CYTOKINE AND CHEMOKINE RESPONSES TO *PLASMODIUM FALCIPARUM* MALARIA VACCINE CANDIDATE ANTIGENS DURING A PERIOD OF INTERRUPTED MALARIA TRANSMISSION IN A HIGHLAND AREA OF WESTERN KENYA

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ABSTRACT

Longitudinal studies of cytokine responses to *Plasmodium falciparum* (*P. falciparum*) antigens have depicted the acquisition and maintenance of clinical immunity, which develops with age. While the balance between cytokines has been shown to be critical in determining the outcome of clinical disease, cytokines have been implicated in altering haemoglobin (Hb) levels in areas of stable malaria transmission. However, to date, the stabilities of antigen-specific cytokine responses associated with protection, whether the responses are age-dependent, the balance between cytokine responses and their effect on Hb in an area of unstable malaria transmission following malaria interruption remains unknown, such as in western Kenya. Insight on the stabilities and balance of cytokine responses to malaria vaccine candidates and their interaction with age and Hb in areas of low unstable malaria transmission would be beneficial as invaluable surrogate markers in evaluating vaccine immunogenicity and efficacy and in informing future vaccine development studies in similar populations. This longitudinal cohort study aimed at measuring the stabilities of antigen-specific cytokine response to multiple *P. falciparum* antigens by age, their effect on Hb concentrations and whether cytokine balance was affected during a period of interrupted malaria transmission. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood, cultured with apical membrane antigen (AMA)-1, circumsporozoite protein (CSP), liver stage antigen (LSA)-1, MB2, merozoite surface protein (MSP)-1 and thrombospondin adhesive protein (TRAP) at 37°C and 5% CO_2 for 5 days. Antigen-specific cytokine (interleukin (IL)-5, IL-6, IL-10, interferon (IFN)-γ and tumor necrosis factor (TNF)-α and chemokine [regulated upon activated, normal T-cells expressed and secreted (RANTES)] responses were assessed in 38 children and 62 adults from Nandi County in western Kenya, during a period of interrupted malaria transmission. McNemar's test was used to compare paired continuous variables, Wilcoxon signed-rank and Fisher's exact test to compare categorical variables, Wilcoxon rank sum test to compare continuous variables and Spearman's rank test to assess associations. Results revealed that levels of IL-6, and RANTES responses to almost all *P. falciparum* antigens were stable (P >0.05), while levels of IL-5, IL-10 IFN-γ, and TNF-α to most *P. falciparum* antigens decreased (P <0.05). No difference was observed between the antigen-specific cytokine responses by age (P>0.05). There were weak to moderate correlation within the cytokines (P<0.05), however, strong correlation between TNF-α and IFN-γ response was observed (P < 0.05). Weak correlations were observed between cytokine (RANTES and TNF-α) levels and Hb levels (P <0.05). There was no difference in the TNF-α:IL-10 cytokine ratios by anemia status (P >0.05). For this population, antigen-specific IL-5, IFN-γ, IL-10, TNF-α decreased, while antigen-specific IL-6 and RANTES responses remained stable after a prolonged period of very low malaria transmission. RANTES and TNF-α weakly correlated with Hb levels. Furthermore, no age-related pattern in the antigen-specific cytokine responses exists. This study shows that *P. falciparum*-specific cytokine/chemokine responses require frequent boosting with antigens to be maintained in this area of unstable transmission. Vaccine development may consider formulating vaccines that could be administered at comparable dosage. Information on the inter-cytokine balance and their relationship with Hb may be used to assess those susceptible to severe disease. Future studies should investigate cellular sources of the cytokines to determine which were impaired by the malaria interruption. Further studies should also investigate antigen-specific IFN-γ, TNF-α and IL- 10 responses as biomarkers of increased population-level susceptibility to malaria after prolonged lack of *P. falciparum* exposure.

CHAPTER ONE: INTRODUCTION

1.1 Background Information

Malaria remains a major public health burden worldwide despite being an entirely preventable and treatable disease. The World Malaria Report 2013 statistics estimated over 200 million cases of malaria and 627,000 deaths in 2012 due to malaria with about 90% of these deaths occurring in sub-Saharan Africa among children less than five years of age (WHO, 2013). Between 2000 and 2012, the scaling up of malaria control strategies, which include indoor residual spraying (IRS) campaigns, use of insecticide-treated bed nets (ITNs) and the introduction of artemisinin-based combination treatments (ACTs) (Bhattarai *et al.,* 2007; Nyarango *et al.,* 2006; Sharp *et al.,* 2007b), has seen a reduction of malaria incidence rates by 25% globally, and by 31% in Africa (WHO, 2013).

Clinical immunity to malaria is not sterile as the malaria parasites are not entirely eliminated and the chances of re-infection remain high (Doolan *et al.,* 2009). Furthermore, clinical immunity to malaria appears to wane quickly in the absence of active malaria transmission (Langhorne *et al.,* 2008b; Teirlinck *et al.,* 2011), and as such, developing a vaccine that can induce both sterilizing and long lasting immunity against malaria is among some of the challenges facing current vaccine development strategies (Achtman *et al.,* 2005). Recent human studies have demonstrated that cellular immune responses to experimental *P. falciparum* malaria can be induced and persist (Roestenberg *et al.,* 2011; Teirlinck *et al.,* 2011). Furthermore, a study in mice showed that cellular immunologic memory was induced and maintained during chronic malaria infection (Stephens and Langhorne, 2010).

The highlands of Kenya is an area of low and unstable malaria transmission with

seasonal epidemics occurring during the rainy season (John *et al.,* 2000). However, following recent malaria interventions, transmission was reportedly interrupted (John *et al.,* 2009) and this could in effect hamper the development of immunity (Smith *et al.,* 2001; Snow and Marsh, 2002).

Identifying stable immunologic correlates of protection against malaria is an important goal of malaria vaccine research (Moormann *et al.,* 2006), as it would influence the understanding of immunologic end-points during malaria vaccine trials. Studies evaluating the stability of cytokine responses to *P. falciparum* malaria in low malaria transmission (Moormann *et al.,* 2009; Wipasa *et al.,* 2011) and high malaria transmission areas (Dent *et al.,* 2009; Moormann *et al.,* 2006) have been carried out. These studies reported stable IFN-γ responses to pre-erythrocytic stage antigen, however, a recent study in an area of low malaria transmission showed that IFN-γ effector response were short-lived (Wipasa *et al.,* 2011). Similarly, IL-10 responses to pre-erythrocytic stage antigen have been shown to be stable (Moormann *et al.,* 2006; Moormann *et al.,* 2009; Wipasa *et al.,* 2011). Despite the numerous studies that have been carried out, the stability of antigen-specific cytokine and chemokine responses to *P. falciparum* malaria vaccine candidate antigens in a setting of interrupted malaria transmission such as in western Kenya, remains unknown. As such the current study aimed at measuring the stabilities of IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES responses to *P. falciparum* MSP-1, MB2, CSP, LSA-1, TRAP and AMA-1 malaria antigens in a highland area of western Kenya during a period of interrupted malaria transmission.

Besides transmission intensity, age has been shown to play an important role in the development of immune responses to malaria (Dent *et al.,* 2009) and in the patterns of clinical disease (Carneiro *et al.,* 2010). While clinical immunity to malaria has been shown to develop with age, the exact age at which clinical immunity is fully acquired however, varies depending on the level of exposure (Gatton and Cheng, 2004). In areas with low unstable malaria transmission, age-related protection from malaria develops slowly or does not occur at all (Noland *et al.,* 2008) and the risk of clinical malaria in children and adults is more or less similar. Age-dependent cellular response and acquisition of protective immunity were reported in areas of stable malaria transmission (John *et al.,* 2004). However, age-related antigen-specific cytokine responses to *P. falciparum* malaria vaccine candidate antigens in an area of unstable malaria such as in western Kenya, following interrupted malaria transmission remain unknown. As such, the current study determined whether the IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES responses to *P. falciparum* MSP-1, MB2, CSP, LSA-1, TRAP and AMA-1 malaria antigens differ by age at baseline in western Kenya.

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The balance in production of pro-inflammatory and anti-inflammatory cytokines is critical in the outcome of disease. Studies have shown that this balance greatly influences the presentation and outcome of clinical malaria (Day *et al.,* 1999; Dodoo *et al.,* 2002a; Lyke *et al.,* 2004; Perkins *et al.,* 2000; Torre *et al.,* 2002b) and determines whether the responses are protective or immunopathologic (Rovira-Vallbona *et al.,* 2012). The correlation of antigen-specific anti-inflammatory and pro-inflammatory cytokine responses to *P. falciparum* malaria vaccine candidate antigens in an area of unstable malaria transmission, such as in western Kenya, during a period when malaria transmission was interrupted however remains unknown. As such, the current study assess the correlation between anti-inflammatory (IL-5 and IL-10) and pro-inflammatory

(IL-6, IFN-γ, TNF-α and RANTES) cytokine response levels to *P. falciparum* malaria antigens in a highland area of western Kenya during a period of interrupted malaria transmission.

Erythropoietic suppression and dyserythropoiesis are among some of the causes of severe malaria anemia (SMA) in humans (Were *et al.,* 2009). TNF-α has been shown to inhibit all stages of erythropoiesis (Dufour *et al.,* 2003) and over-production of pro-inflammatory cytokines IL-6, TNF-α and IFN-γ (John *et al.,* 2006; Kremsner *et al.,* 1995; Lyke *et al.,* 2004; Perkins *et al.,* 2000; Sarangi *et al.,* 2014) and the chemokine RANTES (Were *et al.,* 2006), are associated with anemia. Anti-inflammatory cytokines such as IL-10 and TGF-β have also been thought to protect against bone marrow suppression and erythrophagocytic activity induced by TNF-α (Lamikanra *et al.,* 2007; Omer and Riley, 1998) and thereby affecting erythropoiesis (Zermati *et al.,* 2000). Studies in areas of stable malaria transmission have demonstrated further that low ratios of plasma IL-10/TNF-α is associated with SMA in young children (Othoro *et al.,* 1999). The relationship between antigen-specific cytokine responses to *P. falciparum* malaria vaccine candidate antigens and haemoglobin concentrations in areas with unstable malaria transmission during periods of interrupted malaria transmission remains obscure. As such, the current study assessed whether the levels of secreted cytokines (IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES) have an effect on haemoglobin concentration in a highland area of western Kenya during a period of interrupted malaria transmission.

The IFN-γ, IL-10 and TNF-α were targeted in the current study since *P. falciparum*-specific responses to the cytokines have been correlated with protection from clinical disease (John *et al.,* 2004; Kurtis *et al.,* 1999; Luty *et al.,* 1999), while IL-6 has been shown to be produced in *P. falciparum*-specific responses and further associated with an increased risk of disease (Robinson *et al.,* 2009). IL-5 and RANTES were targeted since

1.3 Objectives of the Study

1.3.1 General Objective

To characterize cytokine and chemokine responses to *P. falciparum* malaria vaccine candidate antigens in a highland area of western Kenya during a period of interrupted malaria transmission.

1.3.2 Specific Objectives

1. To measure the stabilities of IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES responses to *P. falciparum* MSP-1, MB2, CSP, LSA-1, TRAP and AMA-1 malaria antigens in a highland area of western Kenya during a period of interrupted malaria transmission.
2. To determine whether the IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES responses to *P. falciparum* MSP-1, MB2, CSP, LSA-1, TRAP and AMA-1 malaria antigens differ by age at baseline in a highland area of western Kenya during a period of interrupted malaria transmission.
3. To assess the correlation between anti-inflammatory (IL-5 and IL-10) and pro-inflammatory (IL-6, IFN-γ, TNF-α and RANTES) cytokine response levels to *P. falciparum* malaria antigens in a highland area of western Kenya during a period of interrupted malaria transmission.
4. To assess whether the levels of secreted cytokines (IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES) have an effect on haemoglobin concentration in a highland area of western Kenya during a period of interrupted malaria transmission.

1.4 Research Questions

1. What are the stabilities of IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES responses to *P. falciparum* MSP-1, MB2, CSP, LSA-1, TRAP and AMA-1 malaria antigens

during a period of interrupted malaria transmission in an area of unstable malaria transmission?

2. What age-specific differences are there in the IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES responses to *P. falciparum* MSP-1, MB2, CSP, LSA-1, TRAP and AMA-1 malaria antigens at baseline in a highland area of western Kenya during a period of interrupted malaria transmission?
3. What correlations exists between the antigen-specific pro-inflammatory (IL-6, IFN-γ, TNF-α and RANTES) and anti-inflammatory (IL-5, and IL-10) cytokines to *P. falciparum* MSP-1, MB2, CSP, LSA-1, TRAP and AMA-1 malaria antigens secreted during a period of interrupted malaria transmission in an area of unstable malaria transmission?
4. What correlations exist between secreted antigen-specific IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES cytokines to *P. falciparum* MSP-1, MB2, CSP, LSA-1, TRAP and AMA-1 malaria antigens and haemoglobin concentration during a period of interrupted malaria transmission in an area of unstable malaria transmission?

1.5 Justification of the Study

Identifying how the stabilities of *P. falciparum* antigen-specific cytokine responses are affected during a period when malaria was interrupted in areas with unstable malaria transmission would be essential in providing the critical immune end-points during malaria vaccine trials.

Furthermore, understanding whether there is an age-related acquisition of *P. falciparum* antigen-specific cytokine responses during a period when malaria was interrupted would be essential when targeting interventions across the populations with unstable malaria transmission.

To predict disease severity and for future treatment strategies to combat severe syndromes, gaining an understanding of whether the interruption of malaria transmission affects the balance between *P. falciparum* antigen-specific pro- and anti-inflammatory immune responses will be of great importance.

In understanding the effect of interrupted malaria transmission on *P. falciparum* antigen-specific cytokine responses and in turn their relationship with haemoglobin concentrations, provide insight into how malaria anemia develops in populations where malaria transmission is unstable.

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CHAPTER TWO: LITERATURE REVIEW

2.1 The Malaria Parasite

Malaria is a parasitic disease caused by a unicellular protozoan of the genus *Plasmodium* and is transmitted by the bite of an infected female mosquito of the genus *Anopheles*. In humans, malaria infection is caused by five *Plasmodium* species: *Plasmodium falciparum* (*P. falciparum*), *P. ovale, P. malariae*, *P. vivax* and the recently described *P. knowlesi* (Cox-Singh *et al.,* 2008; WHO, 2012). Of these five species, *P. falciparum* is the most severe and life threatening form (Greenwood *et al.,* 2005; WHO, 2012), causing the bulk of mortality and morbidity especially in children under the age of five years, pregnant women particularly during the first pregnancy and in non-immune adults (Hay *et al.,* 2004) and is most prevalent in Africa south of the Sahara and in certain areas of south- east Asia and the western Pacific (WHO, 2005a).

The most competent and efficient malaria vector, *Anopheles gambiae*, occurs exclusively in Africa (Hay *et al.,* 2010). Climatic conditions determine the presence or absence of anopheline vectors (Afrane *et al.,* 2006). Tropical areas of the world have the best combination of adequate rainfall, temperature and humidity allowing for breeding and survival of anophelines (Afrane *et al.,* 2006; Bayoh and Lindsay, 2004). Hence, an effective vaccine against *P. falciparum* would be instrumental in reversing the current malaria mortality and morbidity trends (WHO, 2005a).

2.2 Malaria Life Cycle

The life cycle of the malaria parasite is complex and involves an invertebrate primary host and a vertebrate host (See Appendix 1: The *Plasmodium falciparum* malaria lifecycle). Human malaria is transmitted through the bite of an infective female *Anopheline* mosquito. It has been estimated that less than 100 sporozoites are injected

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per infective bite via the saliva of the mosquito into the skin of the host where they transit at a slow steady rate into the blood stream (Yamauchi *et al.,* 2007) to infect the liver. The sporozoites then invade the hepatocytes and begin a vigorous asexual reproductive cycle called tissue schizogony lasting between 6 - 10 days to yield over 30, 000 merozoites per exoerythrocytic schizont (Doolan *et al.,* 2009). The maturation and rupture of liver stage schizonts is not accompanied by clinical symptoms, however, the released merozoites quickly invade erythrocytes to begin the erythrocytic cycle (blood schizogony) that is responsible for the clinical symptoms of malaria (Miller *et al.,* 2002). Within the erythrocyte, merozoites undergo different phases of maturation and mitotic divisions from rings (early trophozoites), through trophozoites to the schizont stage and finally rupture to release up to 36 merozoites per schizont, which in turn infect new erythrocytes to perpetuate the cycle. The duration of the erythrocytic cycle in *P. falciparum, P. vivax and P. ovale* infection takes 48 hours while *P. malariae* and *P. knowlesi* blood schizogony lasts 72 hours and 12 hours, respectively (Cox-Singh *et al.,* 2008; Cox, 2010; Ng *et al.,* 2008; White, 2008a).

Some erythrocytic parasites develop into male and female gametocyte stages, which can re-infect the mosquito during a blood meal. These gametocytes fuse to form zygotes and go through different phases of differentiations and maturations to form ookinetes, oocysts and finally sporozoites and stored in the salivary gland awaiting release when the mosquito takes another blood meal (Lasonder *et al.,* 2008).

2.3 The Burden of Malaria

2.3.1 Global malaria burden

Malaria is an entirely preventable and treatable mosquito-borne illness. According to WHO, there were 97 countries with ongoing malaria transmission in 2013 (WHO, 2013),

and people living in the poorest countries are the most vulnerable. The World Malaria Report 2013, estimated over 200 million cases and 627,000 deaths due to malaria, in 2012 alone (WHO, 2013). Between 2000 and 2012, the scaling up of malaria control strategies, which include indoor residual spraying (IRS) campaigns, use of insecticide-treated bed nets (ITNs) and the introduction of artemisinin-based combination therapies (ACTs) (Bhattarai *et al.,* 2007; Nyarango *et al.,* 2006; Sharp *et al.,* 2007a), has seen a reduction of malaria incidence rates by 25% globally (WHO, 2013).

2.3.2 Malaria in Africa

Malaria is generally endemic in the tropics, with extension into the sub-tropics. In Africa malaria is mainly extensive, prevalent and is on the increase even in the regions that were previously considered malaria free (Kiszewski and Teklehaimanot, 2004). Stable malaria predominates throughout the continent, however epidemics occur at the fringes of the endemic areas, particularly among communities at the northernmost latitudes, across the arid regions of North Africa, and mostly among the highlands of East, Central and Horn of Africa (Hay *et al.,* 2010). Malaria is still one of the most important causes of morbidity and mortality in developing countries in sub-Saharan Africa, with 90% of the deaths occurring among children less than five years of age (WHO, 2013). Malaria deaths alone are responsible for greater than 10% of Africa's disability-adjusted life years (DALYs) (Alilio *et al.,* 2004). Malaria control strategies has seen a reduction of malaria incidence rates by 31% in Africa (WHO, 2013). Despite the decline in the burden of malaria with the scaling-up of these interventions, there is need for more effort in the prevention and control of the disease in Africa.

2.3.3 Malaria in Kenya

Malaria remains a major public health problem in Kenya, accounting for about 31% of outpatient consultations and 5% of hospital admissions (Okiro *et al.,* 2007). Malaria transmission and infection risk is largely determined by altitude, rainfall patterns and temperature (Cohen *et al.,* 2010). Thus, malaria prevalence varies considerably by season and across geographic regions (Afrane *et al.,* 2006). In Kenya, *Plasmodium falciparum*, which causes the most severe form of the disease, (Ernst *et al.,* 2006). *An. gambiae*, *An. arabiensis* and *An. funestus* are the main malaria vectors driving transmission in Kenya (Amek *et al.,* 2012; Imbahale *et al.,* 2012). In the East African highlands, there is an estimated 34 million people at risk of *P. falciparum* malaria (Ernst *et al.,* 2006).

The Ministry of Health of Kenya introduced new interventions to reduce malaria transmission and improve malaria treatment in the highland areas of western Kenya from 2005 (John *et al.,* 2009). These interventions resulted in reducing both the burden of malaria and its associated mortality and a 44% reduction in mortality in children less than 5 years old (Fegan *et al.,* 2007).

2.4 Clinical Malaria

Clinical manifestations of malaria range from uncomplicated to severe disease and are associated with the multiplication of blood-stage parasites as the merozoites are released into the blood stream of the infected individual (Kwiatkowski *et al.,* 1989). The pathogenesis of malaria-induced fever is thought to be due to the rupturing of schizonts and subsequent release of merozoites into circulation that stimulate the mononuclear cells to secrete TNF-α and other pyrogenic cytokines (Kwiatkowski *et al.,* 1989) including IL-6 (Mshana *et al.,* 1991).

The manifestations of severe and uncomplicated malaria vary geographically but are associated almost exclusively with *P. falciparum* infection (John *et al.,* 2000; Marsh and Kinyanjui, 2006). The intensity of malaria parasite transmission is normally expressed as the entomologic inoculation rate (EIR), the product of the vector-biting rate multiplied by the proportion of mosquitoes infected with sporozoites (Beier *et al.,* 1999). Malaria transmission in Africa is highly variable with EIR ranging from below one to above 1,000 infective bites per person per year in endemic areas (Kelly-Hope and McKenzie, 2009). It is a more direct measure of transmission intensity compared to measures such as malaria prevalence or hospital-based measures of infection (Beier *et al.,* 1999).

In Kenya, a wide range of malaria transmission intensities has been mapped, ranging from areas with low malaria risk to holoendemic areas with continual, repeated exposure to malaria throughout the year (John *et al.,* 2000). Kenya's unstable transmission areas can be divided into areas where transmission potential is limited by low rainfall or low temperature (Alonso *et al.,* 2011). The highlands are prone to unpredictable epidemics of malaria, while in a highly seasonal *P. falciparum* transmission area, the epidemics are more or less predictable (John *et al.,* 2005a). At the Kenyan coast, high incidence of severe malaria occur under conditions of very low levels of transmission by vector populations, and have not been associated with EIRs (Mbogo *et al.,* 1995). In the highland area of western Kenya, transmission previously described as being unstable (Ernst *et al.,* 2006), with seasonal epidemics occurring during or soon after a rainy season has since been reduced to near absent (John *et al.,* 2009) as a result of the up-scaled use of malaria interventions by the Kenyan government (Mulambalah *et al.,* 2011). The effect of interrupted transmission on cytokine dynamics in individuals living in an area of unstable malaria transmission has not been studied and therefore this study

was aimed at describing and characterizing cytokine responses to *P. falciparum* vaccine candidate antigens during a period of interrupted malaria transmission.

2.5 Malaria Control Strategies

2.5.1 Indoor Residual Spraying (IRS)

Indoor residual spraying (IRS) involves the application of a residual deposit of an insecticide onto indoor surfaces where the vectors rest. It is one of the main vector control strategies primarily used in interruption/eradication of malaria transmission (Pluess *et al.,* 2010). IRS is most effective against endophilic mosquito species that rest indoors (Pates and Curtis, 2005) and works by repelling and killing the female mosquitoes (Pluess *et al.,* 2010). IRS has been quite successful in areas where malaria transmission ranges from low to moderate and largely relies on a vectorial mass effect, which leads to a reduction in transmission (Protopopoff *et al.,* 2007a). Having had a long and illustrious history in malaria control its use saw the elimination or massive reduction of malaria in Asia, Russia, Europe, and Latin America by mainly using dichloro-diphenyl-trichlorethane (DDT) (Lengeler and Sharp, 2003; Roberts *et al.,* 2004). A study conducted in Burundi showed that IRS drastically reduced the density of endophilic *Anopheles* (Protopopoff *et al.,* 2007b). Similarly, in an area of high transmission in Uganda, IRS was associated with a reduction in malaria morbidity (Kigozi *et al.,* 2012). However, the greatest impact of IRS on malaria epidemics depends on its timely application at the start of the epidemic and has little or no impact once the peak is reached (WHO, 2005b). Some of the major challenges encountered in the use of IRS include unpredictable funding, poor sustainability infrastructure limitations, insecticide resistance in high malaria transmission areas (Kolaczinski *et al.,* 2007) and environmental concerns with the use DDT (Rogan and Chen, 2005). In

Africa alone approximately 77 million people have benefitted from IRS intervention (WHO, 2012).

