EFFECT OF IRON DEFICIENCY AND IRON DEFICIENCY ANEMIA ON NATURAL IMMUNITY TO MALARIA INFECTIONS AND MALARIA VACCINE RESPONSE IN KILIFI CHILDREN

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Effect of Iron Deficiency and Iron Deficiency Anemia on Natural Immunity to Malaria Infections and Malaria Vaccine Response in Kilifi Children

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This work is dedicated to my husband, Mr. Linus Muthuri and my daughter Keren Pendo for supporting and giving me an easy moment during my studies.

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LIST OF ABBREVIATION

AMA Apical Membrane Protein.

EBA Erythrocyte Binding Antigen.

EDTA Ethylenediaminetetraacetic acid

CRP C-reactive protein

DMT1 Divalent metal transporter 1

FNP Ferroportin.

ELISA Enzyme Link Immunoassay.

MSP Merozoite Binding protein

sTfR Soluble transferrin receptor

TNF-α Tumor necrosis factor-α

TS Transferrin saturation

RON 2 Rhoptry neck protein

Nramp2 Natural Resistance-Associated Macrophage Protein 2

WHO World Health Organization

ABSTRACT

In Kenya, the prevalence of iron deficiency (ID) has been reported to be as high as 35%. ID has been associated with impaired immunity thus increasing susceptibility to infectious diseases. In addition, it has been shown to lower vaccine responses to some childhood vaccines such as measles, diptheria-pertussis and influenza. On the other hand, malaria infection caused by *Plasmodium falciparum* remains a public health problem causing about half a million deaths annually However, little is known about how ID might affect acquisition of natural immunity to malaria and malaria vaccine responses. The objective of this study was to determine the effects of iron deficiency on acquisition of antibodies to malaria and malaria vaccine responses. To achieve these malaria antibodies, iron and inflammatory biomarkers from community-based children were assayed. ID was defined using two definitions a) low ferritin (WHO definition) and b) low transferrin saturation (TSAT) $< 10\%$. Malaria antibodies were measured using enzyme-linked immunosorbent assay (ELISA), including apical merozoite antigen1 (AMA1), merozoite surface antigens 1 and 2 (MSP1 & 2) and schizont in community-based children (n=1,160) and anti-circumsporozoite (CSP) antibodies in RTSS-vaccinated children (n=115). For ME-TRAP-vaccinated children (n=91), interferon gamma (IFN $_{\gamma}$) producing peripheral blood mononuclear cells (PBMCs) were assayed by enzyme-linked immunosorbent spots (ELISPOTs). Student's t-tests, univariate and adjusted multivariate linear regression models were used to determine the effect of ID on natural immunity. The overall prevalence of ID was 33% using the WHO definition and 48% using the low TSAT definition. Using unadjusted linear regression models, there was a significant association between ID (as defined by low ferritin) and AMA1 (β -1.16, 95% CI -1.16; -0.97, $p < 0.001$) and MSP1 (β -0.83, 95% CI -1.02; -0.64, $p < 0.001$). Low TSAT was associated with lower AMA1 (β -0.81, 95% CI -0.99; -0.62, $p < 0.001$) and MSP1 (β -0.45, 95% CI -0.63; -0.28, $p < 0.001$) antibodies in children with TSAT $<10\%$ compared to children with TSAT$>10\%$. In the adjusted model antibody levels to AMA1 (β -0.42, 95% CI -0.59; -0.25, $p < 0.001$) and MSP1 (β -0.40, 95% CI -0.60; -0.20, $p < 0.001$) were significantly lower in ID compared to iron replete (IR) children. However, with the low TSAT definition, only antibodies to AMA1 (β -0.21, 95% CI -0.36; -0.05, $p = 0.008$) remained significantly lower in ID children compared to IR children. were obtained. ID using both definitions was also associated with non-significantly lower antibody responses to the RTSS,S' vaccine at 3, 6 and 14 months after vaccination. However, iron deficient (low TSAT definition) children had higher IFN_{γ} concentrations at 6 months after the ME-TRAP vaccine (β 0.38, 95% CI 0.10; 0.65 $p = 0.01$). In conclusion, ID decreases malaria antibodies acquisition in children, which may decrease their protection and recovery from malaria. Vaccine responses were not significantly affected by ID in children receiving the RTSS,S' vaccine. However, ID children had higher IFN_{γ} responses to the ME-TRAP malaria vaccine. Strategies to prevent and treat iron deficiency may improve natural immunity to malaria for children living in Kenya.

CHAPTER ONE

INTRODUCTION

1.1 Background

Iron deficiency (ID) is the most common micronutrient deficiency in the world affecting approximately one billion people, almost 25% of the total population(Kotze et al., 2009; Oppenheimer, 2001). The main groups at risk are children with a prevalence of roughly 43% and pregnant women at 38% according to a report released in 2015 (WHO, 2015). In Kenya, the prevalence of ID has been reported to be as high as 35% among children (Muriuki et al., 2020). ID is associated with poor child growth with effects such as impaired brain development and long-term impairment of behavioral and cognitive performance(Beard, 2007; Lozoff, 2011). In addition to these defects, ID has been shown to impair the immune system thus increasing susceptibility to infectious diseases (Beard, 2001; Jabara et al., 2016).

Malaria caused by *P. falciparum* is the leading cause of mortality and morbidity particularly in children globally. According to the WHO 2020 malaria report, between 2019 and 2020, estimated malaria cases increased from 213 million to 228 million, and deaths from 534 000 to 602 000 in the WHO African Region (World Health Organization, 2021a). Additionally, Africa accounted for 96% of deaths globally with 80% of all deaths in this region being among children aged under 5 years (World Health Organization, 2021a). Repeated exposure to *P. falciparum* has been shown to be associated with the development of immunity against severe malaria infections however sterile immunity is not usually achieved (Ndungu et al., 2002; Osier et al., 2008). Both cell-mediated immunity and humoral immunity play key roles in malaria infection. Antibodies to merozoites are protective against clinical disease by inhibiting blood stage replication and preventing high-density parasitemia (Richards et al., 2010; Teo et al., 2016). Some of these antibodies include anti-AMA1, anti-EBA $_{175}$, anti-MSP1, anti-MSP2, anti- MSP3 and many others that have been associated with malaria protection

(Beeson et al., 2016). These antibodies differ in the quantity produced, specificity and affinity to malaria antigens (Stirnadel et al., 2000). In cellular immunity, immunity to malaria has been linked to the availability and function of helper CD4+ T cells, cytotoxic CD8+ T cells and γδ T cells that can respond to both the asymptomatic liver stage and the symptomatic blood stage of *P. falciparum* infection (Kurup et al., 2019).

The precise role of iron in immune regulation especially in children vulnerable to ID is not fully known (Das et al., 2014). However, it has been shown to play an important role in the development and function of the immune system. ID affects the capacity to have an adequate immune response that is necessary for immune proliferation and the generation of specific responses to infection (Beard, 2001; Jabara et al., 2016). Many studies have been conducted with the aim of revealing the relationship between iron and immune status however, with conflicting reports. Humoral immunity has been shown to be affected by ID. A study by Jabara et al reported that mutation of transferrin receptor 1 which is important for cellular iron uptake leads to failure to express a 1µ-Cε transcript that is required for immunoglobulin class switching. Consequently, children with the mutated gene had no circulating immunoglobulins (Jabara et al., 2016). Iron has also been reported to be important in B cell proliferations that leads to generation of antibodies (Jiang et al., 2019). Other studies have also reported a decrease of one or more immunoglobulin (Hassan et al., 2016; Tang et al., 2006).In contrast, some studies have reported no difference in immunoglobulin concentrations between ID and IR individuals (Das et al., 2014; Ekiz et al., 2005).

In line with the effects of ID on the immune system many studies have reported the co-occurrence of ID and increased risk of infection. Several chronic diseases have been associated with iron deficiency anemia (IDA) including chronic kidney disease, chronic heart failure, cancer, inflammation and bowel diseases (Jonker & Boele,2014; Lopez et al., 2016). Mean concentrations of serum IgG4 and IgG1, and Pneumococcal specific IgG1, IgG2 antibodies were decreased in children with ID which was thought to lead to recurrent pneumococcal infections (Feng et al., 1994). In addition, Azab et al., reported

that healthy children with IDA were 3.8 times likely to develop stroke compared to healthy children without IDA (Azab et al., 2014).

Despite the huge burden of ID and malaria infection in African children, very little is known about how ID affects immunity to malaria. Malaria infection and ID share the same geographical area and children suffer the largest burden (Matsuzaki-Moriya et al., 2011).A study by Nyakeriga et al reported lower malaria-specific IgG in children with ID compared to children without (Nyakeriga et al., 2004) but the effects of ID on specific malaria antibodies has not been explored. In a mouse model, mice with induced IDA had lower total IgG antibodies against *Plasmodium yoelii* compared to control mice without IDA (Matsuzaki-Moriya et al., 2011). Despite the few studies on ID and malaria immunity, we have some studies that have been carried out exploring the relationship between nutritional status and immunity to malaria. A study carried out in Papua New Guinea showed that wasted children had lower antibodies to MSP1 and MSP2 alleles compared to well-nourished children (Genton et al., 1998). Malnourished children have also been reported to have 33% lower total IgG to schizont antigen compared to non-malnourished children (Fillol et al., 2009).

Public health strategies for malaria in endemic countries aim to prevent transmission of the disease and control the vector (Gachelin et al., 2018). Vaccines play a key role in public health by protecting against infection. There is much effort has been put in to developing various types of malaria vaccines (Burns, 2018; Draper et al., 2018). Two malaria vaccines have been trialed in Kilifi County children: FFM ME-TRAP and RTS,S/AS01E. The FFM ME-TRAP offers protection against malaria infection by stimulating T cells to produce interferon gamma, while RTS,S/AS01E confers protection against the liver stage of *P. falciparum* via anti-circumsporozoite antibodies (Bejon et al., 2006, 2008).

Studies seeking to link iron deficiency and vaccine responses have recently been reviewed (Oppenheimer, 2001; Preston et al., 2021) and the finding have not been

consistent. In a birth cohort, iron supplementation of infants at the time of measles vaccination was shown to improve vaccine response(Stoffel et al., 2020) and iron deficiency has been associated with significantly lower measles vaccine responses in children and individuals with mutations in *TMPRSS6* that leads to increased hepcidin levels were reported to have lower antibody levels against rubella, Hib and anti-*Streptococcus pneumoniae* serotype1 compared to the healthy control group post vaccination (Frost et al., 2021). Low serum iron concentration was associate with non-response in elderly hospitalized patients that received influenza vaccine (Fülöp et al., 1999). In earlier studies the finding differed depending on the vaccine, iron deficient children did not respond to dipheria vaccine (Macdougall et al., 1975) while tetanus, influenza and typhoid vaccine showed no difference between the iron deficient and non-deficient group(Chandra & Saraya, 1975; Crogan et al.,2005; Macdougall et al., 1975). In animal models, lower vaccine responses have been reported in iron deficient piglets (Frost et al., 2021), mice and rats model (Dhur et al., 1990; Jiang et al., 2019; Kochanowski & Sherman, 1985)

The aim of this study was to evaluate the effects of ID on the acquisition of natural immunity against malaria infection in children and on malaria vaccine responses. To achieve this, iron biomarkers and malaria antibodies (AMA1 and MSP1) were measured in 924 community-based children. These results were replicated in a different cohort (n=236) of community-based children. For the effect of ID on malaria vaccine responses we included children vaccinated with the RTSS,ASOE vaccine (n=115) and the ME-TRAP vaccine (n=91).

