
|--------------------------------------------------------------------------------|-----|
| 1. INTRODUCTION | 1 |
| 1.1. The Malaria Problem..... | 1 |
| 1.2. Adaptive Immunity to falciparum Malaria..... | 3 |
| 1.2.1. Mechanisms of Immune Response to Malaria..... | 4 |
| 1.2.2. Antibody Responses and Protection in Malaria..... | 5 |
| 1.2.3. Antibody Longevity | 8 |
| 1.2.4. Age, Level of Endemicity and Malaria Antibodies | 11 |
| 1.2.5. Antigenic Variation and Malaria Immunity..... | 14 |
| 1.2.6. Human Genetics and Malaria Immunity..... | 15 |
| 1.3. Malaria Candidate Vaccines..... | 16 |
| 2. THE RESEARCH QUESTION | 23 |
| 3. MATERIALS AND METHODS..... | 25 |
| 3.1. Study Sites..... | 25 |
| 3.2. Ethical Considerations..... | 288 |
| 3.3. Retrospective Data, Study Populations and Sample Size | 299 |
| 3.4. Study Design and Sampling | 29 |
| 3.5. Antibody Analysis: Recombinant Antigens and ELISA Procedures | 31 |
| 3.6. Data Analysis | 35 |
| 4. RESULTS | 36 |
| 4.1. Retrospective Data and Study Participants | 36 |
| 4.2. Antibody Prevalence and Level | 38 |
| 4.3. Intercorrelations between antibody levels to the different antigens..... | 41 |
| 4.4. Self-Reported Past Malaria Episode and Antibody Response in Shewa Robit.. | 44 |
| 4.5. Antibody Responses and Age | 47 |
| 4.6. Slide-Positivity and Antibody Response in Boditi..... | 54 |
| 4.7. IgG Subclass Distribution | 55 |
| 5. DISCUSSION | 61 |
| 6. CONCLUSIONS..... | 95 |
| 7. RECOMMENDATIONS | 97 |
| 8. REFERENCES | 98 |

LIST OF TABLES

| | |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Table 1 Malaria clinical cases in Shewa Robit and Boditi as reported by the respective Health Centers, 2004-2008. | 37 |
| Table 2 Demographic and self-reported past falciparum malaria episode data for study participants in antibody analysis against <i>P. falciparum</i> blood-stage vaccine candidate antigens, Shewa Robit 2008 (N=228) | 37 |
| Table 3 Age and sex distribution of study participants in Boditi 2009 (N=265). | 38 |
| Table 4 ELISA cutoff OD values for total IgG and subclass antibodies against the different antigens tested for Shewa Robit sera. | 39 |
| Table 5 ELISA cutoff OD values for total IgG and subclass antibodies against the different antigens tested for Boditi sera. | 39 |
| Table 6 Total IgG positivity and level to <i>P. falciparum</i> blood-stage vaccine candidate antigens in Boditi (2009) and Shewa Robit (2008) sera. | 40 |
| Table 7 IgG subclass positivity and level to <i>P. falciparum</i> GLURP-R0 and MSP3 in Boditi (N=265) and Shewa Robit (N=228) sera. | 40 |
| Table 8 Individuals total IgG positive for 1, 2, 3, 4 & ≥ 5 antigens, Shewa Robit 2008 (N=228). | 41 |
| Table 9 IgG prevalence to <i>P. falciparum</i> recombinant antigens and reported past malaria episodes, Shewa Robit 2008 (N = 228) | 45 |
| Table 10 Total IgG prevalence to <i>P. falciparum</i> antigens and reported year of most recent malaria episode, Shewa Robit 2008 (N=177). | 46 |
| Table 11 Pattern of total IgG to <i>P. falciparum</i> antigens and reported past malaria episode, Shewa Robit 2008 (N= 177). | 46 |
| Table 12 Total IgG level respect to reported year of most recent malaria episode, Shewa Robit 2008 (N=177). | 47 |
| Table 13 Total IgG positivity to <i>P. falciparum</i> GLURP-R0, MSP3 and GMZ2 and age groups, Boditi 2009 (N =265). | 47 |
| Table 14 Age-associated pattern in IgG total antibody levels to <i>P. falciparum</i> recombinant antigens tested, Boditi 2009 (N=265) | 48 |
| Table 15 Total IgG positivity to <i>P. falciparum</i> recombinant antigens in age groups, Shewa Robit 2008 (N=228) | 49 |
| Table 16 Age-associated pattern in total IgG levels to <i>P. falciparum</i> antigens, Shewa Robit 2008 | 49 |
| Table 17 Total IgG positivity to <i>P. falciparum</i> GLURP-R0, MSP3 and GMZ2 slide-positivity, Boditi 2009 (N=265). | 54 |
| Table 18 Total IgG level against MSP3, GLURP-R0 and GMZ2 in <i>P. falciparum</i> smear-positive and negative individuals at the time of sample collection, Boditi 2009 (N=265). | 54 |
| Table 19 IgG subclass positivity to <i>P. falciparum</i> GLURP-R0 and MSP3 and smear-positivity at the time of sample collection, Boditi 2009 (N=265) . | 55 |
| Table 20 IgG subclass levels to <i>P. falciparum</i> MSP3 & GLURP-R0 and smear-positivity, Boditi 2009 (N=265). | 55 |
| Table 21 IgG subclass distribution to <i>P. falciparum</i> GLURP-R0 and MSP3 antigens, Shewa Robit 2008 (N=228). | 56 |
| Table 22 Anti-GLURP-R0 and MSP3 IgG subclass level ratios and median of the ratios, Shewa Robit 2008 (N=228) | 57 |

| | |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Table 23 Anti-GLURP-R0 and MSP3 IgG subclass level ratios and median of the ratios, Boditi 2009 (N=265)..... | 58 |
| Table 24 Relative dominance of IgG subclass levels to <i>P. falciparum</i> MSP3 & GLURP-R0 compared at individual level, Boditi 2009 expressed in ratios medians of the ratios | 60 |

LIST OF FIGURES

| | |
|------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1 Generalized life cycle of the malaria parasites..... | 2 |
| Figure 2 Study sites of antibody analysis against <i>P. falciparum</i> blood-stage vaccine candidate antigens in Boditi (2009) and Shewa Robit (2008)..... | 27 |
| Figure 3 Study design of antibody analysis against <i>P. falciparum</i> blood-stage vaccine candidate antigens in Boditi and Shewa Robit. | 31 |
| Figure 4 Correlation between IgG to <i>P. falciparum</i> GMZ2 and GLURP-R0, Boditi 2009..... | 42 |
| Figure 5 Correlation between IgG to <i>P. falciparum</i> GMZ2 and MSP3, Boditi 2009..... | 43 |
| Figure 6 Correlation between IgG levels to MSP2-3D7 and MSP2-FC27, Shewa Robit 2008..... | 43 |
| Figure 7 Correlation between IgG level to AMA1 and age, Shewa Robit 2008 | 50 |
| Figure 8 Correlation between IgG level to MSP2-3D7 and age, Shewa Robit 2008 | 51 |
| Figure 9 Correlation between IgG level to MSP2-FC27 and age, Shewa Robit 2008 | 51 |
| Figure 10 Correlation between IgG level to GLURP-R0 and age, Shewa Robit 2008 | 52 |
| Figure 11 Correlation between IgG level to GLURP-R2 and age, Shewa Robit 2008 | 52 |
| Figure 12 Correlation between IgG level to GMZ2 and age, Shewa Robit 2008..... | 53 |
| Figure 13 Correlation between IgG level to MSP3 and age, Shewa Robit 2008..... | 53 |

LIST OF ABBREVIATIONS

| | |
|---------------|----------------------------------------|
| ALP | Alkaline phosphatase |
| AMA 1 | Apical membrane antigen 1 |
| BSA | Bovine serum albumen |
| CD4+ T | Cluster Differentiation 4 plus T cells |
| CI | Confidence intervals |
| CSP | Circumsporozoite protein |
| ELISA | Enzyme-linked immunosorbent assay |
| EBA175 | Erythrocyte binding antigen 175 |
| Fc γ R | Fc gamma receptor |
| GLURP-R0 | Glutamate-rich protein R0 region |
| GLURP-R2 | Glutamate-rich protein R2 region |
| HRP | Horseradish Peroxidase |
| IFN- γ | Interferon-gamma |
| Ig | Immunoglobulin |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| IRS | Indoor Residual Spraying |
| ITN | Insecticide-Treated Net |
| MCs | Malaria clinical cases |
| LMCs | Latest malaria clinical cases |
| LSA | Liver Stage Antigen |
| MSP1 | Merozoite surface protein 1 |
| MSP3 | Merozoite surface protein 3 |

| | |
|--------------|----------------------------------------------------------------------------|
| MSP2-3D7 | Merozoite surface protein allelic variant 3D7 |
| MSP2-FC27 | Merozoite surface protein allelic variant FC27 |
| OD | Optical density |
| PBS | Phosphate-buffered saline |
| Pf155/RESA | <i>P. falciparum</i> protein 155/Ring-infected erythrocyte surface antigen |
| Pf332 | Plasmodium falciparum protein 332 |
| RDTs | Rapid Diagnostic Tests |
| Seropositive | SP |
| SD | Standard deviation |
| SNNPR | Southern Nations, Nationalities and Peoples Regional State |
| Th | T helper lymphocyte |
| TMB | 3,3',5,5'-Tetramethylbenzidine |
| Treg | T regulatory lymphocyte |
| TRAP | Thrombospondin-related adhesive protein |
| USA | United States of America |
| VSA | Variant surface antigen |
| WHO | World Health Organization |

1. INTRODUCTION

1.1. The Malaria Problem

Malaria is endemic throughout the tropics and sub-tropics with about 40% of the world population being at risk. The disease is prevalent in more than 100 countries and about 90% of the deaths occur in sub-Saharan Africa (Hay et al. 2004). Pregnant women and the elderly are the most vulnerable groups apart from the below five years old children. Malaria affects the health as well as economic growth and development of endemic countries. Although World Malaria Report 2010 (WHO 2010a) indicated that there was a decline in the morbidity and mortality of malaria in several endemic areas in recent times, as a result of scaling up of control efforts, there were about 225 million clinical cases and 781,000 deaths in the year 2009 alone. Because of demographic and environmental changes, globalization and unpredictable climatic fluctuation more people are expected to be at risk of malaria than before.

In Ethiopia, malaria is a primary health concern increasingly becoming an obstacle to the country's poverty reduction program. According to a report by the Federal Democratic Republic of Ethiopia Ministry of Health (FMoH 2007), 75% of the country's total area and an estimated 68% of the total population is at risk of malaria. This same document indicated that annually 5-6 million malaria cases and 70,000 deaths are reported by health facilities notwithstanding many cases that go undetected and unreported due to lack of formal health-care systems.

Human malaria is mainly caused by four species of the protozoan parasites belonging to the genus *Plasmodium* – *Plasmodium falciparum*, *P. malariae*, *P. ovale* and *P.*

There are various malaria control methods. Mosquito larval breeding sites can be targeted for source reduction through environmental management. Breeding sites of mosquitoes can be reduced by community participation especially when water resource development schemes for irrigated agriculture and hydroelectric power generation negatively change the environment. The main modern malaria control methods practiced globally are case management and transmission-blocking through vector-control by insecticide-treated bed nets (ITN) and indoor residual spraying (IRS) (Eisele and Steketee 2011, Skeet 2005). Drug-resistance of the parasite and insecticide-resistance of the vector together with some practical difficulties in implementation prove it challenging to rely on these methods in the fight against the disease (Touré 1999). Developing anti-malaria vaccine remains another approach. An effective malaria vaccine would be integrated into existing control strategies and improve healthcare systems and can make malaria elimination (Feachem et al. 2010) or even possible eradication plans more feasible.

1.2. Adaptive Immunity to falciparum Malaria

Despite the fact that much effort has been exerted to investigate both innate and acquired human immune responses against *P. falciparum* infection and/or clinical disease manifestations, little is understood as to the immune correlates of protection and the appropriate antigenic epitopes that elicit the relevant immunological machinery. The concern increased when two recent blood-stage vaccines in Phase IIb trials failed to confer protection despite eliciting high antibody levels (Ogutu et al. 2009, Sagara et al. 2009), triggering extensive discussion afresh around vaccine candidate selection criteria (Coppel 2009). Although agreement is lacking between studies and the relative relevance of each specific response is little understood different antigens induce different antibody classes and subclasses (Holder 2009).

Major controversies revolve around various study findings in endemic areas (Greenwood and Targett 2009). These include the role of age and level of exposure in the induction and maintenance of malaria protective antibody including the duration, subclass distribution and antigenic targets necessitating more in-depth investigation.

1.2.1. Mechanisms of Immune Response to Malaria

Although it is believed that blood-stage protection is substantially mediated by antibodies, other protective mechanisms are also suggested, including innate immunity, interferon-gamma (IFN- γ) production and T-cell proliferation (Kohler et al. 2003, Sanni et al. 2002, Worku et al. 2001). Furthermore, IFN- γ produced by CD4+ T cells to specific blood-stage antigens has been shown to be associated with protection against re-infection (Luty et al. 1999). CD4+ T-cell secretion of IFN- γ might also help to induce blood-stage-specific antibodies and assist certain mechanisms of clearance of infected red blood cells (Bouharoun-Tayoun et al. 1995).

Specific antigenic epitopes for protective immunity are ill-defined though several antigens have been identified, characterized and tested from various life cycle stages of the parasite with emphasis on blood-stages which are responsible for malarial disease (Metzger et al. 2003, Polley et al. 2006). Potential mechanisms of antibody-mediated action against blood-stage parasites include receptor-blocking to inhibit erythrocyte invasion and/or attachment (cytoadherence) of infected erythrocytes to endothelial cells and/or uninfected erythrocytes thereby avoiding sequestration and rosetting - the main pathogenic surrogate marker of *P. falciparum* associated severe disease - opsonization and complement fixing, antibody-dependent cytotoxic

inhibition, direct killing of freely circulating merozoites before binding (Doolan et al. 2009, Langhorne et al. 2008, Marsh and Kinyanjui et al. 2006).

1.2.2. Antibody Responses and Protection in Malaria

The existence of antibody-mediated malaria immunity was initially reported by Sotiriades in 1917 and then substantiated by Kauders ten years later in 1927 by passively transferring sera into acutely sick malaria patients from a chronically infected patient the outcome of which was clinical improvement in the acute malaria patients (cited in McGregor 1963). Subsequent studies in the Gambia by McGregor and Carrington (1963) and in Thai patients by Sabchareon et al. (1991) have confirmed the classical reports.

Further evidences accumulated through *in vitro*, animal model and immunoepidemiological studies concerning the association of antibodies with malaria protection. However, the results are conflicting. Various field studies recorded the association between both prevalence and levels of antibodies against asexual blood-stage antigens of *P. falciparum* with protection from malaria (Cavanagh et al. 2004, Egan et al. 1996, Metzger et al. 2003). However, other studies reported lack of evidence of association (Dodoo et al. 1999, Okech et al. 2004, Osier et al. 2008) even employing the same antigen epitope targets. Further, immune sera (Sy et al. 1990, Tebo et al. 2001) and purified antibodies (Bolad et al. 2003) that have been reported to inhibit *in vitro* parasite growth were not associated with protective immunity to clinical malaria (Marsh et al. 1989, Perraut et al. 2005). On the contrary purified antibodies that were effective against parasites *in vivo* failed to inhibit parasite growth *in vitro* (Bouharoun-Tayoun et al. 1990).

Thus, humans in endemic areas may develop high levels of antibodies to antigens being targeted by protective mechanisms but these antibodies may not necessarily confer the desired protection. In view of this it was hypothesized that the absence of protection may be because of an imbalance in IgG subclass pattern (Bouharoun-Tayoun and Druilhe 1992). Particularly, owing to the finding that blood monocytes are important in the normal functioning of malaria antibodies (Bouharoun-Tayoun et al. 1990, Lunel and Druilhe 1989), studies geared towards dissecting subclass distribution rather than focusing on just total IgG.

IgG subclasses differ in their structures and mediate different immune effector functions (Nimmerjahn and Ravetch 2008). IgG1 and IgG3 subclass antibodies are termed as cytophilic due to their ability to fix complement. They bind to Fc γ receptors (Fc γ R) on the surface of monocytes, macrophages and neutrophils thereby mediating phagocytosis, opsonization, antibody-dependent-cytotoxicity (ADCC), complement-mediated killing, ADCI and other possible mechanisms of antibody-mediating attack against blood-stage parasites described above (Boucharou-Tayoun et al. 1990, 1992, 1995, Groux and Gysin 1990).

Further, cytophilic subclasses cross the placenta and pass to the fetus more frequently and more efficiently than IgG2 and IgG4 (Deloron et al. 1997). Various immunoepidemiological studies found associations between polarized responses of cytophilic subclasses and clinical protection from malaria (Bouharoun-Tayoun et al 1995, Bouharoun-Tayoun and Druilhe 1992). Also, Bouharoun-Tayoun and Druilhe (1992) observed either an overall low response or an isotype imbalance in non-protected individuals with low level of IgG3 antibodies compared to protected

individuals. In general, blood stage malaria parasites are predominantly attacked by IgG1 and IgG3 (Nebie et al. 2008, Taylor et al. 1995, Tongren et al. 2006). In particular, IgG3 subclass antibody to various blood stage antigens is associated with malaria protection (Cavanagh et al. 2004, Metzger et al. 2003, Soe et al. 2004).

On the other hand, IgG2 and IgG4 subclass antibodies do not fix complement and are thus noncytophilic. They may compete with cytophilic antibodies for antigen recognition and therefore block the protective activity of these antibodies (Bouharoun-Tayoun and Druilhe 1992). The increase of IgG4 against *P. falciparum* variant surface antigen (VSA) particularly in patients with severe malaria may imply this subclass's association with malarial pathology (Yone et al. 2005). In several other studies high noncytophilic subclass responses were implicated in disease progression (Ndunga et al. 2002, Soe et al. 2004) and lower responses with protection (Nasr et al. 2007). Emanating from such observations the importance of ratio of cytophilic to noncytophilic antibodies in immunity to blood-stages of the parasite was suggested for consideration (Groux and Gysin 1990, Bouharoun and Druilhe 1992). Under normal circumstances the estimated percentages of the four IgG subclasses in adult humans is 66, 23, 7 and 4 for IgG1, IgG2, IgG3 and IgG4 respectively (Aucouturier et al. 1985, Yount et al. 1967).

Similarly, in mouse malaria, distinct isotypic patterns of antibody response to *P. chabaudi* were occurred in protected and nonprotected animals (Falanga et al. 1987). In various strains of mice the production of cytophilic classes to *P. yoelii* was found predominantly in strains of mice that developed a mild disease but not in those with severe disease (Taylor et al. 1988).

On the contrary, in a population from Madagascar, IgG1 levels were inversely related to protection and other isotypes had similar tendencies with no clear isotype imbalance (Dubois et al. 1993). Moreover, no particular isotype distribution pattern to crude blood stage antigen of infected erythrocytes could be found associated with protection in a holoendemic area in Senegal (Aribot et al. 1996). Further, there are other studies that reported the possible protective role of the noncytophilic subclasses. IgG2 was higher in one ethnic group, the Fulani, who were considered relatively protected and the IgG1:IgG2, IgG1:IgG3, IgG2:IgG3 ratios were generally high and individually each subclass antibody was high (Israelsson et al. 2008). This is in agreement with the work of Aucan et al. (2000) who reported significant association between protection from malaria and high IgG2 and low IgG4 levels.

Other studies provided additional support observing high IgG2 responses to various *P. falciparum* antigens associated with protection (Giha et al. 2010, Nebie et al. 2008, Roussolhon et al. 2007). At least in one study IgG4 to both MSP1 and GLURP was associated with reduced risk of malaria incidence (Nebie et al. 2008). More recently, significantly higher levels of IgG4 was detected in individuals who have not had clinical malaria compared to those who had at least one attack (Giha et al. 2010). The mechanism of protection by these antibodies may possibly be through agglutination and/or receptor blocking (Doolan et al. 2009, Langhorne et al. 2008).

1.2.3. Antibody Longevity

Whatever the mechanism of immune maturation and the protective efficacy may be, it is widely believed that antigen-specific malaria antibody responses decline rapidly after treatment and in the absence of boost infections (Kinyanjui et al. 2007, Kohler et

al. 2003, Langhorne et al. 2008). Especially, IgM and IgG3 antibodies have relatively shorter half-life and the half-life of IgG1 is around 21 days (Morell et al. 1970, Spiegelberg 1974). Defects in memory B cells to malaria parasite antigens which may account for the short-life of anti-malaria antibodies have been reported (Dorfman et al. 2005, Wykes et al. 2005).

On the other hand, other investigators criticized the widely held assertion and challenged the interpretation of experimental and epidemiological evidences. The early landmark immunological study by Covell and Nicol (1951) reminds that a remarkable tolerance was acquired, against a particular strain of the malaria parasite causing the original infection, provided the attack is allowed to run its course for several days without treatment. Although the authors found that the acquired tolerance had decreased gradually, it conferred protection over a period of years. According to these authors, while a single infection may not produce complete protection to the same strain of the parasite, repeated re-infection may eventually do so. Later serological survey conducted in Thailand, in an area where malaria transmission had been interrupted completely for 30, years provided additional support in this respect (Jacobs et al. 1983).

These reports are further reinforced by later studies in Madagascar which showed that some older people retained immunological memory to Pf155/RESA or CSP1 following 20 years of absence of malaria transmission (Chougnnet et al. 1990, Deloron and Chougnnet 1992). This is similar to antibody responses to *P. vivax* MSP1 antigen which was reported to last for more than 30 years in the absence of reinfection though that observation could possibly be due to reactivation by hypnozoites (Lim et al.

2004). According to Riley et al. (1993), antibody levels to PfMSP1, one of the leading *P. falciparum* candidate vaccine antigens, were remarkably stable irrespective of seasonal changes in transmission. It was reported that MSP2-specific antibodies in naturally exposed individuals in seasonal *P. falciparum* transmission areas where the rate of infectious mosquito bites were highly reduced remained remarkably stable from year to year (Taylor et al. 1996, Rzepczyk et al. 1997). The authors showed that this was true even for anti-MSP2 IgG3 subclass antibody which has relatively a short half-life. The investigators suggested that only infrequent re-infections are enough to maintain high titer anti-MSP2 antibody levels.

A study in a seasonal malaria transmission area in Ghana argues along the same line where MSP1-specific antibody levels and prevalence did not show variation between seasons (Dodoo et al. 1999). Furthermore, a longitudinal study in the Sudan showed that there were some individuals that retained a degree of immunological memory despite the lack of transmission for two consecutive years (Fonjungo et al. 1999). The levels of anti-MSP4 antibodies in Vietnamese did not decrease during the convalescent period (Wang et al. 2001). This stability in antibody level is shared by the response to MSP1 in the same work. It was similarly argued that anti-malaria antibodies persist for up to 10 years even in the absence of periodic or subpatent infection (Struik and Riley 2004, Bouchaud et al. 2006). A recent study that examined antibody response to *P. falciparum* antigens in a seasonal malaria transmission area in Thailand reported a stable and long-lived malaria-specific antibodies and memory B cell population in the absence of frequent boosting (Wipasa et al. 2010).