2.5.2 Insecticide Treated Nets (ITNs)

The use of insecticide treated mosquito nets (ITNs) is effective in the reducing malaria-related morbidity and mortality. In children under the age of 5 years, a reduction in all-cause mortality (Lengeler and Sharp, 2003) and hospital admissions were reported with ITN use (Nevill *et al.,* 1996). Further they have been shown to significantly reduce morbidity and adverse birth outcomes in pregnant women (Gamble *et al.,* 2006; Miller *et al.,* 2007) However, despite the evidence of its benefits, ITN use in sub-Saharan Africa remains relatively low (WHO, 2005b, 2007). Arguably, cost, lack of availability and failure in the distribution systems have been the main barriers to ITN use in Africa (Rhee *et al.,* 2005), prompting some governments to provide highly subsidized nets as well as distributing free insecticide treated nets (Bernard *et al.,* 2009), which translated to increased ITN coverage and consequently a reduction in mortality. According to the *World Malaria Report* of 2012, an estimated 150 million ITNs are needed every year to protect all populations at risk of malaria in sub-Saharan Africa (WHO, 2012).

2.5.3 Intermittent Presumptive Treatment in pregnancy (IPTp)

Intermittent presumptive treatment in pregnancy (IPTp) involves the administration of a curative treatment dose of an effective antimalarial drug, currently being Sufadoxine-pyrimethamine (SP), to all pregnant women whether or not they are infected with the malaria parasite, at predefined intervals during pregnancy (White, 2005). The main objective of IPTp is to reduce or eliminate the adverse effects of malaria on maternal anemia and birth weight, and to prevent severe malaria in the mother (Greenwood, 2004; Menendez *et al.,* 2007). IPTp in conjunction with the use of ITNs was first introduced

in areas of high malaria transmission as a measure to reduce the adverse impact of *P. falciparum* malaria in pregnancy (van Eijk *et al.,* 2004).

2.5.4 Intermittent Presumptive Treatment in infants (IPTi)

Administration of intermittent presumptive treatment to infants (IPTi) has demonstrated a reduction in anemia cases and malaria episodes thus reducing morbidity and mortality in the first year of life (Desai *et al.,* 2003; Munday, 2007). The WHO has recommended that all infants at risk of *P. falciparum* infection countries with moderate to high malaria transmission be administered with SP along with other vaccines through the routine immunization program (WHO, 2010).

2.5.5 Artemisinin-based Combination Therapies (ACTs)

Effective malaria control demands efficacious antimalarials. The existing malaria drugs have tendencies of developing drug resistance and to date three out of the five malaria species known to affect humans (*P. falciparum*, *P. vivax* and *P. malariae*) have been documented to be resistant to most antimalarials (WHO, 2012). The use of two or more drugs with different modes of action in combination is now recommended for *P. falciparum* to give adequate cure rates and delay the onset of drug resistance (Malenga *et al.,* 2005). Artemisinin-based combination therapies (ACTs) are currently the first-line treatments for uncomplicated *P. falciparum* malaria worldwide (Dondorp *et al.,* 2009). The artemisinin compound is meant to reduce the parasite load during the first three days of treatment, while the partner drug is to eliminate the remaining parasites (German and Aweeka, 2008; White, 2008b). Resistance to current therapies has been shown to be high for all antimalarial therapies except artemisinins (Ades, 2011). However, emerging resistance of *P. falciparum* to artemisinin has been reported in four Southeast Asian countries (Noedl *et al.,* 2008; O'Brien *et al.,* 2011). Some of the factors

have been reported to cause this resistance include poor treatment practices, inadequate patient adherence to prescribed antimalarial regimens, and the widespread availability of oral artemisinin-based monotherapies and substandard forms of the drug (WHO, 2012). This resistance could cause dire consequence on public health if it were to spread to or emerge in sub-Saharan Africa (Talisuna *et al.,* 2012).

2.6 Malaria Vaccine Candidates

In spite of many years of dedicated and high quality research, there is still no licensed vaccine to malaria. For a malaria vaccine to be regarded effective, it could either prevent infection altogether or alleviate severe disease and death in those who become infected despite vaccination (Graves and Gelband, 2006). In other studies, it has been argued that an effective malaria vaccine is the only realistic long-term solution for resource-poor countries in curbing malaria (Tongren *et al.,* 2004). While most efforts to develop a malaria vaccine have focused on *P. falciparum* due to the severity of the disease it causes, current vaccine candidates target a single stage of the parasite's life cycle (Hill, 2011). The development of vaccine candidates for malaria has accelerated considerably in the recent past with one having recently reached advanced stage in the clinical trials (Olotu *et al.,* 2011).

2.6.1 Pre-erythrocytic Stage Antigens

The most successful vaccines have targeted the early pre-erythrocytic stages of the malaria parasite (Hill, 2011). This stage consists of the sporozoites and liver stages and is involved in the initiation of infection (Williams and Azad, 2010). Pre-erythrocytic stage vaccines target the sporozoite and the liver-stage parasite to prevent clinical disease (Wipasa *et al.,* 2002).

2.6.1.1 Circumsporozoite Protein (CSP)

The *P. falciparum* CSP antigen is a 58kDa protein that is expressed on the surface of sporozoites (Chenet *et al.,* 2008; Mahajan *et al.,* 2005) with highly polymorphic regions that can induce both humoral and cellular response (Nardin *et al.,* 2000; Wang *et al.,* 1998). The CSP antigen is the most highly characterized malaria vaccine candidate antigens (Singh *et al.,* 2009) and the most advanced malaria vaccine in development, RTS,S/AS01 (Vekemans and Ballou, 2008), is based on it. Cytokine and antibody responses to *P. falciparum* CSP antigen have been associated with protection from infection and clinical disease (John *et al.,* 2005b; Kumkhaek *et al.,* 2005; Migot-Nabias *et al.,* 2000; Reece *et al.,* 2004). Studies on the cytokine responses to CSP have been associated with protection from infection and disease (Kumkhaek *et al.,* 2005; Reece *et al.,* 2004). Importantly, IFN-γ, the most commonly used marker of protection, correlated with protection in individuals immunized with RTS,S (Barbosa *et al.,* 2009; Sun *et al.,* 2003). In holoendemic areas, IFN-γ responses were found to associate with protection from infection and disease (Reece *et al.,* 2004; Todryk *et al.,* 2008). Other studies reported an association between IFN-γ responses to CSP and protection from anemia (Ong'echa *et al.,* 2003).

2.6.1.2 Liver Stage Antigen (LSA)-1

The *P. falciparum* LSA-1 antigen is a 200-kDa protein exclusively expressed by infected hepatocytes (Chowdhury *et al.,* 2009) during schizogony. The LSA-1 is a highly immunogenic, polymorphic protein (Rodriguez *et al.,* 2008) exclusively expressed by infected hepatocytes and conserved among *P. falciparum* isolates (Chenet *et al.,* 2008). Studies have consistently associated cytokine and antibody responses to LSA-1 (John *et al.,* 2005b; John *et al.,* 2000; Kurtis *et al.,* 1999) with protection from infection or clinical disease. Other studies reported the protective role of IFN-γ

responses to LSA-1 against high-density parasitemia (John *et al.,* 2004). Similarly, IL-10 responses to LSA-1 have been shown to be predictive of resistance to re-infection (Kurtis *et al.,* 1999) and it has been reported that the likelihood of reinfection was lowered in individuals who produced IL-10 than in those who did not (John *et al.,* 2000). IFN-γ responses to LSA-1 were also reported to be associated with protection from anemia (Ong'echa *et al.,* 2003) and faster parasite clearance (Luty *et al.,* 1998).

2.6.1.3 Thrombospondin Related Adhesive Protein (TRAP)

The *P. falciparum* TRAP is a 63.3kDa protein essential for sporozoite invasion (Weedall *et al.,* 2007). It is also a malaria vaccine candidate found within the micronemes of the mosquito sporozoite stage (Chowdhury *et al.,* 2009). It is essential for sporozoite invasion (Weedall *et al.,* 2007). It has highly polymorphic T- and B-cell epitopes (Chenet *et al.,* 2008; Escalante *et al.,* 1998). Immunological studies have reported an association between cytokine (Connelly *et al.,* 1997; Luty *et al.,* 1999) and antibody (John *et al.,* 2005b; John *et al.,* 2003) responses to TRAP with protection from infection or clinical disease. A study conducted in Kenya reported an association between T-cell memory against TRAP with resistance to malaria infection (Todryk *et al.,* 2008). IFN-γ-driven TRAP responses were shown to be associated with protection from anemia in children from western Kenya (Ong'echa *et al.,* 2003).

2.6.2 Erythrocytic Stage Antigens

The erythrocytic stage of the malaria parasite is principally the merozoite, which is involved in the invasion of the red blood cell (RBC) and the actual replication of the parasite in the blood (Ballou *et al.,* 2004). The erythrocytic stage is the only stage in the parasite cycle that causes disease. An asexual erythrocytic stage vaccine would prevent or reduce morbidity and mortality by reducing or eliminating the parasite load (Doolan and Hoffman, 1997). Erythrocytic stage vaccines target the antigens on the asexual

parasite along with parasite-derived proteins inserted into the infected erythrocyte membrane.

2.6.2.1 Merozoite Surface Protein (MSP)-1

The MSP-1 is an integral membrane blood stage protein involved in the invasion of erythrocytes and expressed on the surface of the merozoite (Wipasa *et al.,* 2002) via a glycosyl-phosphatidylinosiitol (GPI) anchor (Berzins, 2002). It has highly polymorphic T- and B-cell epitopes (Chenet *et al.,* 2008; Escalante *et al.,* 1998), which make it a target for immune response. The *Pf*MSP-1 is synthesized as a polypeptide of ~200kDa protein that is cleaved into fragments of 83 (N-terminus), 30 and 38 (central portions) and 42 kDa (C-terminus) (Okech *et al.,* 2004). Antibody (Braga *et al.,* 2002) and cellular (Migot-Nabias *et al.,* 1999) immune responses against MSP-1 have been reported to be associated with protection from infection and clinical disease. It is suggested to function at initial attachment onto the RBC (Mitchell *et al.,* 2004). Cellular immune responses to MSP-1 have reported an association with resistance from infection and disease (Migot-Nabias *et al.,* 1999). IL-10 production was associated with faster, significant parasite clearance to asexual stage parasite antigens (Luty *et al.,* 1998).

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2.6.2.2 Apical Membrane Protein (AMA)-1

The AMA-1 is synthesized during late schizogony and is expressed in the schizont stage as an 83-kD protein 622 amino acids in length, which is rapidly processed by cleavage of an N-terminal peptide to a 66-kD protein (Narum and Thomas, 1994), which may be carried on the surface of mature merozoites. It is an integral membrane protein, among the leading malaria vaccine candidates, expressed in the invasive merozoite form of *Plasmodia* species (Triglia *et al.,* 2000), and has highly polymorphic regions (Chenet *et al.,* 2008; Escalante *et al.,* 1998), making it an ideal target for induction of humoral and

cellular immune responses that can protect against malaria. It is believed to function at parasite reorientation and entry into the RBC (Mitchell *et al.,* 2004). It is ccomprised of an ectodomain in which non-overlapping groups of disulfide bonds define four separate domains (pro-domain and domains I, II, and III) (Hodder *et al.,* 1996). In a previous study in mice, it was shown that AMA 1-specific CMI alone could protect against malaria (Xu *et al.,* 2000).

Evidence from immunological studies demonstrates widespread antibody responses to AMA-1 (Nebie *et al.,* 2008). Association between antibody (Osier *et al.,* 2008; Polley *et al.,* 2004) or cell mediated immunity (CMI) with protection from infection and disease have previously been demonstrated (Xu *et al.,* 2000).

2.6.2.3 MB2

The MB2 protein is a ~187-kDa molecule that is expressed in sporozoites, asexual blood stages, and gametocytes (Romero *et al.,* 2004). It has immunogenic and molecular properties important for immune targeting (Nguyen *et al.,* 2009). Anti-MB2 antibodies were detected in individuals from a malaria holoendemic area in Kenya (Nguyen *et al.,* 2009). Since humoral immunity is not sufficient for protection against malaria infection, there is need for studies on cytokine production in response to MB2 in the effort to identify correlates of immunity to this multi-domain antigen. A study conducted in adults living in areas having varying malaria transmission within western Kenya showed that MB2 evoked substantial IL-10 and IFN-γ response (Ochola *et al.,* 2013) as have been previously reported with current malaria vaccine candidates. Despite the antigen-specific cytokine responses described above, the characterization of antigen-specific cytokine responses to *P. falciparum* vaccine candidate antigens during a period of interrupted malaria transmission an area of unstable malaria transmission remain

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unknown.

2.7 Immunity to Malaria

Natural immunity against malaria parasites is provided by both the innate mechanisms and by the development of acquired immunity. This immunity is highly complex and varies with the level of endemicity, genetic makeup, age of the host and parasite stage and species (Lopez *et al.,* 2010). In malaria endemic areas, clinical immunity develops with repeated infections and cumulative exposure as the person ages (Chelimo *et al.,* 2003). It has been presumed that children below 6 months are protected by maternal antibodies, which is however, lost early in childhood and are therefore susceptible to severe disease at 6 months (Riley *et al.,* 2000). However, in areas of low endemicity, where there is infrequent exposure to the parasite, functional immunity against malaria is not fully developed and hence not age-dependent (Hay *et al.,* 2002).

Protective immunity against malaria involves both humoral and cell-mediated immunity, which is principally T-cell dependent (Marsh and Kinyanjui, 2006; Wipasa *et al.,* 2002). However, the acquisition and maintenance of protective immunity largely depends on T-cells (Troye-Blomberg *et al.,* 1994). It is critical that long-term memory response is maintained for protective immunity against malaria infection to be achieved. Studies indicate that naturally acquired immunity to malaria is dependent on age and repetitive *P. falciparum* infections (Langhorne *et al.,* 2008a). Clinical immunity to malaria is not sterile as the malaria parasites are not entirely eliminated and the chances of re-infection remain high (Doolan *et al.,* 2009). Furthermore, clinical immunity to malaria appears to wane quickly in the absence of active malaria transmission (Langhorne *et al.,* 2008a; Teirlinck *et al.,* 2011), and as such, developing a vaccine that can induce both sterilizing and long lasting immunity against malaria is a major challenge to current vaccine

development strategies (Achtman *et al.,* 2005). Recent human studies have demonstrated that cellular immune responses to experimental *P. falciparum* malaria can be induced and persist (Roestenberg *et al.,* 2011; Teirlinck *et al.,* 2011). Furthermore, a study in mice showed that cellular immunologic memory was induced and maintained during chronic malaria infection (Stephens and Langhorne, 2010).

Identifying stable immunologic correlates of protection against malaria is an important goal of malaria vaccine research (Moormann *et al.,* 2006), as it would influence the understanding of immunologic end-points during malaria vaccine trials. Studies evaluating the stability of cytokine responses to *P. falciparum* malaria in low malaria transmission (Moormann *et al.,* 2009; Wipasa *et al.,* 2011) and high malaria transmission areas (Dent *et al.,* 2009; Moormann *et al.,* 2006) have been carried out. These studies reported stable IFN-γ responses to pre-erythrocytic stage antigen, however, a recent study in an area of low malaria transmission showed that IFN-γ effector response were short-lived (Wipasa *et al.,* 2011). Similarly, IL-10 responses to pre-erythrocytic stage antigen have been shown to be stable (Moormann *et al.,* 2006; Moormann *et al.,* 2009; Wipasa *et al.,* 2011). Despite the numerous studies that have been carried out, the stabilities of IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES responses to *P. falciparum* MSP-1, MB2, CSP, LSA-1, TRAP and AMA-1 malaria antigens in a highland area of western Kenya during a period of interrupted malaria transmission remains unknown. As such, the current study measured the stabilities of IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES responses to *P. falciparum* MSP-1, MB2, CSP, LSA-1, TRAP and AMA-1 malaria antigens in a highland area of western Kenya during a period of interrupted malaria transmission.

Immunity to malaria is said to develop with age and frequent exposure (Chelimo *et al.,*

2003; Dent *et al.,* 2009). Numerous studies have demonstrated that besides transmission intensity, age plays a significant role in the development of immune responses to malaria. In areas of stable malaria transmission, clinical immunity to malaria develops by the age of 5 years (Snow *et al.,* 1997), but in areas with low unstable malaria transmission, it has been shown that age-related protection from malaria develops slowly or does not occur at all (Noland *et al.,* 2008). Studies have also reported that in areas of stable malaria transmission, there is an age-dependent antibody response to *P. falciparum* (John *et al.,* 2005a; John *et al.,* 2005b). An age-dependent IFN-γ response to LSA-1 and MSP-1 (Chelimo *et al.,* 2003; John *et al.,* 2004) was also reported in an area of stable malaria transmission. In areas with low unstable malaria transmission, age-related protection from malaria develops slowly or does not occur at all (Noland *et al.,* 2008) and the risk of clinical malaria in children and adults is more or less similar. While clinical immunity to malaria has been shown to develop with age, the exact age at which clinical immunity is fully acquired however, varies depending on the level of exposure (Gatton and Cheng, 2004). Age-dependent cellular response and acquisition of protective immunity were reported in areas of stable malaria transmission (John *et al.,* 2004). However, whether or not the IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES responses to *P. falciparum* MSP-1, MB2, CSP, LSA-1, TRAP and AMA-1 malaria antigens differ by age at baseline in a highland area of western Kenya during a period of interrupted malaria transmission remain unknown. As such, the current study determined whether the IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES responses to *P. falciparum* MSP-1, MB2, CSP, LSA-1, TRAP and AMA-1 malaria antigens differ by age at baseline in a highland area of western Kenya during a period of interrupted malaria transmission.

2.7.1 Humoral immunity against malaria

Humoral immunity to malaria is critical in the resolution of infection and contributes to protection against pre-erythrocytic stage malaria. B cells and antibodies are largely involved in immunity to malaria and antibodies in particular have been shown to be important for the reduction and clearance of parasitaemia (Berzins *et al.,* 1991; Plebanski and Hill, 2000) and the reduction of clinical symptoms in humans (Bouharoun-Tayoun *et al.,* 1990). Antibodies may act by preventing merozoite invasion of red blood cells (Dent *et al.,* 2009), attacking infected RBCs and facilitating phagocytosis, or preventing cytoadhesion of infected RBCs (Mo *et al.,* 2008). Parasite antigens expressed on the surface of infected erythrocytes are important in the development of humoral immunity to the malaria blood stages (Perlmann and Troye-Blomberg, 2002) and antigen-specific *P. falciparum* antibodies have been implicated in having crucial role in controlling parasitemia through antibody dependent cellular inhibition (ADCI) (Aucan *et al.,* 2000; Wipasa *et al.,* 2002). Furthermore, studies in malaria endemic areas of sub-Saharan Africa suggest that immunity to malaria may result from high antibody titers to multiple blood-stage antigenic targets rather than single targets (Nebie *et al.,* 2008; Osier *et al.,* 2008). Immunoglobulin G in particular is a main component of defense against the asexual blood stage of *P. falciparum* (Bouharoun-Tayoun *et al.,* 1990; Druilhe and Perignon, 1994) and the cytophilic IgG1 and IgG3 subclasses have been involved in protection against *P. falciparum* malaria in humans (Jafarshad *et al.,* 2007).

2.7.2 Cell-mediated immunity against malaria

Cellular immune responses induced by *P. falciparum* infection may protect against both pre-erythrocytic and erythrocytic parasite stages. The contribution of T-cell subsets and

their cytokines to the development of natural immunity is essential both in regulating antibody formation and in inducing antibody-independent protection (Winkler *et al.*, 1999). Pre-erythrocytic immunity to *P. falciparum* infection is mediated in part by T-lymphocytes against the liver-stage parasite (Aidoo *et al.*, 2000). The T-cells must recognize parasite-derived peptides on infected host cells in the context of major histocompatibility complex (MHC) antigens. T-cell-mediated immunity appears to target several parasite antigens expressed during the sporozoite and liver stages of the infection (Hoffman *et al.*, 1997).

The $CD4^+$ T-cells are essential for protection against asexual blood stages (Wipasa *et al.*, 2002; Xu *et al.*, 2002), while CD 8^+T-cells also play a key role in protection against pre-erythrocytic stages and liver-stages (Marsh and Kinyanjui, 2006) of malaria infection. To date, the T-cell immune response during a period of low or near absent malaria transmission remains unknown. This study therefore sought to evaluate antigen-specific T-cell immune responses to *P. falciparum* in a period of low or near absent malaria transmission.

In the induction and maintenance of immunity to *P. falciparum*, antigen-specific cytokine production is important. An increasing body of evidence has shown the role cytokines play in the resolution of malaria infection or to a persistent increase in concentrations of malaria parasitemia (Lyke *et al.*, 2004) which leads to complicated malaria such as cerebral malaria (Clark and Alleva, 2009; Day *et al.*, 1999).

IFN-γ is a macrophage-activating factor involved in early immune response to acute uncomplicated malaria (Artavanis-Tsakonas *et al.*, 2003) and has been implicated in both protection against and the pathogenesis of malaria in humans (Nasr *et al.*, 2014). It plays a central role in the anti-malaria immunity and has been shown to appear in the

early stages of malaria infection (Torre *et al.,* 2002a). Its production in response to either liver stage or blood stage peptides was associated with resistance to re-infection with *P. falciparum* among children (Luty *et al.,* 1999). In a previous study, it was shown that increased production of IFN-γ to *P. falciparum* was associated with a reduction in the number of clinical episodes (Robinson *et al.,* 2009).

TNF-α plays a critical central role for both protection and process of malaria pathogenesis. It is a potent immunomodulator and pro-inflammatory cytokine, implicated in the pathogenesis of many inflammatory infections and immune diseases (Kritikos *et al.,* 2010). Increased production of TNF-α predicts a rapid resolution of the parasites and cure from clinical malaria, and its over-production has been linked with malaria pathology (John *et al.,* 2010). TNF-α has a protective role during *P. falciparum* as it is correlated with reduced risk of clinical episodes of malaria in children (Robinson *et al.,* 2009).

IL-6 is yet another important pro-inflammatory cytokine whose protective role has been demonstrated in murine models. It induces IL-1β and TNF-α responses against the pre-erythrocytic stage of the parasite and boosts specific IgG antibodies during the erythrocytic stage of the parasite (Perkins *et al.,* 2011). Though up regulated by TNF-α (Lyke *et al.,* 2004), IL-6 down regulates TNF-α and IFN-γ pathways thereby impeding control of parasite density (Robinson *et al.,* 2009) and acts in concert with other inflammatory mediators to control parasitemia. Elevated levels have been shown to correlate with severe *P. falciparum* infection (Lyke *et al.,* 2004) and increased risk of symptomatic malaria in children (Robinson *et al.,* 2009) and as such, suggested to have a role as an important febrile mediator.

As reported in human studies, IL-10 is a key molecule in the control of inflammatory responses (Angulo and Fresno, 2002; Winkler *et al.,* 1998). It can act directly on $CD4^+$T-cells, inhibiting proliferation and production of IL-2, IFN-γ IL-4, IL-5 and TNF-α, thereby regulating innate and adaptive Th1 and Th2 responses hence an impairment in pathogen control and/or reduced immunopathology (Couper *et al.,* 2008). It inhibits Th1 and $CD8^+$ cells, induces B cell proliferation, down-regulates MHC class II molecule expression on macrophages leading to decreased antigen presentation, prevents T-cell priming and proliferation. *In vitro* studies have shown that IL-10 suppresses the production of *P. falciparum* induced IFN-γ, IL-6, TNF-α and granulocytes-macrophage colony stimulating factor (GM-CSF) by T-cells (Akdis *et al.,* 1999). An association between severe *falciparum* malaria and an inadequate negative feedback response by IL-10 was proposed previously (Ho *et al.,* 1998). Elevated levels of IL-10 was found in individuals with severe *P. falciparum* malaria (Hugosson *et al.,* 2004); (Hugosson *et al.,* 2004; Prakash *et al.,* 2006) and were significantly higher in symptomatic children having uncomplicated malaria, compared to asymptomatic ones (Iriemenam *et al.,* 2009). The plasma concentrations of IL-10 in those with severe malarial anemia were reported to be low when compared with those having cerebral and uncomplicated malaria (Kurtzhals *et al.,* 1998).