1.2 Justification of the study

Despite the homeostatic control of iron in the human body, ID poses a major public health problem especially in children below 5 years. The prevalence of ID has been reported to be as high as 35% in Kenyan children (Muriuki et al., 2020).

Previous studies on iron and immunity report conflicting results for the effects of ID on immunoglobulin production in children (Ekiz et al., 2005; Hassan et al., 2016). However, one small study evaluating the relationship of ID and immunity in *P. falciparum* infected children reported low malaria-specific IgG titers in iron deficient children (Nyakeriga et al., 2004). The mechanism behind this observation remains unknown. Therefore, understanding the relationship between iron status and alteration of malaria immunology is of great importance in helping inform efforts to manage children under five years of age suffering from both malaria and ID (Abbassia Demmouche, 2014) Additionally, few studies have shown that ID lowers response to vaccines in children but its effects on malaria vaccine responses has not been studied. With children being the main targets of the malaria vaccines that are under trial due to their high malaria burden, it is of importance to understand early how ID may affects the efficacy of these malaria vaccines. This will inform policy makers on key decisions such as whether to correct ID before vaccinating the child.

1.3 Statement of the problem

Both ID and malaria are common in sub-Saharan Africa, and the interaction between these conditions is very complex and not clearly (Nyakeriga et al., 2004).They both share a common risk group that includes children under 5 years of age. Due to the high malaria burden in children, they are a key target for the malaria vaccines that are under trial at various phases (Bejon et al., 2006, 2008). ID in children has primarily been linked with depression of maternal iron stores and increased demand of iron for growth with decreased iron intake (Cherayil, 2011; Porto & De Sousa, 2007).

ID has been associated with increased risk of infection. This could be explained by the deleterious effect that it has been shown to have on both adaptive and innate immunity. Some reports have also shown that ID may affect vaccine responses in children. This is a major concern because most vaccines are administered when children are below 5 years of age, and this is the age when about 35% of children have been shown to be ID in Kenya.

Despite the high burden of malaria and ID, few studies have investigated whether ID affects acquisition of natural immunity to malaria. Additionally, currently no malaria vaccine has yet been licensed but RTSS has been recommended for use in malaria endemic regions by the WHO (World Health Organization, 2021b) . Other malaria vaccine candidates are all at different stages of clinical trials. Currently it is not known how iron status will affect the efficacy of these malaria vaccines and therefore understanding this will be of great impact towards the development and rolling out of a malaria vaccine. In addition, understanding the relationship between iron status and alteration of malaria immunology is of great importance in helping inform efforts to manage children under five years of age suffering from both malaria and ID (Abbassia Demmouche, 2014).

1.4 Research question

I. Does iron deficiency and iron deficiency anemia affect acquisition of antibodies (anti- AMA, anti-MSP1&2 and anti-schizont) to malaria infection in Kilifi County children?

II. Does iron deficiency affect malaria vaccine response in Kilifi County children?

1.5 Hypothesis

Null hypothesis: Iron deficiency and iron deficiency anemia does not affect acquisition of antibodies to malaria infection and malaria vaccine responses in Kilifi County children.

Alternative hypothesis: Iron deficiency and iron deficiency anemia influences acquisition of antibodies to malaria infection and malaria vaccine responses in Kilifi County children.

1.6 Objectives

1.6.1 General Objective

To evaluate how the iron deficiency and iron deficiency anemia affects acquisition of antibodies to malaria and malaria vaccine response in Kilifi County children.

1.6.2 Specific objective

1. To evaluate the effect of iron deficiency and iron deficiency anemia acquisition of antibodies to malaria (anti- AMA, anti-MSP1&2 and anti-schizont) among children in Kilifi County.
2. To determine the effects of iron deficiency on malaria vaccine responses among

1.7 Scope of the study

The study focused on the effects of iron deficiency and iron deficiency anemia on acquired malaria antibodies namely AMA1, MSP1, MSP2 and schizont. This included community children in Kilifi County recruited in the ongoing cohort evaluating the history and acquisition of natural immunity to malaria and other infections in children living in Kilifi County. The second objective was a pilot study that focused on children vaccinated with RTS,S or ME-TRAP malaria vaccine candidates. The study evaluated how iron deficiency influenced the response of the two vaccine candidates in the vaccinated children.

CHAPTER TWO

LITERATURE REVIEW

2.1 Iron biology and human health

Iron exists in two oxidative states the ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms. Handling these two forms can be both difficult and dangerous. This is because iron is practically insoluble, both in and outside the body. Under physiologic conditions iron is readily oxidized to the insoluble ferric (Fe^{3+}) (Beard, 2001). In addition, most microbes thrive well on iron, as they need it for their replication and growth. Iron can also catalyze oxidative stress reactions that can damage proteins, lipids and nucleic acids of the host, however these oxidative stress reactions are necessary to kill invading micro-organisms (Cherayil, 2011).

ID and iron overload can have adverse effects on a variety of cell, tissue and organ functions. To overcome this challenge mammals have evolved mechanisms for precise regulation of extra- and intracellular iron levels (Cassat & Skaar, 2013). This enables them to take-up iron and utilize it safely by making use of specific protein complexes, such as transferrin, lactoferrin, ferritin and haeme proteins, while keeping iron away from microbes and not letting it take part in oxidative stress (Nairz et al., 2014). In addition to this, transferrin, which is the major iron transporting molecule is normally only partly saturated (30-40%).This ensures iron is readily bound to transferrin once it appears in the plasma (Yamanishi et al., 2003a).

2.2 Epidemiology and causes of iron deficiency

ID is defined as a condition in which there are no mobilizable iron stores and in which signs of a compromised supply of iron to tissues is note (WHO, 2015). This results from depletion of iron stores and iron absorption cannot keep pace over an extended period with the metabolic demands for iron to sustain growth and to replenish iron loss e.g. in excessive blood loss. The main causes of ID include: low intake of bioavailable iron,

increased iron requirements as a result of rapid growth, pregnancy, menstruation, and excess blood loss caused by pathologic infections, such as hook worm and whipworm causing gastrointestinal blood loss and impaired absorption of iron (Crompton & Nesheim, 2002; Larocque et al., 2005). ID mainly occurs in three sequential stages which includes depletion of iron stores, iron deficiency erythropoiesis and iron deficiency anemia. These stages can be analyzed using biochemical techniques and used in diagnosis of iron deficiency (Kotze et al., 2009).

It is thought that infants have adequate iron stores until they are 4-6 months of age obtained from the maternal transfer and small amount of iron in breast milk (Baker & Greer, 2010; Park et al., 2017). The Institute of Medicine in US estimated that the dietary iron requirements of infants through to about 6 months of age should be 0.27 mg/day, which is within the available iron levels in breast milk. After 6 months, prenatally acquired iron stores are exhausted and the demand for iron increases rapidly due to rapid growth and high nutritional demand. This iron demand rises to 11 mg/day which is way above the amount available in breast milk thus making infants vulnerable to ID (Baker & Greer, 2010).

ID affects more than 2 billion people worldwide, with IDA being one of the top causes of anemia (Kassebaum et al., 2014). ID affects both developing countries and developed countries. Worldwide, over 40% of children have ID and this may exceed 50% in developing countries (Chandra, 2002) In Kenya, Muriuki et al have reported 35 % ID in children under 5 years (Muriuki et al., 2020)

2.3 Iron homeostasis

Iron absorption from the diet takes place in the duodenum and upper jejunum. The amount of iron absorbed is dependent on the amount of iron available in the iron stores (Cassat & Skaar, 2013). In the duodenum gastric acid is produced that lowers the pH thus enabling reduction of Fe^{3+} by ferric reductases to Fe^{2+} that crosses across the apical membrane of enterocytes facilitated by divalent metal ion transporter 1 (DMT1) (Cassat

& Skaar, 2013; Nairz et al., 2014). The amount of iron obtained from the diet accounts for only about 1–2 mg of iron (Finberg, 2011). Macrophages are the main sources of iron in human body. This is obtained from the recycling of red blood cells (RBC) which occurs in the spleen and liver. This accounts for about 25-30mg of iron per day (Soe-Lin et al., 2009).

In the body iron is stored intracellularly with ferritin being the major iron storage protein which is deposited in hepatocytes and macrophages of the reticuloendothelial system (Nairz et al., 2014). Only a small amount of iron, about 0.1% of the body's iron, is found in the plasma compartment but almost all iron is bound to transferrin, transported to cells in need of iron where it is taken up into the cell by transferrin-mediated endocytosis via a cycle called transferrin cycle (Tandara & Salamunic, 2012). The transferrin cycle helps control the access of iron since individual cells can efficiently regulate the entry of iron by regulating transferrin receptor 1 (TfR1) expression according to their iron needs (Richardson et al., 2010).

Iron metabolism is tightly regulated so that the amount of iron entering the circulation from macrophages and iron absorbed from the diet are kept in balance as per body requirement (Andrews & Schmidt, 2007). This is important to help prevent the deleterious effects caused by deficiency or iron overload (Finberg, 2011). Hepcidin, a hormone produced by the liver, plays a central role in the regulation of iron homeostasis. In hyperferremia the liver is stimulated to release hepcidin which binds ferroportin (FPN) protein leading to its internalization and degradation (Nemeth & Ganz, 2009). In case of hypoferremia, hepcidin expression is decreased, resulting in higher levels of FPN which leads to an increase in circulating iron. Overexpression of hepcidin has been associated with anemia of chronic disease, while low hepcidin production results in hereditary hemochromatosis (HFE) with consequent iron accumulation in vital organs (Swinkels\ et al., 2006). A summary of iron homeostasis is shown in figure 2.1 below (Swinkels et al., 2006).

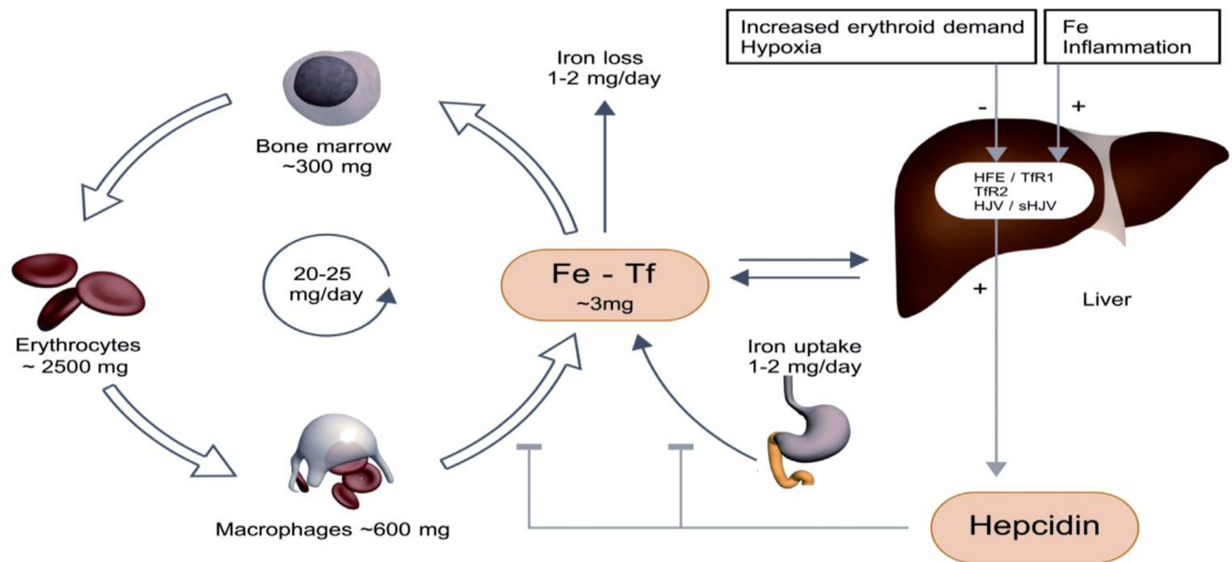

Figure 2.1: Systemic iron regulation

2.4 Pathogen iron acquisition mechanism

In case of an infection the human body withholds iron to prevent the pathogen from accessing it. However, pathogens have developed mechanisms that help them to access this sequestered iron. These mechanisms differ depending on the niche of the organism if it is intracellular or extracellular. Mechanisms used by the pathogens include production of siderophores, heme acquisition system, transferrin or lactoferrin receptors and ferric or ferrous iron transporter (Cassat & Skaar, 2013; Nairz et al., 2013; Nairz et al., 2010)

Plasmodium is an intracellular parasite. During the blood stage the plasmodium invades the RBCs and this creates a unique opportunity for it to obtain iron from haemoglobin. Plasmodium uses haemoglobin as the source of nutrient, but this is dangerous for the parasite since haem is toxic. Plasmodium has therefore developed mechanisms to detoxify the toxic haeme that is released after the breakdown of the haemoglobin in the food vacuole where it is degraded (Shio et al., 2010).The haeme is sequestered into a pigment called hemozoin which is released into the circulation after erythrocytic rupture.