Therefore, as Drakeley et al. (2005) state:

From an immunological viewpoint, the most interesting finding of this study is that once acquired, antibody responses to PfMSP1₁₉ seem to persist for many, many years and indeed be life-long. Data derived from therapeutic malaria infection, isolated malaria outbreaks, and migrants from malaria endemic areas who had not been re-exposed for up to 11 years, all of which suggests that antibody levels to whole parasites or to individual antigens may persist for at least 10 years even in the absence of periodic or subpatent infection.

1.2.4. Age, Level of Endemicity and Malaria Antibodies

Another widely held model of acquired immunity to malaria is slow age-related immune buildup after several years in endemic areas (Day and Marsh 1991). Contributing factors to this perceived slow developing immunity to malaria are not yet clear. It was suggested that protective immunity is dependent upon cumulative exposure to the many parasite variants circulating in the local population as malaria antigens are diverse (Day and Marsh 1991). Further, antigens supposed to elicit protective immunity may be poorly immunogenic and immunologic non-responsiveness may contribute (Good et al. 1988).

On the other hand, it was proposed that helminth infections slow the development of malaria immunity (Roussilhon et al. 2010). Most studies in this another controversial topic are inspired by the observation that while anti-worm immunity is skewed towards Th2/Treg responses, protection from malaria is linked to Th1/Th2 cytokine balance although little is understood (Jackson et al. 2009, Shin et al. 2007). The

mechanism of the possible influence of helminthic infections, which are highly prevalent especially in children in malaria endemic settings, is poorly defined.

The view of slow antibody development in general is questioned irrespective of the mechanisms of the delay. It is argued that protective immunity to severe pathology is achieved relatively rapidly (Baird 1995, Gupta et al. 1999). A work in South East Asia reported the development of malaria immunity as a function of age but independent of cumulative exposure (Baird et al. 1991, Baird et al. 1993). These authors revealed that a trans-migrant population originated from malaria free area and settled in a hyperendemic locality developed strongly reactive anti-*P. falciparum* immunity parallel to that in age- and sex-matched natives relatively quickly within a two year period after brief exposure to the parasite. Though it has been difficult to look into the effect of age on malaria immunity independent of exposure these investigators argued that they managed to do so. This brief exposure/age-dependent protection, as they put it, is contrary to the cumulative exposure/protection model. In the study they analyzed the protective benefit of life-long exposure in the natives versus the uniform two-year period of exposure in the transmigrants. This constituted a major departure from the conventional view regarding how immunity develops to falciparum malaria. Additional support to this view was provided by the work of Wahlgren and colleagues who documented that there was no significant difference in anti-malarial antibody levels between individuals more extensively exposed and those with less exposure (Wahlgren et al. 1986).

In a study undertaken in Madagascar immune responses to two *P. falciparum* antigens did not show correlation with age (Deloron and Chougnet 1992). Further, it was

recorded in the Philippines, using *P. falciparum* merozoite antigen gp195, that although the youngest age group (0-4-year-old age group) had the lowest positivity rate (45%) those in the groups 5-9-year-old had a positivity rate of 90% as well as antibody titers comparable with the older age groups demonstrating the development of anti-malarial immunity early in time (Kramer and Oberst 1992). Further, both prevalence and level of antibody to MSP1 showed no significant difference between individuals living in areas of high and low seasonal malaria transmission areas (Riley et al. 1993). Along the same line anti-AMA1 IgG antibody reactivity was equally very high in all age groups including 2-4-year-old children among naturally exposed Guinea Bissau and Senegalese populations where malaria transmission was moderately endemic and holoendemic, respectively (Thomas et al. 1994). However, the authors observed age-related increase of antibody level in the children from Guinea Bissau though not in Senegal. Antibody response to Pf155/RESA appeared to develop early in life, no significant difference in antibody levels or prevalence could be found in a group of Tanzanian children including below 2 years compared with adults (Siddique et al. 1999). Relatively recent work also could not reveal association between antibody prevalence and age (Okech et al. 2004).

However, age is considered a key variable in malaria morbidity and mortality. In seasonal transmission areas the risk of uncomplicated malaria decreases significantly after 20 years of age (Giha et al. 2000, McGregor and Wilson et al. 1988). In highly endemic areas younger infants experience lower parasite rate compared to older brothers (Deloron et al. 1997). Six month old infants were protected compared to older ones and from six months up to 2 years old suffer much and the impact decreases with age (Bloland et al. 1999).

Reports indicate that infants in endemic areas have high antibody titer which gradually decreases until the age of 3-6 months and then after begin to rise (Duah et al. 2010). The observed better protection of infants in holoendemic areas might be attributed to passively transferred maternal antibodies, and lack or restricted mosquito contact which might result in lower infection rates and lesser parasitemia (Macdonald 1951, White et al. 2010). A study in the Gambia directed towards determining the course, effect and pattern of immune acquisition in children of two groups one kept protected from birth onwards by regular weekly prophylactics and the other without prophylactics but were treated when acutely sick showed significantly higher antibody level in the unprotected group compared to those under prophylaxis by the end of second year. And by the end of third year the unprotected group attained similar antibody level to those in adults (McGregor 1964). But the protected group remained having significantly lower levels up to six years of age. The study was a pioneer that demonstrated that in endemic areas children acquire high antibody levels rather quickly.

1.2.5. Antigenic Variation and Malaria Immunity

Clearly there is nonreplicability in most malaria immunoepidemiological studies. Observing less consistency between immunoepidemiological studies in immune response to *P. falciparum* blood-stage antigens may affect the effectiveness of a future universal vaccine. The relative influence of parasite, host and environmental factors on anti-malaria immunity is poorly illustrated. Nevertheless antigenic variation and human genetic factors stand as the most influential factors.

Although several antigens have been suggested as targets for protective immunity, their role in this context has still not yet been clearly identified. *P. falciparum* is known for its antigenic complexity. This makes the screening of actual antigenic epitopes that trigger protective antibodies in natural settings difficult. Any single *P. falciparum* antigen is composed of several component regions showing variations from within contributing to the divergence of the immune response. For instance, MSP1 has several antigenically distinct component polypeptides with each component polypeptide having its own highly polymorphic and conservative regions that differ in their immunogenicity (Egan et al. 1995, Da Silveria et al. 1999). Similarly, there is a great deal of allelic diversity and antigenic polymorphism in other *P. falciparum* sexual blood-stage antigens including MSP2, MSP3, MSP4, MSP6, MSP7 (Wang et al. 2001, Franks et al. 2003, Pearce et al. 2004). Some allelic variants are predominant in some endemic areas but less frequent in other localities and even individuals. At least according to the cumulative-exposure model discussed above, protective immunity is dependent upon cumulative exposure to many parasite variants circulating in the local population.

1.2.6. Human Genetics and Malaria Immunity

Several studies have documented that a great variation exists between populations and individuals within a population with respect to the clinical outcome of *P. falciparum* infection (Good et al. 1988, Greenwood et al. 1991). Studies suggest that human genes involved in the regulation of adaptive immune responses apart from the long established erythrocyte-related factors are becoming increasingly important (Kwiatkowski 2005).

Studies conducted on monozygotic and dizygotic twin pairs revealed that immune responses to various *P. falciparum* blood-stage antigens had higher concordance in both lymphoproliferative responses and antibody levels in monozygotic than dizygotic twins exposed to similar transmission levels (Jepson et al. 1997, Sjoberg et al. 1992). The studies pointed out that age and sex-matched siblings and unrelated individuals had lower concordance in anti-malaria immune response compared to what was observed for twins irrespective of transmission level.

Further, studies in different West African malaria endemic countries have shown distinct inter-ethnic variation in susceptibility to malaria (Dolo et al. 2005, Pandey et al. 2007). For instance, it was demonstrated that the Fulani ethnic group was relatively protected from clinical malaria compared to its sympatric ethnic groups and had higher anti-malaria-antibody responses (Dolo et al. 2005, Modiano et al. 1999). This relative resistance to malaria has been shown to be independent of classical erythrocyte-related malaria-resistance (Modiano et al. 2001). Some studies have also documented associations between polymorphisms in selected human genetic markers and protection from or susceptibility to clinical malaria (Nasr et al. 2007, Vafa et al. 2007).

1.3. Malaria Candidate Vaccines

An effective vaccine is a long awaited ultimate intervention tool for the control, elimination and possible eradication of malaria. There are no licensed malaria vaccines yet. The delay is attributed partly to relatively low level funding, parasite life cycle complexity and antigenic variation, and insufficient understanding of human immune interaction with the parasite (Ellis et al. 2010, Langhorne et al. 2008).

Broadly, prospective malaria vaccines are categorized into three: pre-erythrocytic, blood-stage and transmission-blocking. Whole parasite vaccine based on radiation-attenuated sporozoite which is found to be effective in protecting monkeys (Jones et al. 2000) is under clinical development (Roestenberg et al. 2009, Billingsley 2010, Butler et al. 2012). Such a vaccine if successful will have a potential to be effective against infection as well as transmission. But, there are concerns pertaining to the immunogenicity and safety of attenuated sporozoites (Richards and Besson 2009). Even if these concerns are cleared and high efficacy level is achieved, factors related to wide scale production, purification and distribution of a whole organism vaccine may prove difficult for implementation in the field.

P. falciparum circumsporozoite protein (CSP) vaccine, RTS,S, which is composed of the central repeat and C-terminal T-cell multi-epitope of the protein fused with hepatitis B virus S-antigen adjuvanted with either AS02 or AS01 (Stoute et al. 1997, Garçon et al. 2007) has passed through Phase III clinical trial with 50% efficacy (Agnandji et al. 2011). This pre-erythrocytic vaccine is the most advanced malaria vaccine with better chance for licensure in the near future. But RTS,S/AS01 efficacy against severe disease in the target age group was found to be minimal (Asante et al. 2011). Moreover, significant rapid waning of vaccine efficacy against clinical malaria and lower vaccine immunogenicity were reported earlier (Abdulla et al. 2008, Aide et al. 2010, Guinovart et al. 2009). RTS,S-induced immunity against pre-erythrocytic stages may result in low dose blood-stage parasitemia thereby contributing towards more effective immune response to clinical malaria (Sutherland et al. 2007, Guinovart et al. 2009). Conversely, this reduced level of blood-stage parasites as result of RTS,S

vaccination may reduce natural anti-blood stage antibodies. A study reported significant reduction in natural blood-stage antibodies in RTS,S-immunized children in Phase IIb trial of RTS,S (Bejon et al. 2011). This may lead to increased risk to malarial disease.

Vaccines developed against gametes, zygotes or ookinets would block-transmission and thus have implications for malaria elimination. Transmission-blocking antibodies against the most promising candidate vaccine antigen, Pfs25, have been recorded in individuals in endemic areas (Langhorne et al. 2008). Such vaccines have the objective of protecting communities from the disease rather than vaccinated individuals directly.

An effective vaccine inducing immunity against the blood-stages looks more convincing as it can confer protection from malarial disease. Thus blood-stage antigens remain to be critical in malaria vaccine research. A highly effective malaria vaccine is expected to be a combined multi-stage, multi-component and as such a blood-stage component must be included. Such a vaccine containing blood-stages would prevent disease as a result of 'leaky' pre-erythrocytic parasites. Further, blood-stage vaccine would confer protection against epidemic malaria in protected populations by pre-erythrocytic vaccines such as RTS,S when naturally acquired immunity wanes. Similarly, transmission-blocking vaccines need to be combined with blood-stage or pre-erythrocytic candidate vaccines. Such combined transmission-blocking candidate vaccines are under preclinical and clinical testing (Crompton et al. 2010). Together with existing malaria control strategies an effective vaccine is required to ultimately eradicate the disease. Thus reducing clinical illness has to be

supplemented by reducing malaria transmission. Vaccines inducing sterile immunity against disease and/or transmission-minimizing would be a priority. Blood-stage vaccines may have a dual role in this approach. These vaccines would not only prevent illness but reduce parasitemia and thus have a role in transmission-reduction. Clinical and animal model studies indicated that reducing parasitemia results in reduced gametocyte production (Richard and Beeson 2009).

Thus the rationale to pursue blood-stage vaccine development is strong in many ways. There are more than 2000 asexual blood-stage antigens (www.plasmodb.org). It is challenging to objectively prioritize which of these antigens and recommend for further development. In most studies the selection criteria were limited by the current knowledge and availability of blood-stage antigens (Campo et al. 2011). Merozoite antigens with proven or suspected role in erythrocyte invasion, cytoadherence, immune evasion and easy targets of antibodies are mostly considered (Silvie et al. 2004, Cowman and Crabb 2006). Several promising blood-stage candidate vaccines are under clinical trials, the most important ones being AMA1, erythrocyte-binding protein 175 (EBA175), MSP1, MSP2, MSP3, GLURP, *P. falciparum* protein 155/ring-infected erythrocyte surface antigen (Pf155/RESA) and GMZ2 (GLURP+MSP3).

AMA1 is expressed in both blood-stages and sporozoites of the parasite and antibodies to it blocked sporozoite invasion of hepatocytes and inhibited *in vitro* erythrocyte invasion (Silvie et al. 2004). The antigen is a polymorphic protein and its major strains that are selected vaccine candidates are the full-length ectodomains of FVO, 3D7 and W2mef (Richards and Beeson 2009).

A 25–30 kDa MSP2 is another polymorphic *P. falciparum* blood-stage vaccine candidate (Clark et al. 1989, Smythe et al. 1988, Smythe et al. 1991). This protein is expressed in trophozoites, schizonts and merozoites. While the N- and C-termini of the protein are conserved among isolates, the central region is variable (Smythe et al. 1990, Thomas et al. 1990). The polymorphic region falls into two major allelic families - serogroup A (3D7-like) and serogroup B (FC27-like) (Fenton et al. 1991, Felger et al. 1997, Hoffmann et al. 2001). But the sequence, number and length of the repeat sequences differ between and within the allelic families (Smythe et al. 1990). The safety and immunogenicity of the antigens was studied in Phase I trial (Sturchler et al. 1995).

A 220-kDa GLURP has a relatively conserved N-terminal nonrepeat gene region containing amino acids 27–500 (fragment R0), a central repeat region with amino acids 500–705 (fragment R1), and a C-terminal immunodominant repeat region of amino acids ranging from 705–1178 (fragment R2) (Theisen et al. 1998, Theisen et al. 2001). Whereas the variation is limited between field isolates across diverse geographic regions for the R0 region a considerable polymorphism exists in the R2 (Felger et al. 1997, McColl and Anders 1997, de Stricker et al. 2000).

MSP3 which is a 48-kDa protein encoded by one member of a multi-gene family is another polymorphic merozoite protein suggested to take part in erythrocyte invasion (Mills et al. 2002, Singh et al. 2009). A major B-cell epitope of MSP3 which is conserved between different *P. falciparum* isolates is the C-terminal region representing amino acids 212–318 (Felger et al. 1997, McColl and Anders 1997). The protein's N-terminal domain showing sequence diversity which had previously been

largely ignored from consideration (Jordan et al. 2011) is defined by two major allelic types 3D7 and K1 (McColl and Anders 1997). Evidences suggest that this domain is more immunogenic than the C-terminal domain *in vivo*, and antibodies against it positively correlated with protection (Jordan et al. 2011, Osier et al. 2007, Polley et al. 2007). A vaccine containing the conserved C-terminal regions was clinically tested and found to be safe and immunogenic (Audran et al. 2005, Druilhe et al. 2005, Sirima et al. 2007).

GMZ2 is a secreted recombinant fusion protein produced in *Lactococcus lactis* from genetically coupled *P. falciparum* GLURP₂₇₋₅₀₀ (non-repeat R0 region) and MSP3₂₁₂₋₃₈₀ (conserved C-terminal) (Theisen et al. 2004). It was shown that GMZ2-immunized mice had higher levels of both GLURP- and MSP3-specific antibodies than mice immunized with either of the component antigens individually or a mix of both (Theisen et al. 2004). The authors reported the *in vitro* parasite-growth inhibiting property of mouse anti-GMZ2 antibody in an attempt to test the protective potential of the hybrid protein. A subsequent preclinical evaluation in *Saimiri sciureus* monkeys demonstrated that the GMZ2 candidate vaccine was immunogenic and conferred partial protection against challenge infection (Carvalho et al. 2005). Phase 1 clinical trials of the candidate vaccine conducted in malaria naïve (Esen et al. 2009) as well as naturally exposed adults and children (Mordmüller et al. 2010, Be'lard et al. 2011, Tamborrini et al. 2011) demonstrated the immunogenicity, tolerability and safety of GMZ2. Currently multi-center phase IIb efficacy trial of the GMZ2 vaccine in the target population (children 1-5 years old) in four sub-Saharan African countries (Burkina Faso, Gabon, Tanzania and Uganda) is in good progress (<http://www.Amanet-trust.org/GMZ2>, retrieved May 10, 2012).

The gene regions of 220-kDa GLURP (de Stricker et al. 2000, Theisen et al. 1995, Theisen et al. 1998, Theisen et al. 2000) and the 48-kDa MSP3 (Huber et al. 1997, McColl and Ander 1997, Singh et al. 2004b, Singh et al. 2009) included in GMZ2 are conserved major B-cell epitopes. While MSP3 is an erythrocytic stage protein GLURP-R0 is expressed in both pre-erythrocytic and erythrocytic stages of *P. falciparum* (Cowman and Crabb 2006). Apart from the conserved nature of the MSP3 and GLURP-R0 domains and their improved immunogenicity in GMZ2 in pre-clinical and clinical trials, both antigens are individually associated with protection from clinical malaria in various studies (Cavanagh et al. 2004, Osier et al. 2007, Tongren et al. 2006). The probable mechanism of protection for MSP3 and GLURP is similar, antibody-dependent cellular inhibition (ADCI) (Theisen et al. 2004).

In single immunisation the vaccine potential of MSP3 and GLURP for multiple B and T helper cell epitopes can be tested. Long synthetic peptides, as both GLURP and MSP3 were used in phase I clinical trials individually (Hermsen et al. 2007, Audran et al. 2005), are difficult and expensive to synthesize in addition to the problem of size restrictions and non-controlled folding (Esen et al. 2009). If the antigenic properties of the subunits could be maintained in a combined fusion form in GMZ2, it would be advantageous as the latter is relatively well-expressed in a well-characterized industrial organism. Anti-GMZ2 response in naturally infected individuals is little explored. Anti-GLURP-R0 and -MSP3 antibody responses may have various forms of possible interactions – antagonistic, synergistic or no interaction under natural settings.

2. THE RESEARCH QUESTION

Apart from a serological survey conducted in the early 1970's (Collins et al. 1971) and a few studies on anti-malaria lymphocyte activation (Kassa et al. 2006, Worku et al. 1997, Worku 2001), there is lack of data on malaria immunity among the population that reside in endemic regions of Ethiopia. In this regard Ethiopia is lagging behind many malaria endemic African countries which have already begun vaccine efficacy trials. To narrow this gap generation of base-line data on naturally acquired immune responses to *P. falciparum* antigens would be necessary. There are several research questions in this respect. Are *P. falciparum* blood-stage vaccine candidate antigens readily recognized in the population? Is there considerably lower immune response in the population in the absence of microscopically detectable current *P. falciparum* infection? Are anti-malaria antibody responses short-lived? Is there a dominance of antibody subclasses that are associated with malaria pathology, in other endemic settings, in the Ethiopian population? Is immune build up against malaria in the Ethiopian setting correlated with exposure?

Hypothesis

Humoral immunity against blood-stage falciparum malaria vaccine candidate antigens under seasonal malaria epidemiological conditions in Ethiopia is long-lived and is characteristically protective.

General Objective

To produce a base-line data on the status of acquired humoral immunity to falciparum malaria blood-stage vaccine candidate antigens, in unstable seasonal malaria transmission settings in Ethiopia.

Specific Objectives

1. To determine the characteristic antibody responses to different *P. falciparum* recombinant blood-stage vaccine candidate antigens and evaluate the relative recognition or immunogenicity of *P. falciparum* vaccine candidates in naturally exposed Ethiopians.
2. To assess the longevity of antibody responses, by using retrospective data, to different *P. falciparum* blood-stage vaccine candidate antigens in individuals in two different malaria epidemic settings in Ethiopia.
3. To determine IgG subclass profile to *P. falciparum* blood-stage candidate vaccine antigens in two different malaria epidemic settings in Ethiopia.
4. To assess the age-related pattern of antibody responses to different *P. falciparum* recombinant antigens in Ethiopia.

3. MATERIALS AND METHODS

3.1. Study Sites

The climate of most Ethiopian malaria endemic regions is Sahelian with small rains ('Belg') in March and April and heavy rain (Kiremt) from June to September followed by a long, dry season from mid-September to February. May is largely a dry month. Malaria in most parts of the country is meso-/hypoendemic and seasonal although stable and perennial transmission occurs largely in western lowlands and river basins. *P. falciparum* is the major species (60-70%) in Ethiopia accounting for most malaria-related mortality and morbidity followed by *P. vivax* (30-40%) as reported by the Federal Democratic Republic of Ethiopia Ministry of Health (FMoH 2007).

This study was undertaken in two malaria endemic localities. These were Boditi in the south and Shewa Robit in north-central Ethiopia. Boditi 370 km away from the capital Addis Ababa along the way to Arba-Minch is the center of Damot Gale District in Wolayta Zone, Southern Nations, Nationalities and Peoples Regional State (SNNPR). The town lies at an altitude of 2059 meters above sea level (masl) with geographical coordinates between latitudes 6°57'32.04''N and longitudes 37° 51'41.55''E. Shewa Robit which is a District town is located in Amhara Regional State, Semiene Shewa Zone, Kewet District about 225 km to the north of Addis Ababa along Dessie road. Figure 2 shows the location map of the two study sites. Mean yearly rainfall of Boditi is 1600 mm and the mean annual maximum and minimum temperatures are 13-25°C (Damot Gale District agricultural office record 2008). The climate of Shewa Robit located at 12°0'47.32''N latitude and 39°37'42.91''E longitude at 1380 masl is warm with 16.5°C mean annual minimum and 31°C maximum temperatures. The mean

annual rainfall is 1007 mm (Amhara regional state Bureau of Agriculture (BoA) 2000).

Damot Gale District was inhabited by 154,610 people (male 75,981; female 78,629) and Boditi town population was 27,684 (population and housing census 2007). The majority in the District are the Wolayta, Omotic people. According to this same census report, while Shewa Robit town had a total population size of 17,575 (male 8874, female 8,701) Kewet District was populated by 118,333 (male 60,911; female 57,422) people. The report showed that the majority of the people in the District belonged to the Amhara ethnic group.