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Regulated upon activation, normal T-cell expressed, and secreted (RANTES), a β-(or CCL-5) chemokine, is a specific chemoattractant for memory T-cells and augments polarization of Th-1 response (Mackay, 2001). It is an important immunoregulatory mediator suppressed in children with malarial anemia (MA) (Were *et al.,* 2009). A study conducted in Ugandan children showed that serum levels of RANTES were lower in children with cerebral malaria and very low levels were associated with mortality (John *et al.,* 2006). Previous studies suggested that elevated levels of RANTES may

provide protection against severe malaria (Ochiel *et al.,* 2005). In western Kenya, RANTES was reported to be suppressed in children with *P. falciparum* malaria (Were *et al.,* 2006) and that in instances of malarial anemia, its suppression is promoted in an IL-10-dependent manner by naturally-acquired hemozoin from monocytes (Were *et al.,* 2009).

Cytokines play an important role in human immune response to malaria and the outcome of *P. falciparum* infection largely relies on the delicate balance between pro-inflammatory and anti-inflammatory cytokines (Dodoo *et al.,* 2002b; Torre *et al.,* 2002a). Studies have shown that this balance determines whether the responses are protective or immunopathologic (Rovira-Vallbona *et al.,* 2012). An imbalance between pro- and anti-inflammatory cytokines could play a role in the establishment of severe malaria syndromes (Kurtzhals *et al.,* 1998; Perkins *et al.,* 2011). While numerous studies have been conducted in areas of stable malaria transmission, the correlation between anti-inflammatory (IL-5 and IL-10) and pro-inflammatory (IL-6, IFN-γ, TNF-α and RANTES) cytokine response levels to *P. falciparum* malaria antigens in a highland area of western Kenya during a period of interrupted malaria transmission remains unknown. As such, the current study assessed the correlation between anti-inflammatory (IL-5 and IL-10) and pro-inflammatory (IL-6, IFN-γ, TNF-α and RANTES) cytokine response levels to *P. falciparum* malaria antigens in a highland area of western Kenya during a period of interrupted malaria transmission.

Cytokines have been shown to play a significant role in malarial anemia (Perkins *et al.,* 2011). Elevated TNF-α level has been found to correlate with high density parasitemia (Perkins *et al.,* 2000) while low plasma IL-10/ TNF-α ratio together with reduced IL-10 levels (Boeuf *et al.,* 2012) have been associated with SMA. Erythropoietic suppression and dyserythropoiesis are among some of the causes of severe malaria anemia (SMA) in

humans (Were *et al.,* 2009). TNF-α has been shown to inhibit all stages of erythropoiesis (Dufour *et al.,* 2003) and over-production of pro-inflammatory cytokines IL-6, TNF-α and IFN-γ (John *et al.,* 2006; Kremsner *et al.,* 1995; Lyke *et al.,* 2009; Perkins *et al.,* 2000; Sarangi *et al.,* 2014) and the chemokine RANTES (Were *et al.,* 2006), is associated with anemia. Anti-inflammatory cytokines IL-10 and TGF-β have also been thought to protect against bone marrow suppression and erythrophagocytic activity induced by TNF-α (Lamikanra *et al.,* 2007; Omer and Riley, 1998) and thereby affecting erythropoiesis (Zermati *et al.,* 2000). A study in an area of stable malaria transmission demonstrated further that low ratios of plasma IL-10/TNF-α is associated with SMA in young children (Othoro *et al.,* 1999). Despite these studies, it remains unknown whether the levels of secreted cytokines (IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES) have an effect on haemoglobin concentration in a highland area of western Kenya during a period of interrupted malaria transmission. As such, the current study assessed whether the levels of secreted cytokines (IL-5, IL-6, IL-10, IFN-γ, TNF-α and RANTES) have an effect on haemoglobin concentration in a highland area of western Kenya during a period of interrupted malaria transmission.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Site and Population

The current study was conducted at a site within Nandi County in Kenya (Figure 3.1). The highland area comprising of Kipsamoite and Kapsisiywa villages, was selected because it lies at an altitude characterized by unstable malaria transmission and periods of malaria epidemics or outbreaks (Ernst *et al.,* 2006; Hay *et al.,* 2002; John *et al.,* 2005a; John *et al.,* 2009).

The study site lies between 0°16'55.64° N to 0°21'52.40° N and 34°59'7.17" E to 35°5'19.90" E (Cohen *et al.,* 2008). The elevation ranges from 1,887 m to 2,100 m above sea level (John *et al.,* 2009) with an EIR of <1 bite/person/year (Rolfes *et al.,* 2012). The area usually has two rainy seasons, short rains from October to December and long rains which begin in March and end in May (Ernst *et al.,* 2006). Temporal variations occur with March being the warmest month and July being the coldest month; the mean monthly temperatures range from 17°C -19°C (Mulambalah *et al.,* 2011).

Malaria transmission in this area often peaks following the long rains from March to May but sometimes may extend up to July (Mulambalah *et al.,* 2011). Previous clinical data described Kipsamoite as an area with sporadic malaria transmission, while Kapsisiywa had a more highly seasonal pattern (John *et al.,* 2009). Conversely, transmission here has been reported to be extremely low or absent due to the interruption with the use of malaria control measures (John *et al.,* 2009).

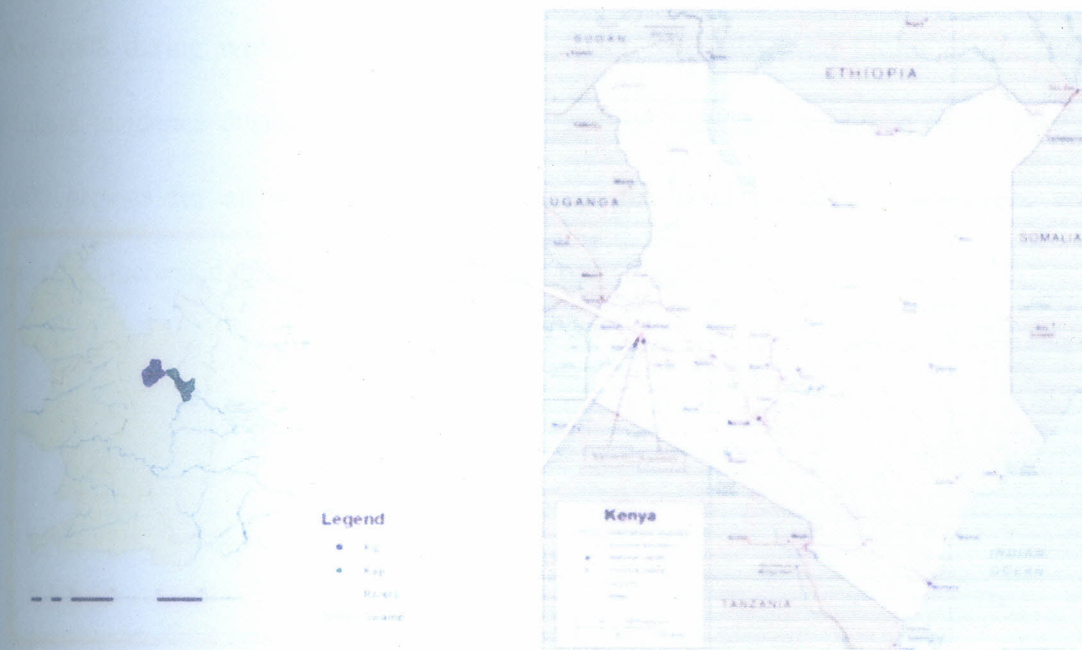


Figure 3.1 Map of study site

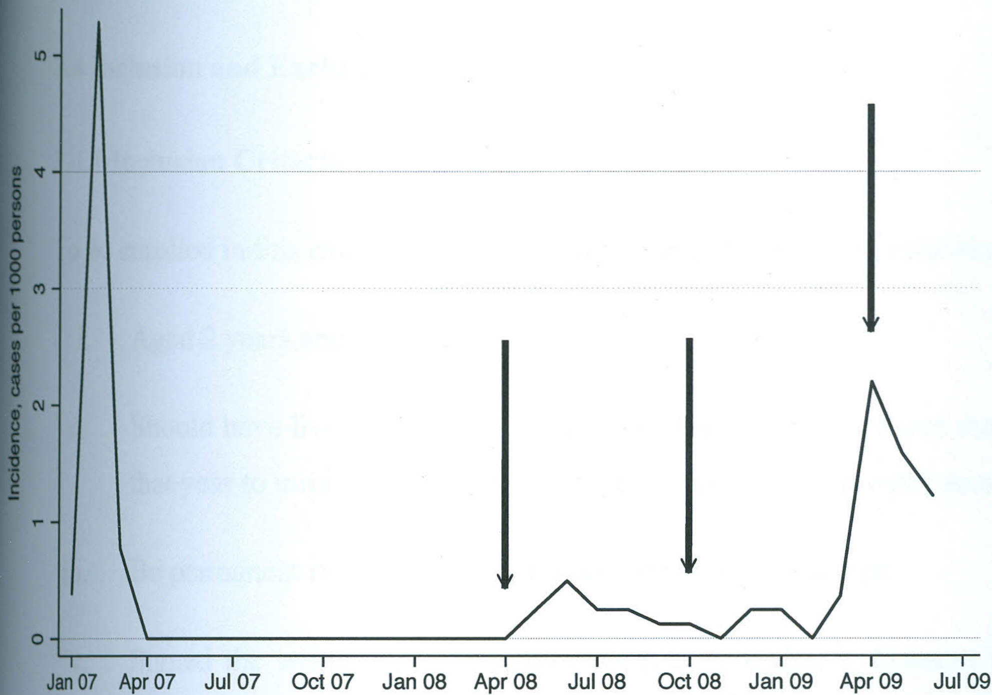
Study sites lie between 0°16'55.64° N to 0°21'52.40° N and 34°59'7.17" E to 35°5'19.90" E.

During the study period, an estimated 3,500 people lived in Kipsamoite and 3,000 in Kapsisiywa villages (Noland *et al.,* 2008). The principal occupations of inhabitants in both villages included subsistence (maize and some vegetables) and cash crop (tea) farming and animal husbandry (cattle, goats, sheep and chicken) (Cohen *et al.,* 2008). Most persons in the study area are of the Kalenjin tribe of the Nandi sub-tribe. Each site has a Kenyan Ministry of Health (MoH) dispensary, capable of microscopy and malaria treatment (Rolfes *et al.,* 2012).

3.2 Study Design

This was a longitudinal cohort study. In the study, healthy individuals from the same study site were recruited and followed over a period of one year. Three venous blood sampling was done at 6-months interval: in April 2008 (when the first samples were taken), in October 2008 (when the second samples were taken) and in April 2009 (when

the last samples were taken), following a 14-month period from March 2007 to April2008 during which no malaria transmission was observed (John *et al.,* 2009). Malaria incidence during this period was very low, with an incidence of <1 case per 1000 persons for all months of study except the final month of collection, when incidence increased to >2 cases per 1000 persons (Figure 3.2).



(John CC unublished data)

Figure 3.2: Malaria incidence in Nandi County 2007 – 2009

Monthly malaria incidence (cases per 1,000 persons) in the study cohort of individuals from Nandi County, western Kenya highlands. Arrows indicate the three sample collection time points for immunological measurements, April 2008, October 2008 and April 2009.

3.3 Sample Size Calculation

Using G*power software, Version 3.1 (Faul *et al.,* 2007), the sample size was calculated with the assumptions that a 80% power would be able to detect a 30% decrease in cytokine response in a population of 6500 individuals while factoring a previous prevalence of 21% (Ochola *et al.,* 2013) and a 10% loss to follow-up.

Power = 80% (0.8); Effect size = 30% (0.3); Prevalence = 21% (0.21); Population = 6,500

Sample size = 0.3x0.8x0.21x90/0.05

= 91 individuals (Rounded up to 100 to after factoring in the 10% loss to follow-up)

3.4 Inclusion and Exclusion Criteria

3.4.1 Inclusion Criteria

To be enrolled in this study, individuals had to meet the following conditions:

i. Aged 2 years and above.

ii. Should have lived in either Kipsamoite or Kapsisiywa for more than 6 months of that year to minimize the effect of exported malaria from other areas.

iii. Be permanent residents of either Kapsisiywa or Kipsamoite.

iv. Signed the written informed consent (See Appendix 3: Consent form) to have their blood drawn.

v. Healthy (not be presenting with any disease).

3.4.2 Exclusion Criteria

To be excluded one had to have met one of the following conditions:

i. Refusal to be part of the study.

ii. Presenting with any disease.

3.5 Blood Sample Collection

Trained field assistants identified the participants by their unique study identification and entered their demographic information, which includes: age, sex, Hb concentration, clinical malaria symptoms, treatments sought and samples obtained from the participant (See Appendix 4: Sample collection form). Qualified phlebotomist from Division of Vector-Borne Diseases in the Ministry of Health drew approximately 15- 20mL of venous blood from adults (> 15 years old) and approximately 5- 6mL from children (≤ 15 years old) into 15mL and 6mL heparinized green top BD Vacutainer tubes (BD, United Kingdom), respectively, by venipuncture. The drawn blood sample was then transported to the University of Minnesota/Kenya Medical Research Institute

(UMN/KEMRI) Malaria Project laboratory located at Centre for Global Health Research (CGHR), Kisian and processed on the same day.

3.6 Haemoglobin Testing

Samples for haemoglobin testing were collected at every time-point in April 2008, October 2008 and April 2009 and recorded in the sample collection form (Appendix 4). Finger-prick blood samples were collected for detection of *Plasmodium* species infection by microscopy and for haemoglobin testing. Haemoglobin concentration was determined by photometry (HemoControl; EKF Diagnostics).

Haemoglobin values were adjusted by 20.8 g/dL for altitude and by 11.0 g/dL if the individual was pregnant, per World Health Organization recommendations (WHO, 2001). Cut-offs for anemia were defined as follows: age 0.5–4.9 years, 11.0 g/dL; age 5–11.9 years, 11.5 g/dL; age 12–14.9 years, 12 g/dL; non-pregnant females ≥15 years, 12.0 g/dL; and males ≥15 years, 13.0 g/dL (WHO, 2001). Individuals found to be anemic were referred to the local dispensary for treatment.

3.7 Malaria Surveillance

Individuals who showed any symptoms of malaria (i.e., fever, chills, severe malaise, and headache) were assessed for malaria by blood smear microscopy. Clinical malaria was defined as symptoms of malaria and a blood smear positive for any *Plasmodium* species. Consenting individuals also provided blood spots on filter paper for detection of *Plasmodium falciparum* by polymerase chain reaction (PCR).

3.7.1 Microscopy for Parasite Detection

Thick and thin blood films were made for malaria microscopy. The blood smears were stained using 5% Giemsa solution at pH 7.2. Parasites in the thick smears were counted

against 200 white blood cells (WBCs) and the counts recorded for *Plasmodium* spp. asexual forms and gametocytes. Two independent readings by trained miscroscopists were done to all slides. In the event of discordant readings, a third reading was done for quality control. Individuals diagnosed with malaria were referred for treatment at the local health centers. Parasite density was estimated by counting the number of parasites per 200 leukocytes in an oil-immersion thick blood film at 100× objective lens magnification. Parasite counts were converted to parasites per 1µl assuming an average of 8,000 leukocytes/µl for each sample.

3.7.2 PCR for *Plasmodium falciparum* Parasitaemia

Blood spots for PCR diagnosis of *P. falciparum* infection were collected on Whatman 903 filter paper (Whatman Corporation, Florham Park, NJ USA) and stored with desiccant in a 20°C freezer until testing in the US. PCR testing was done on all samples from individuals with symptomatic malaria (showed any symptoms of malaria) and malaria positive slides.

Genomic DNA was isolated with a QIAamp 96 DNA blood kit (QIAGEN Inc., Valencia, CA, USA) from dried filter paper blood spots, and *P. falciparum* infection was determined by nested PCR targeting the small subunit RNA gene as previously described (Menge *et al.,* 2008).

3.8 Laboratory Procedures

3.8.1 Peripheral Blood Mononuclear Cell (PBMC) Isolation

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density gradient centrifugation. In this procedure, the anti-coagulated blood was layered carefully onto 5mL of Histopaque (Sigma, St. Louis, MO) in a 15mL tube and then centrifuged at 400×g (1500 rounds per minute (rpm) in Jouan, Thermo Electron Corp.,

USA) for 30 minutes without brakes at room temperature (RT). After the transfer of the plasma, the PBMCs were collected from the buffy coat using a 10mL pipette, and transferred into a 15mL tube. The cells were then washed twice by adding sterile 1×PBS (Sigma, St. Louis, MO), pH 7.0, without calcium or magnesium, to bring a total volume in the tube to 12mL followed by centrifugation for 15 minutes at 240×g (1000 rpm in Jouan) minutes without brakes at room temperature. The supernatants were aspirated off carefully, the pellet broken by gentle flicking of the tube and then resuspended in 2mL of culture medium complete RPMI 1640 (Sigma, St. Louis, MO) supplemented with human type AB heat-inactivated serum (Sigma, St. Louis, MO), gentamicin (Amresco, Solon, OH), HEPES (Gibco, Invitrogen Paisley, Scotland, UK) and L-glutamine (Sigma, St. Louis, MO, USA). The cells were then stained with 10µl of 0.4% Turk's solution in a 1:1 ratio and enumerated using a haemocytometer. The calculations of the cell counts were carried out in Microsoft Excel sheets to determine how to dilute the cells to appropriate concentration of 2×10^5 cells/well for the assay.

3.8.2 Cell Culture

The isolated PBMCs were resuspended in culture medium complete RPMI (cRPMI) 1640. For this study, 2×10^5 cells/well were cultured in 96 well-round-bottom plates after stimulation with the peptides at 10µg/mL each and incubated at 37°C in 5% CO_2, with humidity, for 120 hours (5 days) in a water jacket CO_2 incubator (Thermo Fisher Scientific Inc., USA). The supernatants harvested were stored at -80°C for cytokine testing using the Bio-Plex assay.

3.8.3 Cytokine Testing by Bio-Plex Suspension Array System

The cytokine 6-plex conjugated bead (25×) stock solution required for the assay was vortexed and a working dilution of the anti-cytokine bead (25×) in Bio-Plex Assay

buffer prepared. A 96-well millipore microtiter plates (Millipore corporation, Billerica, MA) were then pre-wetted with 100µL per well of Bio-Plex Assay buffer and aspirated using a millipore vacuum manifold. Fifty µL of the working bead solution was added into the appropriate wells of the plates and then aspirated using a millipore vacuum manifold. A hundred µL of Bio-Plex Wash buffer was added to each well and aspirated. Fifty µL of standard or sample was then added to the appropriate wells and plate placed on a micro-plate shaker (Wilmington, NC) for 30 minutes at RT and gently mixed. The plate was aspirated using a millipore vacuum manifold to remove the supernatant and washed three times with 100µL per well of Bio-Plex wash buffer (The plates were aspirated using a millipore vacuum manifold after each wash. A working dilution of the detection antibody (10×) stock solution was prepared in Bio-Plex Detection Antibody diluent. The working detection antibody solution was then gently vortexed and 25µL added to each well and the plate placed on the microplate shaker for a 30-minute incubation at RT. The buffer was then aspirated, and using 100µL of Bio-Plex wash buffer per well, the plate was washed three times and further aspirated, to remove the wash buffer. A 100 fold working dilution of the streptavidin-PE (100×) stock solution was prepared in Bio-Plex assay buffer and 50µL of the dilution added to each well and incubated for 10-minutes at room temperature on the microplate shaker. The buffer was then removed by aspiration, followed by three washes with 100µL of Bio-Plex wash buffer per well and further aspirated to remove the wash buffer. The beads in each well were then re-suspended with 125µL of Bio-Plex assay buffer per well and briefly mixed and the plate read on the Bio-Plex 200 system (Hercules, CA).

Since the beads are sensitive to light hence the procedure was done away from direct light, and plate covered completely by sealing the plate with aluminum foil when placed

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on the microplate shaker. The Bio-Plex Manager™ software presents data as median fluorescence intensity (MFI) as well as concentration, (pg/mL).

3.9 Antigens and Peptides

The cytokine responses to *P. falciparum* CSP, AMA-1, TRAP, LSA-1, MSP-1 and MB2 were tested using the previously described CSP peptide cs22 amino acid (aa) 378 to 392, aa sequence DIEKKICKMEKCSSV (Reece *et al.,* 2004), AMA-1 peptide PL171 (aa 348 to 366) aa sequence DQPKQYEQHLTDYEKIKEG (Lal *et al.,* 1996; Udhayakumar *et al.,* 2001), TRAP peptide tp6 (aa 51 to 70) aa sequence LLMDCSGSIRRHNWVNHAVP (Flanagan *et al.,* 1999), MSP-1 peptide pool of M1 (aa 20 to 39) aa sequence VTHESYQELVKKLEALEDAV (Parra *et al.,* 2000) and M2 (aa 1467 to 1483) aa sequence GISYYEKVLAKYKDDLE (Udhayakumar *et al.,* 1995), LSA-1 peptide T3 (aa 1813 to 1835), aa sequence NENLDDLDEGIEKSSEELSEEKI (John *et al.,* 2000) and MB2 peptide pool of MB1 (aa 191 to 199) aa sequence SVSSINTNL (Nguyen and James, 2001) and MB2 (aa 119 to 127) aa sequence KPKKKYYEV (Nguyen and James, 2001). All the peptides used as *P. falciparum* antigens have been documented to produce antigen-specific cytokine responses in persons in malaria endemic areas. Conserved regions of vaccine candidate *P. falciparum* antigens, strains 3D7 and/or FVO were used for these peptides. Peptides were synthesized and purified by high-performance liquid chromatography (HPLC) to > 95% purity (Sigma Genosys, St. Louis, MO). Phytohaemagglutinin (PHA) was used as the positive control and phosphate buffered saline (PBS) as the negative control. All peptides and mitogen were used at a concentration of 10μg/mL.

3.10 Data Analysis

Stata Version 12 (Stata Corp, College Station, TX, USA) was used for all data analysis while GraphPad version 5.01 software (GraphPad Systems, Inc., La Jolla, CA, USA) was used to generate graphs. The McNemar's test was used to assess the significance of the differences between two matched categorical variables (frequency of cytokines in same individuals at any two time points) and Wilcoxon signed-rank test was used to assess the significance of the differences between two matched continuous variables (cytokine levels in same individuals at any two time points). A Chi-square (χ^2) test or Fisher's exact test was used to determine the significance of the differences between categorical variables (frequency of cytokines vs. age group), while Wilcoxon rank sum test was used to determine the significance of differences between continuous variables (cytokine levels vs. haemoglobin levels). Spearman's rank correlation test was used to assess correlations between continuous variables (cytokine and haemoglobin levels). Multiple comparisons were adjusted for with the Bonferroni correction. All tests were two-tailed and the significance level was set at *p*-value of 0.05.

3.11 Ethical Considerations and Approval

Ethical approval for the study was obtained from Ethical Review Committee at Kenya Medical Research Institute (KEMRI) and Institutional Review Board at the University of Minnesota, USA (See Appendix 2: Research approval letter). Duly signed informed consent was obtained from each study individuals (in the case of minors, consent was obtained from their parent or guardian) (See Appendix 3: Consent form). Qualified phlebotomists from Division of Vector-Borne Diseases (DVBD) in the Ministry of Health (MoH) carried out venipuncture process under sterile conditions to minimize risk

of infection. All clinical cases among the participants were referred to the clinical officer for further care.

CHAPTER FOUR: RESULTS

4.1 Baseline Demographic and Haematological Characteristics of Study Participants

The cohort consisted of 100 individuals, 52 females and 48 males. Thirty-eight were children (mean [range] of 8.60 [2.07 - 14.69] years) and 62 were adults (36.95 [15.65 - 69.25] years) (Table 4.1). The adults had higher haemoglobin levels (mean [range] of 13.87 [7.1-17.4] g/dL) compared to the children (13.22 [10.8-15.6] g/dL) (p=0.015) (Table 4.1). Asymptomatic *P. falciparum* infection was not present by microscopy at any time point, but was detected in 1 and 2 study participants in October 2008 and April 2009, respectively, by polymerase chain reaction (PCR). No episodes of clinical malaria were observed in the study participants during the entire period of the study (Table 4.1).