The hemozoin is then phagocytosed by phagocytes where it has been shown to have immunoregulatory effects (Olivier et al., 2014).

2.5 Iron and immunity

2.5.1 Iron deficiency and immune functions

Phagocytes play an important role in protecting against invading pathogens. In an infection the phagocytes engulf the pathogen and internalize it in the phagolysosome. Phagolysosomes contain multicomponent enzymes that are activated leading to the ‘‘respiratory burst’’ that kills phagocytosed pathogens (Nairz et al., 2014). These enzymes require iron for their activation. Studies have shown that in patients with ID the oxidative burst is significantly decreased compared to IR controls. This is through the alteration of the formation of oxygen radicals and nitric oxide that are directed against invading pathogens (Hassan et al., 2016; Weiss, 2002). Dendritic cells play an important role in antigen presentation and priming of naïve T lymphocytes in the immune system. In *in vitro* cultures it has been shown that iron deprivation leads to undifferentiated dendritic cells that could not stimulate T cells (Kramer et al., 2002).

Humoral immunity has also been shown to be affected by iron deficiency. A study in iron deficient children reported lower IgG levels in children with ID but they reported no difference in IgA or IgM (Hassan et al., 2016). Jabara et al, showed that children with mutated transferrin receptor 1 could not express 1μ-Cε transcript required for immunoglobulin class switching thus leading to absence of circulating immunoglobulins (Jabara et al., 2016). Other studies have showed similar effects of ID on humoral immunity on different immunoglobulins (Abbassia, 2014; Guzikowska et al., 1989; Nyakeriga et al., 2004; Tang et al., 2006). However, others report no difference in immunoglobulin levels between the iron deficient group and IR. Two studies reported no significance difference in IgG, IgA, IgM and IgG subtypes between the iron deficient and IR group however the studies had small sample sizes (Das et al., 2014; Ekiz et al., 2005; Jabara et al., 2016).

Few studies have focused on the effects of ID on complement proteins. Sobhani et al reported that complement 4 was significantly lower in iron deficient children compared to IR children (Sobhani et al., 2011). Higher levels of serum ferritin were also reported to correlate with higher complement 3 and complement 4 levels (Galan et al., 1988).

Adaptive immunity develops over a period, may be days or weeks, following exposure to a foreign substance. It depends on the activation, proliferation and differentiation of antigen specific B and T lymphocytes (Bonilla & Oettgen, 2010). There is a tightly regulated interaction between antigen-presenting (dendritic) cells and T and B-lymphocytes. This facilitates production of pathogen-specific immunologic effector pathways, generation of immunologic memory, and regulation of host immune homeostasis (Cherayil, 2011). T and B cells express surface transferrin receptor 1(sTfR1) which has been reported to be highly increased during proliferation thus indicating a higher demand for iron (Jabara et al., 2016; Porto & De Sousa, 2007). In an *in vitro* experiment it was shown that the polyclonal proliferation of human B and T lymphocytes can be inhibited by antibodies to TfR1, indicating a requirement for iron uptake during cell division (Kemp et al., 1989). Absence of TfR1 has been reported to cause a complete arrest of T cell differentiation at the triple negative (CD3-CD4- CD8-) stage, but B cell development was less affected and allowed the formation of some IgM+ cells (Ned, Swat, & Andrews, 2003). Both activated and non-activated T lymphocytes synthesize ferritin (Sousa & Salazar, 1989).

The T cell lineage has also been reported to be affected by iron imbalance, however, it is not clear why it is sensitive to iron levels. Hassan et al., reported that patients with ID had higher proportions of circulating immature T-lymphocytes (CD1a+) and lower proportions of mature T-lymphocytes (CD4+ and CD8+) compared to a healthy control group. After iron supplementation for three months, these patients' immunological parameters improved significantly especially in CD1a+ and CD71+ lymphocyte subsets, however, they did not reach the same level as the control group (Hassan et al., 2016). In another study, the mean number of total lymphocytes, CD3 and CD4 subsets, and B-

lymphocytes in children with ID were decreased compared to the IR children. Cytotoxic T cell (CD8+) activity as measured by specific cytotoxicity and cytotoxic capacity was also decreased in iron deficient children (Santos & Falcão, 1990). In contrast, an experiment using an iron-deficient mouse model showed that T- lymphocyte proliferation was not affected however, the iron-deficient mice secreted less IFN-γ than the control mice, which could be due to impaired T-lymphocyte function (Omara & Blakley, 1994). A study involving 32 children with IDA and 29 controls without IDA ranging from 6-24 months of age, found no change in T lymphocyte numbers and distribution of subgroups in cases with IDA (Ekiz et al., 2005). Thibault et al., reported a qualitative rather than a quantitative defect in cell mediated immunity (Thibault et al., 1993).

Cytokines are produced from various cells in the body such as T cells and macrophages among others (Ward et al., 2011). The interaction between iron and cytokines is complex because cytokines regulate iron homeostasis and cytokine production is also regulated by iron (Ward et al., 2011; Weiss, 2002) Jason et al reported increased interferon gamma, interleukin 6 and interleukin 8 in iron deficient children compared to the healthy control group (Jason et al., 2001). Nyakeriga et al reported similar findings but only interleukin 4 was significantly higher in iron deficient children compared to IR (Nyakeriga et al., 2005). However other studies reported lower cytokines in the iron deficient group compared to healthy controls. In two animal model studies, interferon gamma was reported to be significantly lower in the iron deficient animals compared to the controls (Kuvibidila et al., 2010; Kuvibidila & Warrier, 2004).

2.5.2 Iron deficiency and vaccine response.

Vaccine efficacy determination is essential as it helps to determine the success of the immunization program. A study giving tetanus toxoid and diphtheria vaccines to iron deficient children and controls indicated no significance difference in the increase in IgG levels two weeks after immunization in both groups (Bagchi et al., 1980). In a study by

Brussow, children vaccinated against diphtheria and tetanus reported no difference in tetanus antibodies, but diphtheria antibodies were lower in the iron deficient children (Brussow et al., 1995). Two other studies reported normal immunoglobulin titres in iron deficient children (Chandra & Saraya, 1975; Macdougall et al., 1975). However, in animal studies iron deficient animals showed a significantly lower response to the influenza vaccine compared to the control (Dhur et al., 1990).

2.6 Malaria epidemiology and life cycle.

2.6.1 Malaria epidemiology

Malaria is an important cause of illness and death in many parts of the world especially in the Sub-Saharan Africa. According to the WHO 2021 malaria report, between 2019 and 2020, estimated malaria cases increased from 213 million to 228 million, and deaths from 534 000 to 602 000 in the WHO African Region (World Health Organization, 2021a). Additionally, Africa accounted for 96% of deaths globally with 80% of all deaths in this region being among children aged under 5 years (World Health Organization, 2021a). However, despite the high percentage reported, this is likely be an underestimation due poor surveillance method used thus leading to missing out on some cases(Snow et al., 2005). Malaria is transmitted in about 97 countries worldwide. This is mainly influenced by environmental effects primarily the temperatures that determine the ability of the mosquito vector to sustain parasite development (Cowman et al., 2016). There six plasmodial species present a significant health threat for humans including *P. falciparum*, *P.ovale, P. malariae*, *P. vivax* and *P. knowlesi.* Plasmodium falciparum is usually considered the most important in terms of deaths (Cowman et al., 2016).

2.6.2 Malaria life cycle

Plasmodium falciparum has a complex life cycle alternating between Anopheles mosquitoes and a vertebrate. The sporozoites are introduced in the host dermis during a blood meal, where they take about 1-3 hours to exit the site relying on gliding motility and some penetrate the blood vessels to enter the blood stream while others may be

destroyed by host immune system drained by the lymphatic where they induce an immune response (Tavares et al., 2013). The sporozoites in the blood stream then transverse through the sinusoidal barrier to the liver where upon interaction with hepatocytes are converted into "invasive mode". In the hepatocytes invasion the circumsporozoite protein (CSP) which covers the sporozoites, binds to highly proteoglycans (HSPGs) thus activating the processing of the CSP (Herrera et al., 2015).

Once hepatocytes infection has been established, the sporozoites takes about 2-10 days to transform into schizonts each containing about the 40, 000 merozoites. The schizont bunds off and are released into the blood stream where they burst releasing free merozoites that invade the erythrocytes within 2 minutes. The pre-invasion involves an interaction between the erythrocyte and the parasite that leads into parasite actomycin motor-driven deformation of the host cells (Weiss et al., 2015). This step involves erythrocyte binding-like proteins (EBLs) and reticulocyte-binding protein homologs (PfRhs) of the *P. falciparum*. Although their overall function is not well defined, these proteins have been shown to be essential in the overall invasion process (Thamet al., 2012). After the deformation of the RBC the merozoite reorient so that the apical touches the RBC membrane. This involves an atypical PfRh family member called PfRh5 which forms a complex with with PfRipr (Rh5-interacting Protein) (Chen et al., 2011) and CyRPA (cysteine-rich protective antigen) (Reddy et al., 2015). The PfRh5 binds to the host basigin receptor that is essential for the merozoites invasion (Crosnier et al., 2011).

Once the erythrocytes infection is established, the merozoites undergoes cell division to for a schizont that contains about 16-32 merozoites. When the schizont is mature it causes the RBC to burst releasing the merozoites into the circulation. During schizogony in the blood stream, some parasites undergo a developmental switch which initiate them to commit to sexual developments either male or female gametocytes. It takes about 11 days for the gametocytes to mature and become infectious to mosquitoes. The

transmission from human to mosquitoes occurs when a mosquito feeds on the mature gametocytes during a blood meal (Crompton et al., 2010; Joice et al., 2014).

2.7 Immunity to malaria infections.

2.7.1 General Immunity

The human body have developed various mechanisms to protect from invading pathogens. In *P. falciparum* infections both innate and adaptive immunity helps to clear the parasite (Crompton et al., 2014). After sporozoites are deposited, the infection is clinically silent and no evidence of naturally acquired immunity has been described. However, in studies using attenuated whole sporozoites sterilizing immunity was induced and was hypothesized to be due to antibodies. In animal models, the sporozoites have been shown to be drained to the lymph nodes where they interact with antigen presenting cells leading to the priming of CD8+ T cells (Crompton et al., 2014; Tran et al., 2014).