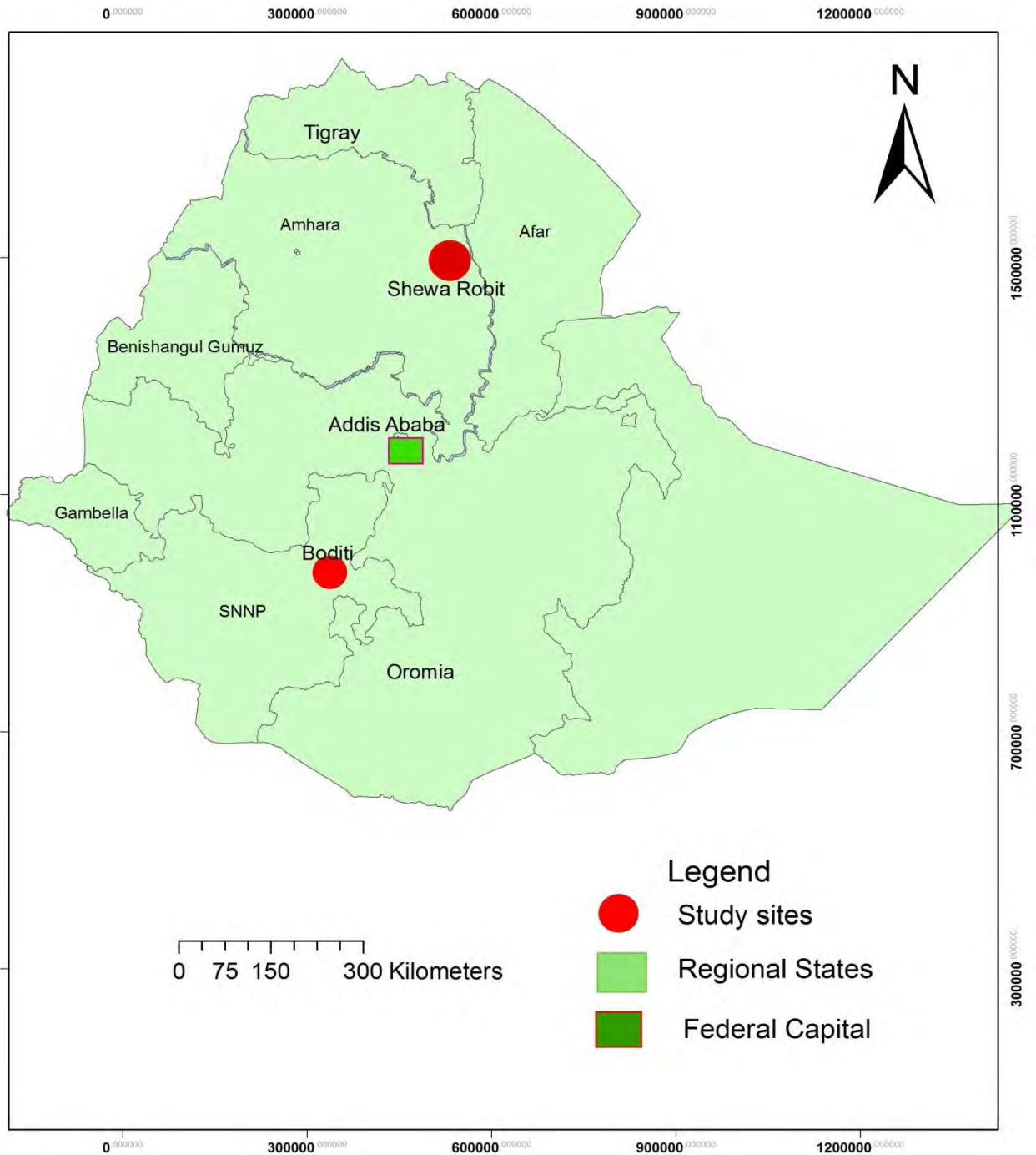


Figure 2 Study sites of antibody analysis against *P. falciparum* blood-stage vaccine candidate antigens in Boditi and Shewa Robit

Both Shewa Robit and Boditi are characterized by tropical climate and receive high rainfall during the main rainy season and are characterized by markedly unstable and seasonal malaria transmission standing among major epidemics-prone areas. Malaria control efforts were well in place in both endemic areas in accordance with the national malaria control policy. Both study sites had one healthcare center each. There were health posts in all the Kebeles of the two Districts to treat suspected malaria cases on the basis of Rapid Diagnostic Test (RDTs) and clinical diagnosis. ITN distribution was 100% at household level in both study sites but poor utilization was reported in Boditi according to health facility information system. IRS was practiced twice per six month regularly. Some delays in IRS accompanied by the reportedly poor ITN usage together with unusual changes in local climatic conditions resulted in low level epidemic eight months preceding the current study in Boditi. The situation is declared epidemics when prevalence is >20% after examining 50 random slides (Personal communication, Damot Gale District health officer). The most recent epidemic in Shewa Robit was in 2005 (local health care system record). Thus it was expected that most individuals in the two towns and their surroundings are exposed to falciparum malaria at least once. However, no data is available pertaining to the dynamics of malaria transmission including annual entomological inoculation rates and prevalence estimates.

3.2. Ethical Considerations

The project was approved by Department of Biology Ethics Review Committee, Addis Ababa University and had a National Ethical Clearance. Informed consent was obtained from adult participants and parents/guardians for children prior to sampling.

Malaria smear-positive cases were treated free of charge in accordance with the national treatment guideline.

3.3. Retrospective Data, Study Populations and Sample Size

Review of malaria incidences in the two study sites for the last five years (2004-2008) prior to the study was made from the respective health facility records. Geographically restricted sections of the two towns (peripheral and central parts) with relatively higher potential for malaria incidence were selected based on pre-survey data from local health facilities and recommendations by healthcare providers. Malaria prevalence approximation was made for the study areas based on population census and retrospective data taking into account cases that were beyond the scope of local healthcare facilities. As malaria prevalence in the areas was not known before, a 25% estimate for the dry season was assumed to give the best sample size (Munyekenye et al. 2005). Accordingly, the sample sizes for the two study sites were calculated with 95% confidence interval (CI) and 5% precision level by the formula (Munyekenye et al. 2005) $n = \frac{z^2(pq)}{d^2}$ (where, n is sample size, z is the critical value of the standard normal distribution (1.96), p the malaria prevalence estimate, $q = 1-p$, and d is the precision level).

3.4. Study Design and Sampling

Community members residing in the study sites for at least two years were invited to take part in the cross-sectional study and consenting members were included in the study. Sociodemographic and other relevant information in the geographic clusters were recorded using questionnaire and interview. In Shewa Robit, study participants were requested to self-report their past malaria episode(s), if any, counting the number

and pointing out the most recent episode. As much as possible, dates of disease episodes and species of malaria parasite were confirmed from the records of local health center for individuals who reported that they had malaria episodes. Cross-sectional parasitological surveys were undertaken to detect concurrent infection in the dry low transmission season (late November 2008 in Shewa Robit and December 2009 in Boditi). Five ml of venous blood was collected from each consenting participant. Malaria RDTs using *CareStart™ malaria HRP2/pLDH(Pf/pan) Combo Cassette Test* (Access Bio, Inc.) was carried out as per the manufacturer's instruction. Thick and thin blood-smears were prepared and microscopically screened for *Plasmodium* parasite infection following the standard procedure (WHO 2010b). Sera were separated and stored at -20°C until use for antibody assay. The flow chart of the study design is shown below (Figure 3).

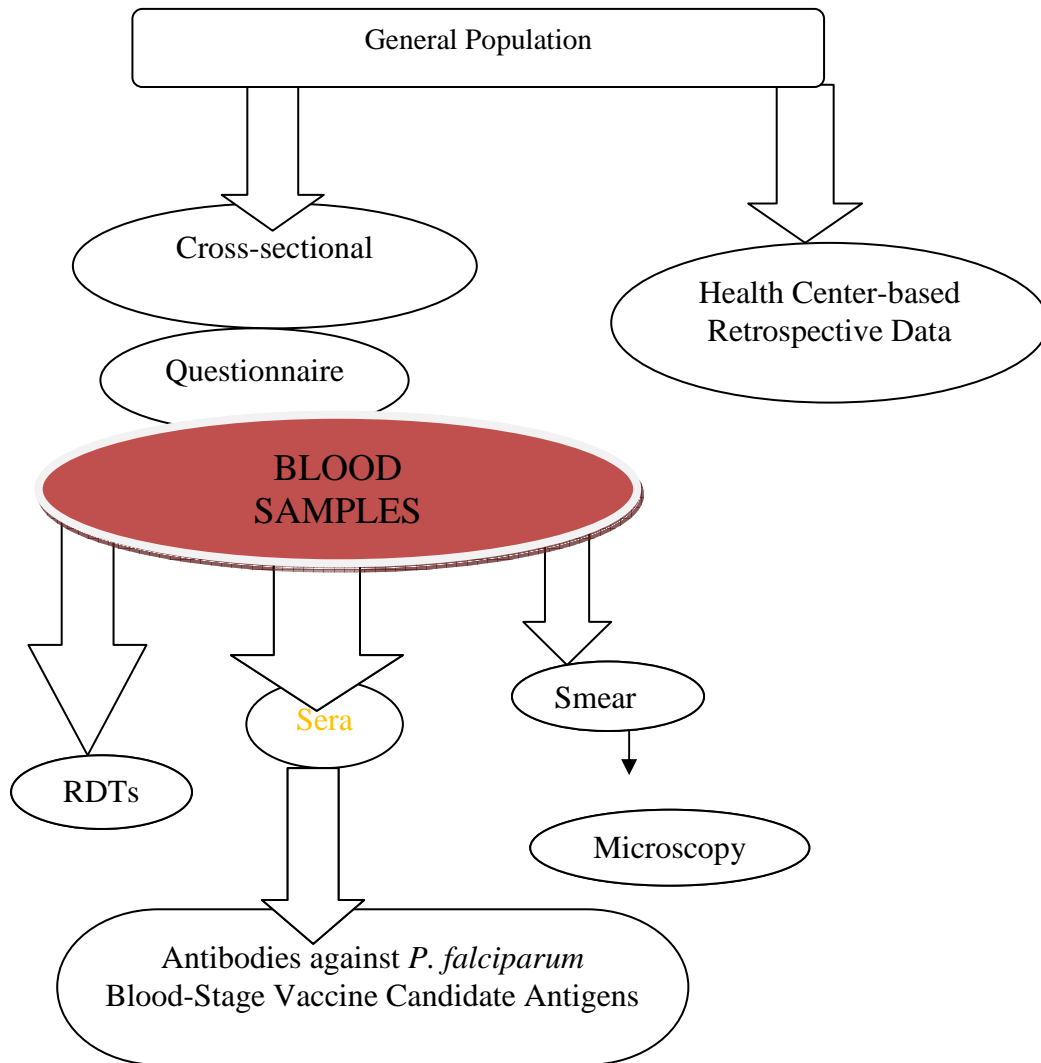


Figure 3 Study design of antibody analysis against *P. falciparum* blood-stage vaccine candidate antigens in Boditi and Shewa Robit.

3.5. Antibody Analysis: Recombinant Antigens and ELISA Procedures

AMA1 gene encoding amino acids 25-545 expressed in *Escherichia coli* as an N-terminal His-tagged recombinant protein (3D7 strain) (Anders et al. 1998, Hodder et al. 2001), MSP2 allelic families (3D7, FC27) (Hoffmann et al. 2001), GLURP-R0 (amino acids 27–500) and -R2 (amino acids 705–1178) regions (Theisen et al. 1998), MSP3 C-terminal region (amino acid 212-380) (Oeuvray et al. 1994) and the GMZ2 vaccine (Theisen et al. 2004.) were used for total IgG analysis. IgG subclasses were

typed using GLURP-R0 and MSP3. GMZ2 was kindly donated by Mr Ce'dric Delsemme (Henogen SA, Belgium), AMA1 and MSP2 by Prof Robin Anders, La Trobe University, Australia; GLURP and MSP3 by Prof Michael Theisen, Statens Serum Institute, Copenhagen, Denmark.

The laboratory work of the study was undertaken in two Phases. The first Phase was done at Department of Immunology, Wenner-Gren Institute, Stockholm University, Stockholm, Sweden. Phase 2 was conducted at Department of Human Parasitology, Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany. In phase 1, total IgG was measured using antigens AMA1, MSP2-3D7, MSP2-FC27 and GLURP-R2 for the Shewa Robit sera. In phase 2, total IgG was determined against GLURP-R0, GMZ2 and MSP3 on both Shewa Robit and Boditi sera. For IgG subclass analysis only GLURP-R0 and MSP3 were used for sera from both sites. So, expectedly there were slight modifications in the assay system in the two phases and data were analyzed separately. Positive high-titer reference serum with confirmed *P. falciparum* infection and pooled negative sera from European donors, never exposed to malaria, were used for assay standardization and seropositivity determination. The test protocol in phase 1 was based on Iriemenam et al. (2009) and that of phase 2 on Esen et al. (2009), both of which are based on the original ELISA procedure (Engvall and Perlman 1971).

Phase 1

Ninety six-well flat-bottomed ELISA plates (Costar, Cambridge, MA, USA) were coated with 1µg/ml solutions of AMA1, MSP2-3D7, MSP2-FC27 or 0.5µg/ml GLURP-R2 in a coating buffer {PBS stock (10x) = 81g NaCl+40g

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} + 5\text{g KH}_2\text{PO}_4 + 1\text{ml } 20\% \text{ NaN}_3$; pH 7.4}. The coating volume was 100 μl /well. The plates were incubated overnight at 4 $^\circ\text{C}$. The plates were blocked for 2 h at 37 $^\circ\text{C}$ by using 100 μl /well blocking buffer {(1.59g $\text{Na}_2\text{CO}_3 + 2.93\text{g NaHCO}_3 + 1000\text{ml dH}_2\text{O}$; pH 9.6), containing 0.5% bovine serum albumin (BSA)}. The plates were washed four times in a washing buffer {PBS (81g $\text{NaCl} + 40\text{g Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} + 5\text{g KH}_2\text{PO}_4 + 1\text{ml } 20\% \text{ NaN}_3$; pH 7.4) with 0.1% Tween(T)-20 and 0.15% Kathon (Mabtech, Nacka, Sweden)} in microplate washer (Skan washer 300, CA, USA). 50 μl /well sera diluted 1:1000 in incubation buffer {PBS (81g $\text{NaCl} + 40\text{g Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} + 5\text{g KH}_2\text{PO}_4 + 1\text{ml } 20\% \text{ NaN}_3$; pH 7.4) with 0.5% BSA} were loaded, in duplicate and incubated for 1 h at 37 $^\circ\text{C}$. To detect bound IgG antibodies alkaline phosphatase (ALP) conjugated to goat anti-human IgG (1:2000) (Mabtech) was added (50 μl /well) and further incubated for 1 h at 37 $^\circ\text{C}$. Finally, the assay was developed by adding 50 μl /well *p*-nitrophenyl phosphate (Sigma–Aldrich GmbH, Steinheim, Germany) in enzyme substrate buffer (97ml $\text{HN}(\text{CH}_2\text{CH}_2\text{OH}) + 101\text{g MgCl}_2 \cdot 6\text{H}_2\text{O} + 100\text{ml HCl (1M)}$, 600ml dH_2O ; pH 9.8) and keeping the plates in dark. After 20 minutes the optical densities (OD) were read (at 405 nm) using VmaxTM Kinetic microplate reader (Molecular Devices, Menlo Park, USA).

Phase 2

Coating was done using high binding microtitre plates (NUNC MaxisorpTM, Germany). The coating concentrations were 0.5 $\mu\text{g/ml}$ for GMZ2 and GLURP-R0 and 1 $\mu\text{g/ml}$ for MSP3 in coating buffer. The coating buffer was PBS tablets {10mM $\text{Na}_2\text{HPO}_4 \cdot 2 \text{ H}_2\text{O}$, 150mM NaCl } (Gibco®, Invitrogen, UK) dissolved in dH_2O , pH 7.4. The coating volume was 100 μl /well. The coated plates were washed four times after overnight incubation at 4 $^\circ\text{C}$. The washing buffer was a mixture of PBS, 0.1% T-

20 and 0.5M NaCl. The plates were blocked using blocking buffer (150µl/well) [PBS (10mM Na₂HPO₄.2 H₂O, 150mM NaCl), 3% non-fat milk powder, 0.1% T-20] and incubated for 1 h shaking gently on a vibrating platform shaker (Heidolph Titramax 1000, Germany) at room temperature. The plates were washed again. Optimum dilutions (1:400 for total IgG and 1:100 for IgG subclasses) were used for Boditi and Shewa Robit sera. The dilutions were in a dilution buffer [PBS (10mM Na₂HPO₄.2 H₂O, 150mM NaCl), 1% non-fat milk, 0.1% T-20, 0.02% NaN₃]. The diluted sera were loaded (100µl/well) and the plates were incubated for 2 h on vibrating platform shaker at room temperature. After washing horseradish peroxidase (HRP) conjugated goat anti-human IgG (Caltag, USA) diluted 1:3000 in dilution buffer was added (100µl/well) and incubated in a room temperature vibrating platform shaker for 1 h to detect malaria-specific IgG. After washing four times, 3,3',5,5'-tetramethylbenzidine (TMB-ONE) (KEM EN TEC, Belgium) was loaded (100µl/well) as a substrate for the HRP and incubated in dark for 20 minutes. OD was read at 450 nm by plate reader (Asys Expert 96, Type G018065). For IgG subclass analysis the same steps were followed except that one additional step of incubation was done with HRP-conjugated goat anti-mouse IgG (1:3000) (Invitrogen, UK) after capturing the subclasses with mouse anti-human monoclonal IgG1 (1:3000) or IgG3 (Skybio, UK), IgG2 (1:5000) (Sigma, Germany) and IgG4 (1:3000) (Caltag, USA).

Samples having a discrepancy of greater than 25% between duplicates were retested. Background was determined from the OD values of blank wells and that was deducted from the mean OD reading of each sample. Seropositivity cutoff threshold was determined as the mean plus 3 standard deviations of readings for the negative-control sera samples.

3.6. Data Analysis

The data was double entered into separate Microsoft Excel spreadsheets. As mean antibody levels were not normally distributed, nonparametric tests were used for analyses. Correlations between OD values of total IgG and subclasses to different antigens were tested by Spearman's rank correlation, and differences in the median ODs with age, self-reported past clinical malaria episodes, and *P. falciparum* slide-positivity were compared using a two-sample Wilcoxon rank sum test. Differences in the prevalence of antibody responses in age groups and differences in seropositivity rates to the different antigens were assessed using the Fisher's exact test. The Kruskal-Wallis and Mann-Whitney tests were used to test for differences in antibody levels between the different antigens and study groups. Multiple comparisons were used to determine significant differences between specific age groups. Statistical analyses were performed using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Two-tailed *p*-values at <0.05 , 95% CI were considered statistically significant.

4. RESULTS

4.1. Retrospective Data and Study Participants

The proportion of average number of symptomatic malaria cases in the two communities during the years prior to the study (2004-2008) was higher for Boditi than Shewa Robit (Table 1). Two hundred twenty eight serum samples were collected from participants in Shewa Robit. The mean age was 28 and there were no individuals below 5 years. All participants were asymptomatic and had no microscopically detectable *P. falciparum* infection at the time of sample collection. Table 2 shows demographic characteristics and questionnaire responses of the study participants in Shewa Robit. Two hundred sixty five serum samples were collected from residents of Boditi with mean age of 23 years. The highest age was 80 and the lowest was 1 year. Age and sex distribution of the study participants in Boditi is shown in Table 3. Forty four individuals were febrile during sample collection. Eleven (25%) among the febrile group were smear-positive for *P. falciparum*. Twelve asymptomatic infections were detected making the total number of positives 23(8.6%). There was 1 *P. vivax* infection.

Table 1 Malaria clinical cases in Shewa Robit and Boditi as reported by the respective Health Centers, 2004-2008.

| | 2004 | | 2005 | | 2006 | | 2007 | | 2008 | |
|----------------------|----------------|----------------|----------------|-----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| | N(%) | | N(%) | | N(%) | | N(%) | | N(%) | |
| | Boditi | Shewa Robit | Boditi | Shewa Robit | Boditi | Shewa Robit | Boditi | Shewa Robit | Boditi | Shewa Robit |
| <i>P. falciparum</i> | 9340 (74.0) | 3959 (81.7) | 8428 (88.8) | 10314 (78.9) | 6452 (79.9) | 3073 (76.9) | 5731 (76.7) | 1563 (71.5) | 2794 (78.9) | 1190 (80.0) |
| <i>P. vivax</i> | 3292 (26.0) | 602 (12.3) | 966 (11.2) | 1564 (21.1) | 1623 (20.1) | 590 (14.8) | 1457 (19.5) | 625 (28.5) | 246 (6.9) | 298 (20.0) |
| Mixed | - | 315 (6.5) | - | - | - | 332 (8.3) | 281 (3.7) | - | 497 (14.1) | - |
| Total | 12632 | 4876 | 9394 | 11878 | 8075 | 3995 | 7469 | 2188 | 3537 | 1488 |

Table 2 Demographic and self-reported past falciparum malaria episode data for study participants in antibody analysis against *P. falciparum* blood-stage vaccine candidate antigens, Shewa Robit 2008 (N=228)

| | Male No (%) | Female No (%) | Total No (%) |
|------------|----------------|------------------|-----------------|
| Age groups | | | |
| 5-14 | 7(3.0) | 13(5.7) | 20(8.7) |
| ≥15 | 181(79.4) | 27(11.8) | 208(91.2) |
| MCs | | | |
| No | 44(19.3) | 7(3.0) | 51(22.4) |
| Yes | 145(63.6) | 32(14.0) | 177(77.6) |
| LMCs | | | |
| 1998-2004 | 28(12.2) | 5(2.2) | 33(14.4) |
| 2005 | 36(15.8) | 9(3.9) | 45(19.7) |
| 2006 | 17(7.4) | 1(0.40) | 18(7.8) |
| 2007 | 30(13.2) | 8(3.5) | 38(16.7) |
| 2008 | 34(14.9) | 9(3.9) | 43(18.8) |

MCs: Malaria cases, LMC: Latest Malaria Case

Table 3 Age and sex distribution of study participants in Boditi 2009 (N=265).

| Age (yr) | Male | | Female | Total |
|----------|------------|-----------|----------|-----------|
| | No. (%) | | No. (%) | No. (%) |
| 0-4 | 22(8.3) | | 22(8.3) | 44(16.6) |
| 5-14 | 51(19.2) | | 36(13.6) | 87(32.8) |
| ≥15 | 40(15.1) | | 94(35.5) | 134(50.6) |
| Total | 113 (42.6) | 152(57.4) | 265(100) | |

4.2. Antibody Prevalence and Level

ELISA cutoff OD values for the different antigen tested are shown in tables 4 and 5 for Shewa Robit and Boditi respectively. Total IgG positivity rates and levels to the different antigens for Shewa Robit and Boditi are indicated in Table 6. Antibody responses to GMZ2, MSP3 and GLURP-R0 were considerably high and at detectable level in most individuals in both sites. Positivity rates and levels to GMZ2 were significantly higher than that to MSP3 ($p = 0.000$) and GLURP-R0 ($p = 0.000$) in both sites. Anti-GLURP-R0 total IgG level was significantly higher than MSP3 ($p = 0.000$) in both study participants. Total IgG positivity rates were higher in Shewa Robit than Boditi for all the three antigens though the differences were insignificant. Positivity rates of IgG1 and IgG3 to GLURP-R0 and all subclasses against MSP3 were significantly higher in Shewa Robit than Boditi ($p < 0.01$) (Table 7). Total IgG levels were significantly higher in Shewa Robit than Boditi for all the three antigens after adjusting for age. MSP3-specific IgG1 and IgG2 titers and IgG4 against MSP3 and GLURP-R0 were significantly higher in Shewa Robit than Boditi ($p \leq 0.01$).