Table 4.1: General Characteristics of Study Participants at Baseline

	Children (n=38)	Adults (n=62)
Gender, N (%)		
Male	23 (60.5%)	25 (40.3%)
Female	15 (39.5%)	37 (59.7%)
Age, years		
Mean ±SD	8.60±3.47	36.95±13.21
Range	2.07 – 14.69	15.65 - 69.25
Hb level, g/dL		
Mean ±SD	13.22±1.13	13.87±2.22*
Range	10.8 – 15.6	7.1 - 17.4
***P. f.* parasitemia**		
Microscopy	0	0
PCR	0	0

*Significant difference in Hb levels between children and adults by Wilcoxon ranksum test; p=0.015; N=number; %=percentage; Hb= haemoglobin (g/dL); SD= Standard deviation; *P.f.= Plasmodium falciparum;* ND= Not determined.

4.2 *P. falciparum* Antigen-specific Cytokine/Chemokine Responses Over Time

In this population, the level of immune challenge was quite low following intense malaria control measures used prior to the period of sample collection.

Overall, IL-5 and IL-10 levels were quite low; IFN-γ and TNF-α levels were moderately low, whereas IL-6 and RANTES levels were relatively high (Figures 4.1 - 4.6). There was an overall decrease in IL-5 levels at 6 months follow-up and more significantly so for LSA-1 (geometric mean (range) pg/mL [0.04 (0 -116.1) vs. 0.01 (0 - 30.5)], $P = 0.0264$), MB2 (0.03 (0 - 72.7) vs. 0.01 (0- 9.3), $P = 0.0384$) and MSP-1 (0.05 (0 – 168.3) vs. 0.1 (0 – 6.42), $P = 0.0048$). A slight increase was observed from time point two to time point three though this increase was not significant (all $P > 0.05$). However, at 12 months follow-up, IL-5 levels were stable as there was no significant change in IL-5 levels to all the antigens (all $P > 0.05$) (Figure 4.1).

Similarly, there was significant decrease in IL-10 levels for AMA-1 (0.18 (0 – 55.5) vs. 0.01 (0- 28.8), $P < 0.00001$), CSP (0.20 (0 – 58.5) vs. 0.01 (0- 5.8), $P < 0.00001$) LSA-1 (0.17 (0 – 81.0) vs. 0.01 (0 - 1.0), $P < 0.00001$), MB2 (0.21 (0 - 79.6) vs. 0.02 (0- 8.9), $P < 0.00001$), MSP-1 (0.20 (0 – 83.4) vs. 0.01 (0- 4.8), $P < 0.00001$) and TRAP (0.21 (0 – 100.5) vs. 0.01 (0- 1.0), $P < 0.00001$) at 6 months follow-up. Similarly, IL-10 levels decreased at 12 months follow-up to all antigens (all $P < 0.00001$). Importantly, there was significant increase in IL-10 response to CSP (0.01 (0- 5.8) vs. 0.03 (236.3), $P = 0.0018$), LSA-1 (0.01 (0- 1.0) vs. 0.02 (12.9), $P = 0.0102$) and TRAP (0.01 (0- 1.0) vs. 0.02 (0 – 53.2), $P = 0.0030$) observed from time point two to time point three (Figure 4.3), corresponding to the apparent increase in malaria cases at that point in time (Figure 3.2).

A significant decrease in IFN-γ levels was observed for AMA-1 (1.397 (0 – 4840.71) vs. 0.145 (0 – 501.9), $P = 0.0012$), LSA-1 (1.3664 (0- 2968.2) vs. 0.2154 (0 – 693.0), $P < 0.00001$), MSP-1 (5.334 (0- 3830.4) vs. 0.1843 (0 – 1324.5), $P < 0.00001$) and TRAP (0.9249 (0- 77198.3) vs. 0.199 (0 – 1168.2), $P = 0.0024$) at 6 months follow-up, though IFN-γ levels were stable at 12 months follow-up for all antigens except the significant reduction seen for MSP-1 (5.334 (0- 3830.4) vs. 0.5926 (0 – 1614.1) $P = 0.0102$) (Figure 4.2).

TNF-α levels decreased significantly for AMA-1 (0.65 (0 - 418.2) vs. 0.12 (0 - 518.6), $P = 0.0060$), CSP (0.61 (0 - 440.2) vs. 0.20 (0 - 51.4), $P = 0.0036$) and LSA-1 (0.99 (0 - 430.7) vs. 0.17 (0 - 410.6), $P = 0.0012$) but were stable for MB2 and MSP-1 (both $P>0.05$) at 6 months follow-up. Conversely, there was a significant increase in the levels of TNF-α responses to TRAP ($P = 0.0030$). At 12 months follow-up, TNF-α responses were stable for all antigens except AMA-1 (0.65 (0- 418.2) vs. 0.13 (0 – 1078.7), $P = 0.0402$) and LSA-1 (0.99 (0- 430.7) vs. 0.20 (0 – 1257.7), $P = 0.0120$) (Figure 4.4).

However, only IL-6 and RANTES were stable at both 6 and 12 months follow up for all antigens, as there was no significant change in the levels over time (all $P > 0.05$) (Figures 4.5 and 4.6).

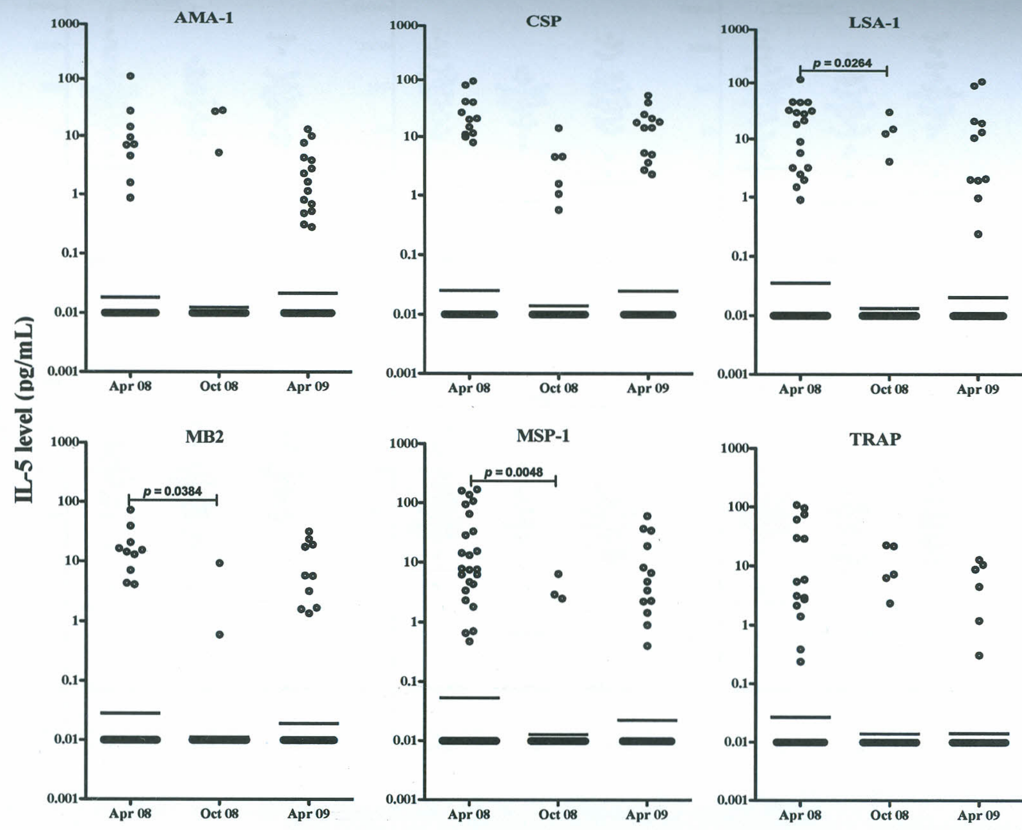

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Figure 4.1: Magnitude of *P. falciparum* antigen-specific IL-5 responses over time.

Levels of IL-5 response to AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP at April 2008, October 2008 and April 2009. Light bars indicate geometric mean levels of cytokines secreted at each time point. Thick bars indicate individuals with no response. Each dot represents an individual.

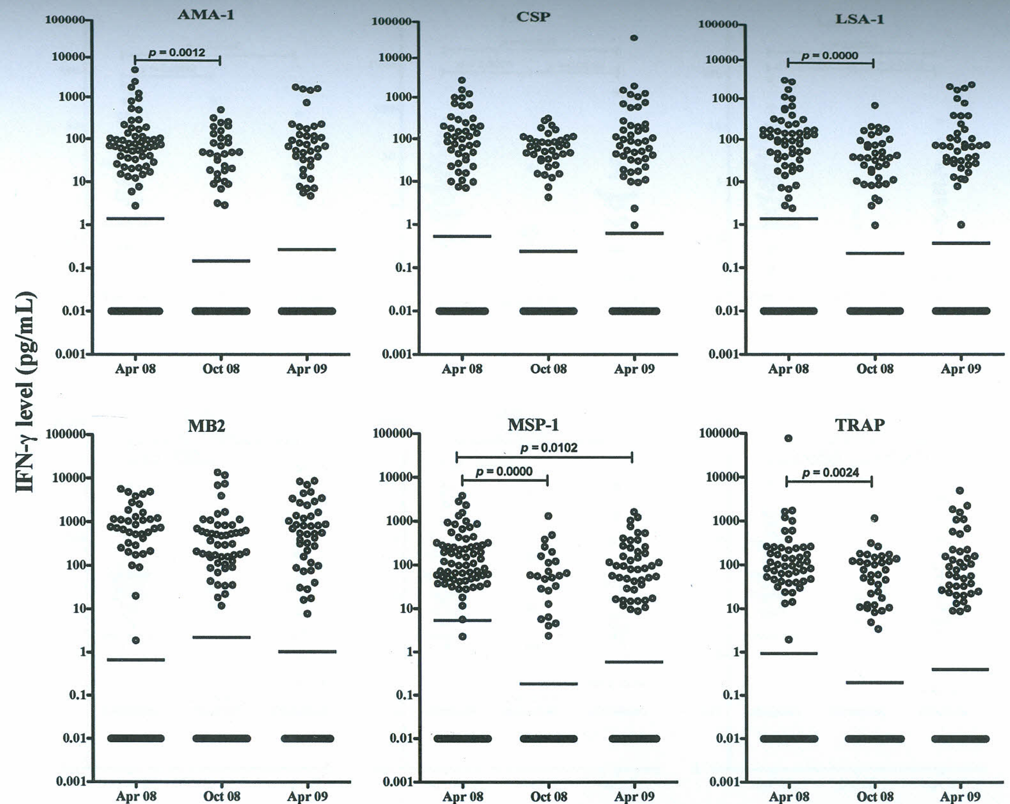

Figure 4.2: Magnitude of *P. falciparum* antigen-specific IFN-γ responses over time.

Levels of IFN-γ response to AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP at April 2008, October 2008 and April 2009. Light bars indicate geometric mean levels of cytokines secreted at each time point. Thick bars indicate individuals with no response. Each dot represents an individual.

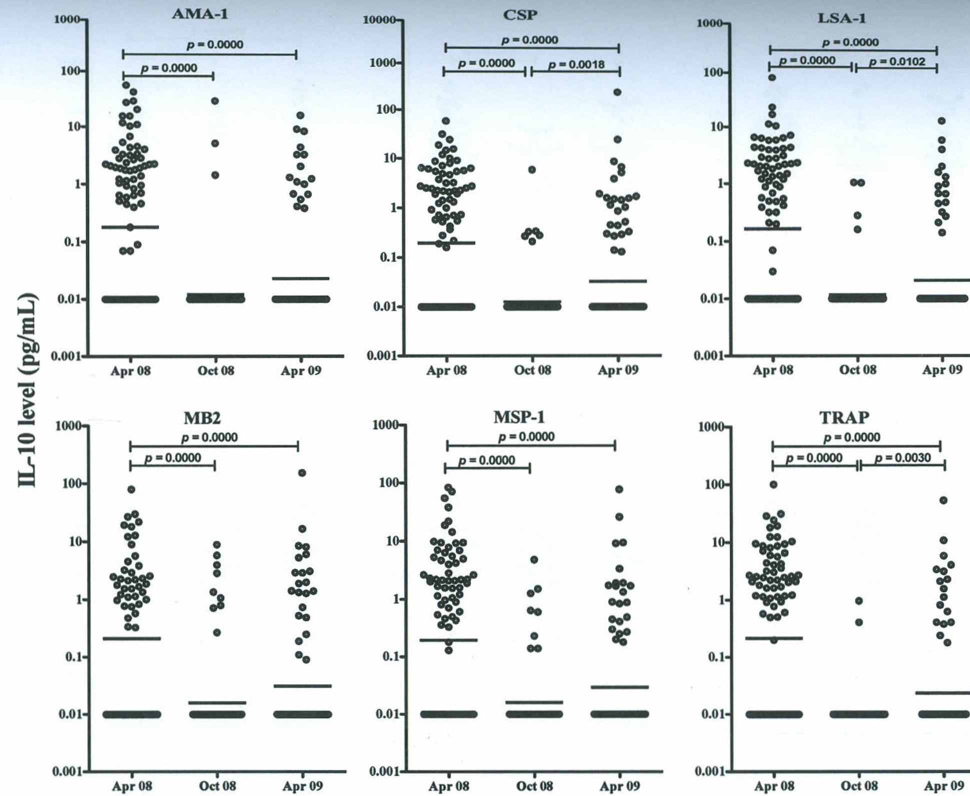

Figure 4.3: Magnitude of *P. falciparum* antigen-specific IL-10 responses over time.

Levels of IL-10 response to AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP at April 2008, October 2008 and April 2009. Light bars indicate geometric mean levels of cytokines secreted at each time point. Thick bars indicate individuals with no response. Each dot represents an individual.

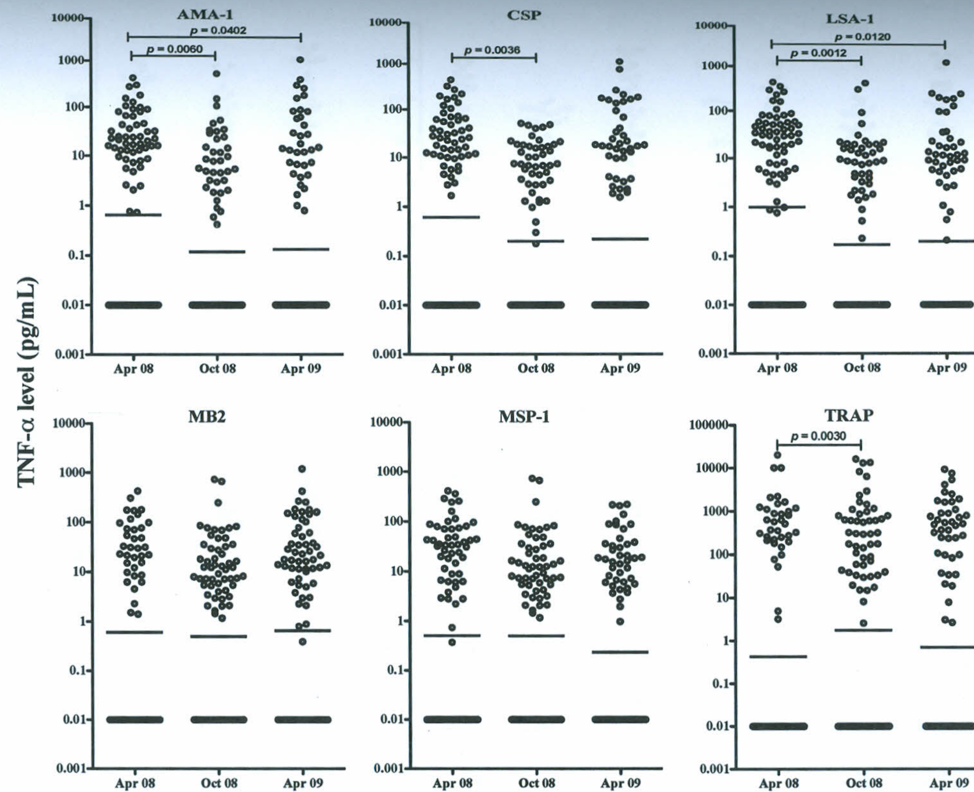

Figure 4.4: Magnitude of *P. falciparum* antigen-specific TNF-α responses over time.

Levels of TNF-α response to AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP at April 2008, October 2008 and April 2009. Light bars indicate geometric mean levels of cytokines secreted at each time point. Thick bars indicate individuals with no response. Each dot represents an individual.

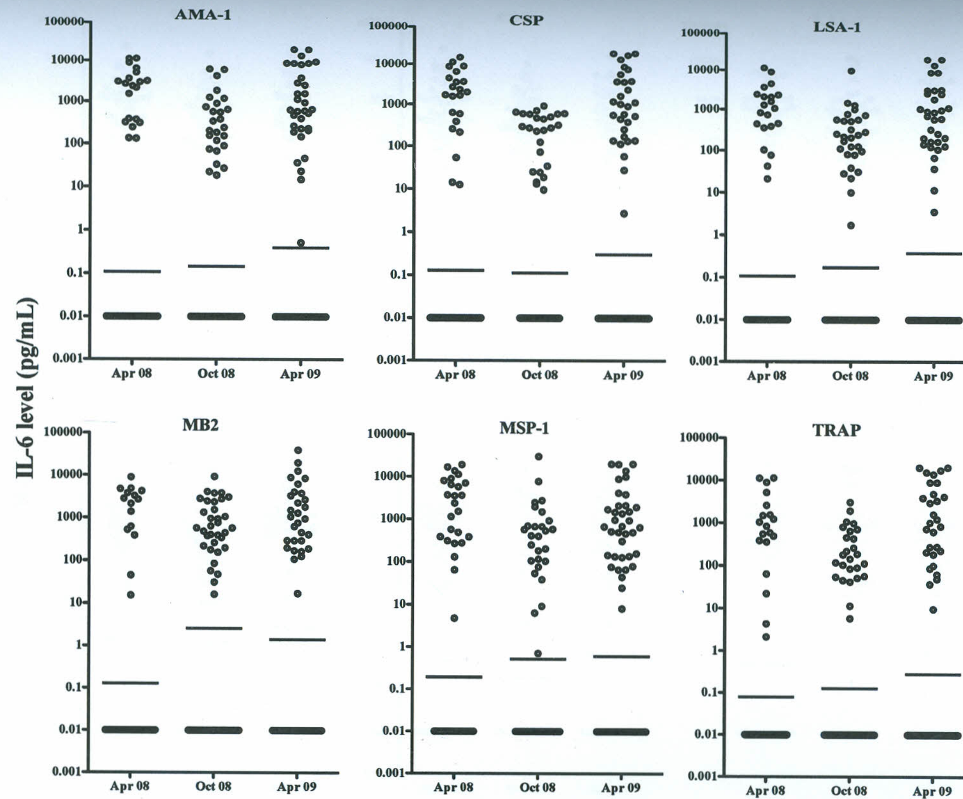

Figure 4.5: Magnitude of *P. falciparum* antigen-specific IL-6 responses over time.

Levels of IL-6 response to AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP at April 2008, October 2008 and April 2009. Light bars indicate geometric mean levels of cytokines secreted at each time point. Thick bars indicate individuals with no response. Each dot represents an individual.

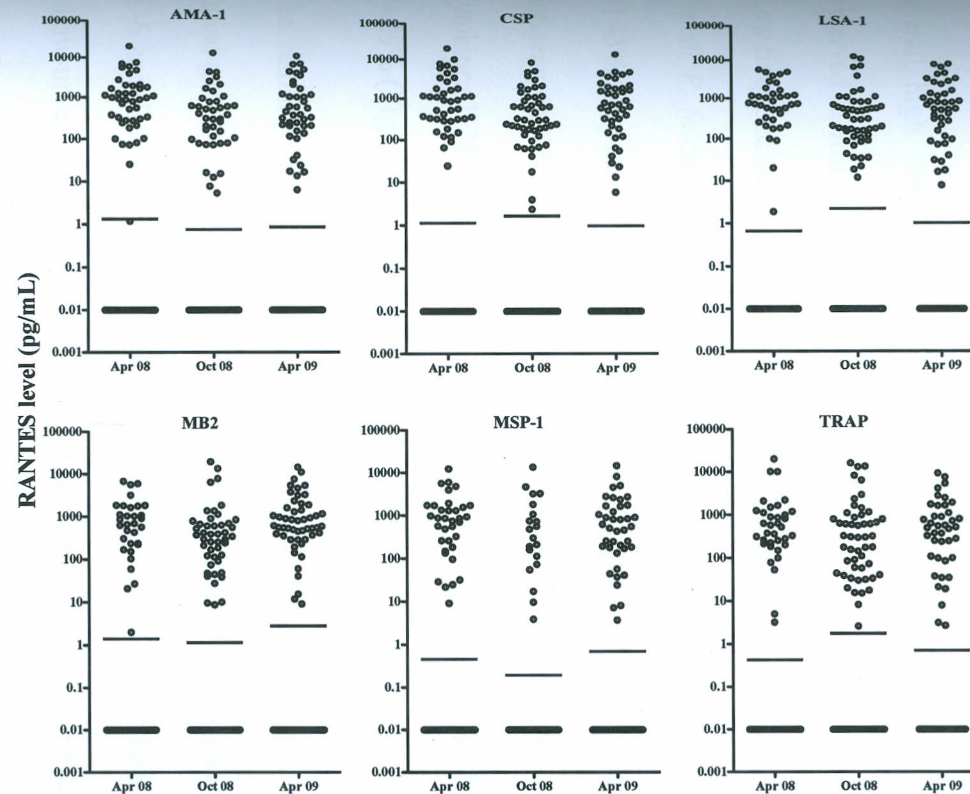

Figure 4.6: Magnitude of *P. falciparum* antigen-specific RANTES responses over time.

Levels of RANTES response to AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP at April 2008, October 2008 and April 2009. Light bars indicate geometric mean levels of cytokines secreted at each time point. Thick bars indicate individuals with no response. Each dot represents an individual.

Blood samples from malaria naïve North Americans (NA) were used to set the cut-off for positive cytokine/chemokine response. Those who had greater than the mean plus 2 standard deviations (SD) of the NA control response were considered responders while those below this cut-off were considered non-responders.

The study showed generally low frequencies (< 20%) of responses to all *P. falciparum* antigens for the pro-inflammatory cytokine IL-5 at initial and follow-up time points ranging between 0 % and 11.43% (Figure 4.7). For the pro-inflammatory cytokine IL-6, overall high frequencies (>20%) of responses to most *P. falciparum* antigens were observed at initial or follow-up time points, ranging between 17% and 50% (Figure 4.8). Low frequencies of responses were also observed for the anti-inflammatory cytokine IL-10 for all *P. falciparum* antigens at initial and follow-up time points ranging from 0 % to 11.43% (Figure 4.9). Generally, *high frequencies of responses to most P. falciparum* antigens at *initial or follow-up time points were observed for the pro-inflammatory* cytokines *IFN-γ ranging from 22% to 68.57% (Figure 4.10), TNF-α, ranging from 14% to* *44.29% (Figure 4.11) and RANTES ranging from 25.71% to 52% (Figure 4.12).*

The stability in the frequency of the *P. falciparum* antigen-specific cytokine responses varied greatly by antigen but did not exhibit any particular pattern. The number of positive IL-6 and RANTES responders were stable to all the antigens at both 6 and 12 months follow-up except for MB2-specific IL-6 response that increased significantly (22.86% vs. 50%, *P* <0.01) (Figure 4.8 and Figure 4.12). At six months follow-up, the frequency of IL-5 response was stable to all antigens at 6 months follow-up except CSP (10% vs. 1%, *P* <0.05) and MSP-1 (11.43% vs. 0%, *P* <0.05). However, at 12-months follow-up, the frequency of IL-5 responses was stable to all the antigens (Figure 4.7). IL-10 responders in the population decreased significantly in response to CSP (8% vs. 0%), MB2 (11.43% vs.