In the liver stage in humans, immunity is not naturally acquired, however artificial administration of attenuated sporozoites in animal models and humans have been shown to induce both innate and adaptive immunity. CD 4+, CD8+, natural killer cells, γδ T cells amongst other responses have been reported (Tran et al., 2014). Upon rupturing of the hepatocytes, thousands of merozoites are released in the peripheral blood. In the blood stage the merozoites are exposed to the immune system before they invade red blood cells. Antibodies to merozoite proteins such as AMA1, MSP and EBA have been shown to play a key role in inhibiting progression of the disease at this stage (Boyle et al., 2015;Osier et al., 2008) Additionally, cytokines produced by CD4+ T cells, macrophages, natural killer cells and γδ T cells play a major role in disease control. The invaded red blood cells burst to release merozoites thus inducing inflammatory substances as reviewed (Crompton et al., 2014; Tran et al., 2014). A summary of the immune response mounted against *P. falciparum* infection is shown in figure 2.1 (Crompton et al., 2014).

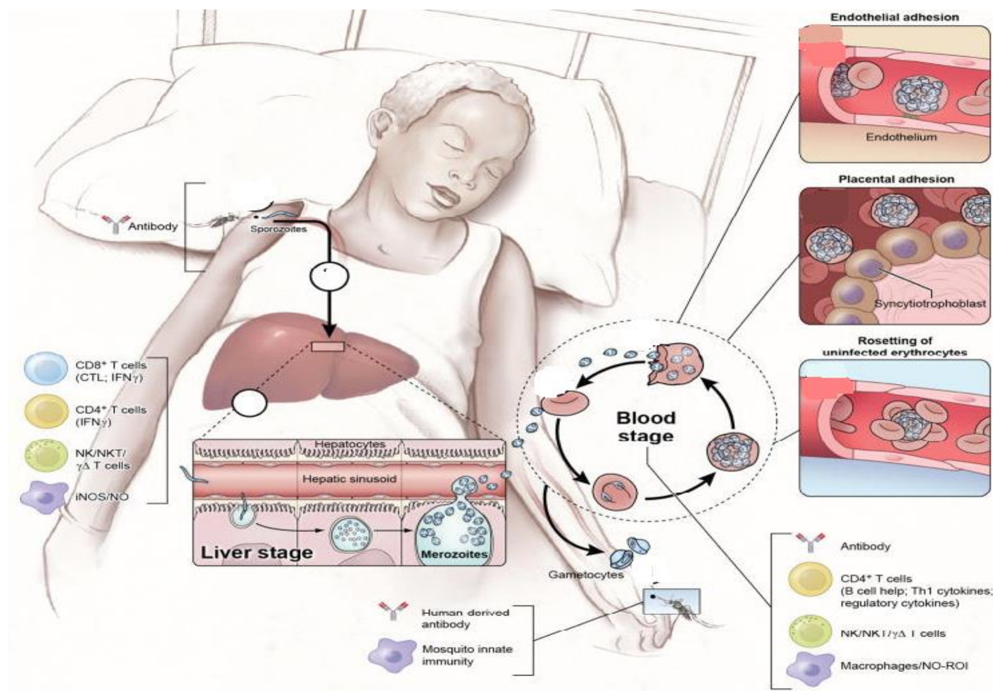

Figure 2.2: Plasmodium falcipa[1]rum lifecycle and immune response at various stage

2.7.2 Malaria antibodies.

Protective immunity against clinical disease takes years to develop and requires persistent exposure to the infection. Naturally acquired immunity has been associated with decreased morbidity and mortality from malaria in older children and adults (Marsh & Kinyanjui, 2006). The protective role of antibodies in malaria infection was first demonstrated by passive transfer studies that demonstrated that human IgG antibodies can protect from blood stage parasites and reduce malaria symptoms (Garnham, 1949). However, in the absence of re-exposure the levels of malaria antibodies rapidly decrease making the immune individual or population susceptible again to malaria infections (Akpogheneta et al., 2010; Marsh & Kinyanjui, 2006). Although malaria antibodies have

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been reported to decline more rapidly in young children, Crompton et al showed that 3-year-old children had slightly higher antibody levels than 2-year old children, which suggests that small proportions of antibodies acquired by 2-year-old children persisted (Crompton et al., 2010). This has been supported by Akpogheneta et al, who also demonstrated that some antibodies persisted for >85 days in children (5-7 years) without persistent exposure to infection, while in younger children the decline was 20-30% of the initial levels. MSP2 has been shown to decrease more rapidly compared to other antibodies such as EBA175, AMA1 and MSP1. The concentrations of these antibodies were reported to increase with age with the exception of MSP1 that did not vary with age (Akpogheneta et al., 2010). However, it is not fully understood to what extent naturally acquired immunity depends upon persistent exposure (Ndungu et al., 2012)

Antibodies produced against blood stage infection play key roles in inhibiting merozoite invasion of RBCs, they arrest merozoite growth in RBCs, opsonize merozoites, interact with complement factors to inhibit invasion and promote neutrophil respiratory burst (Osier et al., 2008; Teo et al., 2016) Although it is not clear how antibodies to merozoites inhibit infection, it has been hypothesized that they inhibit receptor ligand interaction, protein processing or conformational changes that are required by merozoites to aid in invasion of red blood cells. Antibodies to MSP1, AMA1, *P. falciparum* rhoptry proteins4 (PfRH4) and EBA have been shown to inhibit *P. falciparum* from growing in *in vitro* cultures (Chiu et al., 2014; Tran et al., 2014).

Complement fixation is another mechanism exploited by antibodies to control malaria infection. This occurs mainly through the classical pathway. MSP1 and MSP2 have been shown to be the targets for these antibodies (Boyle et al., 2015). The antibody-complement mechanism leads to the formation of Membrane Attack Complexes (MAC) that cause merozoites to rapidly lyse. MAC formation has been associated with protection from malaria in children (Beeson et al., 2016; Boyle et al., 2015)

Opsonization is a mechanism by which the merozoites that are labelled with antibodies are more likely to be taken up by phagocytes. Antibodies to MSP2, MSP3 and glutamate-rich protein (GLURP) have been shown to have high opsonization activity (Mugyenyi et al., 2017; Osier et al., 2014) In longitudinal studies in Kenya and Papua New Guinea opsonic phagocytosis has been associated with protective activity against malaria and also it increases during malaria infection (Hill et al., 2013; Osier et al., 2014)

2.7.3 B cells in malaria infection

B cells have an important role in immunity due to their role in the production of antibodies. They differentiate to form memory B cells (MBCs) that boost immune response in a secondary exposure to a pathogen. Studies have shown that antibody production can be sustained through re-stimulation of MBCs by persistent antigens (Ochsenbein et al., 2000) or by non-proliferating long lived plasma cells (Kurosaki et al., 2015; Slifka & Amanna, 2014). In children because of their immature immune system and the antigenic variation of the malaria parasite, development of effective B-cells occurs after years of repeated malaria exposure (Akpogheneta et al., 2010; Soares & Weiss, 2015). Different B-cell phenotypes have been observed in malaria infection. Atypical memory B cells (aMBCs) are one of the B cell populations found in malaria endemic areas where there are repeated infections. They have been shown to produce protective immunoglobulins against malaria infection (Weiss et al., 2009). Young people in Africa have been shown to have expansion in both the total memory and transitional B-cell populations after experiencing intense infection malaria (Asito et al., 2008). Although *P. falciparum* antibodies may fall to undetectable levels in the absence of persistent exposure, specific memory B cells to malaria have been shown to be maintained in both continually exposed children and in those with low exposure (Bediako et al., 2016).

2.7.4 T cells in malaria infection

It has been shown that T cells have intrinsic reactivity to malaria antigens, can mediate killing of *P. falciparum* merozoites, and expand markedly *in vivo* after malaria infection in previously naïve individuals. Repeated malaria infection during childhood leads to progressive loss and dysfunction of Vδ2 (+) γδ T cells that may facilitate immunological tolerance of the parasite (Jagannathan et al., 2014). $CD4^+$ T-cells have been mainly associated with control of erythrocytic stage parasites, but a small number of animal studies have reported that they have a role in the pre-erythrocytic stage (Oliveira et al., 2008; Overstreet et al., 2011). Th2 $CD4^+$ T-cells produce cytokines that are associated with inflammation in malaria infection. Memory CD8T cells have also been described in malaria infection induced by irradiated whole sporozoites .(Carvalho et al., 2002; Overstreet et al., 2011) Bediako et al showed that *P. falciparum*-specific $CD4^+$ T cells are maintained in children in the absence of continual exposure after observing no significant difference between the proportions of $CD4^+$ T cells in children from an endemic region compared to those from a low malaria region who were historically exposed (Bediako et al., 2016).

2.8 Malaria vaccines

Malaria vaccines are being developed to target the different stages of plasmodium life cycle as shown in Figure 2.2 (Draper et al., 2018). Malaria vaccine candidates are broadly classified as pre-erythrocytic, erythrocytic and transmission blocking vaccines. Pre-erythrocytic malaria vaccine candidates includes RTS,S, R21, Multiple epitope-thrombospondin-related adhesion protein (ME-TRAP) among others. RTS,S is the most advanced malaria vaccine which targets the pre-erythrocytic stage of Plasmodium falciparum infection. RTS,S is a recombinant protein-in-adjuvant vaccine containing the parasite circumsporozoite protein (CSP) fused to the hepatitis B surface antigen (HBsAg), in the adjuvant ASO1B. It primarily induces protection via high antibody responses against the NANP repeat region of the CSP and modest CD4+ T cell (Collins et al., 2017) . In the phase 3 trial that involved 15,460 children in Africa, it showed a

significant short-term protective efficacy of 46% in children and 27% in infant but waned to 28.3% and 18.3% within 48 months in children and infants, respectively (The RTSS Clinical Trials Partnership, 2014). In addition, the children that received a fourth dose, the vaccine provided significant protection against severe malaria and hospital admissions (RTS,S Clinical Trials Partnership, 2015). The World health Organization's (WHO) Strategic advisory group of Experts (SAGE) on immunization and the Malaria Policy Advisory Committee (MPAC) recommended further pilot studies in a Malaria Implementation Programme (MVIP) which are being coordinated by WHO (WHO, 2018). RTS,S/AS01 pilot study aimed to assess whether routine immunization programmes can deliver the four-dose schedule effectively, if the vaccine prevents deaths, and if the safety signals such as bacterial meningitis, death of female children detected in the phase 3 trial were just chance findings (Greenwood & Doumbo, 2016; RTS,S Clinical Trials Partnership, 2015). The RTS,S, pilot study was conducted in Kenya, Malawi and Ghana in children from 6 months of age (WHO, 2019) after which recommended use of RTS,S in malaria endemic countries. ME-TRAP is another pre-erythrocytic malaria vaccine candidate is in the pipeline. ME-TRAP aims to confer protection against malaria infection by induction of T cells that producing interferon gamma (IFNγ) The vaccine is composed of viral vectors simian adenovirus 63 (ChAd63) and Modified Vaccinia Ankara (MVA). In Phase I and II clinical vaccine trials, this vaccine candidate has been shown to induced high numbers of antigen-specific T-cells in humans, and upon controlled malaria infection (CHMI) of malaria naïve adults, it demonstrated 21% sterile efficacy and a delay to parasitaemia in a further 36% of vaccinees (Ewer et al., 2013). 67% reduction in malaria infection was measured in ChAd63.MVA ME-TRAP vaccinated adults in a malaria endemic region of coastal Kenya (Ogwang et al., 2015).