The differences in total IgG positivity rates between all other antigens tested in Phase 1 ELISA were significant except when AMA1 and MSP2-3D7 were compared. While anti-AMA1 IgG level was not significantly different from the level against MSP2-3D7 ($p = 0.072$) and GLURP-R2 ($P = 0.091$) it was significantly lower than that to MSP2-FC27 ($p = 0.000$). GLURP-R2 level was significantly lower than the level to MSP2-3D7 ($p = 0.010$) and MSP2-FC27 ($p = 0.000$). IgG level to MSP2-FC27 was significantly higher than MSP2-3D7 ($p = 0.000$).

Table 4 ELISA cutoff OD values for total IgG and subclass antibodies against the different antigens tested for Shewa Robit sera.

| Abs | <i>P. falciparum</i> Recombinant Antigens | | | | | | |
|-----------|-------------------------------------------|----------|----------|---------|----------|-----------|----------|
| | AMA1 | GLURP-R0 | GLURP-R2 | GMZ2 | MSP2-3D7 | MSP2-FC27 | MSP3 |
| Total IgG | 0.092889 | 0.076627 | 0.041121 | 0.07285 | 0.097075 | 0.097483 | 0.073045 |
| IgG1 | - | 0.046635 | - | - | - | - | 0.069662 |
| IgG2 | - | 0.063896 | - | - | - | - | 0.077536 |
| IgG3 | - | 0.050761 | - | - | - | - | 0.041388 |
| IgG4 | - | 0.018584 | - | - | - | - | 0.025136 |

Table 5 ELISA cutoff OD values for total IgG and subclass antibodies against the different antigens tested for Boditi sera.

| Abs | <i>P. falciparum</i> Recombinant Antigens | | |
|-----------|-------------------------------------------|--------|----------|
| | GLURP-R0 | GMZ2 | MSP3 |
| Total IgG | 0.080283 | 0.0836 | 0.081929 |
| IgG1 | 0.033821 | - | 0.025014 |
| IgG2 | 0.024841 | - | 0.014551 |
| IgG3 | 0.035481 | - | 0.024264 |
| IgG4 | 0.014125 | - | 0.012878 |

Table 6 Total IgG positivity and level to *P. falciparum* blood-stage vaccine candidate antigens in Boditi and Shewa Robit sera.

| | Positivity rate (%) ¹ | | ² Median OD values (±SD) ² | |
|-----------|----------------------------------|---------------------|--------------------------------------------------|---------------------|
| | Boditi (n=265) ³ | Shewa Robit (n=228) | Boditi (n=265) | Shewa Robit (n=228) |
| GMZ2 | 240(90.6) | 226(99.1) | 0.416(1.159) | 0.540(0.903) |
| GLURP-R0 | 226(85.3) | 206(90.3) | 0.284(1.092) | 0.366(0.510) |
| MSP3 | 216(81.5) | 215(94.3) | 0.264(0.940) | 0.272(0.633) |
| AMA1 | - | 148(64.9) | - | 0.122(0.166) |
| MSP2-3D7 | - | 150(65.8) | - | 0.121(0.166) |
| MSP2-FC27 | - | 186(81.6) | - | 0.198(0.636) |
| GLURP-R2 | - | 96(42.1) | - | 0.110(0.352) |

¹Fisher's exact test was used to test the significance between differences in positivity rates for any two antigens. ²Differences between the medians were analyzed by Kruskal-Wallis test ($P<0.05$). ³Boditi sera were tested for GMZ2, GLURP-R0 and MSP3 only.

Table 7 IgG subclass positivity and level to *P. falciparum* GLURP-R0 and MSP3 in Boditi (N=265) and Shewa Robit (N=228) sera

| | GLURP-R0 | | | | MSP3 | | | |
|------|---------------------------------------|--------------------|-------------------------------------|--------------------|---------------------------------------|------------------|-------------------------------------|--------------------|
| | Positivity rate (no. (%) ¹ | | ² Median OD values (±SD) | | Positivity rate (no. (%) ¹ | | ² Median OD values (±SD) | |
| | Boditi | Shewa Robit | Boditi | Shewa Robit | Boditi | Shewa Rob | Boditi | Shewa Robit |
| IgG1 | 171(64.5) | 192(84.2) | 0.302(1.186) | 0.337(0.812) | 146(55.1) | 209(91.7) | 0.140(0.970) | 0.230(0.589) |
| IgG2 | 203(76.6) | 178(78.1) | 0.246(0.652) | 0.324(0.573) | 89(33.6) | 213(93.4) | 0.055(0.328) | 0.279(0.337) |
| IgG3 | 147(55.5) | 171(75.0) | 0.135(0.928) | 0.197(0.671) | 108(40.8) | 146(64.0) | 0.096(0.513) | 0.107(0.245) |
| IgG4 | 73(27.5) | 72(31.5) | 0.018(0.136) | 0.067(0.37) | 32(12.1) | 74(32.4) | 0.026(0.203) | 0.088(0.103) |

¹Fisher's exact test was used to test the significance between differences in positivity rates for the two antigens. ²Differences between the medians were analyzed using a Kruskal-Wallis test ($P<0.05$).

For Boditi sera, 189(71.3%) individuals were total IgG positive against all the three antigens and 18(6.8%) did not have detectable IgG level against any of the antigens.

For Shewa Robit a large number of individuals were total IgG positive for one antigen and not for the other although most individuals positive for any other antigen were also positive for GMZ2, MSP3 and GLURP-R0 concurrently. While one individual responded to GMZ2 only, more than 97% were positive against more than 3 of the seven antigens tested. Particularly, some individuals were positive to one of the two allelic variants of MSP2 but not to the other. A substantial number of individuals (about 20%) were positive for MSP2-FC27 but not for MSP2-3D7. Similarly, 51% of

individuals positive for GLURP-R0 were not so for GLURP-R2. Conversely, few individuals were positive for MSP2-3D7 or GLURP-R2 but not MSP2-FC27 or GLURP-R0, respectively. Anti-AMA1 and-GLURP-R2 responses were relatively the least frequently detected though both antigens were well-recognized by the test sera. There was no serum sample that was not total IgG positive at least against one antigen. Totally, over 70% of the study participants were total IgG positive for five or more antigens (Table 8).

Table 8 Individuals total IgG positive for 1, 2, 3, 4 & ≥ 5 antigens, Shewa Robit 2008 (N=228).

| No. of Ags | No. of seropositives (SP) | % |
|------------|---------------------------|------|
| 1 | 1 | 0.44 |
| 2 | 5 | 2.2 |
| 3 | 22 | 9.6 |
| 4 | 39 | 17.1 |
| ≥ 5 | 161 | 70.6 |

Different proportions of individuals had higher/lower IgG responses (above/below the mean value) for the various antigens tested (data not shown). An individual who showed high response for one antigen had not necessarily high response for others.

4.3. Intercorrelations between antibody levels to the different antigens

For both Boditi and Shewa Robit, there was significant intercorrelation between total IgG and subclass responses to GLURP-R0, GMZ2 and MSP3 at different levels of strength. IgG response to GLURP-R0 was significantly correlated with its response to GMZ2 ($P = 0.000$, $R = 0.906$) and MSP3 ($P = 0.000$, $R = 0.757$). There was a positively significant correlation between IgG response to GMZ2 and MSP3 ($P =$

0.000, $R = 0.826$) although it was weaker than the strength for GLURP-R0 and GMZ2 (Figures 4-5) in both sites. In general, the correlation between IgG to GLURP-R0 and IgG subclasses to the same antigen was stronger than that to MSP3. Further, there was stronger tendency for the correlations between IgG to GMZ2 and subclass responses to GLURP-R0 than MSP3 in both sites. Total IgG responses to AMA1, MSP2-3D7, MSP2-FC27 and GLURP-R2 were significantly positively correlated with each other. Particularly, anti-MSP2-3D7 and MSP2-FC27, the two allelic variants of MSP2, responses have shown the strongest correlation ($P = 0.000$, $R = 0.651$) in this regard (Figure 6). However, anti-GLURP-R0 and GLURP-R2 total IgG levels were not significantly correlated.

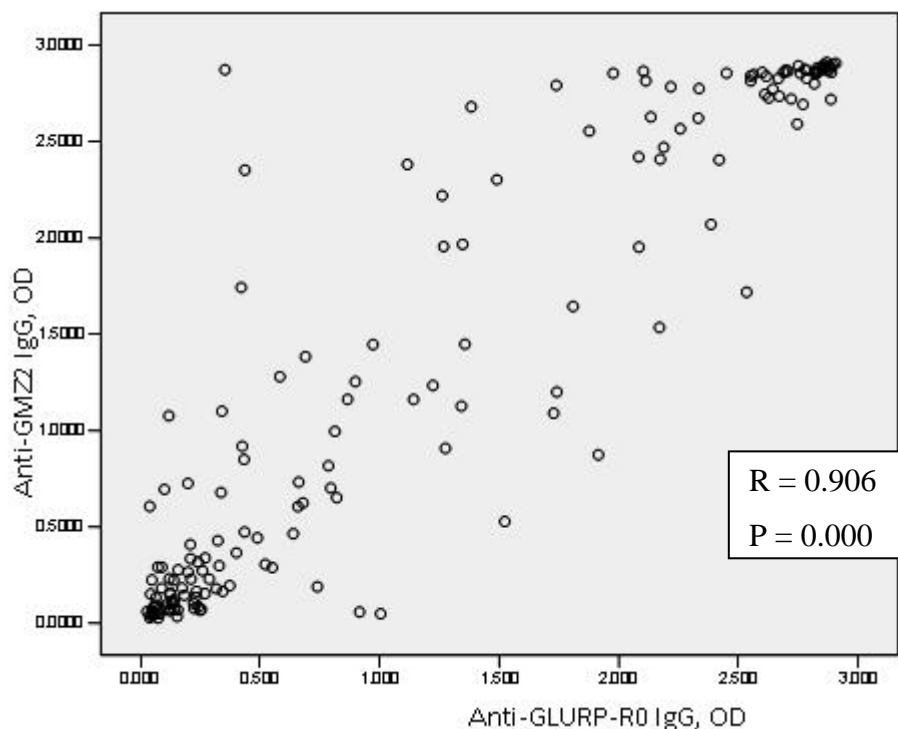


Figure 4 Correlation between IgG to *P. falciparum* GMZ2 and GLURP-R0, Boditi 2009.

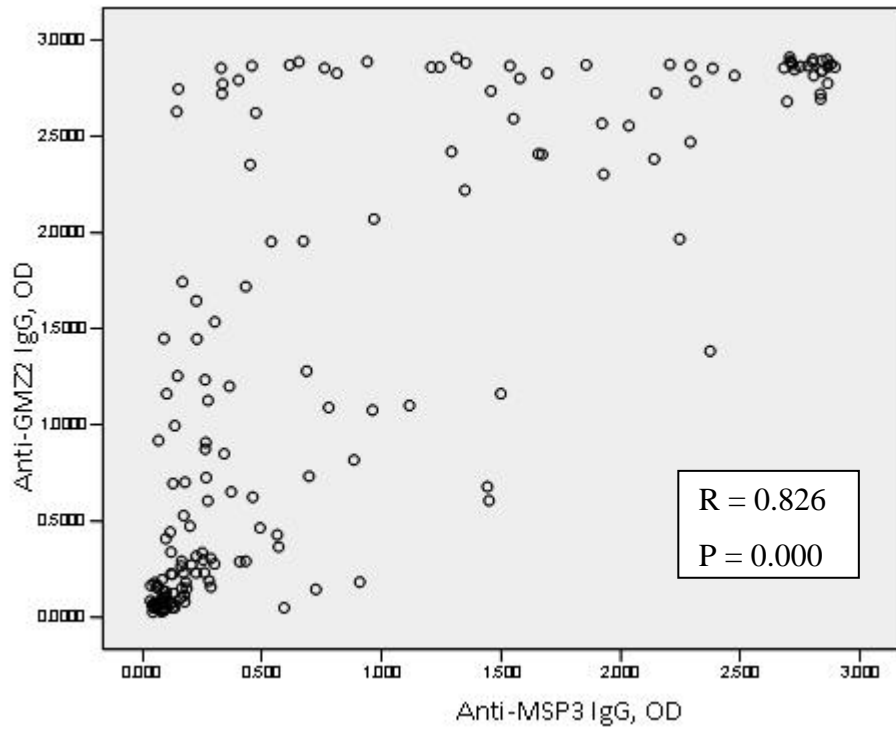


Figure 5 Correlation between IgG to *P. falciparum* GMZ2 and MSP3, Boditi 2009.

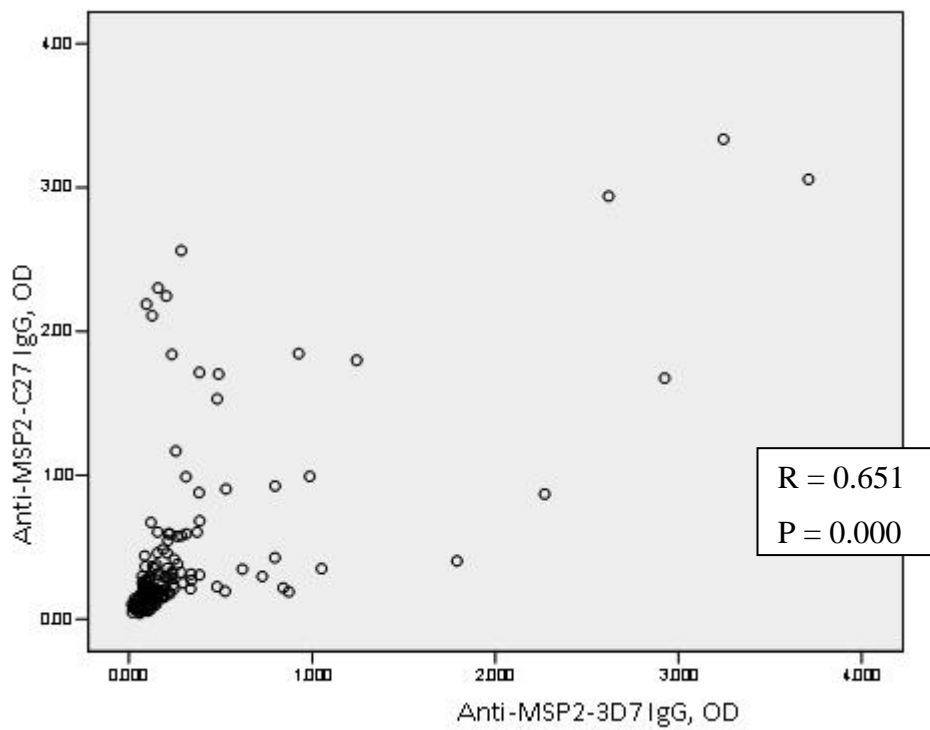


Figure 6 Correlation between IgG levels to MSP2-3D7 and MSP2-FC27, Shewa Robit 2008.

4.4. Self-Reported Past Malaria Episode and Antibody Response in Shewa Robit

Fifty one (22%) of the study participants reported that they had not been exposed to clinical malaria episodes in their life time in spite of residing in endemic area(s). The rest 177 (78%) participants reported at least one clinical malaria episode with laboratory confirmed *P. falciparum* infection. The subgroups among the exposed were those who had experienced a clinical episode once (71/177(40.1%) and more than once (106/177(59.9%). Total IgG positivity to the different antigens differed between exposed and unexposed individuals (Table 9). While to AMA1 the unexposed group had significantly higher positivity than the exposed ($p = 0.008$); for MSP2-3D7 ($p = 0.000$), MSP2-FC27 ($p = 0.000$) and GLURP-R2 ($p = 0.037$) the opposite was noticed. For GLURP-R0, GMZ2 and MSP3 no significant difference was found between the two groups although the unexposed had slightly higher prevalence for GLURP-R0, GMZ2 and the opposite for MSP3 was observed.

The difference in IgG positivity rate to the antigens between those who reported malaria episode once and more than once was less marked. However, the rate to AMA1 ($p = 0.001$) (higher in once), MSP2-FC27 ($p = 0.000$) (higher in more than once) and GLURP-R0 ($p = 0.000$) (higher in more than once) indicated significance between the two groups. Anti-AMA1 positivity rate was the highest among those who reported their most recent episode in 1991-2004 (Table 10). Similarly, anti-AMA1 antibody level was significantly higher in individuals who reported that they were unexposed to malaria episode in their life time than the exposed ($p = 0.000$) (Table 11). On the other hand, the highest median antibody level against this antigen was for individuals whose latest reported episode was in 2008, the year of sample collection (Table 12). Antibody level was also significantly higher in the group that reported

exposure to malaria episode than those not exposed for MSP2-3D7 and MSP2-FC27. IgG levels to these antigens were significantly higher in individuals who reportedly had episodes more than once compared to those who had reported only one episode.

IgG subclass positivity against MSP3 and GLURP-R0 was equally distributed among those reported malaria episode and not, and those who experienced the episode(s) once and more than once for all subclass antibodies characterized. But IgG3 positivity against GLURP-R0 was significantly higher in those who reported episode once than those more than once ($P = 0.026$). IgG4 positivity to both MSP3 and GLURP-R0 was the highest, though not significant, in the group who reported their latest episode sometime from 1991-2004. The proportion of individuals who reported unexposed to malaria episode but were IgG positive was high and varied with antigen. It was 80.3, 52.9, 64.7, 86.2, 29.4, 100 and 92.1 percent respectively; for AMA1, MSP2-3D7, MSP2-FC27, GLURP-R0, GLURP-R2, GMZ2 and MSP3.

Table 9 IgG prevalence to *P. falciparum* recombinant antigens and reported past malaria episodes, Shewa Robit 2008 (N = 228)

| Clinical malaria episode | n | <i>P. falciparum</i> antigens | | | | | | |
|--------------------------|-----------|-------------------------------|---------------------|----------------------|---------------------|---------------------|-----------------|-----------------|
| | | AMA1 No. (%) | MSP2-3D7 No. (%) | MSP2-FC27 No. (%) | GLURP-R0 No. (%) | GLURP-R2 No. (%) | GMZ2 No. (%) | MSP3 No. (%) |
| Unexposed | 51(22.4) | 41(80.3) | 27(52.9) | 33(64.7) | 44(86.2) | 15(29.4) | 51(100) | 47(92.1) |
| Exposed | 177(77.6) | 107(60.4) | 123(69.4) | 153(86.4) | 162(91.5) | 81(45.7) | 176(99.4) | 168(94.9) |
| Single | 71(40.1) | 35(49.2) | 48(67.6) | 58(81.6) | 62(87.3) | 32(45.0) | 71(100) | 66(92.9) |
| Multiple | 106(59.9) | 72(67.9) | 75(70.7) | 95(89.6) | 100(94.3) | 49(46.2) | 105(99.0) | 102(95.2) |
| Total | 228 | 148(64.9) | 150(65.8) | 186(81.6) | 206(90.4) | 96(42.1) | 227(99.4) | 215(94.3) |

Fisher's exact test was used to test the significance between the proportions between the groups.

Table 10 Total IgG prevalence to *P. falciparum* antigens and reported year of most recent malaria episode, Shewa Robit 2008 (N=177).

| | | <i>P. falciparum</i> antigens | | | | | | |
|-----------------------------|-----|-------------------------------|---------------------|----------------------|---------------------|--------------------|-----------------|-----------------|
| Year of latest past episode | n | AMA1 No. (%) | MSP2-3D7 No. (%) | MSP2-FC27 No. (%) | GLURP-R0 No. (%) | GLUR-R2 No. (%) | GMZ2 No. (%) | MSP3 No. (%) |
| 1991-2004 | 33 | 23(69.6) | 25(75.7) | 30(90.9) | 30(90.9) | 17(51.5) | 33(100) | 31(93.9) |
| 2005 | 45 | 25(55.5) | 31(68.8) | 41(91.1) | 43(95.5) | 18(40.0) | 45(100) | 44(97.7) |
| 2006 | 18 | 10(55.5) | 14(77.7) | 17(94.4) | 17(94.4) | 10(55.5) | 18(100) | 16(88.8) |
| 2007 | 38 | 22(57.8) | 21(55.2) | 29(76.3) | 31(81.6) | 13(34.2) | 37(97.3) | 35(92.1) |
| 2008 | 43 | 27(62.7) | 32(74.4) | 36(83.7) | 41(95.3) | 23(53.4) | 43(100) | 42(97.6) |
| Total | 177 | 107(60.4) | 123(69.4) | 153(86.4) | 162(91.5) | 81(45.7) | 176(99.4) | 168(94.9) |

Fisher's exact test was used to test the significance between the proportions between the groups.

Table 11 Pattern of total IgG to *P. falciparum* antigens and reported past malaria episode, Shewa Robit 2008 (N= 177).

| | | <i>P. falciparum</i> antigens | | | | | | |
|-----------|-----|-------------------------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Condition | n | AMA1 | MSP2-3D7 | MSP2-FC27 | GLURP-R0 | GLURP-R2 | GMZ2 | MSP3 |
| Unexposed | 51 | 0.128 (0.165) | 0.103 (0.454) | 0.124 (0.520) | 0.276 (0.538) | 0.105 (0.170) | 0.490 (0.897) | 0.263 (0.616) |
| Exposed | 177 | 0.119 (0.167) | 0.130 (0.472) | 0.215 (0.538) | 0.252 (0.504) | 0.113 (0.385) | 0.572 (0.907) | 0.278 (0.640) |
| Single | 71 | 0.089 (0.175) | 0.129 (0.518) | 0.197 (0.533) | 0.356 (0.413) | 0.113 (0.378) | 0.441 (0.803) | 0.260 (0.509) |
| Multiple | 106 | 0.126 (0.161) | 0.131 (0.441) | 0.267 (0.541) | 0.321 (0.553) | 0.112 (0.391) | 0.616 (0.964) | 0.294 (0.711) |

Median OD values (\pm SD) for total IgG against the antigens are shown. Differences between the medians were analyzed by Mann-Whitney U test ($P < 0.05$).

Table 12 Total IgG level respect to reported year of most recent malaria episode, Shewa Robit 2008 (N=177).

| <i>P. falciparum</i> recombinant antigens | | | | | | | | |
|-------------------------------------------|---|---------|----------|-----------|----------|----------|---------|---------|
| Latest episode | n | AMA1 | MSP2-3D7 | MSP2-FC27 | GLURP-R0 | GLURP-R2 | GMZ2 | MSP3 |
| 1991-2004 | 3 | 0.129 | 0.129 | 0.228 | 0.356 | 0.136 | 0.441 | 0.279 |
| | | (0.105) | (0.413) | (0.370) | (0.336) | (0.397) | (0.861) | (0.279) |
| | 4 | 0.106 | 0.109 | 0.200 | 0.258 | 0.097 | 0.602 | 0.250 |
| 2005 | 5 | (0.188) | (0.194) | (0.485) | (0.547) | (0.289) | (0.756) | (0.461) |
| | 1 | 0.099 | 0.145 | 0.238 | 0.321 | 0.140 | 0.781 | 0.268 |
| 2006 | 8 | (0.128) | (0.199) | (0.787) | (0.293) | (0.208) | (0.414) | (0.260) |
| | 3 | 0.107 | 0.138 | 0.199 | 0.331 | 0.087 | 0.520 | 0.284 |
| 2007 | 8 | (0.108) | (0.262) | (0.191) | (0.631) | (0.133) | (1.025) | (0.729) |
| | 4 | 0.130 | 0.134 | 0.222 | 0.373 | 0.137 | 0.516 | 0.336 |
| 2008 | 3 | (0.230) | (0.806) | (0.725) | (0.523) | (0.580) | (1.085) | (0.905) |

Median OD values (\pm SD) for total IgG against the antigens are shown. Differences between the medians were analyzed using a Kruskal-Wallis test ($P < 0.05$).