0%) and TRAP (9% vs. 0%) (All *P* <0.05) at 6 months follow-up but were stable for all the antigens at 12 months follow-up (Figure 4.9). Positive IFN-γ responses were stable for all the antigens but significantly decreased in response to AMA-1 (52% vs. 27%) and MSP-1 (68.57% vs. 27.14%) at 6-months follow-up (all *P*<0.01). Similarly, at 12 months follow-up, AMA-1 (52% vs. 32%) and MSP-1 (68.57% vs. 44.29%) decreased significantly (all *P*<0.05) (Figure 4.10). Positive TNF-α responders in the study population decreased significantly for all antigens except MB2 (AMA-1 (41% vs. 14%), CSP (42% vs. 21%), LSA-1 (43% vs. 19%), MSP-1 (40% vs. 18.57%)) at 6 months follow-up and to AMA-1 (41% vs. 22%) and LSA-1 (43% vs. 23%) at 12-months follow-up (all *P*<0.05) (Figure 4.11).

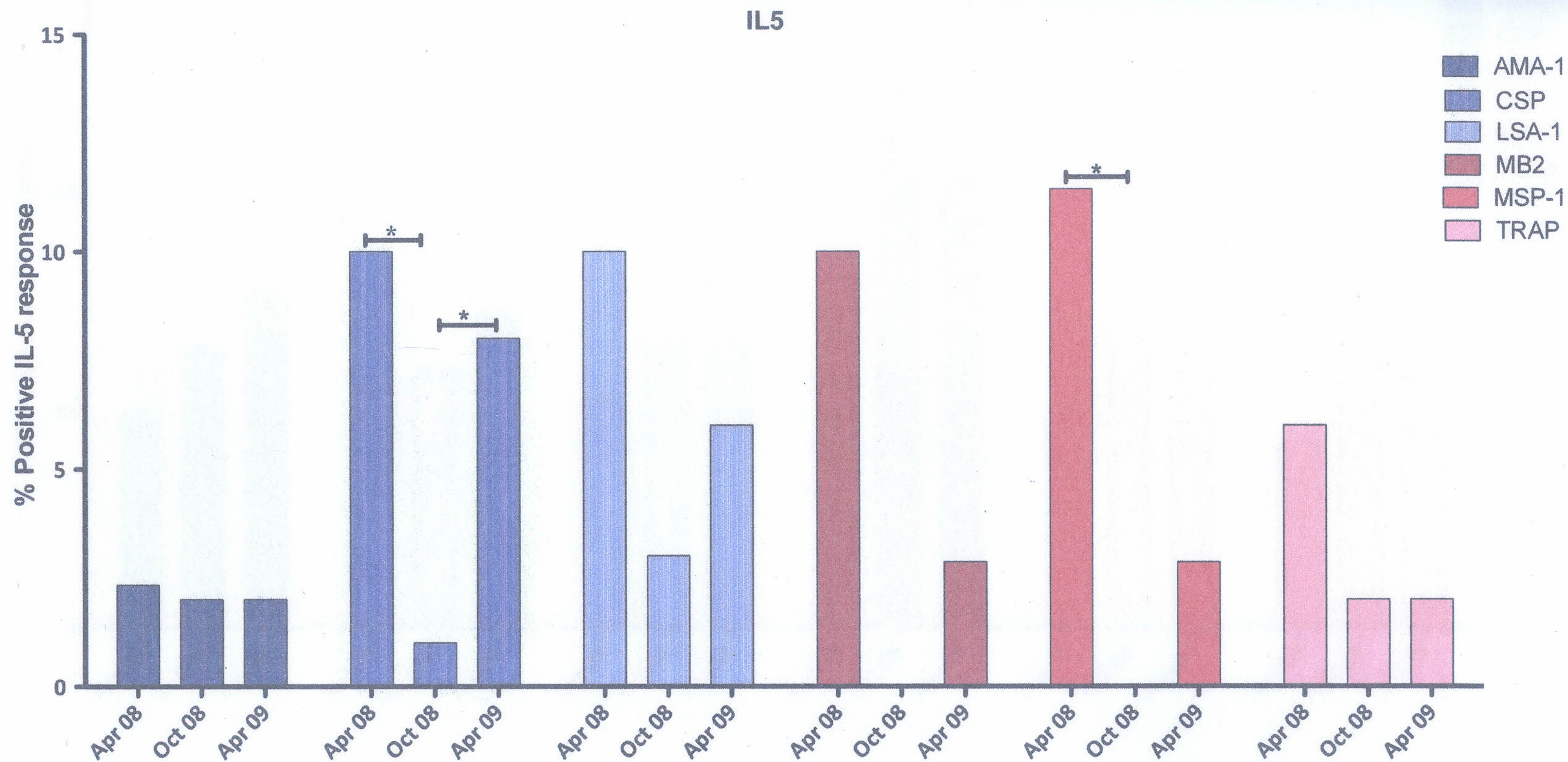

Figure 4.7: Frequency of positive IL-5 responses over time.

IL-5 responders to *P. falciparum* AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP antigen over time. * $P \leq 0.05$ by two-tailed McNemar's test for matched pairs (with Bonferroni correction).

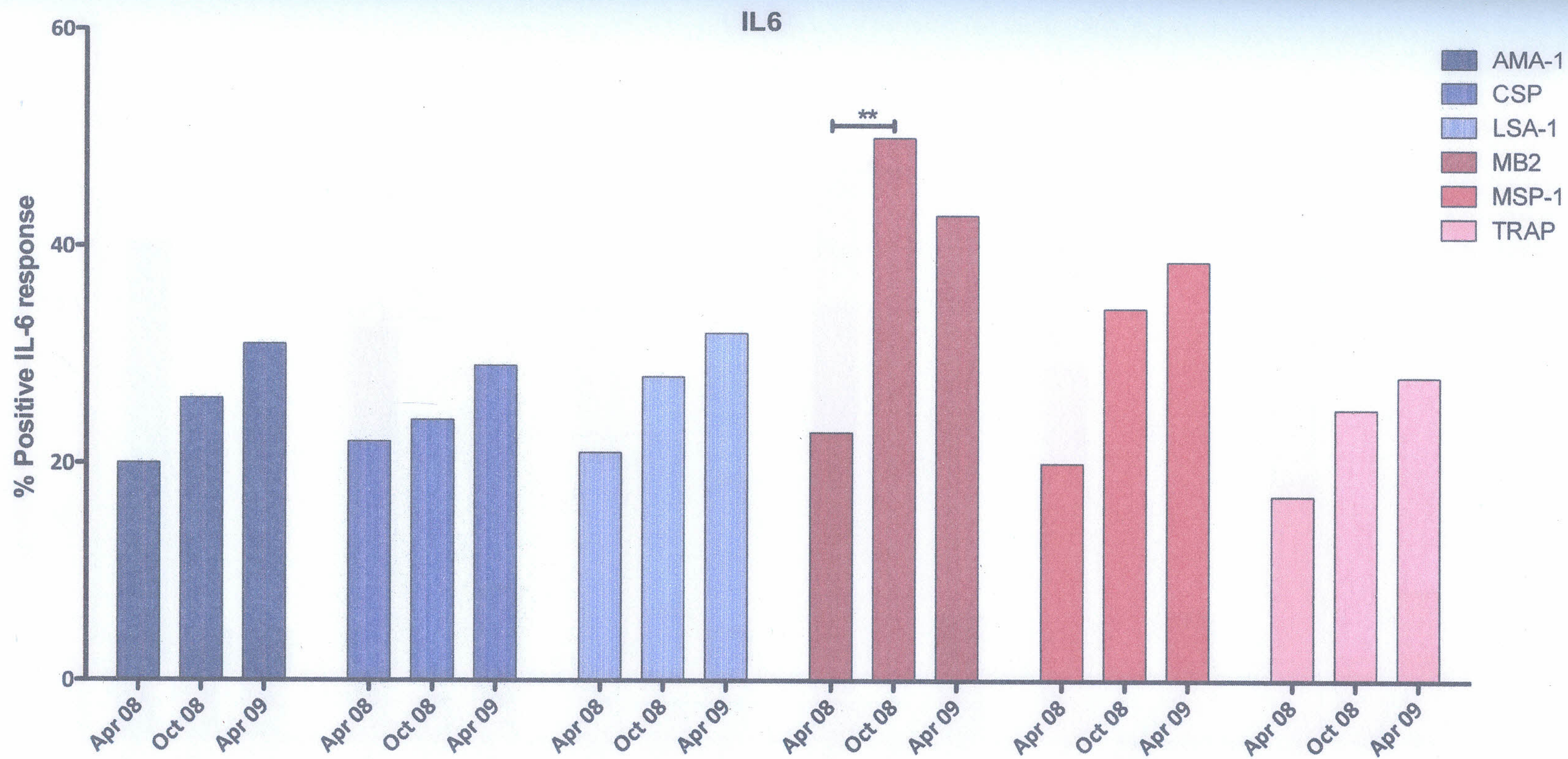

Figure 4.8: Frequency of positive IL-6 responses over time.

IL-6 responders to *P. falciparum* AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP antigen over time. **$P \leq 0.01$ by two-tailed McNemar's test for matched pairs (with Bonferroni correction).

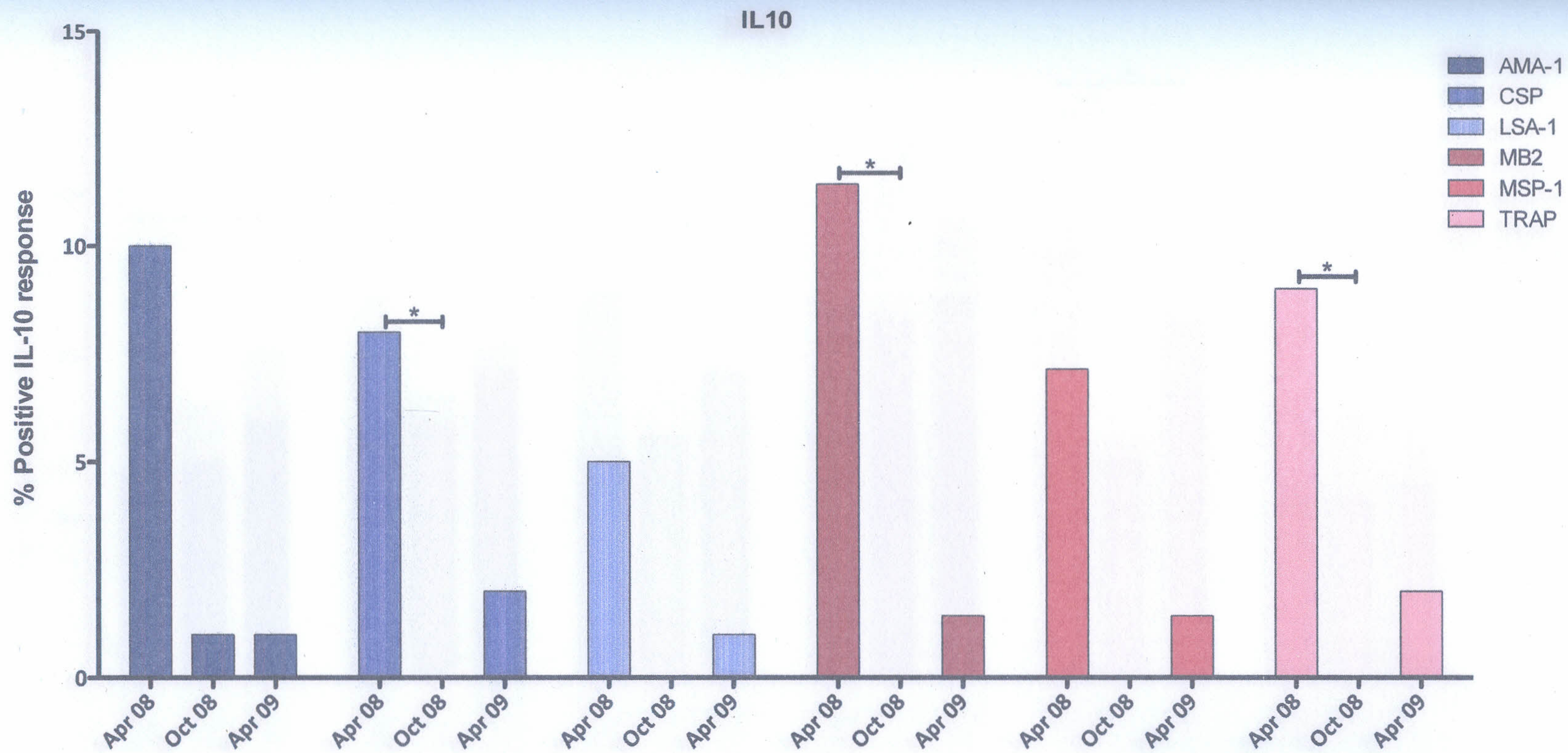

Figure 4.9: Frequency of positive IL-10 responses over time.

IL-10 responders to *P. falciparum* AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP antigen over time. * $P \leq 0.05$ by two-tailed McNemar's test for matched pairs (with Bonferroni correction).

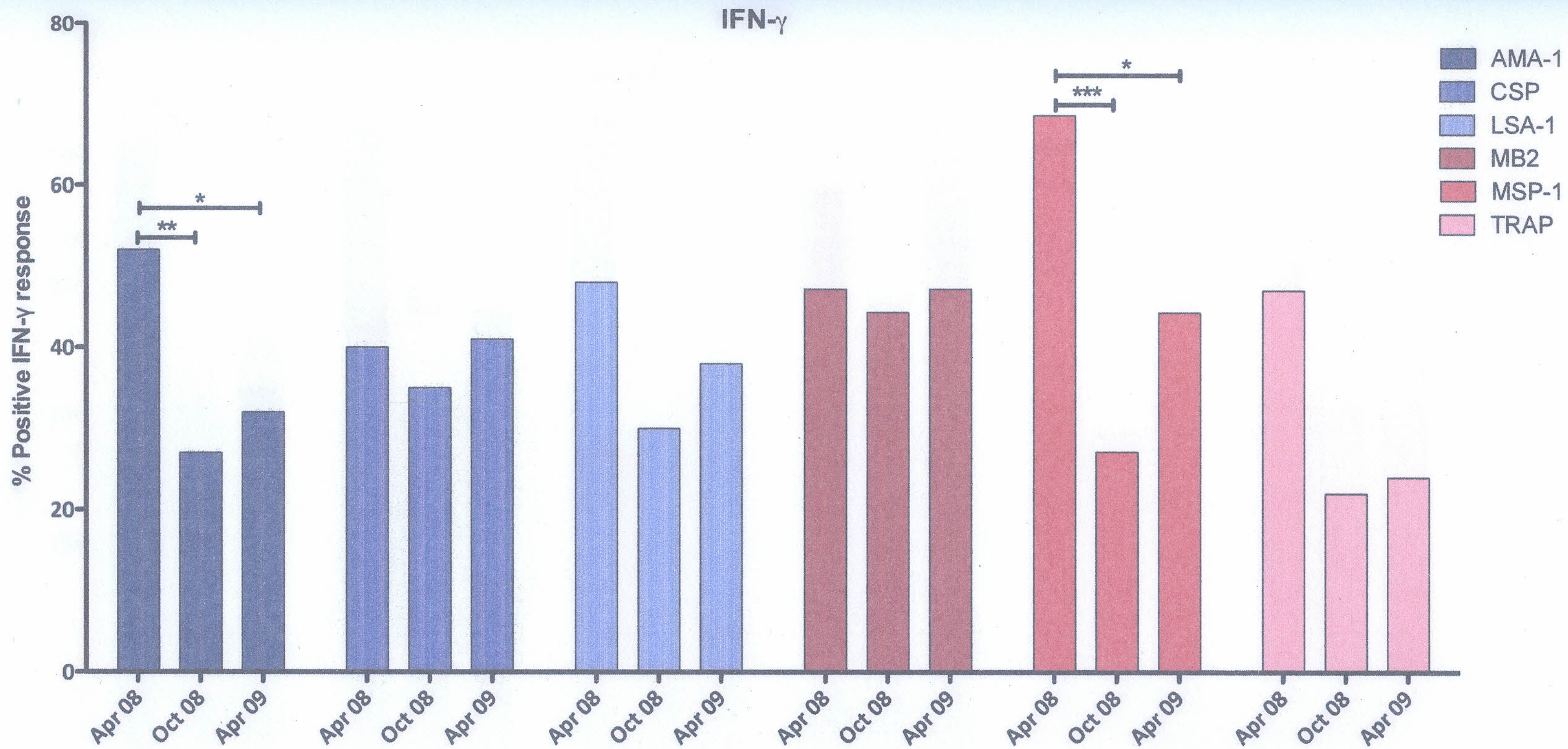

Figure 4.10: Frequency of positive IFN-γ responses over time.

IFN-γ responders to *P. falciparum* AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP antigen over time. * $P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$ by two-tailed McNemar's test for matched pairs (with Bonferroni correction).

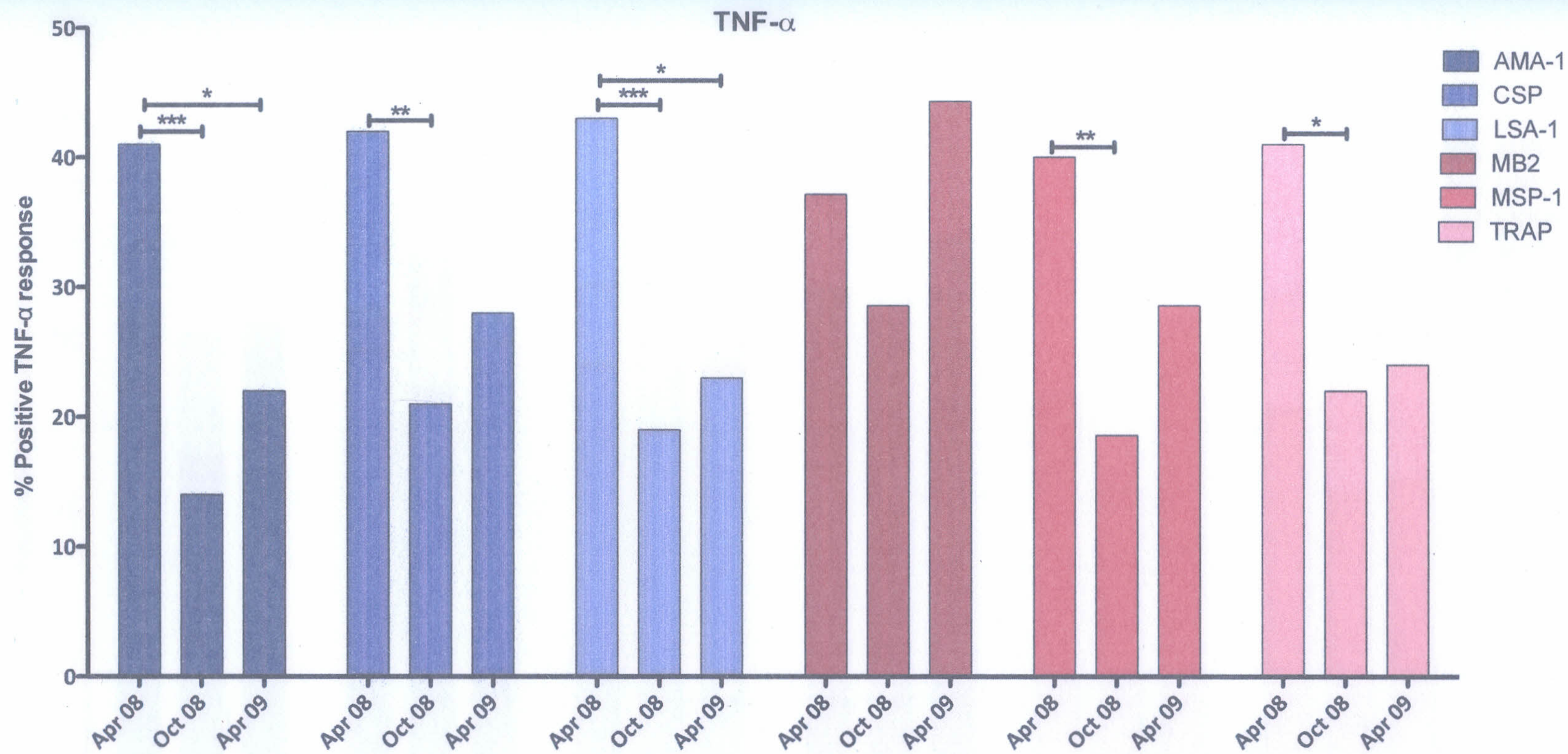

Figure 4.11: Frequency of positive TNF-α responses over time.

TNF-α responders to *P. falciparum* AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP antigen over time. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$ by two-tailed McNemar's test for matched pairs (with Bonferroni correction).

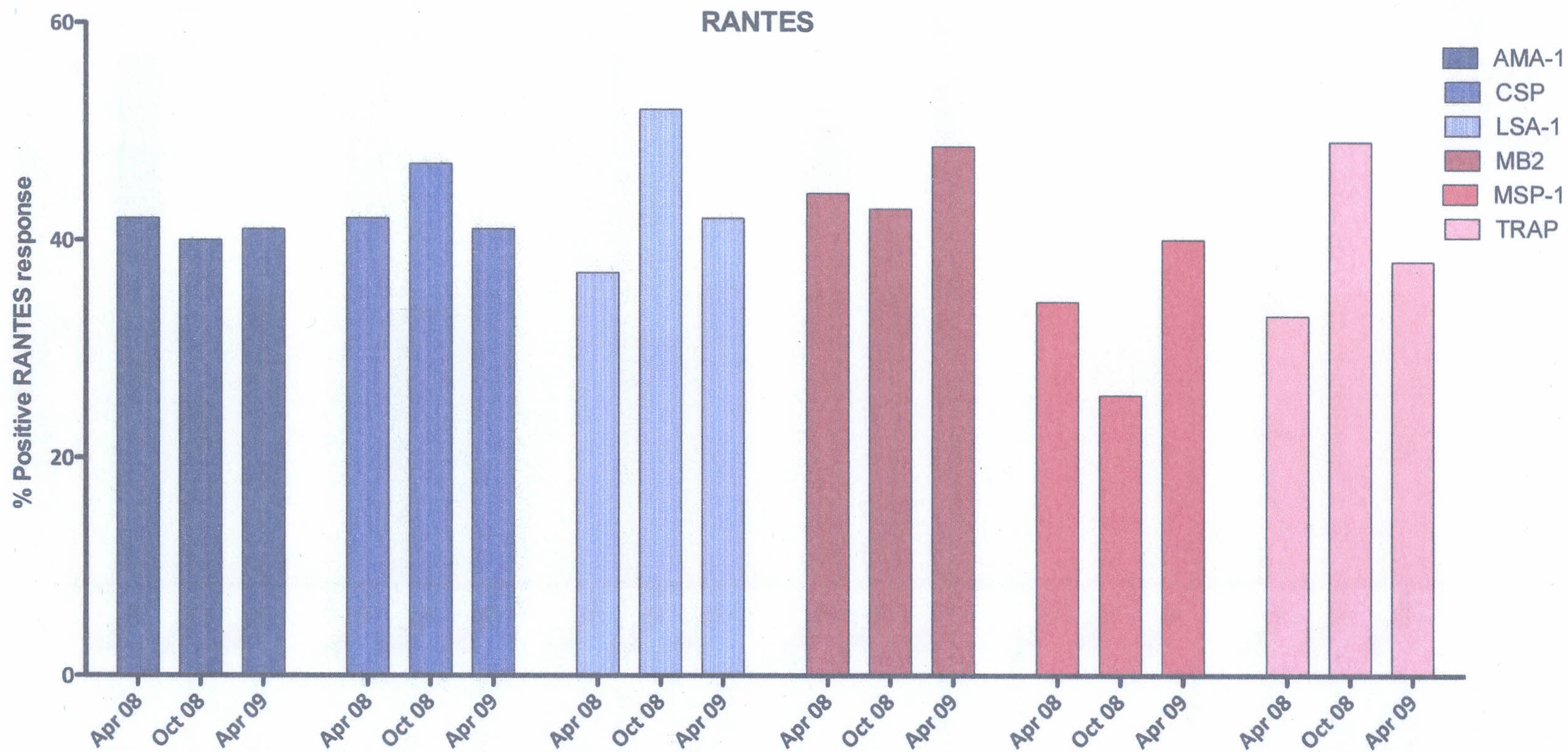

Figure 4.12: Frequency of positive RANTES responses over time.