P. falciparum reticulocyte-binding protein homolog 5 (*Pf*RH5) is the most advanced malaria vaccines targeting the blood-stage parasite (Draper et al., 2018).. In the first clinical trial assessing RH5 antigen vaccine in UK adults, using viral vectors (the

replication-deficient chimpanzee adenovirus serotype 63 (ChAd63) and the attenuated orthopoxvirus modified vaccinia virus Ankara (MVA)), encoding RH5 from the 3D7 clone of *P. falciparum* showed that the vaccine induced greater antibodies response compared to the natural immune response observed malaria exposed Africans adults (Payne et al., 2017). The RH5 vaccine induced antibodies also exhibited cross-strain functional in GIA in vitro, targeted linear and conformational epitopes within RH5 and interestingly inhibited a key interaction within the RH5 invasion complex (Alanine et al., 2019; Payne et al., 2017). Other vaccine candidates targeting the erythrocytic stage are in the pipeline including PfAMA1, GLA-SE among others. Transmission blocking vaccine are those targeting the sexual stages do not directly prevent infection or clinical symptoms within the host, but rather impact the parasite's life cycle in the mosquito vector aiming to prevent sporozoite development in the mosquitoes and hence preventing onward transmission. There are a couple of antigens that have been identified as candidate vaccine target however most clinical trials are in their early phases. Some of these antigens include Pfs48/45, Pfs230 and Pfs25 (Draper et al., 2018; Kapulu et al., 2015).

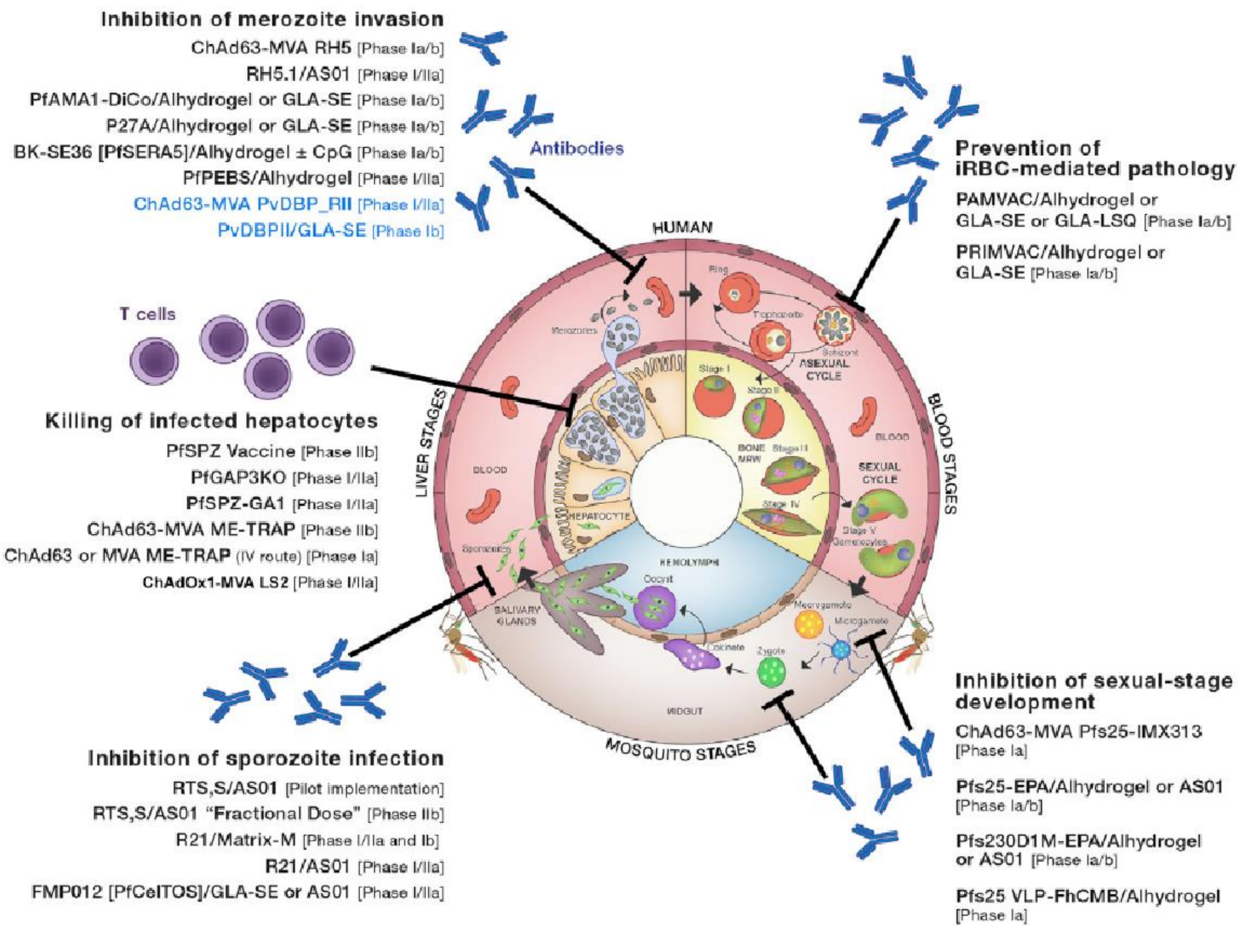

Figure 2.3: Malaria vaccine candidates in clinical developments

CHAPTER THREE

METHODOLOGY

3.1 Study area

The study was conducted in Kilifi County, on the coast of Kenya. There were four cohorts, two immunology cohorts Ngerenya and Junju, and two vaccine cohorts RTSS and ME-TRAP. Junju and Ngerenya are villages located within 20 km of each other, with Junju lying on the southern side and Ngerenya on the northern side of an Indian Ocean creek. The county has a population of 1,109,735. It covers an area of 12,245.90 km2 (4,728.17 sq mi) (Kenya National Bureau of Statistics KNBS, 2009). The residents are Mijikenda people mainly farmers and fishermen. The region has two rainy seasons: the "long" rains in May–July and the "short" rains in November. RTSS and ME-TRAP are vaccine cohorts composed of community-based children in Kilifi County who were recruited to be vaccinated with RTS,S or ME-TRAP malaria vaccines respectively.

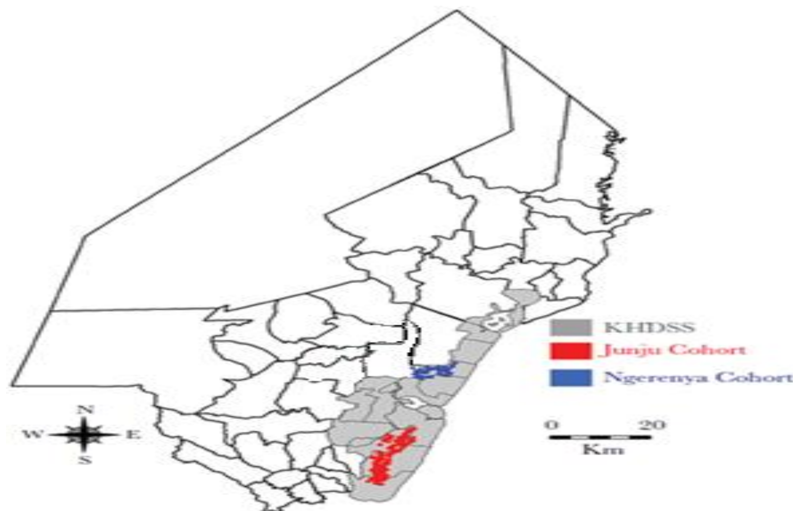

Figure 3.1: Kilifi County map showing the study cohorts

3.2 Study design

The study was nested within an ongoing rolling cohort evaluating the history and acquisition of natural immunity to malaria and other infections in children living in

Kilifi County (Mwangi et al., 2005). The first objective evaluating effects of iron deficiency and iron deficiency anemia on malaria antibodies utilizes a cross-sectional study design. Iron markers were measured at the same time point as the malaria antibodies. The second objective evaluating effects of iron deficiency on malaria vaccines response utilizes a longitudinal study design. Iron was measured at the screening time point while vaccine responses were measured at screening, 3-, 6- and 14-months post vaccination for RTSS cohort, and at screening, 1 week, 3 weeks and 6 months post vaccination in ME-TRAP cohort. Figure 3.2 shows the study summary.

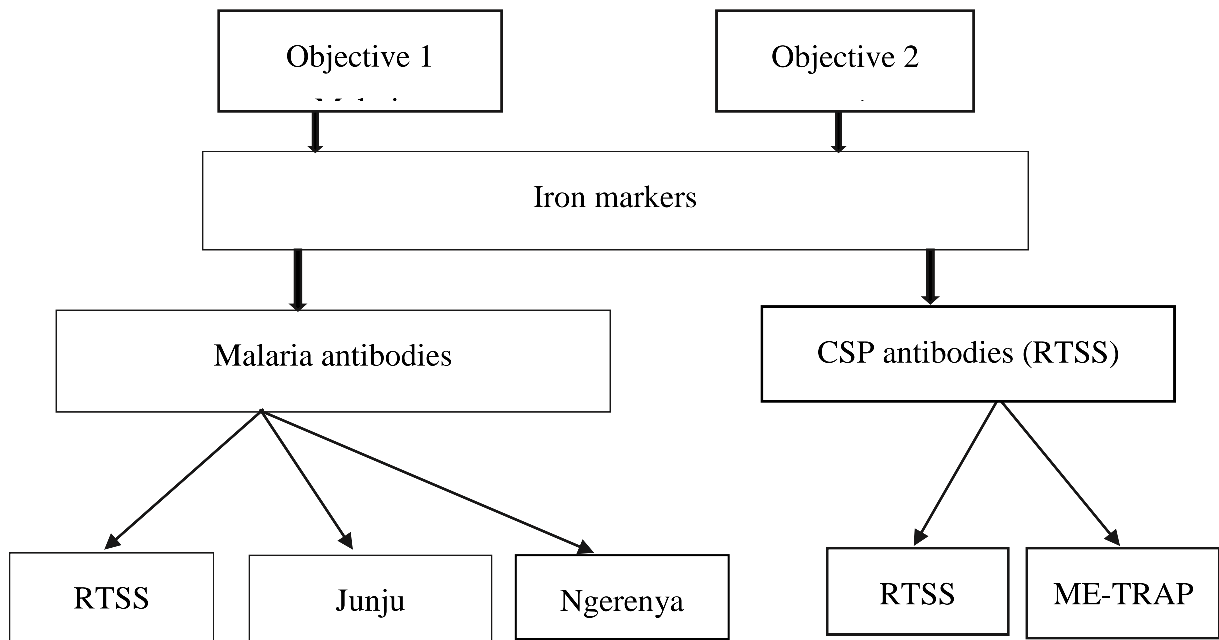

Figure 3.2 Study summary schema

3.3 Study population

The study consisted of four cohorts Junju and Ngerenya (immunology cohorts) and RTSS and ME-TRAP (vaccine cohorts) as shown in Figure 3.2. Junju and Ngerenya cohorts are villages found in Kilifi County along the Kenyan Coast. The area experiences two seasonal peaks in malaria transmission (June to August and November to December) but there is low transmission throughout the year (Mwangi et al., 2005; O'Meara et al., 2008). Children residing in randomly selected households were recruited, at birth, and remained under surveillance until their 15th birthday (Mwangi et al., 2005). The RTSS cohort was the RTS, S/AS01E vaccine trial against malaria that was conducted between 2007 and 2008 and included children between 0-17 months. These children were then monitored for 8 years (Bejon et al., 2008). The ME-TRAP cohort is a vaccine trial cohort conducted in community-based children in Kilifi in between 2005 to 2006. The participating children were 1-6 years old and healthy (Bejon et al., 2006). Extensive and detailed records of the number and dates of malaria episodes for each child over the period they are enrolled in the cohort are maintained. Blood samples collected from the study participants had the plasma was separated and stored at -80 ^{0}C.