4.5. Antibody Responses and Age

For Boditi there was age-related increase in total IgG positivity rate (Table 13). There was significant difference between age groups in total IgG positivity to GMZ2 ($p = 0.005$), MSP3 ($p = 0.015$) and GLURP-R0 ($p = 0.000$). Total IgG levels also showed significant age-based increase for GMZ2 ($p = 0.000$), MSP3 ($p = 0.001$) and GLURP-R0 ($p = 0.000$) (Table 16). There was no significant difference between sexes in IgG response to the antigens tested.

Table 13 Total IgG positivity to *P. falciparum* GLURP-R0, MSP3 and GMZ2 and age groups, Boditi 2009 (N =265).

| Age groups (Years) | n | GLURP-R0 No. (%) | MSP3 No. (%) | GMZ2 No. (%) |
|--------------------|-----|------------------|--------------|--------------|
| 0-4 | 44 | 31(70.5) | 30(68.1) | 36(81.8) |
| 5-14 | 79 | 69(87.3) | 69(87.3) | 69(87.3) |
| ≥ 15 | 142 | 126(88.7) | 117(82.4) | 135(95.1) |
| Total | 265 | 226(85.3) | 216(81.5) | 240(90.6) |

Fisher's exact test and multiple comparisons were used to test the significance between the various proportions between the age groups.

Table 14 Age-associated pattern in IgG total antibody levels to *P. falciparum* recombinant antigens tested, Boditi 2009 (N=265)

| | | <i>P. falciparum</i> recombinant antigens | | |
|------------|-----|-------------------------------------------|---------------|---------------|
| Age groups | n | GLURP-R0 | MSP3 | GMZ2 |
| (Years) | | OD(\pm SD) | OD(\pm SD) | OD(\pm SD) |
| 0-4 | 44 | 0.150 (0.878) | 0.148 (0.724) | 0.237 (0.786) |
| 5-14 | 79 | 0.197 (1.036) | 0.180 (0.784) | 0.342 (0.927) |
| \geq 15 | 142 | 0.551 (1.190) | 0.465 (1.080) | 0.722 (1.115) |
| Overall | 265 | 0.284 (1.159) | 0.264 (0.940) | 0.416 (1.092) |

* Median OD (\pm SD) values for Boditi sera (1:400 dilution) for different age groups (years) are shown. Differences between the medians were analyzed using a Kruskal-Wallis test. n indicates the number of individuals in each age group.

The general antibody titer of total IgG was significantly positively correlated for GMZ2 ($R = 0.328$, $P = 0.000$), MSP3 ($R = 0.221$, $P = 0.000$) and GLURP-R0 ($R = 0.352$, $P = 0.000$) with increasing age. All IgG subclass antibodies against MSP3 and GLURP-R0 were strongly and significantly correlated with age except IgG3 response to MSP3. The R and P values for correlations between age subclass antibody levels against MSP3 were IgG1 ($R = 0.175$, $P = 0.004$), IgG2 ($R = 0.462$, $P = 0.000$), IgG3 ($R = 0.021$, $P = 0.733$), IgG4 ($R = 0.288$, $P = 0.000$) and against GLURP-R0 IgG1 ($R = 0.299$, $P = 0.000$), IgG2 ($R = 0.456$, $P = 0.000$), IgG3 ($R = 0.244$, $P = 0.000$), and IgG4 ($R = 0.354$, $P = 0.000$).

For Shewa Robit, the prevalence of total IgG to the seven antigens varied with specific antigen and age group (Table 15). The age group over 14 years old had greater number of positive individuals than those under 14 for AMA1, MSP2-3D7, GLURP-R0, GLURP-R2 and MSP3. For MSP2-FC27 more individuals were positive

in the age group 5-14 years old. For GMZ2 the prevalence was almost the same in both age groups. But significant difference was found only for MSP2-FC27 ($p = 0.029$) and GLURP-R0 ($p = 0.006$). Total IgG median antibody levels to the antigens tested were higher for the older age group but it was significant only for GLURP-R0 ($p = 0.000$) (Table 18).

Table 15 Total IgG positivity to *P. falciparum* recombinant antigens in age groups, Shewa Robit 2008 (N=228)

| | | <i>P. falciparum</i> antigens | | | | | | |
|---------|-----|-------------------------------|-----------|-----------|-----------|----------|-----------|-----------|
| Age | | AMA1 | MSP2-3D7 | MSP2-FC27 | GLURP-R0 | GLURP-R2 | GMZ2 | MSP3 |
| (Years) | n | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) |
| 5-14 | 20 | 10(50.0) | 11(55.0) | 20(100) | 18(90.0) | 5(25.0) | 20(100) | 18(90.0) |
| ≥15 | 208 | 138(66.3) | 139(66.8) | 166(89.4) | 196(94.2) | 91(43.7) | 207(99.5) | 197(94.7) |
| Overall | 228 | 148(64.9) | 150(65.8) | 186(81.5) | 206(90.4) | 96(42.1) | 227(99.6) | 215(94.3) |

Fisher's exact test was used to test the significance between the proportions between the age groups.

Table 16 Age-associated pattern in total IgG levels to *P. falciparum* antigens, Shewa Robit 2008

| | | <i>P. falciparum</i> antigens | | | | | | |
|---------|-----|-------------------------------|--------------|--------------|--------------|--------------|--------------|--------------|
| Age | | AMA1 | MSP2-3D7 | MSP2-FC27 | GLURP-R0 | GLURP-R2 | MSP3 | GMZ2 |
| groups | n | | | | | | | |
| (Years) | | | | | | | | |
| 5-14 | 20 | 0.091(0.055) | 0.103(0.049) | 0.172(0.105) | 0.082(0.690) | 0.094(0.088) | 0.175(0.635) | 0.371(0.801) |
| ≥15 | 208 | 0.125(0.173) | 0.128(0.488) | 0.199(0.599) | 0.286(0.492) | 0.111(0.366) | 0.276(0.634) | 0.559(0.910) |
| Overall | 228 | 0.122 (0.166) | 0.121(0.469) | 0.198(0.636) | 0.366(0.510) | 0.110(0.352) | 0.272(0.633) | 0.540(0.903) |

*Median OD (\pm SD) for Shewa Robit sera for different age groups (years) are shown. Differences between the medians were analyzed by Mann-Whitney U test; n indicates the number of individuals in each age group.

When general total IgG antibody titer was considered anti-AMA1 response was negatively associated with age though the relationship was insignificant ($R = -0.016$, $P = 0.813$). Against GLURP-R2 total antibody titer increased with increasing age but the association was insignificant ($R = 0.025$, $P = 0.749$). For all other antigens tested in Shewa Robit total IgG level positively significantly correlated with age. The strongest positive correlation with age was recorded for anti-MSP3 IgG response ($R = 0.384$, $P = 0.000$) followed by MSP2-FC27 ($R = 0.360$, $P = 0.000$), MSP2-3D7 ($R = 0.248$, $P = 0.000$), GMZ2 ($R = 0.203$, $P = 0.002$) and GLURP-R0 ($R = 0.149$, $P = 0.027$) (Figures 7-13). All IgG subclass titers to GLURP-R0 were significantly correlated with age. Similar positive correlation with age was found for all subclass antibody level against MSP3 although it was significant only for IgG2.

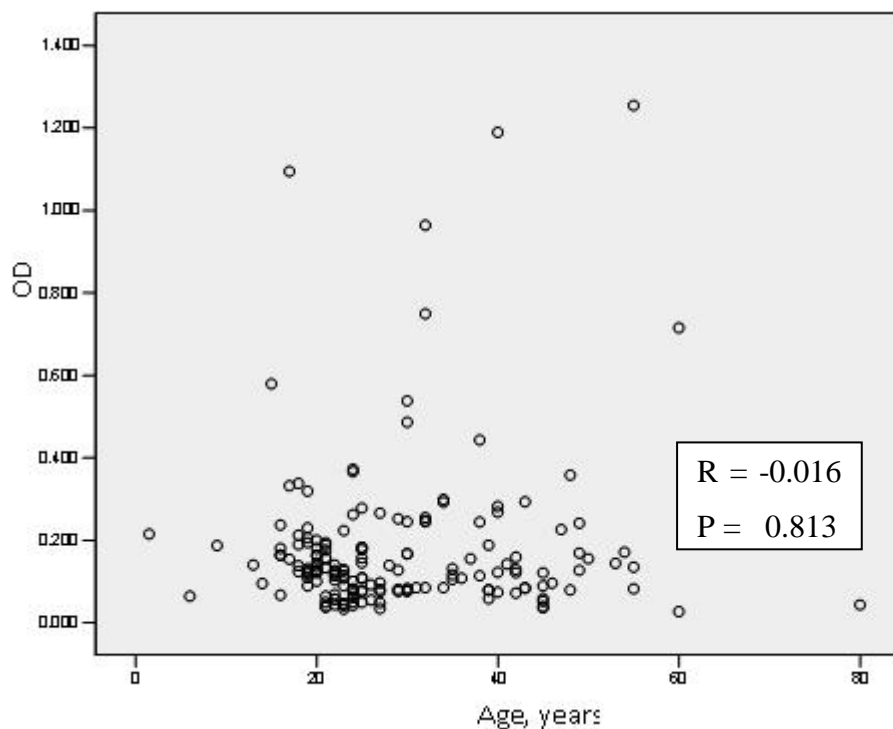


Figure 7 Correlation between IgG level to AMA1 and age, Shewa Robit 2008

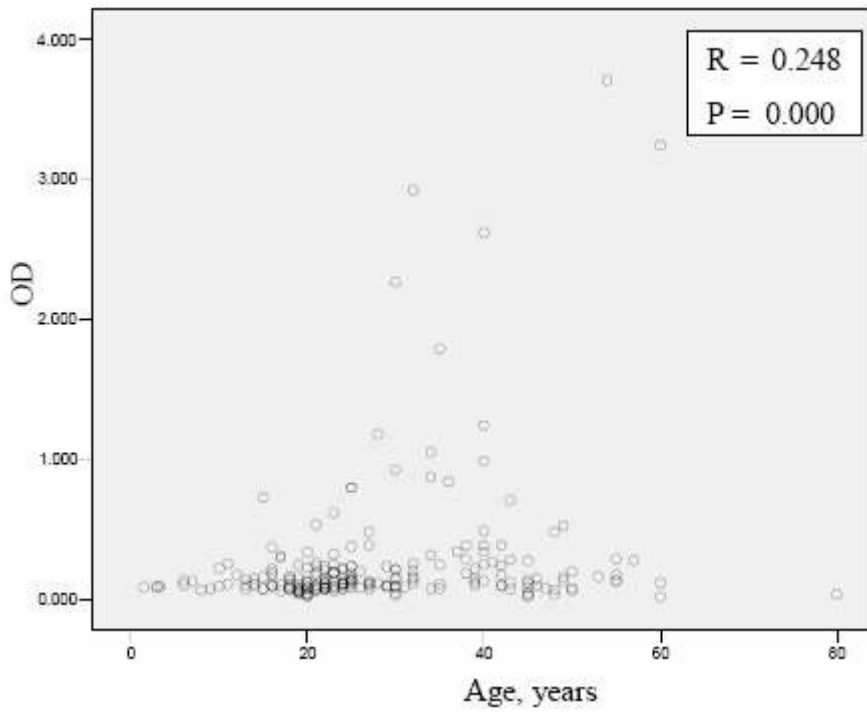


Figure 8 Correlation between IgG level to MSP2-3D7 and age, Shewa Robit 2008

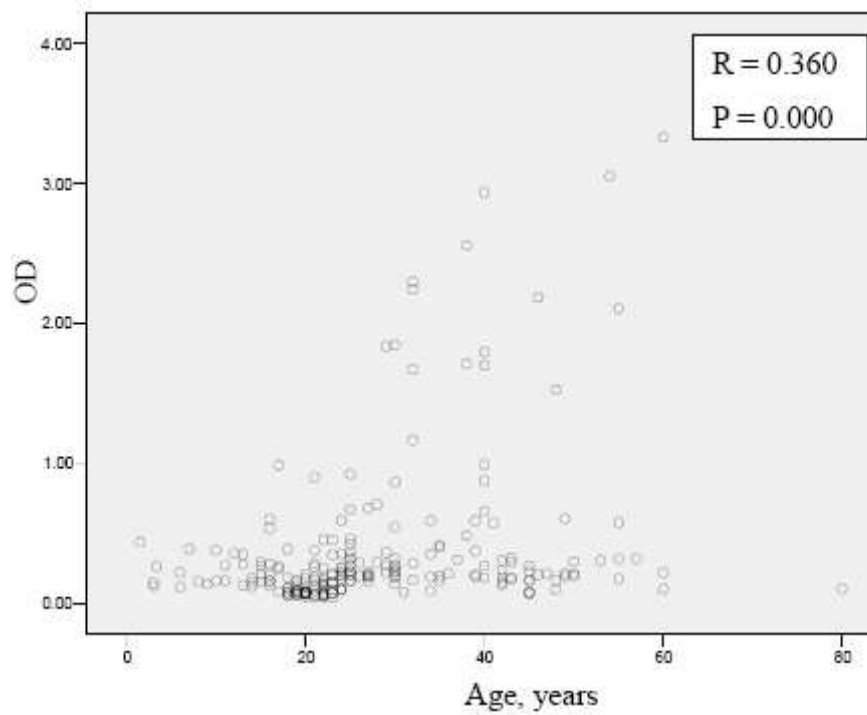


Figure 9 Correlation between IgG level to MSP2-FC27 and age, Shewa Robit 2008

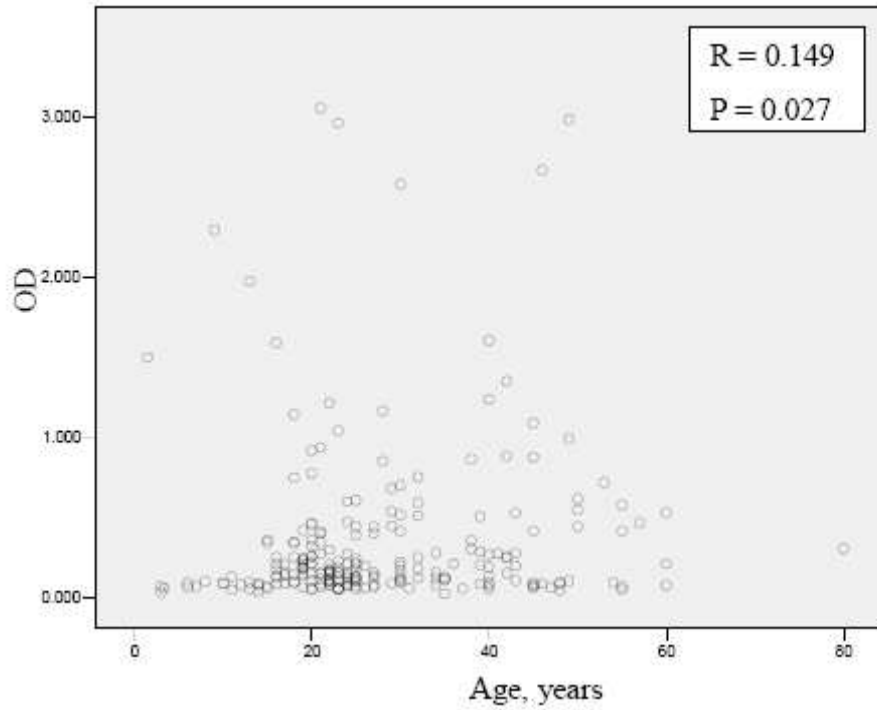


Figure 10 Correlation between IgG level to GLURP-R0 and age, Shewa Robit 2008

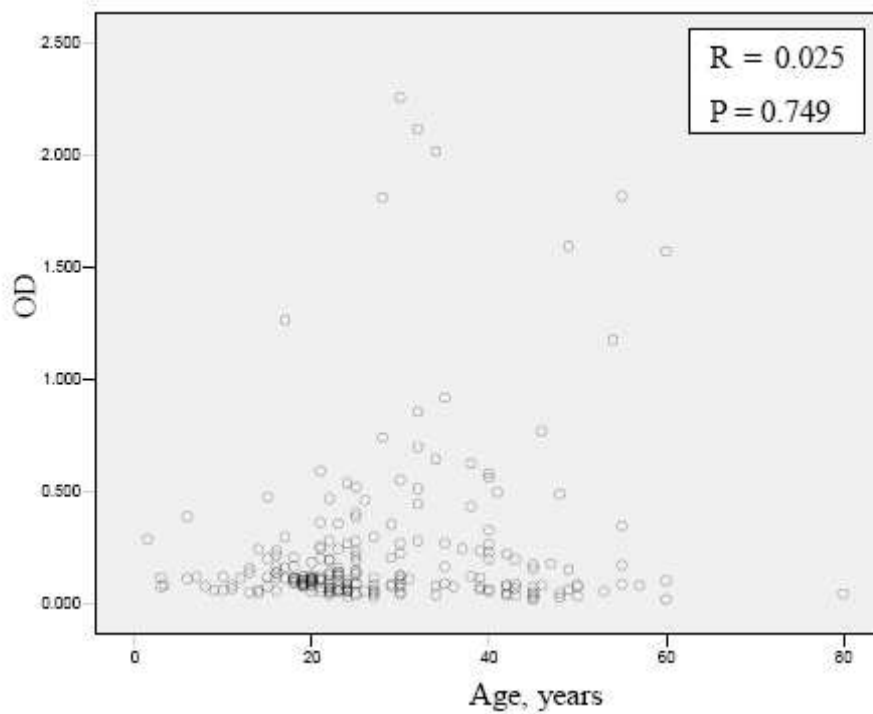


Figure 11 Correlation between IgG level to GLURP-R2 and age, Shewa Robit 2008

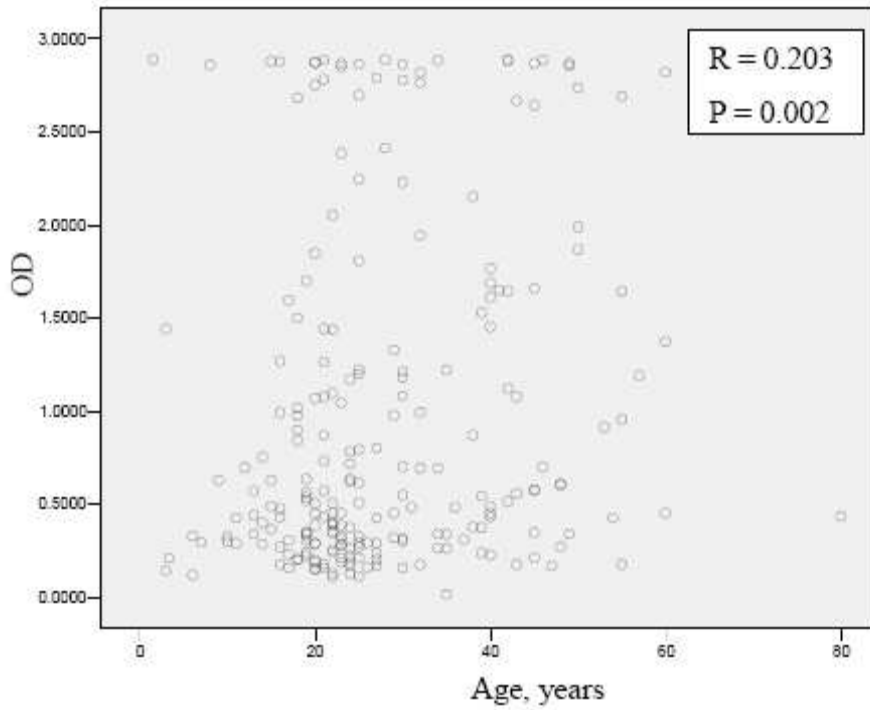


Figure 12 Correlation between IgG level to GMZ2 and age, Shewa Robit 2008

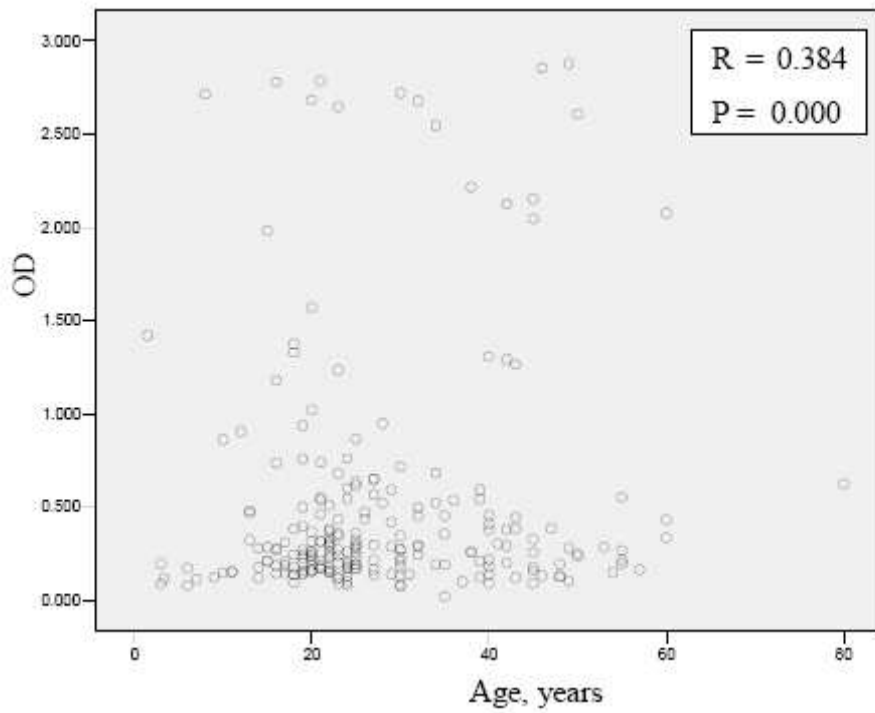


Figure 13 Correlation between IgG level to MSP3 and age, Shewa Robit 2008

4.6. Slide-Positivity and Antibody Response in Boditi

Although it was insignificant, total IgG positivity (Table 17) and level (Table 18) to the antigens tested were higher in the blood-film-negative group for *P. falciparum* infection than in the slide-positives at the time of sample collection. IgG2 ($P = 0.035$) and IgG3 ($P = 0.048$) positivity to GLURP-R0 (Table 19) and IgG2 ($P = 0.004$) level to MSP3 (Table 20) were significantly higher in smear-negatives than in the positives. IgG3 level against GLURP-R0 was also slightly higher in smear-negative individuals than in the positives.