RANTES responders to *P. falciparum* AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP antigen over time.

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4.3 Correlation Between *P. falciparum* Antigen-specific Cytokine/Chemokine Response and Age

In order to assess the correlation between the production of antigen-specific cytokine/chemokine and age of individuals from a highland area of Kenya during the period of interrupted malaria transmission, the cytokine/chemokine responses were stratified by age and compared. The individuals in the study were divided into four groups: 0 to 5 years, 6 to 15 years, 16 to 40 years and >40 years as per previous studies (Noland *et al.,* 2008). There were 34 children (individuals below 15 years of age) and 66 adults (individuals above the age of 15).

Correlations were seen between the antigen-specific cytokine/chemokine responses. At baseline, there were no correlations observed between *P. falciparum* antigen-specific cytokine responses and age except for the TRAP-specific IL-5 responses, which had a significantly weak negative correlation with age (r_s = -0.2339, P = 0.0192) (Table 4.3). This demonstrates that TRAP-specific IL-5 responses decreased marginally with age.

When the frequencies of positive responses were assessed by age, IL-5 had overall low frequencies (< 6 %), children between the ages of 5 and 15 years had more responders than the other age groups, but this difference was not statistically significant (P>0.05) (Figure 4.13). The frequencies of IL-6 responders were similarly low (<12%) and those between the ages of 5 and 15 years had high frequencies in relation to the other age groups for all the *P. falciparum* antigens tested, but the difference was not statistically significant (P>0.05) (Figure 4.14). Those with positive IL-10 responses were few (<6%) and those in the ≥5 to <15 age bracket had relatively higher for CSP, LSA-1, MSP-1 and TRAP compared to other age groups though the differences observed were not statistically significant (P>0.05) (Figure 4.15). The frequencies of IFN-γ were relatively moderate in

the population (>12%). Older children above 15 years of age and adults below the age of 40 had more responders to AMA-1, MSP-1 and TRAP (>20%) relative to other age groups. However, the differences observed between the age groups were not significant ($P>0.05$) (Figure 4.16). Frequencies of TNF-α responses were relatively high to all *P. falciparum* antigens tested (>15%). However, frequencies of responses to TRAP were significantly higher in the older children (>15 years) and adults below 40 years of age ($P<0.05$) (Figure 4.17). Similarly, the frequencies of RANTES responses to all antigens tested except MSP-1 were high (>15%) for individuals between the ages of 15 and 40 years. However, the difference was not significant. ($P>0.05$) (Figure 4.18). Overall, individuals between the ages of 15 and 40 elicited more responses in comparison to the other age groups.

Table 4.2: Correlation Between Antigen-Specific Cytokine /Chemokine Levels and Age in April 2008

	IL-5		IL6		IL10		IFN-γ		TNF-α		RANTES	
Antigen	r_s	p^a	r_s	p	r_s	P	r_s	P	r_s	P	r_s	p
AMA-1	-0.1147	0.2559	-0.0841	0.4056	0.0723	0.4747	0.0062	0.9515	0.0606	0.5493	0.0733	0.4687
CSP	-0.1727	0.0858	-0.1483	0.1408	0.0789	0.4350	0.0021	0.9838	0.1046	0.3003	-0.1302	0.1968
LSA-1	-0.0552	0.5853	-0.1183	0.2409	-0.0257	0.7994	0.1148	0.2554	0.0318	0.7535	-0.0217	0.8305
MB2	-0.1773	0.1419	-0.1167	0.3361	0.0660	0.5870	-0.0887	0.4653	0.0418	0.7312	0.1300	0.2833
MSP-1	-0.0581	0.5660	-0.1037	0.3046	-0.1147	0.2559	0.0543	0.5917	-0.0288	0.7763	-0.1553	0.1230
TRAP	**-0.2339**	**0.0192**	0.0281	0.7811	0.0899	0.3736	0.0726	0.4731	0.0491	0.6276	0.0794	0.4325

[a]Spearman's rank correlation test (Values not adjusted for multiple comparisons). r_s = Spearman correlation coefficient. Values in bold indicate significant correlations.

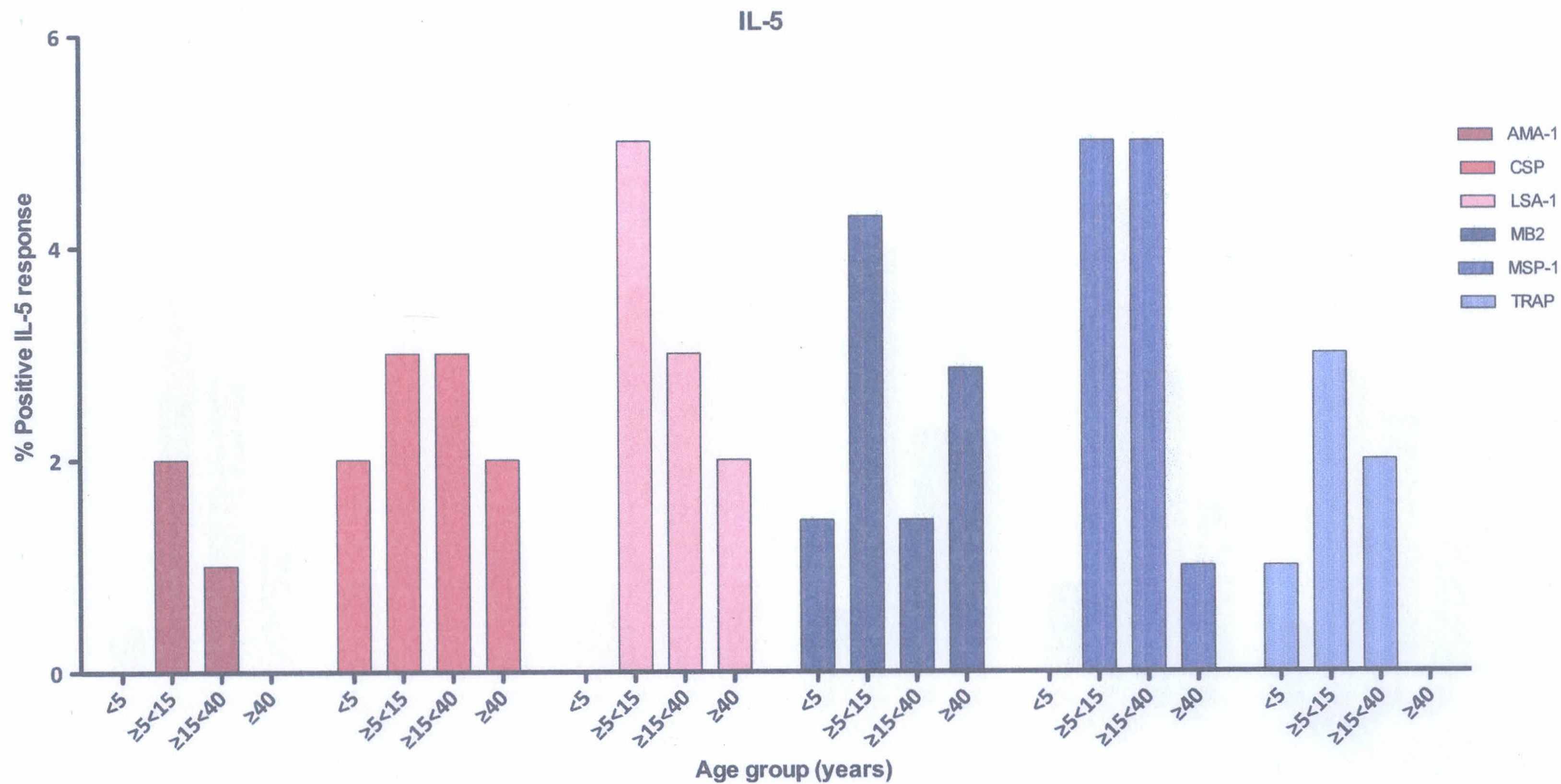

Figure 4.13: Frequency of positive IL-5 responses by age in April 2008 (Baseline).

IL-5 responders to *P. falciparum* AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP antigens.

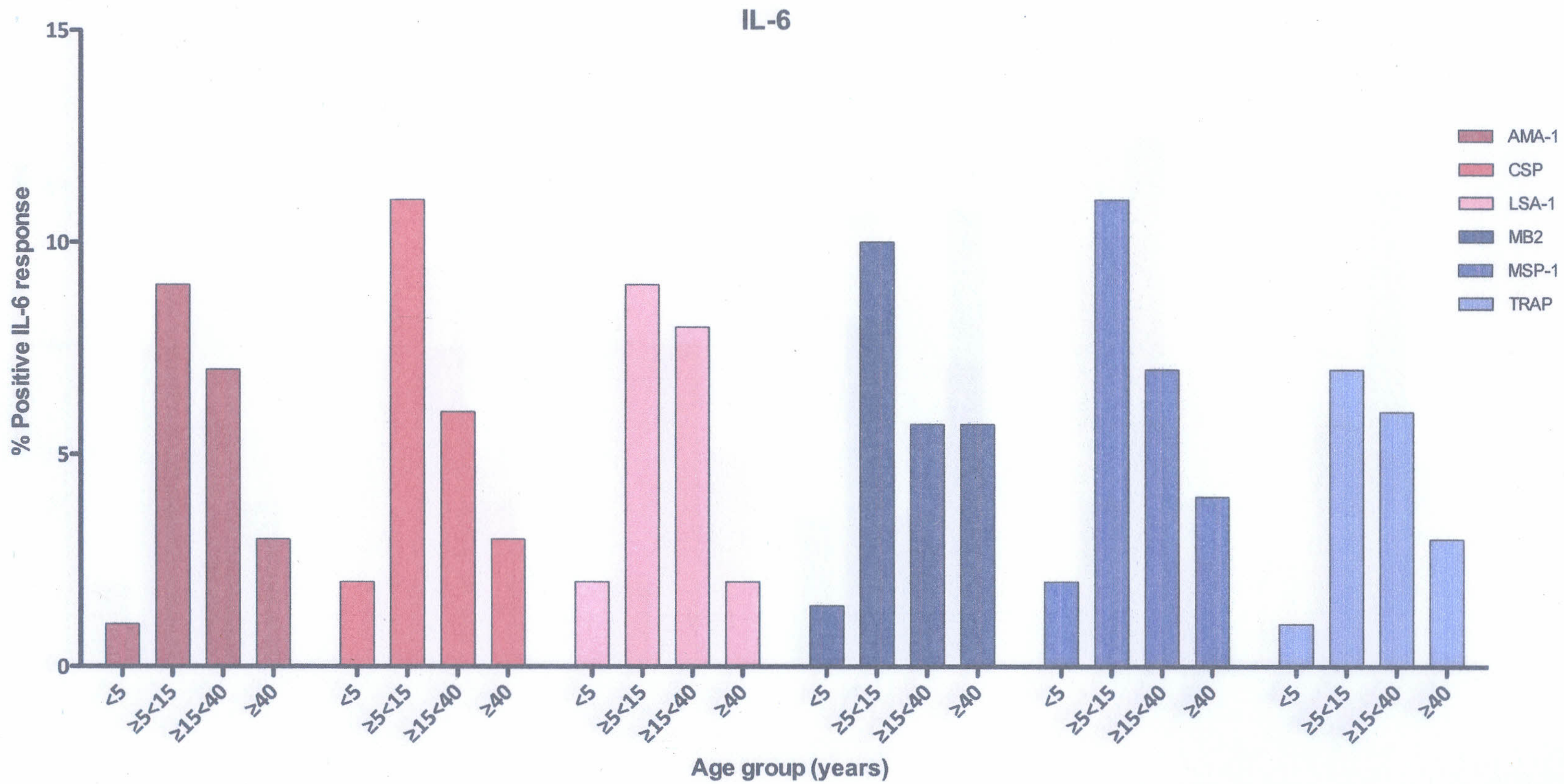

Figure 4.14: Frequency of positive IL-6 responses by age in April 2008 (Baseline).

IL-6 responders to *P. falciparum* AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP antigens.

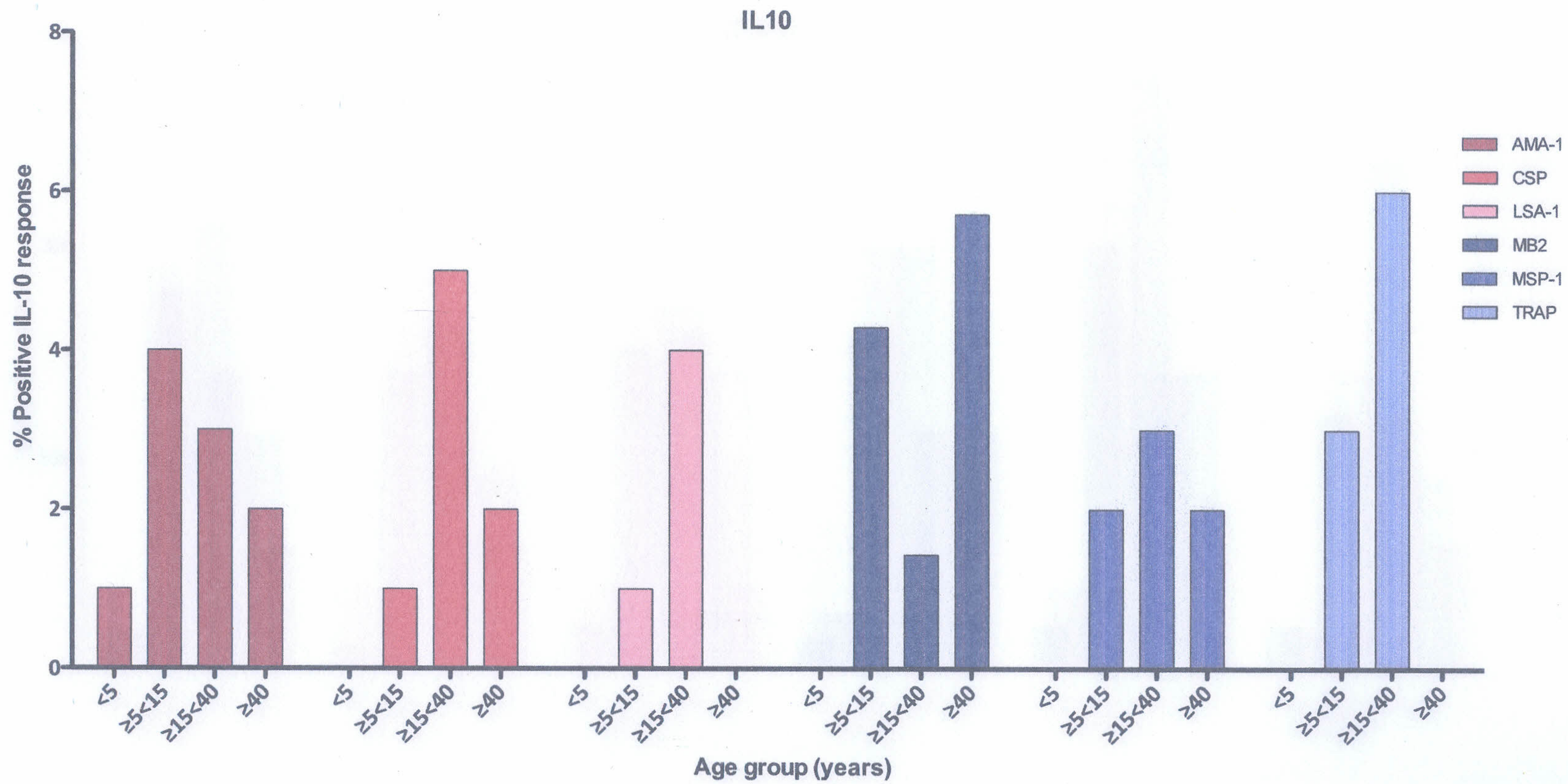

Figure 4.15: Frequency of positive IL-10 responses by age in April 2008 (Baseline).

IL-10 responders to *P. falciparum* AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP antigens.

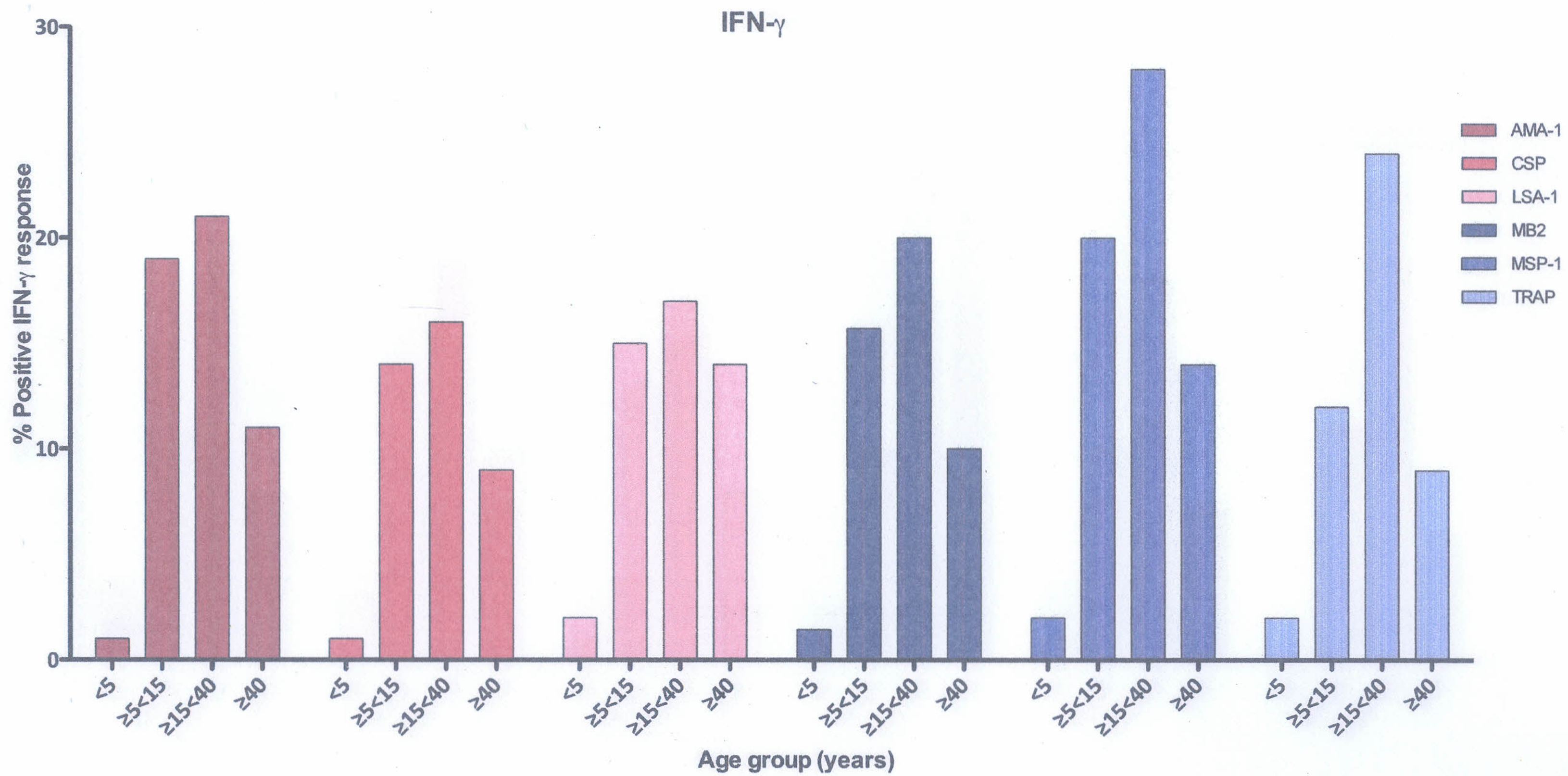

Figure 4.16: Frequency of positive IFN-γ responses by age in April 2008 (Baseline).

IFN-γ responders to *P. falciparum* AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP antigens.

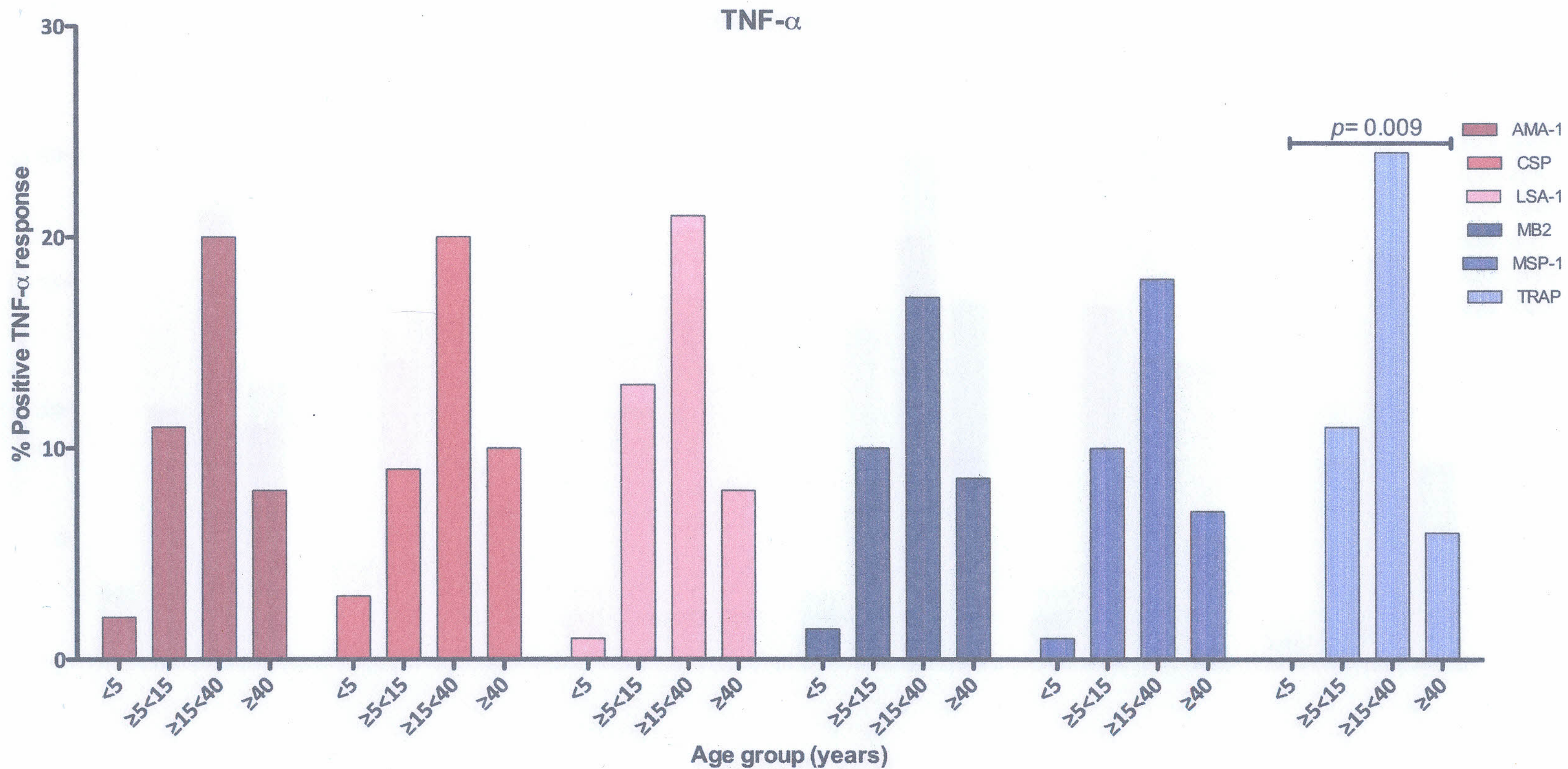

Figure 4.17: Frequency of positive TNF-α responses by age in April 2008 (Baseline).

TNF-α responders to *P. falciparum* AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP antigens. *P* value by Fischer's exact test.