3.3.1 Inclusion criteria and exclusion criteria

All the children in the study cohort below 8 years were included in the study and those above 8 years were excluded. Children in the vaccine cohorts that were vaccinated with rabies vaccine were excluded.

3.4 Sample size calculation

Considering the specific objective one as the primary objective of the study; for iron status to affect antibody production, there should be a correlation between iron levels and malaria antibody levels; to evaluate the correlation, correlation coefficient statistic was used. Sample size was therefore calculated based on the expected correlation

coefficient between iron levels and antibody levels/malaria vaccine response using the formula for correlation studies (Zou, 2012).

$$n \geq \left\{\frac{[Z_{\alpha/2} + Z_{1-\beta}]}{C}\right\}^2 + 3$$

Where;

n is the minimum required number of children.

Z_ (α/2) is the standard normal critical value at α-level of significance for a two-sided test (α=0.05, Z_(α/2) =1.96).

Z_ (1-β) is the standard normal critical value for 1-β power (1-β=0.80, Z_(1-β) = 0.84).

C is the effect size; C=0.5*ln ((1+r)/(1-r)) =0.1 (based on standard correlation values defined by (Moinester & Gottfried, 2014)

Based on this formula and defined parameters, a minimum of 787 samples were used for the study for each objective. However, a number greater than the minimum could be included in the study.

3.5 Sampling methods.

The study included community-based children in four cohorts: Junju, Ngerenya, ME-TRAP and RTSS. Stratified sampling with proportional allocation was used to obtain the sample for this study since the study was nested within a larger cohort. Participants were selected from each stratum (cohort) using simple random sampling. Table 3.1 summarizes the sampling procedure that was used.

Table 3.1: Sampling procedure

Cohort	Population	Sample
Junju	702	582
Ngerenya	307	236
ME-TRAP	92	91
RTSS	383	342

3.6 Data collection

In Junju and Ngerenya (immunology cohorts) cross sectional bleeds are conducted in March each year, just before the long rains, which marks the beginning of the bi-annual malaria transmission seasons for the year. 10 ml (5 ml for children aged 6 months to 1 year) is taken from each child and is used for a full blood count, malaria blood film and immunological assays in accordance with SSC 1131 and 3149 protocols (Mwangi et al., 2005; Ndungu et al., 2015). Iron biomarkers were measured from a single cross-sectional bleed based on the availability of plasma samples archived at -80^{0}C between 2010 and 2015. In the RTSS cohort, venous blood was drawn before administration of the first vaccine and active surveillance began 2.5 months after the vaccine and continued throughout the follow up period. Another sample was taken 3, 6 and 14 months after the first vaccine (Bejon et al., 2008). In the ME-TRAP cohort, the baseline sample was obtained before vaccination, then follow up was conducted and samples collected at one week, 3 and 6 months after vaccination (Bejon et al., 2006). The archived samples were used to measure malaria antibodies and iron markers. For the RTS,S cohort, malaria antibodies were measured in the screening sample before vaccination.

3.6.1 Iron analyses

Various iron and inflammation markers were analyzed using the methods shown in Table 3.2.

Table 3.2: Iron markers measurement

Variable	Technique of measurement
Iron	iron calorimetric assay
Transferrin	chemiluminescent microparticle immunoassay
Ferritin	chemiluminescent microparticle immunoassay
CRP	MULTIGENT CRP Vario assay [CRPVa]
sTFR	enzyme linked immunosorbent assay
Hepcidin	enzyme linked immunosorbent

Iron assays were performed on plasma samples collected during cross-sectional surveys and stored at -80^{0}C in the KWTWRP laboratory biobank. We assayed various iron biomarkers which included plasm iron (MULTIGEN iron calorimetric assay), ferritin (Chemiluminescent microparticle Immunoassay), soluble transferrin receptor (sTfR) (BioVendor RD194011100 Human sTfR ELISA), transferrin (Chemiluminescent Microparticle Immunoassay) and hepcidin (DRG Hepcidin 25 [bioactive] HS ELISA Kit). Since iron biomarkers are affected by inflammation, C-reactive protein, a marker of inflammation, was also assayed using MULTIGENT CRP Vario assay (CRPVa).

3.6.2 Malaria natural immunity antibody analysis

Anti-AMA1 and anti-MSP1 antibodies were assayed using ELISA. Briefly, the test was optimized to determine the antigen dilution that was optimal to cover the polystyrene 96-well microtiter plate (Dynex Technologies Inc). This was conducted using various dilutions of the antigen obtained via serial dilution. Serum antibodies to purified recombinant *P. falciparum* (Glutathione S transferase fusion proteins) antigens have been previously described (Osier et al., 2008). IgG was measured towards a panel of merozoite antigens using recombinant antigen homologues to 3D7. Previous studies

have shown that antigen encoded by the 3D7 genotype are well recognized across the globe in different populations (Fowkes et al., 2010)

Procedure:

For the Junju and RTSS cohorts AMA1 and MSP1 antibodies were analyzed in duplicate. Sera from unexposed individuals (n=5) in the United Kingdom (UK) and positive controls obtained from Pooled hyperimmune serum (PHIS) (n=1) were run in each plate. One phosphate buffered saline (PBS) blank was included in each plate as a plate control to allow for the standardization of values thus accounting for any plate to plate variation.

Individual wells of Dynex Immunolon 4HBX ELISA plate (Dynex Technologies Inc.) were coated with 1 µg/ml recombinant *P. falciparum* antigen per 100 µl of carbonate coating buffer. Plates were incubated overnight at 4^0C and washed the next morning three times in PBS-Tween (PBS-0.05% Tween 20). They were then blocked for 3 hours at room temperature with 1% skimmed milk in PBS-Tween (blocking buffer), washed again 3 times in PBS-Tween and this was then followed by incubation overnight with 100ul of test serum (1/1,000 dilution in blocking buffer). Plates were then washed six times and incubated for 3 hours at room temperature with 100ul of horseradish peroxidase-conjugate rabbit ant-human IgG (Dako Ltd) at a 1/5,000 dilution in blocking buffer followed by six times final wash. A reaction was detected using OPD (o-phenylenediamine) dihydrochloride substrate (sigma) 100 µl per well. The reaction was stopped with 25ul of 2M H_2SO_4 per well, and absorbance was read at 492nm. To generate standard units of specific IgG reactivity additional wells were coated with purified myeloma-derived human IgG (MIG) (The Binding Site, United Kingdom) that were serially diluted. The optical density (OD) obtained from MIG were used to generate a standard curve that was interpolated to calculate the individual antibody concentration. IgG antibodies against whole *P. falciparum* schizont extract and against the 3 AMA1 alleles (3D7, W2mef and HB3) and 2 MSP2 alleles (3D7 and FC27) were

assayed by standard enzyme-linked immunosorbent assay in the Ngerenya cohort as previously described (Mugyenyi et al., 2013)

3.6.3 CS antibodies ELISA assays for RTS,S/AS01E vaccine

ELISA was used to measure the antibodies to *P. falciparum* circumsporozoite repeat region (anti-CS antibodies) in RTS,S/AS01E malaria vaccinated children. The anti-CS antibodies were analyzed at screening, 3 months, 6 months and 14 months after vaccination. The ELISA plates were adsorbed with the recombinant antigen R32LR that contains the sequence [NVDP (NANP)15]2LR. This assay was conducted at the Centre for Vaccinology (CEVAC; Ghent, Belgium) as described by Bejon et al and Olotu et al. The results were reported in EU/ml (Bejon et al., 2008; Olotu et al., 2013).

3.6.4 ELISpot assays for ME-TRAP vaccine response

ELISpots used Millipore MAIP S45 plates and Mab Tech antibodies according to the manufacturer's instructions for the ME-TRAP malaria vaccinated children. The *ex vivo* assay was used to measure the circulating PBMCs that were producing IFN γ. In *ex vivo* experiments freshly isolated peripheral blood mononuclear cells (PBMCs) were incubated with peptide for 18-20 hours before developing. The peptides used were multiple epitope (ME) string and thombospondin-related adhesion protein (TRAP) peptides that were pooled together as described by Bejon et al (Bejon et al., 2006). The spot forming cells were counted by enzyme-linked immunosorbent spots (ELISPOTs) plate reader (Autoimmun Diagnostika, version 3.0). Non- recombinant vaccinia (Westrn Reserve/ WR strain) was added to the wells at a ratio of 3 plaques forming unit (pfu) per PBMC to help measure anti-vector immunity. Phytohaemagglutinin (20 µg/ml, Sigma-Aldrich) was used as the positive control while PBMC cultured in media alone were used as negative controls.

Cultured ELISPOTs was used to measure the resting PBMCs that were capable of producing IFN γ upon stimulation. In cultured ELISPOTs, PBMCs were incubated with TRAP and ME string peptides in a 24 well plate. On the 3rd and 7th day, 250ul of culture supernatant was replaced with an equal amount of fresh culture medium

containing 210IU/ml recombinant IL 2. On the 9th day the cells were washed three times and left overnight before performing the ELISpot assay (Bejon et al., 2006)

3.7 Case definitions

ID was defined using two definitions a) low ferritin as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation (CRP > 5mg/L) in children < 5 years or < 15µg/L in children ≥ 5 years (Atkinson et al., 2014; WHO, 2015) b) transferrin saturation (TSAT) <10% (calculated as iron in µmol/L / (transferrin in g/L x 25.1) x 100) (Yamanishi, Iyama, Yamaguchi, Kanakura, & Iwatani, 2003b). Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 4 years or hemoglobin < 11.5 g/dL in children above 4 years while IDA was defined as low ferritin and anemia (World Heath Organization, 2001) The malaria exposure index estimates a distance-weighted local prevalence of malaria infection within a kilometer radius around an index child. The malaria exposure index had previously been calculated (Olotu et al., 2012). IR was used to define children that did not fall in the ID category.

3.8 Data managements and statistical analysis

Data obtained from the laboratory and the KDHSS were stored in password-protected MS Excel spread sheet. Data analyses were conducted using STATA version 15.0 for Windows (StatCorp. College Station, TX). The difference in geometric means of antibody levels between the iron deficient and IR children was determined using two-tailed Student t-test. For univariable model and multivariable models, iron biomarkers (except haemoglobin) and malaria antibodies were log transformed to assume normal distribution. Multiple linear regression models were used to estimate the effect of ID on natural immunity to malaria, adjusting for sex, age, inflammation and malaria exposure index. All tests were performed at 95% level of confidence. A p-value of < 0.05 was considered significant.

Multiple linear regression model

The model we used took the following form:

$$\boldsymbol{Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \cdots + \beta_P X_P}$$

Where;

Y – the outcome variable i.e. malaria antibody level

β_i – partial regression coefficient – average effect of X_i (predictor) on Y (outcome), adjusting for the other predictors.

X_i – Predictors e.g. iron status, age

β_0 – regression intercept – average antibody level in the absence of all predictors.