Table 17 Total IgG positivity to *P. falciparum* GLURP-R0, MSP3 and GMZ2 slide-positivity, Boditi 2009 (N=265)

| | <i>P. falciparum</i> | |
|----------|----------------------|-----------------------|
| | Positive (n = 23) | Negative (n = 242) |
| | No. (%) | No. (%) |
| GMZ2 | 18 (78.2) | 210 (86.7) |
| MSP3 | 16 (69.5) | 200 (82.6) |
| GLURP-R0 | 18 (78.2) | 208 (85.9) |

Fisher's exact test was used to test the significance between the proportions between the two groups.

Table 18 Total IgG level against MSP3, GLURP-R0 and GMZ2 in *P. falciparum* smear-positive and negative individuals at the time of sample collection, Boditi 2009 (N=265)

| <i>P. falciparum</i> | n | <i>P. falciparum</i> antigens | | |
|----------------------|-----|-------------------------------|---------------|---------------|
| | | GMZ2 | MSP3 | GLURP-R0 |
| Negative | 242 | 0.435 (1.160) | 0.216 (0.945) | 0.272 (1.089) |
| Positive | 23 | 0.236 (1.130) | 0.164 (0.865) | 0.186 (1.086) |

Median OD values (\pm SD) for total IgG against the antigens are shown. Differences between the medians were analyzed by Mann-Whitney U test ($P < 0.05$).

Table 19 IgG subclass positivity to *P. falciparum* GLURP-R0 and MSP3 and smear-positivity at the time of sample collection, Boditi 2009 (N=265)

| <i>P. falciparum</i> antigens | | | | | | | | | |
|-------------------------------|-----|------------|-----------|------------|-----------|------------|------------|------------|----------|
| | | MSP3 | | | | GLURP-R0 | | | |
| <i>P. falciparum</i> | n | IgG1 | IgG2 | IgG3 | IgG4 | IgG1 | IgG2 | IgG3 | IgG4 |
| | | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) |
| Negative | 242 | 134 (55.4) | 85 (35.0) | 99 (40.9) | 28 (11.6) | 159 (65.7) | 190 (78.5) | 140 (57.9) | 70(28.9) |
| Positive | 23 | 11 (47.8) | 5 (21.7) | 9 (39.1) | 4 (17.4) | 12 (52.1) | 13 (56.5) | 9 (39.1) | 3(1.3) |
| Total | 265 | 145 (54.7) | 90 (34.0) | 108 (40.8) | 32 (12.1) | 171 (64.5) | 203 (76.6) | 149 (56.2) | 73(27.5) |

Median OD values (\pm standard deviation) for IgG subclasses against the antigens are shown. Differences between the medians were analyzed by Mann-Whitney U test ($P<0.05$).

Table 20 IgG subclass levels to *P. falciparum* MSP3 & GLURP-R0 and smear-positivity, Boditi 2009 (N=265)

| <i>P. falciparum</i> antigens | | | | | | | | | |
|-------------------------------|--|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| | | MSP3 | | | | GLURP-R0 | | | |
| <i>P. falci</i> | | IgG1 | IgG2 | IgG3 | IgG4 | IgG1 | IgG2 | IgG3 | IgG4 |
| Negative | | 0.146(0.974) | 0.057(0.341) | 0.066(0.488) | 0.026(0.212) | 0.372(1.190) | 0.256(0.636) | 0.145(0.937) | 0.019(0.130) |
| Positive | | 0.095(0.942) | 0.021(0.094) | 0.060(0.726) | 0.025(0.050) | 0.165(1.136) | 0.156(0.817) | 0.041(0.813) | 0.015(0.187) |

Median OD values (\pm standard deviation) for IgG subclasses against the antigens are shown. Differences between the medians were analyzed by Mann-Whitney U test ($P<0.05$).

4.7. IgG Subclass Distribution

IgG subclass positivity rates and titers against MSP3 and GLURP-R0 for both study populations are indicated in Table 7. In Shewa Robit, anti-MSP3 IgG2 was more prevalent (93.4%) than IgG1 (91.7%) followed by IgG3 (64.0%) and IgG4 (32%) was the least. The differences were significant in all cases ($P<0.05$). IgG2 ($P = 0.001$) and IgG1 ($P = 0.023$) levels to MSP3 were significantly higher than IgG3. Although the difference was insignificant anti-MSP3 IgG2 level was higher than IgG1. Anti-MSP3

IgG2 titer surpassed that of IgG1 in 53.5% and IgG3 in 80.7% of study participants. For the same antigen 35(15.3%) individuals had higher levels of IgG4 than IgG1, 31(13.6%) had higher IgG4 than IgG2 and 96(42.1%) individuals had higher IgG4 levels than IgG3 (Table 21). Overall, in about one half of the study participants the sum of IgG1 and IgG3 (cytophilic subclasses) was greater than IgG2 and IgG4 (noncytophilic), and in another half IgG2 and IgG4 was greater than IgG1 and IgG3 (Table 22). Thus, the dominance of the cytophilic over noncytophilic and vice-versa was dependent on the individual study participant tested.

Table 21 IgG subclass distribution to *P. falciparum* GLURP-R0 and MSP3 antigens, Shewa Robit 2008 (N=228)

| <i>P. falciparum</i> antigens | | | | | |
|-------------------------------|-------|---------------|---------------|--------------|--------------|
| Subclass | Ratio | GLURP-R0 | | MSP3 | |
| | | <1 No. (%) | ≥1 No. (%) | <1 No (%) | ≥1 No (%) |
| IgG1/IgG2 | | 43(18.9) | 185(81.1) | 122(53.5) | 106(46.5) |
| IgG1/IgG3 | | 9(3.9) | 219(96.1) | 48(21.1) | 180(78.9) |
| IgG1/IgG4 | | 3(1.3) | 225(98.7) | 35(15.4) | 193(84.6) |
| IgG2/IgG3 | | 66(28.9) | 162(71.1) | 44(19.3) | 184(80.7) |
| IgG2/IgG4 | | 16(7.1) | 212(92.9) | 31(13.6) | 197(86.4) |
| IgG3/IgG4 | | 16(7.1) | 212(92.9) | 96(42.1) | 132(57.9) |

Table 22 Anti-GLURP-R0 and MSP3 IgG subclass level ratios and median of the ratios, Shewa Robit 2008 (N=228)

| Antigens | GLURP-R0 | | MSP3 | | GLURP-R0/MSP3 | | Median OD (\pm SD) of the ratios | | |
|---------------------|----------|-----------|-----------|-----------|---------------|-----------|-------------------------------------|--------------|---------------|
| | <1 | \geq 1 | <1 | \geq 1 | <1 | \geq 1 | GLURP-R0 | MSP3 | GLURP-R0/MSP3 |
| | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) | | | |
| IgG1+IgG3:IgG2+IgG4 | 35(15.4) | 193(84.6) | 113(49.6) | 115(50.4) | - | - | 2.390(3.858) | 1.018(1.760) | |
| IgG1:IgG1 | - | - | - | - | 14(6.1) | 214(93.9) | - | - | 3.669(3.687) |
| IgG2:IgG2 | - | - | - | - | 69(30.3) | 159(69.7) | - | - | 1.59(1.224) |
| IgG3:IgG3 | - | - | - | - | 49(21.5) | 179(78.5) | - | - | 2.441(3.766) |
| IgG4:IgG4 | - | - | - | - | 159(69.7) | 69(30.3) | - | - | 0.684(1.572) |

However, for Boditi sera anti-MSP3 subclass response was predominantly composed of IgG1 and IgG3, with significantly higher IgG1 ($P = 0.000$). Anti-MSP3 IgG2 and IgG4, were significantly lower than IgG1 and IgG3 ($P = 0.000$). Anti-MSP3 IgG2 was significantly higher than IgG4 ($P = 0.001$). Thus, against MSP3 the cytophilic antibodies were predominantly prevalent compared to noncytophilic ones in Boditi. The median antibody levels were significantly higher for IgG1 than IgG3 which was also significantly higher than IgG2 and which was in turn significantly higher than IgG4 ($P < 0.05$). The ratio of MSP3-specific IgG1 to IgG2 was >1 for 81.5% of the study participants (Table 25). Fourteen (5.3%) individuals had higher IgG4 levels than IgG1. IgG3 was higher than IgG2 in 50.6% of the sera tested. Thus individually as well as in a combined way cytophilic antibodies had higher antibody titers than noncytophilic ones although the relative dominance of each subclass largely varied between individuals and some sera that were IgG1-positive were IgG3-negative and vice versa, and IgG2/IgG4 containing sera lacked detectable IgG1/IgG3 in some instances.

Table 23 Anti-GLURP-R0 and MSP3 IgG subclass level ratios and median of the ratios, Boditi 2009 (N=265)

| Antigens | <i>P. falciparum</i> antigens | | | | | | |
|-------------------------|-------------------------------|-----------|-----------|-----------|---------------------|----------------------------------|------|
| | Ratio | GLURP-R0 | | MSP3 | | Median (\pm SD) of the ratios | |
| | | <1 | >1 | <1 | >1 | GLURP-R0 | MSP3 |
| | No. (%) | No. (%) | No. (%) | No. (%) | | | |
| Antibodies | | | | | | | |
| IgG1:IgG2 | 112(42.2) | 153(57.7) | 49(18.5) | 216(81.5) | 1.270(9.803) | 2.797(17.572) | |
| IgG1:IgG3 | 71(26.8) | 194(73.2) | 53(20.0) | 212(80.0) | 1.600(11.134) | 2.529(11.686) | |
| IgG1:IgG4 | 7(2.6) | 258(97.4) | 14(5.3) | 251(94.7) | 10.024(35.362) | 5.435(22.944) | |
| IgG2:IgG3 | 111(41.8) | 154(58.1) | 134(50.6) | 131(49.4) | 1.507(9.148) | 0.948(4.188) | |
| IgG2:IgG4 | 7(2.6) | 258(97.4) | 80(30.2) | 185(69.8) | 8.718(20.743) | 1.914(5.162) | |
| IgG3:IgG4 | 15(5.7) | 250(94.3) | 66(24.9) | 199(75.1) | 5.074(16.644) | 2.253(12.786) | |
| (IgG1+IgG3):(IgG2+IgG4) | 92(34.7) | 173(65.3) | 49(18.5) | 216(81.5) | 2.193(\pm 8.178) | 2.973(10.777) | |

IgG1 prevalence to GLURP-R0, in Shewa Robit, was significantly higher than IgG2 followed by IgG3 and IgG4. The differences were significant ($P < 0.01$). Most sera having detectable IgG3 and IgG4 levels were usually positive for IgG1 and IgG2 (predominantly expressed subclasses). Similarly the median level of IgG1 to GLURP-R0 was the highest followed by IgG2, IgG3 and IgG4 with significant differences ($P < 0.01$). IgG1 titer to GLURP-R0 was higher than IgG2, IgG3 and IgG4 to the antigen in 81.1, 96.1 and 98.7 percent of the study participants, respectively (Table 21). Only 3(1.3%) individuals had anti-GLURP-R0 IgG4 level stronger than that of IgG1. But the strength of IgG2 reactivity to GLURP-R0 was higher in 71.1% of the participants compared to IgG3 and 92.9% compared to IgG4 response. Greater proportion of the population had higher IgG2 level than IgG3 to GLURP-R0 compared to MSP3. IgG subclass responses against GLURP-R0 were dominantly composed of a mixture cytophilic (IgG1) and noncytophilic (IgG2) subclasses. IgG4 reactivity to GLURP-R0 was low in the majority and high in limited number of individuals. Its level was higher in 16(7%) individuals compared to IgG2 and

33(14%) compared to IgG3 against GLURP-R0. The ratio of cytophilic to noncytophilic subclasses was >1 for 84.6% of the study participants indicating the dominance of cytophilic subclasses over noncytophilic.

For Boditi, on the other hand, anti-GLURP-R0 IgG2 prevalence (76.6%) was significantly higher than IgG1 (64.5%), which was significantly higher than IgG3 (55.5%) ($P \leq 0.01$). IgG4 prevalence (27.5%) was the lowest. There was elevated anti-GLURP-R0 IgG2 prevalence in Boditi. The median anti-GLURP-R0 IgG1 level was significantly higher than all other subclass levels including IgG2 for the antigen ($P = 0.000$) (Table 7). IgG2 and IgG3 levels to GLURP-R0, both significantly higher than IgG4 ($P = 0.000$), were not significantly different from each other though IgG2 concentration was higher than IgG3. Overall, against GLURP-R0 the response was a mixture of cytophilic and noncytophilic isotypes in Boditi study participants. The predominant expression of anti-GLURP-R0 subclasses in most individuals was indicated in the IgG1 to IgG2 ratio which was >1 for 57.7% of the study participants (Table 24). In 58.1% IgG2 to IgG3 ratio was >1 . Seven (2.6%) individuals had higher IgG4 level than IgG1.

For Shewa Robit sera, while IgG1 and IgG2 positivity rates were significantly higher for MSP3 than GLURP-R0 the opposite was observed for IgG3 ($P \leq 0.01$) (Table 5). Anti-GLURP-R0 and MSP3 IgG4 prevalence were not significantly different. IgG subclass positivity rate to GLURP-R0 was significantly higher than to MSP3 for all corresponding subclasses in Boditi sera ($P \leq 0.01$) (Table 5). Antibody levels were significantly higher for GLURP-R0 than MSP3 for all the corresponding subclasses in both sites in the majority of the study participants with exception of IgG4 ($P = 0.000$).

The difference between IgG2 levels to GLURP-R0 and MSP3 was more pronounced, in 87.9% of the study participants IgG2 response to GLURP-R0 was higher than against MSP3 in Boditi. However, there were inter-individual differences as to the relative dominance of a specific subclass response to GLURP-R0 over MSP3 and vice-versa in both study localities. There were 14(6.1%), 69(30.3%), 49(21.5%) and 158(69.7%) individuals with higher anti-MSP3 levels than anti-GLURP-R0 for IgG1, IgG2, IgG3 and IgG4, respectively, in Shewa Robit (Table 23). In Boditi; IgG1, IgG2, IgG3 and IgG4 antibody levels against MSP3 were higher than the respective subclass responses against GLURP-R0 in 40.8, 12.1, 31.3 and 60.0 percent of the study participants, respectively (Table 24).

Table 24 Relative dominance of IgG subclass levels to *P. falciparum* MSP3 & GLURP-R0 compared at individual level, Boditi 2009 expressed in ratios medians of the ratios

| Ratio | <1 No. (%) | >1 No. (%) | Median OD(\pm SD) of the ratios |
|---------------------------|---------------|---------------|------------------------------------|
| IgG1(MSP3):IgG1(GLURP-R0) | 157(61.3) | 108(40.8) | 0.906(3.825) |
| IgG2(MSP3):IgG2(GLURP-R0) | 233(87.9) | 32(12.1) | 0.261(1.259) |
| IgG3(MSP3):IgG3(GLURP-R0) | 182(68.7) | 83(31.3) | 0.565(11.538) |
| IgG4(MSP3):IgG4(GLURP-R0) | 106(40.0) | 159(60.0) | 1.195(5.370) |

5. DISCUSSION

No report on naturally acquired anti-GMZ2 antibody response exists so far. Therefore, the significantly high total IgG prevalence and levels detected in both sites of the present study for GMZ2 than either of its two component domains indicates the enhanced immunogenicity of GMZ2 in natural infections. The findings suggest that people in endemic areas have high antibody responses to the GMZ2 vaccine candidate antigen in the absence of microscopically detectable infection. The higher total IgG and subclass antibody levels against GLURP-R0 than MSP3 in both study sites indicates the better immunogenicity of GLURP-R0 compared to MSP3. The findings are in line with data from pre-clinical and clinical evaluations of the GMZ2 vaccine candidate antigen. Pre-clinical studies in monkeys (Carvalho et al. 2005) and mice (Theisen et al. 2004) documented higher total IgG prevalence and level against GMZ2 compared to GLURP-R0 and MSP3. The same studies showed that total IgG prevalence and titers were significantly higher for GLURP-R0 than MSP3.

Initial human trials in malaria-naïve (Esen et al. 2009) and naturally exposed individuals (Mordmüller et al. 2010) have also shown that anti-GMZ2 responses were stronger than the responses to its component antigens, with GLURP-R0 being relatively more immunogenic than MSP3. But a later phase Ib GMZ2 clinical trial in naturally primed children revealed higher responses to MSP3, compared to what was recorded by both Esen et al. (2009) and Mordmüller et al. (2010), against both low (30 mg) and high (100 mg) GMZ2 doses (Be'lard et al. 2011). In a similar GMZ2 clinical trial, MSP3-specific antibody prevalence comparatively increased, where the immunogenicity of the protein was enhanced using virosomal preparation compared

to the alum adjuvanted formulation (Tamborrini et al. 2011). The authors speculated that virosomal preparation balanced the immunogenicity between the two GMZ2 domains as the display of the hybrid on virosome surface may increase the accessibility of MSP3 B-cell epitopes to antibodies.

Studies that examined anti-GLURP-R0 and -MSP3 natural responses separately had recorded varying total IgG prevalence for the two antigens. In one such report, anti-MSP3 total IgG prevalence was higher than that for GLURP-R0 in a hyperendemic area in Myanmar (Soe et al. 2004). This observation is in agreement with the present findings in Shewa Robit, but in contrast to the relative prevalence against the two antigens in Boditi. Also, a study from Venezuelan Amazon where malaria transmission was very low and considered controlled (Metzger et al. 2009) recorded higher total IgG prevalence against GLURP-R0 than MSP3 (Baumann et al. 2012). Similarly, total IgG and subclass levels were significantly higher against GLURP-R0 than MSP3 in both Boditi and Shewa Robit. These findings are supported by a laboratory study of *P. falciparum* laboratory lines and field isolates from a wide range of epidemiological conditions (Africa, Asia and Latin America) which revealed a highly conserved nature of B-cell epitopes within GLURP and MSP3 (de Stricker et al. 2000, Huber et al. 1997, McColl and Anders 1997).

The high antibody responses to MSP3 in the present study support the findings of previous studies from other endemic areas. High anti-MSP3 antibody responses were documented among naturally exposed individuals in Kenya (Osier et al. 2007), the Gambia (Polley et al. 2007), Senegal (Courtin et al. 2009, Demanga et al. 2010) and Peru (Jordan et al. 2011). Also, several studies, at different levels of malaria

endemicity, that examined total IgG response against GLURP, in general and specific regions in particular, recorded strong antibody reactivity. A high IgG positivity rate was recorded in a holoendemic area from Liberia against the C-terminal 783 amino acids of the 220-kD GLURP₄₈₉₋₁₂₇₁ (containing both R1 (489-705) and R2 (705-1179) regions) (Hogh et al. 1992). In a seasonal malaria transmission locality in the Gambia, IgG positivity rate reached 95% for the 220-kD GLURP region (Dziegiel et al. 1993). High total IgG level to GLURP₂₅₋₅₁₄ (includes the R0 region) was also detected in a stable seasonal transmission area in Senegal (Courtin et al. 2009).

When IgG responses against GLURP-R0 and other regions of the peptide were compared there is apparent disparity between the findings. The lower anti-GLURP-R2 IgG prevalence detected in the present study, compared to GLURP-R0, differs from other observations in Myanmar (Soe et al. 2004) and Brazil (Pratt–Ricchio et al. 2005). The R0 region has limited variation between isolates from diverse geographic areas in contrast to the R2 repeat region which has been shown to have considerable polymorphism (Borre et al. 1991, de Stricker et al. 2000). Therefore, the R2 strain may not be common in the study population or the strain-specific antibody induced may be short-lived, and was at undetectable level at the time of sampling. Secondly, human genetic differences between the study populations might have contributed to the variation. The higher antibody reaction to GLURP-R0 than to GLURP-R2 in the present study suggests that antibodies induced to GLURP-R0 may be more stable, the antigen is more immunogenic and/or this particular region of GLURP is more prevalent in the study population.

The lower anti-AMA1 IgG prevalence recorded in the present study indicates apparent disparity from findings of other studies. For instance, a very high IgG positivity rate was observed against AMA1 in sera from Guinea-Bissau and Senegal (Thomas et al. 1994) and among Kenyan children (Chelimo et al. 2005). This disparity may be attributable to differences in malaria transmission levels since the reports of high anti-AMA1 antibody prevalence come from areas of high malaria transmission (holoendemic areas) and the present findings is from meso-/hypo-endemic epidemiological condition. However, equally high antibody prevalence to AMA1 was detected in a seasonal transmission area (Courtin et al. 2009). On the contrary, the prevalence of IgG response to AMA1 was substantially lower, compared to the findings in this study, in a low transmission setting from Thailand (Wipasa et al. 2010) showing that endemicity level is a less likely explanation for the variation.

Thus, the seroprevalence data from various studies on AMA1 are less consistent. Several factors are potential sources of the variation. Among these; presence/absence of active infection and its duration and the level of parasitemia, time of recent infection, chronicity or persistence of subpatent or submicroscopic asymptomatic infections, etc. may make it difficult to compare results from different studies. More importantly antigen preparation or constructs used can be major source of differences as AMA1 is a polymorphic protein with great strain variations (Richards and Beeson 2009). Furthermore, differences in the binding specificity and sensitivity of some ELISA reagents, dilution factors, positive and negative control sera and overall test system, host genetics, etc. may account for the apparent disparity.

Detection of high anti-MSP2 IgG positivity rate and level in this study is in agreement with reports of other studies. In an adult population in a seasonal transmission area in the Gambia the anti-MSP2 antibody prevalence was very high, reaching 95% (Taylor et al. 1995). Specifically, 81% of the individuals tested were positive for 3D7 and 86% were for serogroup FC27 being consistent with the present study though the value for 3D7 was lower here. More than 90% prevalence against the full-length proteins of both 3D7 and FC27 of MSP2 was observed in studies in Solomon Islands (Rzepczyk et al. 1989, Rzepczyk et al. 1997) and Papua New Guinea (Al-Yaman et al. 1994, Al-Yaman et al. 1995). Both allelic forms of MSP2 are found in Solomon Islands and Papua New Guinea according to these authors. Anti-MSP2-FC27 total IgG level was significantly higher than anti-MSP2-3D7 in a study in Sudan (Iriemenam et al. 2009) lending support to the present study. MSP2-specific IgG was detected in all individuals in general from Burkina Faso (Aucan et al. 2000) and to MSP2-3D7 specifically in Senegalese children (Courtin et al. 2009). On the other hand, a study from Thailand measured only 15% IgG positivity rate against MSP2, in individuals with history of past infection (Wipasa et al. 2010).

The two allelic variants of MSP2 are very distinct proteins structurally apart from the C-terminal sequence and a short conserved sequence at the N-terminus (Metzger et al. 2003). It was reported that unlike other *P. falciparum* loci, both MSP2 allelic types commonly occur in most populations around the globe (Anderson et al. 2000, Hoffmann et al. 2001) but no information is available as to the occurrence and the relative distribution of the two serogroups in Ethiopia. The current serologic result, the first of its kind in the country, confirmed the occurrence of both allelic variants probably in varying relative abundance at least in the study participants.