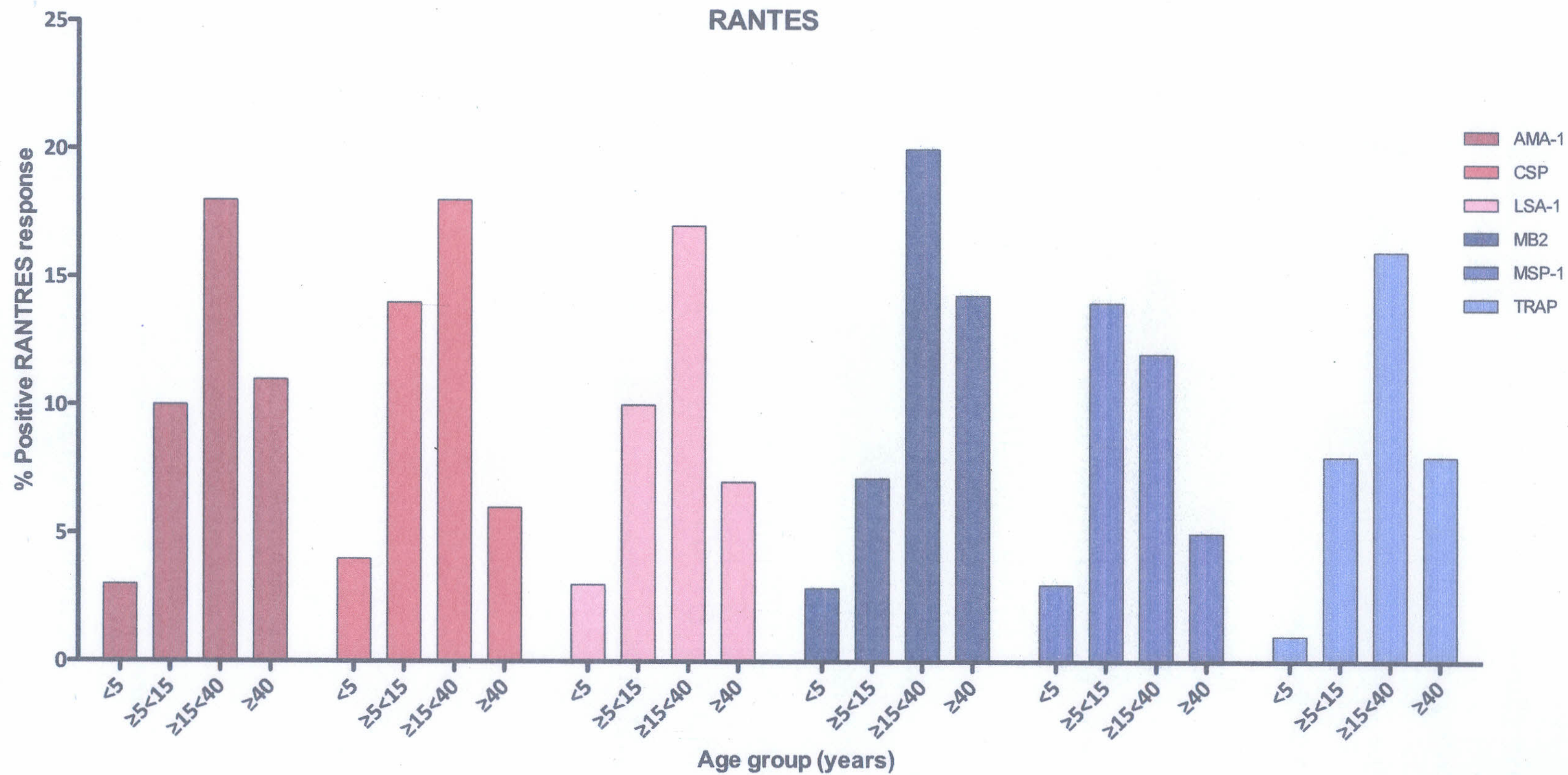

Figure 4.18: Frequency of positive RANTES responses by age in April 2008 (Baseline).

RANTES responders to *P. falciparum* AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP antigens.

4.4 Correlations Between Individual *P. falciparum* Antigen-specific Cytokine/Chemokine Responses

During *P. falciparum* infection, complex interactions between cytokine networks occur. Cytokines are known to act concomitantly rather than individually (Feghali and Wright, 1997). Cellular responses to most of the *P. falciparum* antigens positively correlated with each other. At baseline, significant correlations varied from weak ($r_s = 0.2061$) to moderate ($r_s = 0.5603$). For the AMA-1-induced cytokine responses, IL-6 correlated weakly with IFN-γ ($r_s = 0.2520$, $P < 0.05$), TNF-α ($r_s = 0.2426$, $P < 0.05$) and RANTES ($r_s = 0.2738$, $P < 0.01$), IFN-γ correlated with IL-10 ($r_s = 0.2177$, $P < 0.05$), TNF-α ($r_s = 0.2962$, $P < 0.01$) and RANTES ($r_s = 0.2165$, $P < 0.05$), while TNF-α correlated weakly with IL-10 ($r_s = 0.3581$, $P < 0.001$) but moderately with RANTES ($r_s = 0.5179$, $P < 0.01$) (Table 4.3).

The CSP-induced IL-5 responses correlated weakly with IL-10 ($r_s = 0.2061$, $P < 0.05$) and TNF-α ($r_s = 0.2570$, $P < 0.01$); IL-10 also correlated weakly with IFN-γ ($r_s = 0.2124$, $P < 0.05$) and moderately with TNF-α ($r_s = 0.4949$, $P < 0.001$); IL-6 correlated weakly with IFN-γ ($r_s = 0.3132$, $P < 0.01$) and RANTES ($r_s = 0.2365$, $P < 0.05$); IFN-γ correlated moderately with TNF-α ($r_s = 0.4739$, $p < 0.001$) but weakly with RANTES ($r_s = 0.3550$, $P < 0.001$); and TNF-α correlated weakly with RANTES ($r_s = 0.2353$, $P < 0.05$) (Table 4.3).

LSA-1-specific IL-6 responses correlated weakly with IFN-γ ($r_s = 0.2640$, $P < 0.01$) and TNF-α correlated weakly with IL-6 ($r_s = 0.2000$, $P < 0.05$), IFN-γ ($r_s = 0.3277$, $P < 0.001$) and RANTES ($r_s = 0.2019$, $P < 0.05$), but moderately with IL-10 ($r_s = 0.4448$, $P < 0.001$) (Table 4.3).

The MB2-induced cytokine IFN-γ responses correlated weakly with IL-6 ($r_s = 0.2640$, $P < 0.01$), IL-10 ($r_s = 0.3884$, $P < 0.001$), TNF-α ($r_s = 0.3605$, $P < 0.01$) and RANTES ($r_s = 0.3407$, $P < 0.01$), while TNF-α correlated moderately with IL-10 ($r_s = 0.5603$, $P < 0.001$) (Table 4.3).

The MSP-1-induced IL-6 responses correlated weakly with IFN-γ ($r_s = 0.2235$, $P < 0.05$), though TNF-α correlated moderately with IL-10 ($r_s = 0.4660$, $P < 0.001$) and weakly with IFN-γ ($r_s = 0.2733$, $P < 0.01$) (Table 4.3).

The TRAP-induced IFN-γ responses correlated weakly with IL-6 ($r_s = 0.3004$, $P < 0.01$), IL-10 ($r_s = 0.2960$, $P < 0.01$) and TNF-α ($r_s = 0.3883$, $P < 0.001$), TNF-α correlated weakly with IL-10 ($r_s = 0.3408$, $P < 0.001$) and RANTES ($r_s = 0.2636$, $P < 0.01$), similarly RANTES correlated weakly with IL-6 ($r_s = 0.2823$, $P < 0.01$) but negatively so with IL-5 ($r_s = 0.2028$, $P < 0.05$) (Table 4.3).

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Table 4.3: Correlations Between Individual Cytokine/Chemokine Levels in April 2008.

tigen / Cytokine	IL-5	IL-6	IL-10	IFN-γ	TNF-α
MA-1	r_s	r_s	r_s	r_s	r_s
IL-6	-0.1557				
IL-10	0.1060	0.1070			
IFN-γ	0.1693	**0.2520***	**0.2177***		
TNF-α	0.0295	**0.2426***	**0.3581*****	**0.2962****	
RANTES	0.0150	**0.2738****	0.0359	**0.2165***	**0.5179*****
SP					
IL-6	-0.0496				
IL-10	**0.2061***	-0.0726			
IFN-γ	0.1716	**0.3132****	**0.2124***		
TNF-α	**0.2570****	0.1879	**0.4949*****	**0.4739*****	
RANTES	-0.0328	**0.2365***	0.1234	**0.3550*****	**0.2353***
SA-1					
IL-6	0.0818				
IL-10	0.0942	-0.0360			
IFN-γ	0.1134	**0.2640****	0.0537		
TNF-α	-0.0155	**0.2000***	**0.4448*****	**0.3277*****	
RANTES	-0.1452	0.1397	0.0581	0.0833	**0.2019***
B2					
IL-6	0.0478				
IL-10	0.1551	0.1321			
IFN-γ	0.0539	**0.3162****	**0.3884*****		
TNF-α	-0.0201	0.1724	**0.5603*****	**0.3665****	
RANTES	-0.0043	0.1017	0.1288	**0.3407****	0.2131
SP-1					
IL-6	-0.0252				
IL-10	0.1050	0.0535			
IFN-γ	0.1502	**0.2235***	0.1908		
TNF-α	0.0309	0.1533	**0.4660*****	**0.2733****	
RANTES	-0.1471	0.1186	-0.1238	0.1773	0.1371
RAP					
IL-6	-0.0594				
IL-10	0.0118	0.0059			
IFN-γ	0.0132	**0.3004****	**0.2960****		
TNF-α	-0.1457	0.0944	**0.3408*****	**0.3883*****	
RANTES	**-0.2028***	**0.2823****	0.0550	0.1169	**0.2636****

r_s = Spearman correlation coefficient. Asterisks indicate P values, as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

At 6 months follow-up, there was strong correlation between IFN-γ and TNF-α [AMA-1 (r_s = 0.7248, $P < 0.001$), CSP (r_s = 0.7821, $P < 0.001$), LSA-1 (r_s = 0.7816, $P < 0.001$), MB2 (r_s = 0.7669, $P < 0.001$), MSP-1 (r_s = 0.6645, $P < 0.001$) and TRAP (r_s = 0.7892, $P < 0.001$)]; IL-6 and IFN-γ [AMA-1 (r_s = 0.6488, $P < 0.001$), MB2 (r_s = 0.6997, $P < 0.001$) and MSP-1 (r_s = 0.6228, $P < 0.001$)] and IL-6 and TNF-α [MB2 (r_s = 0.6490, $P < 0.001$)] (Table 4.4).

Moderate correlation was observed between IL-5 and IL-10 [MB2 (r_s = 0.4488, $P < 0.001$)]; IL-6 and IFN-γ [CSP (r_s = 0.4766, $P < 0.001$), LSA-1 (r_s = 0.5597, $P < 0.001$) and TRAP (r_s = 0.5982, $P < 0.001$)]; IL-6 and TNF-α [AMA-1 (r_s = 0.4748, $P < 0.001$), CSP (r_s = 0.4997, $P < 0.001$), LSA-1 (r_s = 0.5591, $P < 0.001$), MSP-1 (r_s = 0.4768, $P < 0.001$) and TRAP (r_s = 0.5717, $P < 0.001$)]; IFN-γ and RANTES [MB2 (r_s = 0.4652, $P < 0.001$) and TRAP (r_s = 0.4083, $P < 0.001$)] and TNF-α and RANTES [AMA-1 (r_s = 0.4073, $P < 0.001$), MB2 (r_s = 0.4526, $P < 0.001$) and TRAP (r_s = 0.4042, $P < 0.001$)] (Table 4.4).

Weak correlations was observed between IL-5 and IL-10 [AMA-1 (r_s = 0.3195, $P < 0.01$), LSA-1 (r_s = 0.2083, $P < 0.05$) and TRAP (r_s = 0.3045, $P < 0.01$)]; IL-5 and IFN-γ [CSP (r_s = 0.3561, $P < 0.001$), MB2 (r_s = 0.2037, $P < 0.05$) and TRAP (r_s = 0.3514, $P < 0.001$)]; IL-5 and TNF-α [CSP (r_s = 0.2575, $P < 0.01$), MB2 (r_s = 0.2062, $P < 0.05$) and TRAP (r_s = 0.2745, $P < 0.01$)], IL-5 and RANTES [CSP (r_s = 0.7821, $P < 0.001$)]; IL-6 and RANTES [TRAP (r_s = 0.2154, $P < 0.05$)], IL-10 and IFN-γ [AMA-1 (r_s = 0.2924, $P < 0.01$) and TRAP (r_s = 0.2700, $P < 0.01$)]; IL-10 and TNF-α [AMA-1 (r_s = 0.3131, $P < 0.01$), LSA-1 (r_s = 0.2980, $P < 0.01$), MB2 (r_s = 0.3448, $P < 0.001$), MSP-1 (r_s = 0.2492, $P < 0.05$) and TRAP (r_s = 0.2532, $P < 0.05$)]; IL-10 and RANTES [MSP-1 (r_s = 0.3066, $P < 0.01$)]; IFN-γ and RANTES [AMA-1 (r_s = 0.3966, $P < 0.001$), CSP (r_s = 0.2532, $P < 0.05$), LSA-1 (r_s = 0.3647, $P < 0.001$) and MSP-1 (r_s = 0.3675, $P < 0.01$] and TNF-α and RANTES [LSA-1 (r_s = 0.3222, $P < 0.001$) and MSP-1 (r_s = 0.3745, $P < 0.01$)] (Table 4.4).

Table 4.4: Correlations Between Individual Cytokine/Chemokine Levels in October 2008.

Antigen	Cytokine	IL-5	IL-6	IL-10	IFN-γ	TNF-α
AMA-1		r_s	r_s	r_s	r_s	r_s
	IL-6	0.0013				
	IL-10	**0.3195****	**0.3137****			
	IFN-γ	-0.0037	**0.6488*****	**0.2924****		
	TNF-α	0.0152	**0.4748*****	**0.3131****	**0.7248*****	
	RANTES	0.0385	0.1791	0.1721	**0.3966*****	**0.4073*****
CSP						
	IL-6	0.0422				
	IL-10	0.1315	0.1368			
	IFN-γ	**0.3561*****	**0.4766*****	0.1831		
	TNF-α	**0.2575****	**0.4997*****	0.1891	**0.7821*****	
	RANTES	**0.2779****	0.0298	0.1588	**0.2532***	0.1863
LSA-1						
	IL-6	0.1049				
	IL-10	**0.2083***	**0.2392***			
	IFN-γ	0.0803	**0.5597*****	0.1987		
	TNF-α	0.0692	**0.5591*****	**0.2980****	**0.7816*****	
	RANTES	-0.0150	0.1214	0.1090	**0.3647*****	**0.3222****
MB2						
	IL-6	0.0895				
	IL-10	**0.4488*****	0.1495			
	IFN-γ	**0.2037***	**0.6997*****	0.1571		
	TNF-α	**0.2062***	**0.6490*****	**0.3448*****	**0.7669*****	
	RANTES	0.1761	0.3427***	0.0462	**0.4652*****	**0.4526*****
MSP-1						
	IL-6	-0.0671				
	IL-10	0.1744	0.0223			
	IFN-γ	-0.1527	**0.6228*****	0.1640		
	TNF-α	-0.0762	**0.4768*****	**0.2492***	**0.6645*****	
	RANTES	-0.1313	0.0313	**0.3066****	**0.3675****	**0.3745****
TRAP						
	IL-6	0.0855				
	IL-10	**0.3045****	0.1094			
	IFN-γ	**0.3514*****	**0.5982*****	**0.2700****		
	TNF-α	**0.2745****	**0.5717*****	**0.2532***	**0.7892*****	
	RANTES	0.1009	**0.2154***	**0.2049***	**0.4083*****	**0.4042*****

r_s = Spearman correlation coefficient. Asterisks indicate P values, as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

At 12 months follow-up, strong correlations were observed between IL-5 and IL-10 [AMA-1 (r_s = 0.6748, $P < 0.001$) and MB2 ($r_s = 0.6534$, $P < 0.001$)] and IFN-γ and TNF-α [AMA-1 (r_s = 0.6580, $P < 0.001$), CSP ($r_s = 0.6300$, $P < 0.001$), LSA-1 ($r_s = 0.7270$, $P < 0.001$), MB2 (r_s = 0.7344, $P < 0.001$), MSP-1 ($r_s = 0.7306$, $P < 0.001$) and TRAP ($r_s = 0.7355$, $P < 0.001$)] (Table 4.5).

Moderate correlation was observed between IL-5 and IL-10 [CSP $r_s = 0.5436$, $P < 0.001$), IL-5 and IFN-γ [AMA-1 ($r_s = 0.5090$, $P < 0.001$) and CSP ($r_s = 0.4412$, $P < 0.001$)]; IL-6 and IFN-γ [AMA-1 ($r_s = 0.4199$, $P < 0.001$), CSP ($r_s = 0.4962$, $P < 0.001$), LSA-1 ($r_s = 0.4538$, $P < 0.001$), MSP-1 ($r_s = 0.4123$, $P < 0.001$) and TRAP ($r_s = 0.5072$, $P < 0.001$)]; IL-6 and TNF-α [AMA-1 ($r_s = 0.5639$, $P < 0.001$), CSP ($r_s = 0.4974$, $P < 0.001$), LSA-1 ($r_s = 0.45831$, $P < 0.001$), MSP-1 ($r_s = 0.4421$, $P < 0.001$) and TRAP ($r_s = 0.5557$, $P < 0.001$)]; IL-10 and IFN-γ [AMA-1 (r_s = 0.5246, $P < 0.001$) and CSP ($r_s = 0.5101$, $P < 0.001$)]; IL-10 and TNF-α [AMA-1 ($r_s = 0.4236$, $P < 0.001$), LSA-1 ($r_s = 0.4668$, $P < 0.001$) and MB2 ($r_s = 0.6645$, $P < 0.001$)] (Table 4.5).

Weak correlations was observed between IL-5 and IL-10 [LSA-1 ($r_s = 0.2994$, $P < 0.01$) and TRAP ($r_s = 0.3494$, $P < 0.001$)]; IL-5 and IFN-γ [CSP ($r_s = 0.4412$, $P < 0.001$), LSA-1 (r_s = 0.2322, $P < 0.05$), MB2 ($r_s = 0.2255$, $P < 0.05$) and TRAP ($r_s = 0.2737$, $P < 0.01$)]; IL-5 and TNF-α [AMA-1 ($r_s = 0.3721$, $P < 0.001$), CSP ($r_s = 0.2687$, $P < 0.01$), LSA-1 ($r_s = 0.2707$, $P <$ 0.01), MB2 ($r_s = 0.3911$, $P < 0.001$) and TRAP ($r_s = 0.3147$, $P < 0.01$)]; IL-5 and RANTES [TRAP ($r_s = 0.2125$, $P < 0.05$)]; IL-6 and IFN-γ [MB2 ($r_s = 0.3517$, $P < 0.001$)]; IL-6 and TNF-α MB2 ($r_s = 0.2679$, $P < 0.01$); IL-6 and RANTES [AMA-1 ($r_s = 0.2876$, $P < 0.01$), CSP (r_s = 0.3398, $P < 0.001$) and MSP-1 ($r_s = 0.2417$, $P < 0.05$)]; IL-10 and IFN-γ [LSA-1 ($r_s = 0.3312$, P < 0.001), MB2 ($r_s = 0.3029$, $P < 0.01$), MSP-1 ($r_s = 0.3925$, $P < 0.001$) and TRAP ($r_s = 0.3743$, $P < 0.001$)]; IL-10 and TNF-α [CSP ($r_s = 0.3667$, $P < 0.001$), MSP-1 ($r_s = 0.3600$, $P < 0.001$) and TRAP ($r_s = 0.3896$, $P < 0.001$)]; IL-10 and RANTES [AMA-1 ($r_s = 0.2768$, $P < 0.01$), CSP ($r_s = 0.2503$, $P < 0.05$), LSA-1 ($r_s = 0.2710$, $P < 0.01$) and TRAP ($r_s = 0.2901$, $P < 0.01$)];

IFN-γ and RANTES [AMA-1 (r_s = 0.2697 P < 0.01), CSP (r_s = 0.2788, P < 0.01), MB2 (r_s = 0.3103, P < 0.01) and MSP-1 (r_s = 0.2055, P < 0.05)] and TNF-α and RANTES [AMA-1 (r_s = 0.3796 P < 0.001), CSP (r_s = 0.3756, P < 0.001), LSA-1 (r_s = 0.2162, P < 0.05), MB2 (r_s = 0.3443, p < 0.001) MSP-1 (r_s = 0.3067, P < 0.01) and TRAP (r_s = 0.2012, P < 0.05)]. Very weak correlations were also observed between RANTES and IL-5 [MB2 (r_s = 0.1979, P < 0.05)] and between RANTES and IL-10 [MB2 (r_s = 0.1984, P < 0.05)] (Table 4.5).

Table 4.5: Correlations Between Individual Cytokine/Chemokine Levels in April 2009.

Antigen	Cytokine	IL-5	IL-6	IL-10	IFN-γ	TNF-α
AMA-1		r_s	r_s	r_s	r_s	r_s
	IL-6	0.1724				
	IL-10	**0.6748*** **	0.1684			
	IFN-γ	**0.5090*** **	**0.4199*** **	**0.5246*** **		
	TNF-α	**0.3721*** **	**0.5639*** **	**0.4236*** **	**0.6580*** **	
	RANTES	0.1368	**0.2876** **	**0.2768** **	**0.2697** **	**0.3769*** **
CSP						
	IL-6	0.1063				
	IL-10	**0.5436*** **	0.1650			
	IFN-γ	**0.4412*** **	**0.4962*** **	**0.5101*** **		
	TNF-α	**0.2687** **	**0.4974*** **	**0.3667*** **	**0.6300*** **	
	RANTES	0.1515	**0.3398*** **	**0.2503* **	**0.2788** **	**0.3756*** **
LSA-1						
	IL-6	0.0853				
	IL-10	**0.2994** **	0.0185			
	IFN-γ	**0.2322* **	**0.4538*** **	**0.3312*** **		
	TNF-α	**0.2707** **	**0.4583*** **	**0.4668*** **	**0.7270*** **	
	RANTES	0.0882	0.1905	**0.2710** **	0.1133	**0.2162* **
MB2						
	IL-6	-0.0345				
	IL-10	**0.6534*** **	-0.0493			
	IFN-γ	**0.2255* **	**0.3517*** **	**0.3029** **		
	TNF-α	**0.3911*** **	**0.2679** **	**0.4831*** **	**0.7344*** **	
	RANTES	**0.1979* **	0.1631	**0.1984* **	**0.3103** **	**0.3443*** **
MSP-1						
	IL-6	-0.0622				
	IL-10	0.1702	0.0056			
	IFN-γ	0.1803	**0.4123*** **	**0.3925*** **		
	TNF-α	-0.0294	**0.4421*** **	**0.3600*** **	**0.7306*** **	
	RANTES	-0.1505	**0.2417* **	0.1110	**0.2055* **	**0.3067** **
TRAP						
	IL-6	0.0510				
	IL-10	**0.3494*** **	0.0160			
	IFN-γ	**0.2739** **	**0.5072*** **	**0.3743*** **		
	TNF-α	**0.3147** **	**0.5557*** **	**0.3896*** **	**0.7355*** **	
	RANTES	**0.2125* **	-0.0105	**0.2901** **	0.1768	**0.2012* **

r_s = Spearman correlation coefficient. Asterisks indicate P values, as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

4.5 *P. falciparum* Antigen-specific Cytokine/Chemokine Responses and Haemoglobin Concentration

To further examine the association between secreted cytokines and anemia, the relationship between peripheral cytokines and Hb concentrations was determined for the entire population (n = 100). Overall, there was no pattern in the correlations observed though a few associations were observed between cytokine levels and Hb concentrations. Though IL-5, IL-6, IL-10 and IFN-γ responses were not significantly associated with Hb concentration; TNF-α response to CSP at baseline was correlated weakly with the Hb concentration (r_s = 0.2170; P = 0.0310) and to CSP and MB2 at 12 months follow-up was positively correlated with the Hb concentration (r_s = 0.2409; P = 0.0158 and r_s = 0.2451; P = 0.014, respectively) (Table 4.6), albeit weak. Similarly, RANTES response to AMA-1, CSP, LSA-1 and MSP-1 at 12 months follow-up was weakly correlated with Hb concentration (r_s = 0.2746; P = 0.0057, r_s = 0.2132; P = 0.0332, r_s = 0.2436; P = 0.0146 and r_s = 0.2313; P = 0.0206, respectively) (Table 4.6).