Re-writing the above model using variables from our data:

$$\mathrm{Y} = \beta_0 + \beta_1 \text{Iron status} + \beta_2 \text{age} + \beta_3 \text{sex} + \beta_4 \text{cohort} + \beta_5 \text{malaria episodes (exposure index)}$$

3.9 Ethical considerations

The study received ethical approval from the Kenya Medical Research Institute (KEMRI) Ethical Review Committee (KEMRI/SERU/CGMR-C/046/3257). Written informed consent was obtained from the parent/ guardian of all the children before being allowed in the study, which also allowed storage, shipment and testing of bio-samples in future studies. All procedures were conducted in accordance with the Declaration of Helsinki and Good Clinical and Laboratory Practices.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Effects of iron deficiency and iron deficiency anemia on acquisition of malaria antibodies

4.1.1 Participant's characteristics

Total of 1160community-based children from RTSS, Ngerenya and Junju were included in this study. Table 4.1 shows the summary of the study participants' characteristics. Gender was evenly distributed between groups based on iron status. The RTSS cohort had the youngest children with a median age of 12.6 (range, 9.1-16.8) months, while Ngerenya had the oldest children with a median age of 48.7 (range, 26.3-67.8) months. Children in the RTSS cohort had the least exposure to malaria with a median of 0.09 (range, 2.17^{-14}-0.32) compared to Junju and Ngerenya children as shown in the Table 4.1. Using the low ferritin definition, the prevalence of ID was 20% (111/552) in Junju, 46% (153/335) in RTSS and 56% (134/236) in Ngerenya. Using the low TSAT definition, the prevalence of ID was 40% (220/554) in Junju and 61% (205/335) in RTSS (not available for Ngerenya). Anemia prevalence was 62% (332/516) in Junju and 44% (36/81) in Ngerenya (Hb not available for RTSS). Prevalence of IDA was 18% (90/488) in Junju and 31% (25/81) in Ngerenya (not available for RTSS cohort). Age, inflammation and *P. falciparum* parasitemia were associated with a significant increase in malaria antibodies (Table 4.2).

Table 4.1: Participant's characteristics

	Junju (n=582)	RTSS (n=342)	Ngerenya (n=236)
	no./total %	**no./total %**	**no./total %**
Median age months (IQR)	27.7 (19.0, 51.7)	12.6 (9.1-16.8)	48.7 (26.3, 67.8)
Sex: Females	284/582 (48.8)	168/342 (49.1)	103/236 (43.64)
Inflammation [a]	180/564 (31.9)	95/339 (28.02)	22/236 (9.3)
Malaria parasitemia	202/582 (34.7)	3/152 (0.01)	18/124 (14.5)
Median exp ind (IQR)	0.5 (6.16^{-11}, 0.75)	0.09 (2.17^{-14}, 0.32)	0.5 (0.29, 0.71)
Iron deficiency [b]			
Low ferritin	111/552 (20.0)	153/335 (45.7)	134/236 (56.8)
TSAT<10%	220/554 (39.7)	205/335 (61.2)	n/a
Anaemia [c]	322/516 (62.4)	n/a	36/81 (44.4)
IDA[d]	90/488 (18.4)	n/a	25/81 (30.9)
Biomarkers	**n (gmean (95% CI))**	**n (gmean (95% CI))**	**n (gmean (95% CI))**
Ferritin (µg/L)	552 (32.68 (30.02; 35.58))	335 (17.41 (15.70; 19.30))	236 (18.49 (16.26; 21.03)
Hepcidin (µg/L)	546 (7.01(6.35; 7.73))	298 (5.70 (4.92; 6.58))	236 (3.77 (3.03; 4.68))
sTfR (mg/L)	573 (18.22 (17.58; 18.87))	339 (18.04 (17.35; 18.67))	232 (49.56 (47.28; 51.95))
Haemoglobin (g/dL)	516 (10.15 (10.00; 10.30))	n/a	81 (10.29 (9.96; 10.63))
TSAT (%)	568 (2.65 (2,60-2.70))	337 (2.87 (2.81; 2.92))	n/a

IQR; interquartile range, Median exp ind; median malaria exposure index, TSAT; transferrin saturation, IDA; Iron deficiency anemia, sTfR; soluble transferrin saturation

[a] Inflammation was defined as C-reactive protein > 5mg/L

[b] Iron deficiency was defined using two definitions: 1) Low ferritin defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation in children < 5 years or < 15µg/L in children ≥ 5 years; and 2) Transferrin saturation < 10% (not available for (n/a) Ngerenya cohort).

[c] Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 4 years or hemoglobin < 11.5 g/dL in children above 4 years (hemoglobin not available for (n/a) RTSS cohort and in Ngerenya available for 81 children only)

[d] Iron deficiency anaemia was defined as low ferritin and anaemia (not available for (n/a) RTSS cohort)

Table 4.2: Univariable linear regression showing the association between covariates and AMA1 and MSP1 antibodies for the Junju and RTSS cohorts

Iron biomarker	n	coefficient	[95% Conf. Interval]	P value
AMA1				
Sex: female	924	0.01	-0.01; 0.04	0.19
Age in months	924	8.64	7.89; 9.29	<0.001
Inflammation	903	0.04	0.02; 0.07	<0.001
Mal exp index	833	-0.002	-0.02; 0.02	0.84
Parasitemia	734	0.15	0.13; 0.17	<0.001
MSP1				
Sex: female	924	0.01	-0.01; 0.04	0.33
Age in months	924	6.04	5.20; 6.88	<0.001
Inflammation	903	0.04	0.01; 0.06	0.002
Mal exp index	833	0.01	-0.01; 0.03	0.33
Parasitemia	734	0.11	0.08; 0.13	<0.001

Mal exp index: Malaria exposure index, parasitemia

4.1.2 Combined cohorts (RTSS and Junju)

Since RTSS and Junju cohort were assayed using the same protocols for iron markers and malaria antibodies, they were combined to obtain an overall effect of ID and IDA on malaria antibodies. In the combined cohort geometric means of AMA1 and MSP1 antibodies were significantly lower in iron deficient children compared to IR children using low ferritin and low TSAT definitions. Children with IDA had significantly lower AMA1 and MSP1 antibodies, however, anemic children had non-significantly lower compared to the non-anemic children as shown in Figure 4.1 and Table 4.3 for individual cohorts (RTSS and Junju).

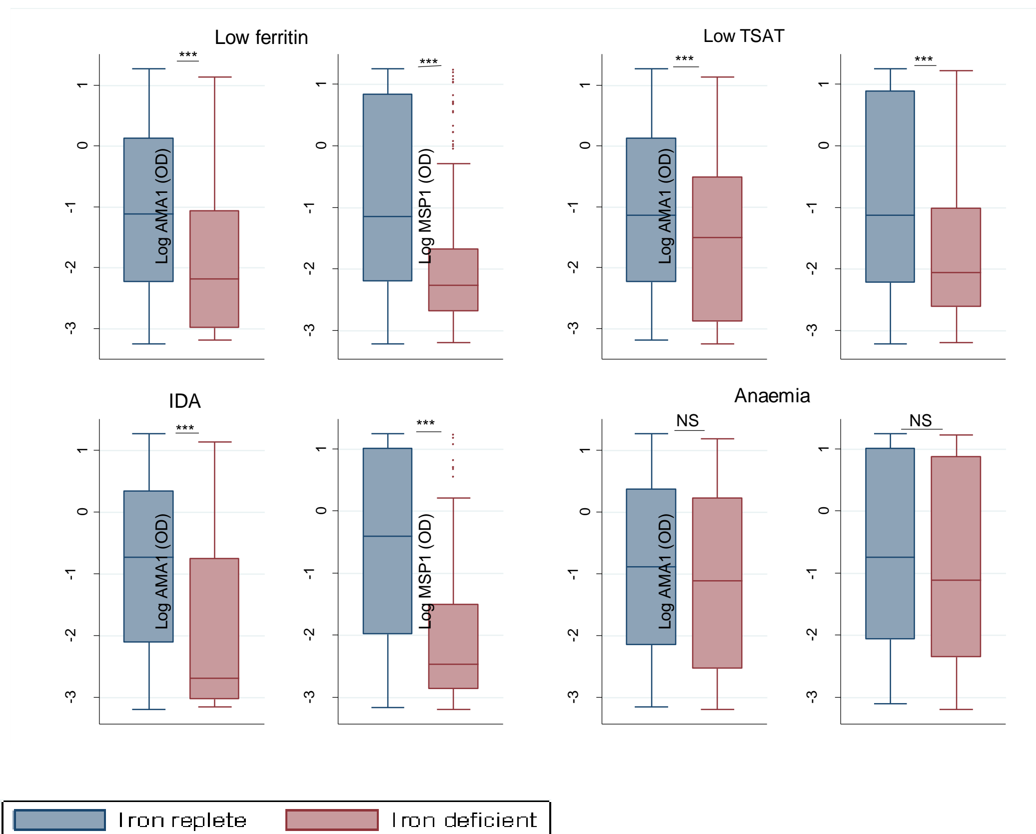

Figure *4*.1: Box plot showing the geometric mean AMA1 and MSP1 antibodies by ID, IDA and anemia

Iron deficiency was defined using two definitions: 1) Low ferritin defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation in children < 5 years or < 15µg/L in children ≥ 5 years; and 2) Transferrin saturation < 10%; Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 4 years or haemoglobin < 11.5 g/dL in children above 4 years; IDA was defined as low ferritin and anaemia; apical merozoite antibodies 1, MSP1; Merozoite surface protein 1, IDA; iron deficiency anaemia. P value was calculated using student t test. *** P < 0.001, ns; not significant

Table 4.3 : Geometric means of AMA1 and MSP1 antibodies by iron deficiency as defined by low ferritin, low TSAT, IDA and anemia for individual cohorts

Antibody	Iron status	n	Geometric mean	[95% Conf. Interval]	P value
Junju cohort					
ID, low ferritin					
AMA1	Replete	441	0.68	0.59; 0.78	<0.001
	Deficient	111	0.15	0.12; 0.19	
MSP1	Replete	441	0.45	0.40; 0.51	<0.001
	Deficient	111	0.15	0.11; 0.19	
ID, TSAT<10%					
AMA1	Replete	334	0.70	0.60; 0.82	<0.001
	Deficient	220	0.30	0.25; 0.37	
MSP1	Replete	334	0.45	0.39; 0.52	<0.001
	Deficient	220	0.27	0.22; 0.33	
IDA					
AMA1	Replete	412	0.57	0.49; 0.66	<0.001
	Deficient	91	0.13	0.11; 0.17	
MSP1	Replete	412	0.42	0.37; 0.48	<0.001
	Deficient	91	0.14	0.11; 0.18	
Anemia					
AMA1	Replete	168	0.49	0.39; 0.62	0.32
	Deficient	365	0.43	0.36; 0.50	
MSP1	Replete	168	0.39	0.32; 0.48	0.21
	Deficient	365	0.33	0.29; 0.38	
RTSS cohort					
ID, low ferritin					
AMA1	Replete	182	0.14	0.12; 0.15	0.21
	Deficient	153	0.12	0.11; 0.14	
MSP1	Replete	182	0.18	0.15; 0.21	0.23
	Deficient	153	0.16	0.13; 0.19	
ID, TSAT<10%					
AMA1	Replete	130	0.13	0.12; 0.15	0.39
	Deficient	205	0.12	0.11; 0.14	
MSP1	Replete	130	0.17	0.14; 0.21	0.98
	Deficient	205	0.17	0.15; 0.20	

ID; Iron deficiency: ID was defined using two definitions: 1) Low ferritin defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation in children < 5 years or < 15µg/L in children ≥ 5 years; and 2) Transferrin saturation < 10%; Anemia was defined as hemoglobin < 11g/dL in children aged 0 to 4 years or hemoglobin < 11.5 g/dL in children above 4 years; IDA was defined as low ferritin and anemia. P value calculated using T test. In RTSS cohort IDA and anemia is not evaluated because hemoglobin measurements were not available

In further analyses using unadjusted linear regression models, there was a significant association between ID (as defined by low ferritin) and AMA1 (β -1.16, 95% CI -1.16; -0.97, $p < 0.001$) and MSP1 (β -0.83, 95% CI -1.02; -0.64, $p < 0.001$) antibodies indicating that ID is associated with lower concentrations of malaria antibodies (Figure 4.2 and Figure 4.3 respectively). Low TSAT <10% was significantly associated with lower AMA1 (β -0.81, 95% CI -0.99; -0.62, $p < 0.001$) and MSP1 (β -0.45, 95% CI -0.63; -0.28, $p < 0.001$) antibody concentrations compared to children with TSAT>10%. Children with IDA had significantly lower AMA1 (β -1.44, 95% CI -1.77; -1.11, $p < 0.001$) and MSP1 (β -1.10, 95% CI -1.41; -0.80, $p < 0.001$) antibodies compared to children without IDA. While anemic children had non-significantly lower AMA1 (β -0.14, 95% CI -0.43; 0.14, $p = 0.32$) and MSP1 (β -0.17, 95% CI -0.43; 0.09, $p = 0.20$) compared to non-anemic children. Ferritin, Hb, TSAT and hepcidin showed significant positive associations with AMA1 and MSP1 antibodies indicating an increase in these iron biomarkers leads to an increase in malaria antibodies (Figure 4.2 and Figure 4.3 respectively).