The inter-individual variability in antibody response to a specific antigen observed among the study participants in the present study was also reported from other malaria endemic areas (Aribot et al. 1996, Perraut et al. 2002). Although the overall trend was higher immunogenicity of GLURP-R0 compared to MSP3 in the present study and Baumann et al. (2012), and the reverse in Soe et al. (2004) considerable inter-individual variations were noticed in all studies. In the present study, an individual who showed high response for one antigen had not necessarily high response for other antigens suggesting that no predisposing host factors are responsible to produce high antibodies consistently and this may be due to different antigen characteristics. A possible antagonistic effect of IgG response against one antigen on the other in some individuals may be suggested.

Level of exposure to a certain parasite strain may also account for the inter-individual difference. As a substantial number of individuals were positive for MSP2-FC27, but not MSP2-3D7, and similarly, 51% of sera positive for GLURP-R0 were not so for GLURP-R2, an allele-specific IgG response was observed in the study. The induction of allele-specific natural antibodies to various *P. falciparum* recombinant antigens was similarly revealed in other studies (Polley et al 2006, Polley et al 2007, Kimbi et al 2004). Combination B malaria vaccine (containing a combination of Pf155/RESA, MSP1 and the 3D7 allele of MSP2) trial in Papua New Guinea showed allele-specific vaccine-induced MSP2 antibodies (Genton et al. 2002, Fluck et al. 2004). The lack of significant correlation between anti-GLURP-R0 and GLURP-R2 antibody levels observed in the present study provides additional support in this regard. No correlation between IgG responses against recombinant 783 C-terminal amino acid

residue containing two areas of repeated sequences (GLURP₄₈₉₋₁₂₇₁) and a synthetic peptide representing the major glutamate-rich repeat sequence from the Pf155/RESA (EENV)₆ was found (Hogh et al. 1992). The correlation between antibody levels to the MSP2 allelic families in the present study may be explained by exposure to both allelic families locally. Contrarily Taylor et al. (1995) found weak correlations between antibody responses to the two allelic variants of MSP2. Similarly, other investigators found little evidence of correlation between IgG subclass responses to the two alleles (Stanisic et al. 2009). Recording significant correlation between anti-AMA1 and MSP2 total IgG responses in the present study also differs from some studies (Wipasa et al. 2010) but agrees with others (Stanisic et al. 2009).

From studies on other *P. falciparum* blood-stage antigens strong associations were demonstrated. For example, total IgG and subclass antibodies to the central repeats of Pf155/RESA were highly intercorrelated (Dubois et al. 1993). Further, IgG antibodies against crude extract, Pf155/RESA, MSP1 and MSP2 were correlated (Aucan et al. 2000). In another study, intercorrelations between IgG subclass antibodies against crude blood stage antigens of *P. falciparum* were also observed (Tangteerawatana et al. 2007). Anti-crude antigen responses revealed significant correlations between IgG and IgE as well as IgG and IgM (Israelsson et al. 2008). Anti-MSP1 IgG responses were significantly correlated with anti-AMA1 and MSP2 responses (Stanisic et al. 2009). Apart from the strong correlation of IgG subclass responses to the two AMA1 variants Stanisic et al. (2009) found that high responders for a specific subclass to AMA1-3D7 were significantly more likely to be high responders for the same subclass than high responders for a different subclass to AMA1-W2mef. Anti-Pf332-

C231 total IgG and subclass levels were correlated at variable levels of strength (Giha et al. 2010).

The extensive intercorrelations of IgG levels to blood-stage antigens in the present study suggest a link or similarity and cross-reactivity between the antigens apart from the effect of exposure to multiple parasite strains and allelic forms. To this effect, a study has shown extensive antibody cross-reactivity among different sequence variants, for instance, within the MSP2 serogroups (Franks et al. 2003) though studies that found weak correlations reported this as lack of cross-reactive subclass antibodies to the antigens (Taylor et al. 1998). More recently, a significant positive correlation between responses to the 3D7 and K1 class domains of MSP3 were also observed, where at least some of these responses were suggested to be truly cross-reactive as was confirmed by competition ELISAs (Jordan et al. 2011). Such extensive intercorrelations between responses to a panel of blood-stage vaccine candidate antigens together with the over 70% positivity rate recorded for more than five antigens in the present study has important implications for current vaccine design efforts in which multi-stage, multi-antigen combination vaccine approaches where strain or allele-transcending formulations are being envisaged (Heppner et al. 2005). In general, the relative strength of antibody responses to different antigens vary based on populations and individuals. Simultaneous assessment of responses to a panel of *P. falciparum* antigens can reveal the possible antagonistic, synergistic or no interactions between the responses to the multiple antigenic epitopes.

In the present study, in the absence of active transmission, especially in Shewa Robit where slide positivity rate was zero, strong immune responses were recorded for most

antigens in the majority of study participants. This suggests that malaria immunity is long-lived. A significantly lower number of positive individuals had shown low reactivity to some antigens which may reflect gradual decay of antibodies relating to individual genetic background. Early reversion to seronegativity of a number of seropositive children at the time of an epidemic in Panama and El Salvador in a six month time (Warren et al. 1976) was suggested to be due to very early clearance of parasitemia by curative treatments and thus very brief exposure of the immune system to parasite antigens. The present finding supports earlier observation that antibodies can last over years in people in endemic areas who have been naturally exposed to *P. falciparum* infection (Jacobs et al. 1983). This has been reinforced by the report from a longitudinal study in a stable seasonal malaria transmission locality in Ghana where no significant variation between seasons in anti-MSP1 antibody level and prevalence was reported (Dodoo et al. 1999).

Additional evidence also exists from a study on African immigrants from malaria endemic areas in the past but who were living in Europe for at least four consecutive years, had lower parasitemia, less frequent severe disease, accelerated parasite clearance and high antibody levels (Bouchaud et al. 2006). The investigators interpreted the findings as an evidence for long-term persistence of antibodies in the absence of reinfection. Further, antibodies to *P. falciparum* variant surface antigens (VSA) were detectable and were not short-lived (Elliott et al. 2007). Other studies had also reported the long-lived nature of antibodies (Drakely et al. 2005, Collins et al. 1968, Luby et al. 1967). A more recent study demonstrated that although antibody longevity is age-dependent and there were substantial variations within individuals, anti-AMA1, EBA175, MSP1 and MSP2 total IgG and subclass responses once

induced generally remained stable and persisted for several years in the absence of boosting (Akpogheneta et al. 2008).

Age-related antibody durations were also reported from other studies (Branch et al. 2000, Taylor et al. 1996). The authors suggested that age, in unrelated way to the effect of cumulative exposure to repeated infection, influences antibody longevity by determining the differentiation and regulation of long or short-lived plasma cells. A negative association between the human puberty-related hormone, dehydroepiandrosteron sulphate, and antibody responses to schistosomiasis was observed (Abebe et al. 2003). Similar intrinsic age-related factors may play a role in malaria as well. Age-related difference probably as a result of intrinsic factors on immune maturation with age was evidenced in infant mice that lacked long-lived plasma cells (Pihlgren et al. 2006). But in Akpogheneta et al. (2008), adults and children with persistent asymptomatic malaria infection, antibody declined equally slowly implying that both factors – antigen persistence and immunogenic maturity play a part in determining antibody durability. From Peru, in a low transmission area, investigators reported the rapid development and maintenance of anti-MSP1 antibody (Torres et al. 2008). Further, anti-MSP1 was stable over time (Dent et al. 2009). More recently, in Thailand where malaria transmission was considered so low and that repeated infection became less common, antibodies and memory B cells were maintained for years without evidence for reinfection (Wipasa et al. 2010).

Although the present study was cross-sectional in its design, the information gathered through interviews produced substantial amount of past history of the Shewa Robit study participants by the assistance of local health facility record system that

maintained malaria cases by passive case detection. Thus those who were grouped as reportedly unexposed were equivalent to the 'protected group' in longitudinal studies like Dubois et al. (1993) and Wipasa et al. (2010).

The absence of significant difference between those that reported clinical malaria episode and those who did not, for anti-GLURP-R0, GMZ2 and MSP3 antibody response, is consistent with a study in Madagascar where total IgG and subclass responses were similar in individuals considered protected and susceptible (Dubois et al. 1993). According to these authors subjects who never developed clinical malaria were considered protected irrespective of their parasitological status and those who experienced at least one clinical attack were nonprotected. Thus the protected group might have been free of parasites or they harbored less parasitemia and were asymptomatic. Most studies adopted this description to categorize their study participants as clinically immune and susceptible. But, a few other studies (Egan et al. 1996, Metzger et al. 2003) excluded parasite-negative individuals during follow-up from the 'protected' group, as they found it difficult to distinguish protection due to immunity from lack of exposure to infective mosquito bites (Bejon et al. 2009).

Anti-AMA1 antibody response, both positivity rate and level, were significantly higher among individuals who were unexposed in their life time than the exposed. Further, IgG seropositivity rate for this antigen was greater in those who had clinical episode once than more than once. Anti-AMA1 IgG seropositivity rate was the highest among those who reported their most recent malaria clinical attack long time ago in the years 1991-2004. But the highest median titer for this antibody level was for individuals whose latest episode was in the year 2008, the year of sample

collection. These findings taken together suggest that protective AMA1-specific malaria antibody remains stable at detectable level for longer period but the level declines, though not significantly, overtime. Similarly, Giha et al. (2010) observed significantly lower IgG total and subclass levels to Pf332-C231 in individuals exposed to clinical malaria at least once compared to the unexposed.

Literature pertaining to the correlation between anti-AMA1 antibody and risk of symptomatic malaria is not concordant. No correlation between IgG antibody levels to AMA1 and malaria related fever or parasite density was observed (Thomas et al. 1994). Similarly, antibody responses to individual antigens did not correlate with immune status but combined recognition of AMA1 and the two allelic variants of MSP2 were significantly associated with protection (Gray et al. 2007). Moreover, no association between IgG total to AMA1 and risk of reduced malaria incidence as well as the level of parasitemia was found (Nebie et al. 2008). Similarly, high IgG3 response to AMA1 was significantly associated with protection but IgG total against AMA1 was weakly associated with protection (Stanisic et al. 2009). For AMA1-specific IgG3 Courtin et al. (2009) could not find association with protection. Contrastingly, antibody responses to AMA1 were associated with protection (Dodoo et al. 2008, Polley et al. 2004). More recent investigations also reported that IgG total as well as IgG1 antibody levels to AMA1 were associated with protection (Osier et al. 2008, Iriemenam et al. 2009). Similarly, *in vitro* parasite growth inhibition was observed for anti-AMA1 antibody responses (Courtin et al. 2009).

Lack of significant difference between exposed and unexposed groups in antibody responses to GLURP-R0, GMZ2 and MSP3 may also suggest the longer durability

and protective role of antibody responses to these antigens. In the absence of detectable infection by microscopy antibody prevalence and level against these antigens was maintained high and stable. Individuals who reported past clinical episode once and more than once had no significant differences in their antibody immune responses against most antigens. Further, no significant difference was observed between groups of individuals whose most recent clinical malaria episodes ranged from 1991 through 2008. There was no indication of decline in antibody positivity in those whose clinical episode was long time ago (1991-2004). After one year, antibody titer to GMZ2 remained high in the first clinical trial in malaria naïve adults (Esen et al. 2009). In the absence of boost infection anti-GMZ2 antibodies in immunized adults and children lasted 6-12 months (Mordmüller et al. 2010, Be'lard et al. 2011) and then started waning. When natural MSP3- and GLURP-R0-specific IgG subclass levels were considered, no major change in the levels of specific IgG1 against the two antigens before and after five years was reported (Soe et al. 2004). But the report indicated that the protection status of some individuals was associated with IgG3 to GLURP-R0 at one time point (before five years) and IgG3 to MSP3 after five years suggesting changes between individuals in their response to the antigens and the dynamics (or complementing effect) of responses to these antigens overtime.

Studies relating to naturally acquired anti-GMZ2 antibodies with protection from clinical malaria are currently lacking. The present study suggests that both antigens, GLURP-R0 and MSP3, are important for the induction of protective antibodies against malaria from natural infections providing additional rational for combining the two antigens in a hybrid vaccine formulation. Thus GMZ2 has high potential as a candidate vaccine compared to other blood-stage candidate vaccine antigens.

However, it remains unclear whether the GMZ2 vaccines would be effective in the heterogeneous epidemiological settings of Ethiopia as there is evidence that vaccine-induced antibodies may not be as potent as those naturally acquired (Metzger et al. 1999). But the induction of functional antibodies, cytophilic subclasses, following natural exposure in the present study signals that the GMZ2 vaccine would likely be effective at least through boosting of the pre-existing antibodies. The role of challenge infections in boosting vaccine stimulated-antibodies for parasitemia control was noticed in pre-clinical study in monkeys (Carvalho et al. 2005). Furthermore, there was evidence from phase I clinical trial in naturally exposed adults that the GMZ2 vaccine boosted pre-existing natural immunity (Mordmüller et al. 2010).

Several studies reported the association between anti-GLURP-R0 antibodies and protection from symptomatic malaria (Courtin et al. 2009, Meraldi et al. 2004, Nebie et al. 2008, Soe et al. 2004). In other studies, however, no clear association between IgG response to GLURP and clinical immunity was observed. Studies that specifically targeted the R0 region reported significant correlation between anti-GLURP-R0 IgG1 and IgG3 antibodies and protection (Dodoo et al. 2008, Iriemenam et al. 2009, Nebie et al. 2008). Conversely, others reported no association between reduced risk of malaria and antibody response to GLURP-R0 (Lusingu et al. 2005).

IgG3 to MSP3 was associated with protection (Meraldi et al. 2004, Soe et al. 2004). Other studies also reported the association of antibody levels to MSP3 with protection (Nebie et al. 2008, Osier et al. 2008, Roussilhon et al. 2007). Protected individuals with low IgG3 responses to MSP3 were found to have a strong IgG3 response to GLURP-R0 and vice-versa (Soe et al. 2004). This suggests the complimentary and

compensating advantage by the response to one antigen for the lower response against the other. On the other hand, most protected individuals had high IgG3 responses against both MSP3 and GLURP (Soe et al. 2004).

For MSP2-3D7, MSP2-FC27 and GLURP-R2 the falciparum malaria exposed group had significantly higher IgG prevalence than the unexposed. The median antibody level was also significantly higher in the exposed than unexposed for MSP2-3D7 and MSP2-FC27. Further, IgG levels to MSP2-3D7 and MSP2-FC27 were significantly higher in those individuals who experienced clinical episode more than once than those who had only once. Antibodies to these antigens may need more boosting to be maintained, thus those who experienced clinical attacks had relatively higher and more stable responses. However, in a seasonal and unstable setting in the Gambia it was shown that total IgG antibodies to MSP2 did not significantly fluctuate between transmission seasons in protected adults (Taylor et al. 1996). Antibody responses to MSP1 remained high in both dry and wet seasons (Omosun et al. 2005). Alternatively, the exposed having relatively higher overall responses to the two allelic variants of MSP2 may suggest that antibodies to these antigens are less protective.

It was proposed that the breadth (number of important targets to which antibodies were made) and magnitude (antibody level measured in a random serum sample) of the antibody response were important predictors of protection from clinical malaria (Osier et al. 2008). The authors have shown that while antibody levels to AMA1, MSP2 and MSP3 were inversely related to the probability of developing malaria, levels to MSP1 and EBA17 were not suggesting that a particular combination of antigens is relevant to achieve protection.

While IgG3 antibodies to MSP2-3D7 were inversely correlated with protection IgG1 to MSP2-FC27 was associated with protection (Taylor et al. 1998). Further, antibody response to these antigens did not correlate with protection (Gray et al. 2007). More studies that analyzed the association between antibody responses to MSP2-3D7 and MSP2-FC27 that showed no evidence of a reduced risk of symptomatic disease included (Scopel et al. 2007, Stanisic et al. 2009). Similarly, although some investigators demonstrated the protection of mice immunized with peptides from conserved regions of MSP2 from challenge with the murine malaria *P. chabaudi* (Saul et al. 1992) others failed to confirm in other model systems (Pye et al. 1991).

Several others reported the association of total IgG, IgG1 and IgG3 to MSP2 with protection (Clark et al. 1989, Gray et al. 2007, Osier et al. 2008). In addition, *in vitro* parasite growth inhibition was observed for anti-MSP2 antibodies (Clark et al. 1989, Courtin et al. 2009, Eppinge et al. 1988).

Those reported unexposed but antibody positive individuals might have been asymptotically infected and because of their higher response might have controlled parasitemia and thus were protected. Similar findings were documented in Sudan and Brazil both in hypoendemic unstable transmission setting like the current study (Giha et al. 2010, Pratt–Riccio et al. 2005). The persistence of chronic low-grade asymptomatic infection with *P. falciparum* among individuals in hypoendemic unstable malaria transmission areas was detected using the polymerase chain reaction (PCR) (Roper et al. 1996).

Since most of the study adult population have been permanently or for a significant period of time residing in or at least migrated to an endemic area their chance of remaining uninfected was very unlikely. In unstable seasonal transmission setting the role of submicroscopic asymptomatic infection in induction of protective and stable antibodies was emphasized in other studies as mentioned above (Giha et al. 2010, Pratt–Riccio et al. 2005) and the findings in the present study are in line with these reports. Thus, some individuals might have induced and maintained protective immunity and thereby might be protected from secondary infections. In summary, the documentation of high level antibody responses in individuals without history of clinical exposure may imply asymptomatic subpatent, submicroscopic infection and that may imply the potential importance of such infections in the induction and maintenance of high level protective immunity as it was also suggested by Giha et al. (2010) and Baumann et al. (2012).

Individuals in the group might have been sick sometime in the past but failed to remember and report their clinical episodes and reported as unexposed. In fact there may be some limitation regarding the reliability of donor-reported information. Definitely there may be some individuals who failed to recall the number and date of clinical episodes they experienced. However, it is likely that residents in endemic areas acquire malaria immunity somehow in the past following their first and second infection and then could report less episodes in the more recent years or may not even be exposed to clinical attack at all. Conversely, younger people living for a shorter time in the area, for instance settlers might have experienced more episodes relatively recently. They might have been misdiagnosed, false negative for *P. falciparum* and were categorized among *P. vivax* positives.

Alternatively, they might have been genuinely *P. falciparum*-unexposed but were *P. vivax* positive and because of cross-reaction they became *P. falciparum* seropositive. This is likely that a significant number of study populations reporting prior infection only with *P. vivax* were seropositive for *P. falciparum* antigens (Jangpatarapongsa et al. 2006, Wipasa et al. 2010). Thus, undiagnosed prior infection with *P. falciparum* and/or cross-reactivity of antibodies to the two species in the past might also explain why the unexposed were seropositive. It might be just due to lower parasitemia and could not induce clinical disease. Whatever the explanation might be, detecting high antibody prevalence and level in this group suggests that a significant proportion of study participants had malaria-specific antibodies that readily recognized blood stage antigens of *P. falciparum* in Ethiopia in the absence of detectable parasitemia by microscopy.

Detection of high and comparable antibody levels and seropositivity rates in individuals exposed once and those who had multiple clinical episodes, but some years back, may again suggest either submicroscopic low level persistent infection capable of boosting the immune response or simply the long-lived nature of antibody responses in the absence of persistent infection or re-infection for some antigens. The possibility of acquiring high antibody levels following primary infection was also reported (Wahlgren et al. 1986, Elliott et al. 2007). Similar results were observed in Thailand with nearly similar study design, population characteristics and setting (Wipasa et al. 2010). These investigators found high anti-AMA1, MSP1 and MSP2 antibody responses in individuals with no known prior episode of malaria infection for six years in the past. They demonstrated that most seropositive individuals

remained so after long time. The authors further estimated the half-life of IgG, for instance, to AMA1 as 10.4 years.

The very low number of unexposed and seronegative individuals might have simply not been bitten by infectious mosquito for various reasons [individual differences in applying personal intervention procedures, odor, etc], or the group might have lost their immunity through time. Those who reported exposed but were seronegative probably lost their antibodies. Alternatively, in the past they were probably misdiagnosed for *P. falciparum*. Further, the duration and intensity of their infection might have been short and low not allowing their immune response enough exposure to the parasite antigens. Because of induction of antibodies to polymorphic epitopes that did not cross-react with the antigens used in this study (test system), or due to immunological nonresponsiveness. Similarly, in a study in Thailand some of the study population who reported prior infection (or clinical episode) was devoid of memory B cell and/or antibodies (Wipasa et al. 2010).

IgG subclass positivity against MSP3 and GLURP-R0 was fairly equally distributed among unexposed and exposed groups and exposed once and more than once for all the subclass antibodies characterized. This further suggests the stability of malaria-specific antibodies. But IgG3 seropositivity rate against GLURP-R0 was significantly higher in those who were exposed once than those who were exposed more than once. This suggests that those who were exposed only once had maintained higher IgG3 response following primary infection and were relatively protected.

IgG4 positivity to both MSP3 and GLURP-R0 was the highest, though not significant, in individuals whose latest clinical episode was in the years 1991-2004. This subclass antibody was more prevalent and persistent in those who experienced clinical episode long time ago. It needs future investigation whether this may indicate the protective role of IgG4 against severe clinical malaria. While low IgG4 levels were reported to have protection in one study (Aucan et al. 2000) others emphasized the inhibitory/blocking effect of IgG2 and IgG4 on the activities of protection-associated cytophilic antibodies. In another study anti-GLURP-R0 IgG4 level was significantly associated with increased frequency of clinical malaria attacks (Soe et al. 2004). At least in one study high IgG4 responses to MSP1 and GLURP were associated with reduced risk of malaria incidence (Nebie et al. 2008). More recently, significantly higher levels of IgG4, to crude extract antigens, was detected in individuals who did not have had malaria compared to those who had experienced at least one clinical malaria attack (Giha et al. 2010). It was shown that GLURP-specific IgG4 was detected at significantly lower levels in HbAS (sickle cell trait) children (Maya et al. 2006) suggesting the possible impact of erythrocyte-based human genetic factors in IgG4 expression at least against some blood-stage *P. falciparum*. This may possibly imply the link between adaptive malaria immunity and the established erythrocyte-related mechanism of protection against malaria. Human genetic studies are required to evaluate this in Ethiopian context.

As a whole, there was no significant difference in IgG subclass prevalence and level between individuals whose most recent malaria clinical episodes were in different years. Similarly no relationship was observed between antibody responses to GLURP-R0 and GLURP-R2 and reported number of previous malaria episodes in another

study (Pratt–Riccio et al. 2005). In a prospective study in seasonal unstable transmission locality in The Sudan total IgG and subclass levels to Pf332-C231 were similar between donors who did not and those who experienced from one to seven malaria episodes (Giha et al. 2010).

The findings and accompanying arguments in interpretation are more plausible given that sample collection was not during peak transmission season; the setting is a seasonal and unstable malaria transmission area. In addition, most of the study participants were devoid of malaria signs and symptoms. Moreover, currently there is prompt treatment of suspected and confirmed malaria cases and extensive intervention activities resulted in reduced transmission potential countrywide as there is significant decline (>20%) in malaria transmission and incidence in several places across endemic areas globally in the last few years (Murray et al. 2012, WHO 2010a). Taken together, the data support previous studies that reported the occurrence of long-lived and stable malaria antibody response once induced in the absence of microscopically detectable parasitemia and re-infection as described above.