Table 4.6: Correlation Between Antigen-Specific Cytokine /Chemokine Levels and Haemoglobin Concentrations

	IL-5		IL6		IL10		IFN-γ		TNF-α		RANTES	
Apr 2008	r_s	p^a	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
AMA-1	0.1102	0.2774	-0.1766	0.0804	0.0882	0.3853	0.0523	0.6072	0.0234	0.8179	-0.0266	0.7937
CSP	0.0707	0.4867	-0.0895	0.3782	0.1559	0.1234	0.1242	0.2208	**0.2170**	**0.0310**	-0.0048	0.9622
LSA-1	0.0299	0.7690	-0.1624	0.1082	0.0329	0.7465	0.0475	0.6409	0.0890	0.3810	-0.0166	0.8708
MB2	0.2179	0.0700	-0.1173	0.3333	0.0996	0.4122	0.0778	0.5218	0.2105	0.0802	0.1024	0.3988
MSP-1	0.0757	0.4564	-0.1106	0.2758	0.666	0.5123	0.0433	0.6706	0.1013	0.3186	-0.0406	0.6898
TRAP	-0.0280	0.7833	-0.0348	0.7327	0.0031	0.9756	-0.0111	0.9133	0.0826	0.4165	0.0974	0.3375
Oct 2008												
AMA-1	-0.0226	0.8236	-0.0517	0.6092	0.0383	0.7051	-0.0215	0.8321	0.0138	0.8918	-0.0459	0.6502
CSP	-0.0375	0.7109	-0.1776	0.0771	-0.1365	0.1756	-0.0835	0.4087	-0.0726	0.4731	0.0872	0.3882
LSA-1	0.0983	0.3306	-0.0892	0.3776	-0.1519	0.1313	-0.1719	0.0872	-0.0444	0.6608	0.0609	0.5475
MB2	0.0520	0.6072	-0.1668	0.0971	-0.0396	0.6956	-0.1321	0.1901	-0.0759	0.4531	-0.5014	0.6112
MSP-1	0.0054	0.9647	-0.1378	0.2554	-0.1003	0.4086	-0.0008	0.9948	-0.0923	0.4473	-0.1345	0.2670
TRAP	0.0040	0.9683	-0.0361	0.7213	-0.1242	0.2184	-0.0200	0.8437	-0.0112	0.9117	-0.0818	0.4186
Apr 2009												
AMA-1	0.0345	0.7335	0.1484	0.1406	0.1193	0.2370	0.1074	0.2877	0.1754	0.0809	**0.2746**	**0.0057**
CSP	-0.0094	0.9260	0.1744	0.0827	0.1704	0.0901	0.2221	0.0264	**0.2409**	**0.0158**	**0.2132**	**0.0332**
LSA-1	-0.0033	0.9736	0.0614	0.5443	0.1037	0.3044	0.0286	0.7777	0.1059	0.2945	**0.2436**	**0.0146**
MB2	0.0328	0.7458	0.0718	0.4776	0.1275	0.2062	0.1576	0.1173	**0.2451**	**0.0140**	0.1375	0.1727
MSP-1	0.0509	0.6148	0.1693	0.0923	0.0224	0.8247	0.1575	0.1176	0.0816	0.4197	**0.2313**	**0.0206**
TRAP	0.0332	0.7428	0.0770	0.4464	0.0812	0.4222	0.0810	0.4231	0.1193	0.2373	0.0870	0.3896

[a]Spearman's rank correlation test (Values not adjusted for multiple comparisons). r_s = Spearman correlation coefficient. Values in bold indicate significant correlations.

As the balance between pro- and anti-inflammatory cytokine responses is more important than a single cytokine level, the association between the TNF-α/IL- 10 ratio and Hb concentration was further evaluated. The TNF-α to IL- 10 ratio was calculated for each participant. Cutoffs for anemia were defined as follows: age 0.5–4.9 years, 11.0 g/dL; age 5–11.9 years, 11.5 g/dL; age 12–14.9 years, 12 g/dL; non-pregnant females ≥15 years, 12.0 g/dL; and males ≥15 years, 13.0 g/dL (WHO, 2001). Individuals who had Hb concentrations below the cut off were considered anemic and those above non-anemic. Overall, the TNF-α/IL- 10 ratios were relatively low compared to earlier studies on the same. Statistical differences in the mean ratios between the anemic and the non-anemic were then evaluated. No significant relationships were seen between TNF-α:IL- 10 ratios and Hb in the anemic vs. non-anemic (geometric mean (range): 1.149 (0.000348 – 26913) vs. 2.850 (0.0000423 – 77602), respectively (Table 4.7).

Table 4.7: Comparisons Between TNF-α:L- 10 Ratios and Haemoglobin Concentrations

		TNF-α:IL- 10 ratio, geometric mean (range) pg/mL		
		Anemic	Non-anemic	*P**
Apr 08		(n=11)	(n= 89)	
	AMA-1	16.179 (0.00535 – 9082)	2.919 (0.00035 – 31730)	0.2948
	CSP	2.719 (0.00368 – 1461)	3.175 (0.00041 – 16586)	0.8634
	LSA-1	1.401 (0.00241 – 3061)	7.307 (0.00230 – 16665)	0.1460
	MB2	1.320 (0.00990 – 17491)	3.205 (0.000779 – 4822)	0.2878
	MSP-1	1.248 (0.00495 – 484)	2.850 (0.00069 – 10038)	0.4293
	TRAP	1.149 (0.00034 – 1291)	4.451 (0.000409 – 4978)	0.3668
Oct 08		(n=15)	(n=85)	
	AMA-1	8.203 (1.000 – 774)	10.121 (1.000 – 51865)	0.8197
	CSP	19.292 (0.0476 – 4024)	15.292 (0.037 – 5143)	0.7609
	LSA-1	22.339 (1.000 – 1611)	13.339 (1.000 – 29528)	0.6447
	MB2	104. 070 (1.000 – 8593)	24.883 (0.037 – 66530)	0.1123
	MSP-1	5.517 (1.000 – 1035)	8.981 (0.00207 – 10825)	0.6378
	TRAP	2.902 (1.000 – 3441)	10.989 (1.000 – 25525)	0.5993
Apr 09		(n=10)	(n=90)	
	AMA-1	12.940 (1.000 – 17633)	5.242 (0.00310 – 28994)	0.5269
	CSP	4.910 (0.00116 – 4081)	7.071 (0.000042 – 77602)	0.8453
	LSA-1	11.961 (1.000 – 17633)	9.356 (0.01492 – 12799)	0.9227
	MB2	7.415 (1.000 – 13283)	23.788 (0.00164 – 15932)	0.2768
	MSP-1	21.843 (1.000 – 2990)	6.996 (0.00105 – 13841)	0.1719
	TRAP	16.090 (0.0556 – 26913)	7.541 (0.000188 – 13247)	0.6597

*Wilcoxon rank sum test; n= number; cut off for anemia set as per WHO recommendations (WHO, 2001).

CHAPTER FIVE: DISCUSSION

This study set out to assess the cellular immune response to multiple *P. falciparum* antigens in an area of unstable malaria transmission in the highlands of Kenya and more particularly to establish whether these cellular responses were stable over a period of one year following a 14-month period when malaria transmission was interrupted. The study further investigated the role age plays in the development and persistence of cellular responses as well as the effect these responses have on the haemoglobin concentrations of individuals in this population. This study demonstrates that indeed even after the interruption of malaria transmission in an area of unstable malaria, individuals were still able to mount *P. falciparum* antigen-specific cytokine responses. Besides, it emerged that the stability of these responses were not antigen-specific but rather without a specific pattern. Further, it emerged that these responses were not age-specific nor did they influence the outcome of haemoglobin concentrations in the population.

5.1 Stability of *P. falciparum* Antigen-specific Cytokine Responses Over Time

Clinical immunity to malaria has been shown to wane quickly in the absence of active malaria transmission (Langhorne *et al.,* 2008a; Teirlinck *et al.,* 2011), but the reasons for this loss of clinical immunity are not well characterized. The present study shows that *P. falciparum*-specific cytokine responses associated with protection against clinical malaria in previous studies (IFN-γ, IL-10, TNF-α) decrease within 24 months of reduction of malaria incidence to low levels (<2 cases/1000 person-months), but responses associated with an increased risk of clinical malaria (IL-6) are unchanged over this time period.

Previous studies have shown that responses of the pro-inflammatory cytokines IFN-γ and TNF-α and the anti-inflammatory cytokine IL-10 to parasitized red blood cells (pRBC) or to specific *P. falciparum* antigens are associated with protection from clinical malaria. Studies

from Kenya (John *et al.,* 2004; Todryk *et al.,* 2008) and Gabon (Luty *et al.,* 1999) showed an association of IFN-γ responses to *P. falciparum* pre-erythrocytic (CSP, LSA-1, TRAP) and blood stage (MSP-1) antigens with protection from malaria, while a study from Papua New Guinea showed an association of IFN-γ responses to pRBC (D'Ombrain *et al.,* 2008; McCall and Sauerwein, 2010) with protection from malaria. Similarly, TNF-α responses to LSA-1 (Migot-Nabias *et al.,* 2000) and pRBC (Ramharter *et al.,* 2004; Robinson *et al.,* 2009), and IL-10 responses to LSA-1 (Kurtis *et al.,* 1999) and pRBC (Couper *et al.,* 2008) have been associated with protection from malaria. Murine studies and *in vitro* testing suggest that these cytokines could play a role in host defense against malaria. Murine models show that IFN-γ can work at the sporozoite level by inducing nitric oxide synthase (Seguin *et al.,* 1994), at the liver stage by inhibiting parasite growth and development in hepatocytes (Tsuji and Zavala, 2001) and at the blood stage by increasing the phagocytic action of macrophages (Yoneto *et al.,* 1999). IL-10 can affect parasite clearance (Hugosson *et al.,* 2004), can act on monocytes/macrophages to inhibit antigen presentation and down-regulate excessive inflammation (Li *et al.,* 1999), or may regulate TNF-α (Ho *et al.,* 1995), IL-12 (Luty *et al.,* 2000) or IFN-γ (Winkler *et al.,* 1998), all of which can adversely affect the parasite. TNF-α can inhibit hepatic development of *P. falciparum* (Depinay *et al.,* 2011; Mordmuller *et al.,* 1997), mediate parasite killing by macrophages (Bouharoun-Tayoun *et al.,* 1995). Alternatively, antigen-specific IL-10, TNF-α and IFN-γ responses could be surrogate markers for other protective immune responses. In either case, the findings suggest that antigen-specific IL-10, TNF-α and IFN-γ responses should be further studied as potential markers of loss of clinical protection from malaria in populations that have had a prolonged absence of *P. falciparum* exposure.

An earlier study documented that IL-6 responses to pRBC were associated with an increased risk of clinical malaria (Robinson *et al.,* 2009), and excess IL-6 has been associated with

more severe disease in malaria (Rovira-Vallbona *et al.,* 2012). It is unclear how IL-6 production would lead to increased risk of malaria. One might predict the opposite effect, as high levels of IL-6 can lead to iron deficiency (through induction of hepcidin, which blocks release of iron from macrophages (Nemeth *et al.,* 2004), and iron deficiency is associated with protection from malaria. There are no studies published to date on *P. falciparum* antigen-specific RANTES responses in individuals in malaria endemic areas, but studies of serum RANTES levels show that they are decreased in severe disease (John *et al.,* 2006) and remain decreased in children with prior severe illness (Ochiel *et al.,* 2005), suggesting that children with severe malaria have an impaired ability to produce RANTES. In the present study, antigen-specific RANTES responses were maintained in the absence of high-level malaria transmission, suggesting that impaired ability to produce RANTES may not be a primary factor in susceptibility to malaria in this highland population.

In the present study, malaria was interrupted for approximately a year prior to the first collection, and was present subsequently at very low levels (<1 malaria case per 1000 person months) until the month of final collection. In addition, within the cohort, rates of asymptomatic parasitemia by microscopy and PCR during collection times were low (<3%). The presence of two individuals with asymptomatic parasitemia at the last collection suggests that at that time, the study area had low but not completely absent transmission. This could potentially explain the slight increase in some responses (notably IL-10 responses to some antigens) at the last collection.

An earlier study assessing cellular immune responses after experimental human malaria induced with *P. falciparum*-infected mosquitoes demonstrated that IFN-γ responses to whole sporozoites or to *P. falciparum*-infected RBC remained present 14 months after even a single infection (Teirlinck *et al.,* 2011), while a second study of cellular immune responses in naturally exposed individuals in Thailand documented short-lived IFN-γ effector

responses to *P. falciparum* schizont extract (*Pf*SE) but long-lived IL-10 memory T cell responses (>6 years) to *Pf*SE (Wipasa *et al.,* 2011). This study assessed responses to peptides from vaccine-candidate antigens, while the experimental human study assessed responses to the whole sporozoite, and the Thai study assessed responses to schizont extract, which also contains many antigens. These differences make it difficult to compare findings of the studies. The cytokine responses in this study are more specific than in those previously studied (Teirlinck *et al.,* 2011; Wipasa *et al.,* 2011), but are likely weaker and less frequent, since the number of epitopes presented are far fewer. The findings of this study are relevant to considerations for multiple epitope or multiple antigen vaccines, but are likely not as relevant to, for example, a whole sporozoite vaccine. In the present study, antigen-specific IFN-γ and IL-10 levels decreased in the absence of transmission. These findings are similar to those of a study conducted in a different highland area of Kenya (John *et al.,* 2000), but differ from studies conducted in a malaria holoendemic area of Kenya (Moormann *et al.,* 2006), and in the same highland area as in the present study at two time points of much higher malaria incidence (Moormann *et al.,* 2009). In those studies, IL-10 levels did not differ significantly over time. Taken together, the findings of this study suggest that some minimum level of malaria transmission is required for persistence of responses.

However, the level of malaria transmission required to sustain cytokine responses that have correlated with protection from malaria, such as IFN-γ, TNF-α, and IL-10 responses, is not known. Based on previous studies and the current study, it appears that in populations of low transmission, the absence of clinical malaria incidence is a crude indicator that malaria transmission is below the required level, and that malaria incidence at some level between 2 cases/1000 person-months (the maximum for this period of study) and 15 cases/1000

person-months (the minimum at the times of collection in the past study) reflects the necessary transmission level to sustain IFN-γ and IL-10 responses.

5.2 Lack of Correlation Between *P. falciparum* Antigen-specific Cytokine/ Chemokine Responses and Age

Age plays an essential role in the development of natural immunity to *P. falciparum* malaria, besides exposure to infection. While clinical immunity to malaria has been shown to develop with age, the exact age at which clinical immunity is fully acquired however, varies depending on the level of exposure (Gatton and Cheng, 2004). In areas of stable high-level malaria transmission, clinical immunity develops earlier in children from those areas experiencing less frequent episodes of malaria after the age of 5 years (Gatton and Cheng, 2004), while people in areas of unstable low-level malaria transmission may never develop clinical immunity (Noland *et al.,* 2008). In Gabon and Papua New Guinea, both malaria endemic areas, age-related increases in T-cell immune response to *P. falciparum* (Winkler *et al.,* 1999), and *P. vivax* (Xainli *et al.,* 2002) were reported. More recent data from Mozambique suggests that the ability to regulate pro-inflammatory and anti-inflammatory responses varied by age and/or exposure (Moncunill *et al.,* 2013).

The current study shows that cellular immune response to the six *P. falciparum* antigens was not influenced by the age of the individuals producing them. This study is consistent with studies conducted in areas of similar transmission pressures in Mali and Uganda, which have suggested that the risk for uncomplicated malaria may remain constant with age (Dicko *et al.,* 2007). However, it contrasts other studies, which have demonstrated an age-related acquisition of clinical immunity (Gbedande *et al.,* 2013; Gerritsen *et al.,* 2008) and studies by our group in this highland area (Kipsamoite) in a period of higher transmission (John *et al.,* 2004; John *et al.,* 2000; Rolfes *et al.,* 2012). This may have been due to the absence of persistant exposure to the malaria parasite and hence no acquisition of clinical immunity that

would normally develop by age of 5 years. The findings of this study therefore demonstrates that for the development of cellular immune responses to *P. falciparum* AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP antigens in this population, repeated and prolonged exposure is essential in all age groups alike.

5.3 Associations Between *P. falciparum* Antigen-specific Cytokine Responses

Cytokines have been shown to be involved in the control of *P. falciparum* infection (Hoffman *et al.,* 1997), however, the excessive production of pro-inflammatory cytokines may be pathological (Mackintosh *et al.,* 2004). Pro-inflammatory cytokines are regulated by anti-inflammatory cytokines such as IL-10 or transforming growth factor (TGF)-β1, which have been shown to be a key factor in preventing acute pathology (Perkins *et al.,* 2000). The balance between pro-inflammatory and anti-inflammatory cytokines is thought to determine the outcome of *P. falciparum* infection (Day *et al.,* 1999; Prakash *et al.,* 2006).

Overall, this study shows weak or moderate correlations within cytokine responses, with exception of TNF-α and IFN-γ. During the early stages of the host's immune response to *P. falciparum* malaria, pro-inflammatory cytokines such as TNF-α and IFN-γ are secreted (Robinson *et al.,* 2009). Both have been shown to play a protective role during malaria infection (D'Ombrain *et al.,* 2008; Mordmuller *et al.,* 1997) and a recent study suggested that their synergistic action assist in the effective clearance and killing of the parasite to resolve malaria infection (Sinha *et al.,* 2010). Importantly, these findings are consistent with the present study, which demonstrates a strong correlation between the two pro-inflammatory cytokines, confirming the synergistic action between TNF-α and IFN-γ responses to *P.falciparum.*

Despite being protective, the overproduction of these two pro-inflammatory cytokines has been associated with severe manifestations of the disease (Boeuf *et al.,* 2012; Lyke *et al.,*

2004; Perkins *et al.,* 2000) and have been shown to contribute to bone marrow suppression, dyserythropoiesis, and erythrophagocytosis thereby exacerbating malarial anemia pathogenesis (Clark and Cowden, 2003). Conversely, no one presented with clinical disease, and as such, this study could not ascertain whether the associations between these antigen-specific cytokines and the resulting outcomes were affected by the interruption of malaria transmission in an area of unstable malaria transmission.

5.4 Effect of *P. falciparum* Antigen-specific Cytokine Responses on Haemoglobin Concentrations

P. falciparum infection is believed to be a major contributing factor to the etiology of severe anemia witnessed in malaria endemic areas (Desai *et al.,* 2005; Koram *et al.,* 2000; Perkins *et al.,* 2011), in spite of anemia having several other causes. In malaria-endemic areas, the intensity of *P. falciparum* transmission correlates with the incidence of severe anemia as well as with age -specific rates of anemia (Desai *et al.,* 2005). More particularly, cytokine response to malaria infection has been implicated affecting haemoglobin concentration and thus the development of malarial anemia (Biemba *et al.,* 2000; Ekvall, 2003). Two separate studies conducted in a malaria-endemic area of Kenya found that reduced circulating levels of RANTES were associated with SMA in children (Ochiel *et al.,* 2005; Were *et al.,* 2006) and the suppression of erythropoiesis (Were *et al.,* 2006). Consistent with these findings, the current study showed high RANTES levels, which correlated positively with haemoglobin levels. This suggests that there was no suppression of erythropoiesis and thus no likelihood of SMA cases in the population. Elevated levels of TNF-α have been associated with inhibition of erythropoiesis and SMA in young children with *P. falciparum* malaria (Boeuf *et al.,* 2012). Several studies have found high circulating levels of pro-inflammatory cytokines TNF-α and IFN-γ to be associated with SMA (Biemba *et al.,* 2000; Kurtzhals *et al.,* 1998). Furthermore, these elevated levels have been associated with low

haemoglobin levels (Boeuf *et al.,* 2012). This study is consistent with the findings of these studies as it demonstrated positive correlation between the levels of TNF-α and haemoglobin levels, suggesting that erythropoiesis was not inhibited and that the occurrence of SMA was unlikely in the population. The observation of RANTES and TNF-α levels weakly correlating with haemoglobin levels suggests that this population might be protected against SMA.

Previous studies have revealed decreased Hb levels with elevated TNF-α levels (Sarangi *et al.,* 2014). Studies have also shown that high levels of TNF-α in relation to IL-10 levels were associated with severe anemia, whereas a high IL-10/TNF-α ratio was associated with protection (Boeuf *et al.,* 2012; Othoro *et al.,* 1999). Though the levels were relatively lower, the present study demonstrates high TNF-α/IL-10 ratios consistent with the previous study in Ghana, with no significant ratio differences being observed between the anemic and non-anemic individuals (Boeuf *et al.,* 2012). The lower IL-10 levels in relation to the TNF-α levels suggests that this population is predisposed to severe disease as demonstrated by a previous study conducted in Kenyan children (Othoro *et al.,* 1999) though this study may be due to the relatively few cases of anemia (<16) detected in the study.

CHAPTER SIX: SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of Findings

1. a) Levels of IL-6, and RANTES responses to almost all *P. falciparum* antigens were stable, while levels of IL-5, IL-10 IFN-γ, and TNF-α to most *P. falciparum* antigens decreased during the period when malaria transmission was interrupted.

   b) Frequencies of positive IL-6, and RANTES responders to almost all *P. falciparum* antigens were stable, while frequencies of positive IL-5, IL-10 IFN-γ, and TNF-α to most *P. falciparum* antigens decreased significantly during the period when malaria transmission was interrupted.

2. No correlation was observed between the levels of cytokines secreted in response to the *P. falciparum* antigens (AMA-1, CSP, LSA-1, MB2, MSP-1 and TRAP) and age at baseline during the period of interrupted malaria transmission.

3. There were weak or moderate correlations within the cytokines with exception of TNF-α and IFN-γ response that had strong correlations to all *P. falciparum* antigens.

4. a) There was weak correlation between the *P. falciparum*-specific RANTES and TNF-α responses with haemoglobin concentrations during the period of interrupted malaria transmission.

   b) There was no difference in the TNF-α:IL-10 ratio between the anemic and the non-anemic groups.

6.2 Conclusions

1. *P. falciparum*-specific cytokine responses, previously documented to be correlates of protection against clinical malaria (IL-5, IFN-γ, IL-10, TNF-α) decrease, while *P. falciparum*-specific cytokine responses associated with increased risk of clinical malaria (IL-6) and suppression of erythropoiesis (RANTES) are unchanged after a prolonged period of very low malaria transmission.

2. Acquisition of *P. falciparum* antigen-specific cytokine/chemokine response during periods of interrupted malaria transmission in an area of low malaria transmission is not age-specific at baseline.

3. Weak to moderate correlation between the cytokines, with strong correlation between IFN-γ and TNF-α to *P. falciparum* antigen even after periods of interrupted malaria transmission.

4. There is no correlation between antigen-specific cytokine responses or TNF-α:IL-10 ratios and haemoglobin concentration in an area of low malaria transmission.

6.3 Recommendations from The Current Study

In the light of the results of this study, the following are the recommendations:

1. For T-cell (cytokine/chemokine) responses to be maintained in this area of unstable transmission, frequent boosting with antigens will be necessary.

2. When developing a malaria vaccine for this population, an ideal vaccine may be administered at a comparable dosage.

3. Assessment of the relationships between antigen-specific pro- and anti-inflammatory cytokines alone is not adequate and may need to be done in the context of clinical malaria outcomes to better inform vaccine development studies.

4. In this population, antigen-specific cytokine response in relation to haemoglobin concentration may not be adequate as a marker for sever disease or malaria complication

6.4 Recommendations for Future Research

1. Future studies should investigate the cellular sources of antigen-specific cytokines to determine which ones would be affected by the interruption of malaria.
2. Future studies should investigate antigen-specific IFN-γ, TNF-α and IL-10 responses as biomarkers of increased population-level susceptibility to malaria after prolonged lack of *P. falciparum* exposure.
3. Future studies should investigate how *P. falciparum* antigen-specific cytokine responses relate to clinical disease.
4. Future studies should evaluate the difference in antigen-specific cytokine responses during high and low transmissions.

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