The age-related changes in total IgG level in Shewa Robit that varied for a specific antigen is consistent with other reports. Chelimo et al. (2005) reported that the pattern of age-dependent difference in IgG response frequencies varied by antigen. The absence of significant difference in both anti-AMA1 prevalence and level between the age groups and lack of significant association between age and antibody titer agrees with some reports (Nebie et al. 2008, Wipasa et al. 2010) and departs from others countries (Akpogheneta et al. 2008, Courtin et al. 2009, Stanistic et al. 2009). Some investigators have shown that anti-AMA1 IgG response is acquired rapidly in early

life (Courtin et al. 2009). Thus lack of significant age-dependent pattern for this antibody response may not be surprising. Alternatively, as age and exposure are interdependent the more exposed grownups may have significantly higher antibody levels and prevalence to the antigen than less exposed people (Stanisic et al. 2009).

But the general negative relation of anti-AMA1 IgG with age, though insignificant, in this study might be because children had undetectable subpatent infection that increased the antibody response and adults even with lower parasite density might have lowered antibodies. Some intrinsic age-influenced factors related to the parasite genotype or allelic variant may regulate the induction and maintenance of IgG response to this antigen. Anti-AMA1 antibody develops early in life and cumulative exposure does not significantly influence this specific antibody response in certain settings (Courtin et al. 2009) explaining the absence of significant age-dependent changes in antibody response to this particular antigen.

Age-related increase observed for other *P. falciparum* recombinant antigens tested in Shewa Robit was in agreement with other studies. The prevalence of total IgG to MSP2 serogroup antigens in the Gambia correlated positively with age (Metzger et al. 2003, Taylor et al. 1998). Later studies on MSP2 also confirmed earlier findings (Akpogheneta et al. 2008, Courtin et al. 2009, Osier et al. 2008). Stanisic et al. (2009) reported antibody responses to MSP2 significantly increased with age and exposure. These authors demonstrated that both level and prevalence of the predominant antibody subclasses were significantly higher in the older children although anti-MSP2 IgG1 level was unrelated to age. According to Scopel et al. (2006) and Tongren et al. increasing age (and therefore malaria exposure) leads to an increasing anti-

MSP2 antibody prevalence and levels in more exposed individuals than less exposed ones. But, other investigators reported no age-related change in anti-MSP2 IgG level (Wipasa et al. 2010).

Various other studies reported age-specific positivity rate to different regions of GLURP (Dziegiel et al. 1993, Hogg et al. 1992) in agreement with current study. Studies further revealed age-related increase in total IgG as well as subclass responses to GLURP-R0 and GLURP-R2 in two similar seasonal transmission areas (Nebie et al. 2008, Pratt–Riccio et al. 2005). In the present study the differences between age groups were significant for both antibody level and prevalence to GLURP-R0 being consistent with the above studies. Observing the strongest association between anti-MSP3 total IgG titer with age in the present study is consistent with a study that reported significantly increased IgG prevalence to both the conserved and allele-specific epitopes of MSP3 with age (Osier et al. 2007). It was shown that total IgG and subclass responses to MSP3 significantly increased with age (Courtin et al. 2009, Nebie et al. 2008, Segeja et al. 2010) in keeping with the present study for both study sites. Similarly increased age-dependence of total IgG, IgG1 and IgG3 antibody prevalence and levels were recorded for GLURP (Maya et al. 2006). Total IgG and subclass levels against all GLURP regions were significantly associated with age (Soe et al 2004).

Several other studies in different levels of endemicity reported similar as well as contrasting results for other blood stage antigens of *P. falciparum*. Malaria-specific antibody titer to crude antigens increased with age (Israelsson et al. 2008, Giha et al. 2010, Leorati et al. 2008, Spencer et al. 1981). To the same antigen another study

found high IgG level in very young children and that level remained maintained in older patients in apparent contradiction to most reports (Calissano et al. 2003). Anti-CSP, EBA175, thrombospondin-related adhesive protein (TRAP) and liver stage antigen 1 (LSA1) IgG prevalence in a holoendemic area in Kenya increased with age (Chelimo et al. 2005). In another study antibody acquisition to EBA175 was age- and exposure-dependent (Akpogheneta et al. 2008, Toure et al. 2006). A study which measured antibody responses to *P. falciparum* gp195 in The Philippines has found the lowest seropositivity rate in the age group 0-4 years old (Krame and Oberse 1992). Antibody prevalence and level to *P. falciparum* heat shock protein was positively correlated with age (Alexander e al. 1997). Similarly, antibody responses to VSA varied based on transmission intensity and age (Vestergaard et al. 2008).

The results recorded for MSP1 are more conflicting compared to other antigens. Anti-MSP1 subclass levels as well as ratios increased with age (Aucan et al. 2000). Further, infants less than 300 days old and pregnant women had the lowest cytophilic to noncytophilic IgG ratio (Branch et al. 2000). By 300 days of age infants and their mothers had similar subclass levels and composition. Older infants with parasitemia only 1 or 2 times detected had the highest cytophilic to noncytophilic ratio. An age-based increase in anti-MSP1 total IgG and subclasses was recorded (Omosun et al. 2005). Total IgG and subclass responses to MSP1 increased with age in a seasonal transmission area (Nebie et al. 2008). A study conducted in Kenya that compared anti-MSP1 and EBA175 levels between individuals in stable and unstable transmission areas could not find significant difference (Noland et al. 2008).

But in another setting antibody titers against MSP1 increased with age (Courtin et al. 2009). Further, antibody response to MSP1 significantly increased with age and exposure, both level and prevalence of the predominant antibody subclasses were significantly higher in the older children (Stanisic et al. 2009). Similarly, the breadth of antibody specificity to MSP1 increased with age (Osier et al. 2008). On the contrary, a longitudinal study in a seasonal but stable transmission locality in Ghana reported no significant variation between age groups in anti-MSP1 antibody level and prevalence (Dodoo et al. 1999). Further, there was no age-related change in the antibody titers to MSP1 (Wipasa et al. 2010). In another study IgG antibody prevalence to MSP1 in a holoendemic area in Kenya was similar across age groups (Chelimo et al. 2005). Further, antibody response to the antigen was not significantly associated with age (Akpogheneta et al. 2008). But, age is one of key factors that influence the presence, absence, levels and type of malarial antibodies in general (Baird et al. 1995).

Other important confounding factors in this connection, as indicated above, are duration and intensity of the infection, previous experience with the parasite strain, other infections, human genetics, nutritional status, etc. For instance, a longer duration of infection (infections that were not terminated quickly by treatment) particularly in children resulted in higher seropositivity rates in El Salvador (Spencer et al. 1981). In primary infections less consistent and usually lower antibody response was observed compared to the response in those known to have experienced a previous malaria attack (Spencer et al. 1981). But these and similar earlier reports are inconsistent with the finding in this study. No significant difference was observed in antibody response between those experienced reportedly multiple past *P. falciparum* episodes and those

who had only their primary clinical attack suggesting that previous experience (or cumulative exposure) is less important for the maintenance of malaria-specific antibody at least in the current study population. Also, in this context the duration of infection may be less important in antibody response induction and maintenance as most of the individuals tested had access to effective presumptive treatment schemes which were made readily available through extensive network of treatment posts in addition to regular campaigns of control efforts that reduce man-mosquito contact and thus lower multiple infections and make parasitemia less.

The impact of intervention aimed at targeting vector control reduces force of infection and has implications for malaria immunity (Snow et al. 1996, Snow and Marsh 2002). Furthermore, the study areas were characterized by markedly unstable seasonal transmission. These all supposed to significantly reduce the duration as well as intensity of infection (the parasitemia). Moreover, samples were collected almost during the dry season. Participants were microscopy and RDTs-negative and had no clinical signs suggestive of malaria. Further, most had their recent infections three years back in 2005, the year in which the last epidemic occurred in the area (Shewa Robit). In spite of all these conditions that are assumed to delay the acquisition and persistence of antibody responses, the study evidenced higher seropositivity rate and antibody levels and overall higher reactivity in most sera analyzed against most of the antigens tested.

The results are in agreement with other studies. High IgG total concentration was measured for relatively protected Liberian adults and primarily infected Swedish patients demonstrating that primary infection can result in antibody levels comparable

to that is in the residents in endemic areas (Wahlgren et al. 1983). This is in line with the finding of the present study that people who experienced malaria clinical episode only once had higher seropositivity rate and antibody levels suggesting the protective role of the antibodies. Further, when sera from individuals who experienced more extensive exposure and those with lesser exposure to *P. falciparum* were analyzed, no significant antibody titers were observed between the two groups (Wahlgren et al. 1986). A study which revealed the development of age-dependent acquired malarial immunity within a two-year period of exposure in hyperendemic setting (Baird et al. 1991) corroborates this finding.

Similarly, antibody responses detected by Riley et al. (1993) to MSP1 showed no significant difference between individuals living in areas of low and high transmission of seasonal malaria. Additionally, age-dependent, but only after brief exposure (after 1-2 years), development of malaria specific immunity was observed in a transmigrant population, originated from malaria free area, in a hyperendemic area (Baird et al. 1993). In another study (Branch et al. 2000) antibodies to MSP1 were age- but not multiple exposures with parasitemia-dependent for their development. These findings argue contrary to the most popular model for malaria immunity which hypothesizes that protection increases with age as a cumulative product of exposure to multiple parasite antigens repeatedly over years of life.

The risk of severe malaria is known to vary in age- and transmission-dependent manner. In areas of higher endemicity severe malaria is frequently associated with children but in lower transmission areas adults are also susceptible although children may suffer more. But in general it is widely perceived that nonimmune adults and

children suffer equally from severe malaria as a result of primary exposure. However, there are only few studies that addressed the rate of severe malaria among first exposed individuals in age-specific manner. Such studies evidenced higher risk of severe malaria among greater than 40-years old nonimmune people than youngsters (Schwartz et al. 2001, Mühlberger et al. 2003).

In light of these apparently conflicting observations it was suggested that generally some intrinsic age-related factors influence the development, maintenance, protective and harmful aspects of malarial immune response leading to differing disease outcomes (Baird 1995, 1998, Baird et al. 1991, Baird et al. 1993, Dondrop et al. 2008). The age-related increase in antibody response to most of the malaria antigens tested in the present study at least for Shewa Robit appears to discount the role of cumulative exposure to the parasite and as such possible gradual maturation of the immune system. Thus other intrinsic age-related factors in immune maturation may influence the immune response in the population. However, for Boditi participants where the number of previous self-reported malaria episode was not captured it is hard to assess the role of cumulative exposure to the parasite variants.

Detecting higher total IgG and subclass positivity and levels to all the three antigens tested in blood-film-negative group for *P. falciparum* infection compared with smear-positives suggests that slide-negative individuals were relatively protected due to their high antibody responses. Nonetheless, in another study, no relationship was observed between antibody responses to GLURP-R0 and GLURP-R2 and presence or absence of *P. falciparum* infection and the level of parasitemia (Pratt–Ricchio et al. 2005). No significant association was found between anti-MSP3 IgG1 levels and *P. falciparum*

slide-positivity although individuals with *P. falciparum* infection had significantly higher total IgG and IgG3 levels (Segeja et al. 2010). Regardless of age, individuals with active *P. falciparum* infection had higher IgG level against crude parasite antigen than parasite-negatives (Titangi et al. 2002). The prevalence of IgG to MSP2 serogroup antigens in the Gambia correlated positively with the presence of parasitemia at the time of sampling (Metzger et al. 2003). Moreover, it was indicated that IgG1 and IgG3 levels to AMA1, EBA175, MSP1 and MSP2 were significantly higher in parasite harboring individuals than slide-negative ones for *P. falciparum* infection (Akpogheneta et al. 2008, Stanistic et al. 2009). Such reports may suggest the influence of recent boost infections that resulted in relatively elevated antibody responses in one hand and the lower protective efficacy of the antibodies in parasite control on the other.

The elevated anti-MSP3 IgG2 prevalence and level in Shewa Robit was in contrast to most other studies in various settings that observed either predominantly IgG3 or its coexpression with IgG1 to the antigen (Courtin et al. 2009, Druilhe et al. 2005, Stanistic et al. 2009). The relative abundance of IgG4 also shows the overexpression of noncytophilic subclasses against MSP3 suggesting the possible protective efficacy of these antibodies in the study participants who were asymptomatic. The predominant expression of anti-GLURP-R0 IgG1 is in support of other reports (Courtin et al. 2009, Iriemenam et al. 2009, Nebie et al. 2008, Stanistic et al. 2009). But the higher strength of IgG2 reactivity to GLURP-R0 in the majority of the study participants compared to IgG3 demonstrated skewing towards IgG2 in apparent contradiction with the above same studies. However the overall dominance of cytophilic subclasses over the

noncytophilic shows the functionality of GLURP-R0-specific IgG subclasses explaining the better protected status of Shewa Robit participants.

In Boditi, anti-MSP3 subclass response was predominantly composed of IgG1 and IgG3 indicating the induction of functional antibodies against the antigen. This dominance of MSP3-specific IgG1 and IgG3 isotypes goes in agreement with studies from other settings (Courtin et al. 2009, Nebie et al. 2008, Stanisic et al. 2009). For GLURP-R0 it was a mixture of cytophilic and noncytophilic IgG subclasses with higher IgG1 level, indicating the presence of protection-associated subclass.

In both study sites IgG subclass antibody level to GLURP-R0 was significantly higher than that to MSP3 for all corresponding subclasses characterized in most individuals indicating the increased relative immunogenicity and protective potential of GLURP-R0 compared to MSP3. In general, against both GLURP-R0 and MSP3, the ratio of cytophilic to noncytophilic antibodies was >1 in the majority of the study participants in both sites indicating considerable isotype imbalance in keeping with other studies (Courtin et al. 2009, Nebie et al. 2008). Against other antigens of the parasite similar biased subclass distribution was noted. A study in Nigerian patients with acute malarial disease published slightly lower IgG2, IgG3 and IgG4 but elevated IgG1 levels (Salimonou et al. 1982). Another earlier study that characterized IgG subclass pattern to *P. falciparum* crude antigens showed the detection of all the four subclasses with frequently elevated levels of IgG1 and IgG3 (Wahlgren et al. 1983).

In another similar study, against a crude antigen IgG1 and IgG3 were co-prevalent in almost all sera tested (Wahlgren et al. 1986) and relative concentrations of the

subclasses paralleled those of total subclass concentration, i.e. IgG1>IgG2>IgG3>IgG4. In a later study, while the coexpression of IgG1 and IgG3 was observed in more than 90% of the cases, in one and two thirds of IgG1- or IgG3-containing samples IgG2 and IgG4 were undetected respectively (Dubois et al. 1993). According to this same study, all IgG2-positive samples were also positive for IgG1 and IgG3 but IgG4 was absent in nearly 50% of the samples. The two dominant subclasses to crude extract antigens were also IgG1 and IgG3 with the former significantly higher than the latter (, Israelsson et al. 2008). But Nasr et al. (2007) recorded IgG1 as the predominant subclass followed by IgG2. Similarly, *in vitro* experiments showed that sensitized B cells from individuals in endemic areas produced all four IgG subclasses to crude antigen preparations but to a defined antigen only IgG1 and/or IgG3 were produced (Garraud et al. 2002).

In a highly endemic area in Solomon Islands IgG3 was the dominant subclass to MSP2 (Rzepczyk et al. 1997). In the Gambia anti-MSP2 antibodies were predominantly IgG1 and IgG3 and age-based polarization to IgG3 dominance was observed (Akpogheneta et al. 2008, Taylor et al. 1998). Similar reports included (Scopel et al. 2005, Tongren et al. 2006). IgG3 was also a dominant subclass to MSP4 (Wang et al. 2001). Similarly, skewing of anti-MSP1 antibodies towards IgG1 and IgG3 was observed with the former higher than the latter in most cases (Akpogheneta et al. 2008, Dodoo et al. 1999, Egan et al. 1996, Omosun et al. 2005). IgG subclass antibodies to other major blood-stage-antigens like AMA1, CSP, EBA175 were predominantly IgG1 and IgG3 (Akpogheneta et al. 2008, Chelimo et al. 2005). More studies confirmed that antibody subclasses to AMA1, MSP1 and MSP2 were predominantly mixed IgG1 and IgG3, with IgG1 being predominant for AMA1 and

MSP1 and IgG3 for MSP2 (Courtin et al. 2009, Iriemenam et al. 2009, Stanisic et al. 2009). The above authors observed variable level of predominance which varied between different individuals and also depended on the inducing antigen involved, which is consistent with the present study.

Factors that control the preferential induction of polarized subclass responses are not well-understood. Cytokines and B cell activators are involved in inducing Ig class-switching in a model system (Coffman et al. 1993) but little is known concerning *in vivo* mechanisms in human malaria (Garraud et al. 2002, Tongren et al. 2006). It was suggested that some bacterial antigens are known to induce IgG2 expression (Lane et al. 1986, Lane and MacLennan 1986) and allergens or helminths stimulate IgG4 and IgE production (Garraud et al. 1995). Polysaccharides like dextran or levan contribute towards IgG2 elevation (Yount et al. 1968) especially in response to other parasitic diseases such as schistosomiasis (Dunne et al. 1988). When there is polarized Th2-type response, IL4 and IL13 direct the production of IgG4 and IgE (Garraud et al. 2003). Specifically to malaria, different antigens are known to induce different IgG subclass profiles with varying relative levels which also vary for different populations and individuals in different endemic localities (Metzger et al. 2003, Tongren et al. 2006).

Differing speculations are presented as to the role of antigen properties and human genetic determinants in IgG subclass distribution. It was suggested that antigen characteristics independent of other factors can induce antibody class/subclass-switching in B cells (Garraud et al. 2002). Familial correlation of IgG subclass responses to *Plasmodium falciparum* antigens was observed indicating the role of

human genetic determinants (Aucan et al. 2001). The possible influence of erythrocyte-based human genetic markers in malaria-specific IgG subclass distribution was suggested (Maya et al. 2006, Ntoumi et al. 2005).

Apart from the nature of the antigen cumulative exposure to the antigen, and the maturity of the immune system (i.e., the age of the individual) could independently influence IgG1/IgG3 class switching (Taylor et al. 1998, Tongren et al. 2006). However, Scopel et al. (2006) could not confirm the effect of cumulative exposure and age though concerning the role of antigens they provided additional evidence. Also, the authors could not find the influence of FcγRIIA on IgG subclass distribution during malaria infection contrary to other authors like Ntoumi et al. (2005). It was suggested that intrinsic antigen nature determines subclass expression in unrelated manner to host-related factors (Stanisic et al. 2009). Micronutrient deficiencies were implicated in Th1/Th2 cytokine imbalance related to Ig isotype switch. A study reported a nonspecific decline in cytokine production associated with iron deficiency, which is largely linked to malaria, in children (Mbugi et al. 2010).

Significantly lower falciparum-specific antibody response was noticed in Boditi than Shewa Robit. This may be because of nutritional factors, genetic differences of the two populations, parasite strain variation and level of transmission. During the surveys it was noticed that there was severe food shortage in Boditi. Boditi is a highland fringe at an altitude of 2059 masl. The seasonality of malaria in highlands is more pronounced (particularly above 1800 masl (FMoH 2007) with characteristic lower immunity compared to warm hotbeds of malaria transmission. A classical serological survey in Ethiopian highlands indicated that high *P. falciparum*-specific

responses were obtained for people living at elevations of 1828 masl and less compared to those at 1920 masl or above (Collins et al. 1971). Differences in employing control methods such as treatment seeking behavior and access to prompt and effective treatment schemes, appropriate ITN usage, factors in relation to human sweat components, vicinity to mosquito breeding sites, regularity in IRS and overall variations of control methods account for heterogeneity in exposure to infective mosquito bites (White et al. 2010, White et al. 2011) making even low level boost infections more infrequent thereby possibly contributing towards relatively lower immune responses in Boditi study participants.

In Shewa Robit (at an elevation of 1380 masl) an irrigated tobacco farmland created ideal pools of mosquito breeding sites. This can be a potential source of submicroscopic subpatent infections that maintained higher immunity compared to Boditi. The Shewa Robit study participants who had stronger functional antibodies were asymptomatic and had no microscopically detectable infection suggesting that they were relatively better protected compared to the Boditi. It was possible to have some picture about symptomatic malaria in the two communities during the years prior to the study. The higher average number of malaria cases for Boditi than Shewa Robit further suggests that the antibody responses by the Shewa Robit community were better associated with protection from clinical malaria.

6. CONCLUSIONS

1. Detection of high antibody response in microscopically-negative individuals suggests that people in seasonal malaria transmission areas of Ethiopia acquire and maintain immune responses to *P. falciparum* blood-stage antigens in the absence of microscopically detectable infection. The documentation of high level antibody responses in individuals without history of self-reported malaria episode as well as microscopically smear-negative individuals implies the potential importance of low-grade asymptomatic submicroscopic infections in the induction and maintenance of high level protective immunity.
2. High antibody prevalence and level detected against GMZ2 suggests that this antigen could be a more relevant blood-stage malaria vaccine candidate.
3. Though few sera did not show reactivity to GLURP-R0 and MSP3 in both study sites, most were positive for all IgG1, IgG2 and IgG3 subclasses suggesting the these antigens are readily recognized in the populations.
4. The ratio of cytophilic to noncytophilic antibodies against GMZ2 subunits, GLURP-R0 and MSP3, was >1 in the majority of the study population in both study sites implying the stimulation of functional IgG subclasses.
5. The Boditi population is at a higher relative risk of symptomatic malaria than age-matched individuals in Shewa Robit. The higher antibody responses in Shewa Robit than in Boditi suggest variations in the level of malaria

transmission and in the relative abundance of specific parasite strains in the respective areas.

6. The highest antibody prevalence rates and titers were among adults and varied between antigens, but without evidence of dependence on frequency of reported past exposure to clinical malaria. Intrinsic (biological) age-related factors may play a role in malaria-specific immune maturation.

7. Extensive intercorrelations between anti-blood-stage antigen IgG levels against different antigens is an indication of cross-reactions and/or exposure of the study populations to different strains of *P. falciparum*.

7. RECOMMENDATIONS

1. Because of varying malaria epidemiological conditions, parasite and human genetic variabilities, data from vaccine efficacy trials from other African settings may not be generalisable to the situation in Ethiopia. Therefore, naturally acquired immunity to malaria must be independently assessed in Ethiopia.
2. The protective efficacy of naturally stimulated antibodies against the GMZ2 vaccine candidate antigen requires more investigation through a prospective study.
3. In view of age-associated antibody pattern observed, the possible effect of human maturation hormones on malaria antibody development needs to be addressed.
4. The possible synergistic or antagonistic interactions between malaria-specific antibody responses against a combination of different antigenic epitopes require further investigation.
5. Countrywide malaria survey has to be conducted in Ethiopia to determine the level of endemicity in the country.

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DECLARATION

I, the under signed, declare that this thesis is my original work and has not been presented for a degree in any other university.

Hassen Mamo, candidate

Signature _____