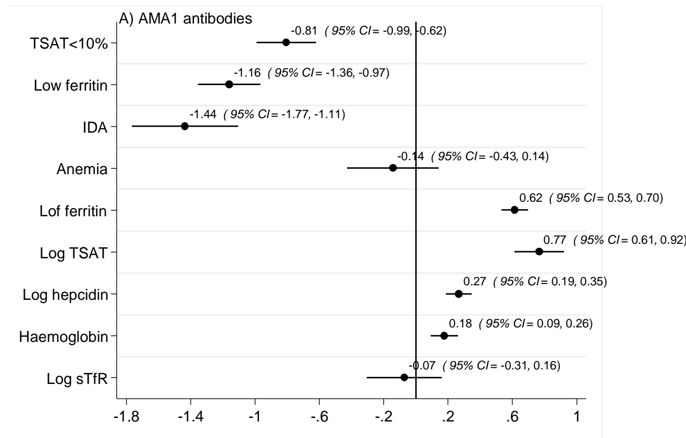

Figure *4*.2: Forest plot showing the unadjusted association between AMA1 antibodies and iron deficiency and iron biomarkers in the combined cohort (Junju and RTSS cohorts).

Iron deficiency (ID) was defined using two definitions: 1) Low ferritin defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation in children < 5 years or < 15µg/L in children ≥ 5 years; and 2) Transferrin saturation < 10%; Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 4 years or haemoglobin < 11.5 g/dL in children above 4 years; IDA was defined as low ferritin and anaemia; transferrin saturation <10%, log TSAT; log transferrin saturation, log sTfR; log soluble transferrin receptors

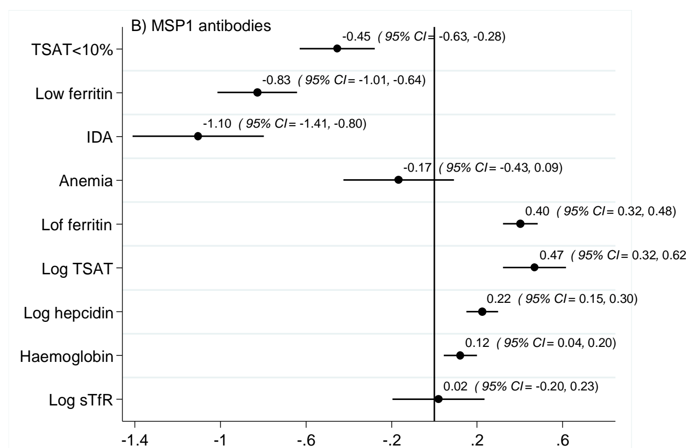

Figure 4.3: Forest plot showing the unadjusted association between MSP1 antibodies and iron deficiency and iron biomarkers in the combined cohort (Junju and RTSS cohorts).

Iron deficiency (ID) was defined using two definitions: 1) Low ferritin defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation in children < 5 years or < 15µg/L in children ≥ 5 years; and 2) Transferrin saturation < 10%; Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 4 years or haemoglobin < 11.5 g/dL in children above 4 years; IDA was defined as low ferritin and anaemia; transferrin saturation <10%, log TSAT; log transferrin saturation, log sTfR; log soluble transferrin receptors

Age, sex, inflammation, study site and malaria exposure were possible confounders in this study. Linear regression models were fitted adjusting for these confounders. In an adjusted model antibody levels to AMA1 (β -0.42, 95% CI -0.59; -0.25, $p < 0.001$) and MSP1 (β -0.40, 95% CI -0.60; -0.20, $p < 0.001$) were significantly lower in iron deficient children compared to IR children Figure 4.4 and Figure 4.5 respectively). However, using a low TSAT definition, only antibodies to AMA1 (β -0.21, 95% CI -0.36; -0.05, $p = 0.008$) remained significantly lower in iron deficient compared to IR children. IDA was also associated with significantly lower AMA1 (β -0.57, 95% CI -0.85; -0.28, $p < 0.001$) and MSP1 (β -0.64, 95% CI -0.97; 0.32, $p < 0.001$) antibodies in children with IDA compared to those without IDA. Anemic children had lower levels of AMA1 and MSP1 antibodies compared to non-anemic children, but this was not significant (Figure 4.4 and Figure 4.5 respectively).

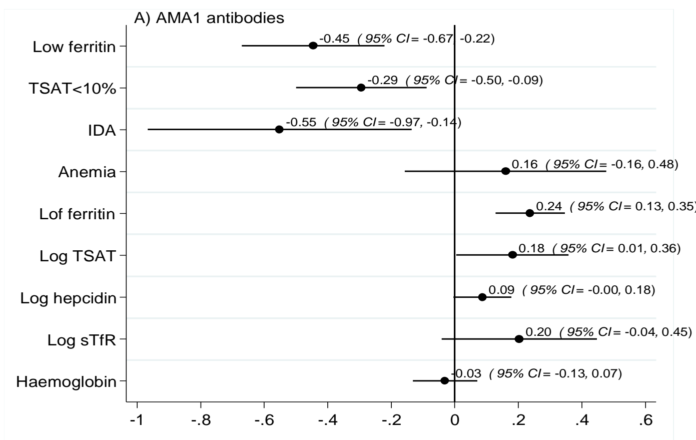

Figure 4.4: Forest plot showing the adjusted association between AMA1 and MSP1 antibodies and iron deficiency and iron biomarkers in the combined cohort (Junju and RTSS cohorts).

Confounders adjusted for included: age, sex, inflammation and malaria exposure index. Iron deficiency (ID) was defined using two definitions: 1) Low ferritin defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation in children < 5 years or < 15µg/L in children ≥ 5 years; and 2) Transferrin saturation < 10%; Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 4 years or haemoglobin < 11.5 g/dL in children above 4 years; IDA was defined as low ferritin and anaemia; transferrin saturation <10%, log TSAT; log transferrin saturation, log sTfR; log soluble transferrin receptors

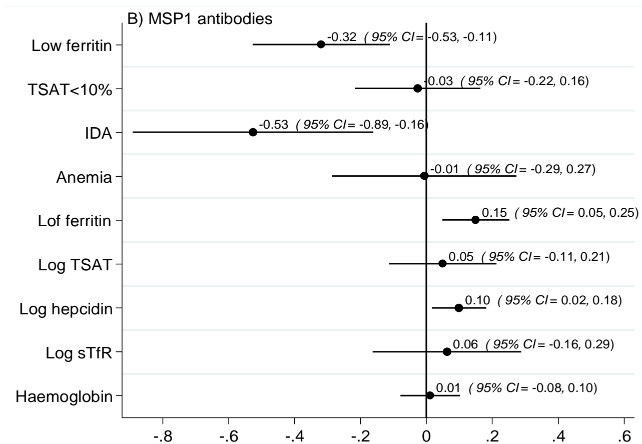

Figure 4.5: Forest plot showing the adjusted association between MSP1 antibodies and iron deficiency and iron biomarkers in the combined cohort (Junju and RTSS cohorts).

Confounders adjusted for included: age, sex, inflammation and malaria exposure index. Iron deficiency (ID) was defined using two definitions: 1) Low ferritin defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation in children < 5 years or < 15µg/L in children ≥ 5 years; and 2) Transferrin saturation < 10%; Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 4 years or haemoglobin < 11.5 g/dL in children above 4 years; IDA was defined as low ferritin and anaemia; transferrin saturation <10%, log TSAT; log transferrin saturation, log sTfR; log soluble transferrin receptors

Individual cohorts i.e., Junju cohort and RTSS cohorts showed similar results to the combined cohorts indicating lower AMA1 and MSP1 antibodies in children with ID and IDA, but this was only significant for the Junju cohort (Table 4.4 and 4.5).

Table 4.4: Linear regression (unadjusted and adjusted) showing the association between AMA1 and MSP1 antibodies and iron deficiency and iron biomarkers in the Junju cohort

				Unadjusted			Adjusted
Iron biomarker	**n**	**Coefficient**	**[95% Conf. Interval]**	**p value**	**Coefficient**	**[95% Conf. Interval]**	**p value**
ID, Low ferritin							
LogAMA1	552	-1.52	-1.81; -1.22	<0.001	-0.62	-0.90; -0.35	<0.001
LogMSP1		-1.14	-1.41; -0.86	<0.001	-0.68	-0.98; -0.37	<0.001
ID, TSAT<10%							
LogAMA1	554	-0.84	-1.09; -0.59	<0.001	-0.19	-0.42; 0.04	0.10
LogMSP1		-0.50	-0.74; -0.26	<0.001	-0.13	-0.38; 0.13	0.32
IDA							
LogAMA1	488	-1.49	-1.81; -1.15	<0.001	-0.57	-0.86; -0.27	<0.001
LogMSP1		-1.12	-1.41; -0.82	<0.001	-0.64	-0.96; -0.31	<0.001
Anemia							
LogAMA1	516	-0.24	-0.53; 0.05	0.10	0.15	-0.08; 0.38	0.20
LogMSP1		-0.21	-0.47; -0.82	0.12	-0.01	-0.27; -0.31	0.91
Log ferritin							
LogAMA1	552	0.71	0.60; 0.82	<0.001	0.29	0.17; 0.42	<0.001
LogMSP1		0.44	0.33; 0.55	<0.001	0.27	0.13; 0.40	<0.001
Log TSAT							
LogAMA1	554	0.81	0.61; 1.01	<0.001	0.22	0.03; 0.40	0.02
LogMSP1		0.52	0.33; 0.71	<0.001	0.16	-0.05; 0.37	0.13
Log hepcidin							
LogAMA1	546	0.39	0.28; 0.49	<0.001	0.12	0.03; 0.22	0.01
LogMSP1		0.28	0.18; 0.38	<0.001	0.15	0.04; 0.26	0.01
Hb							
LogAMA1	516	1.83	1.05; 2.62	<0.001	0.07	-0.58; 0.72	0.83
LogMSP1		1.14	0.43; 1.87	0.002	0.18	-0.55; 0.91	0.63
Log sTfR							
LogAMA1	573	-0.22	-0.52; 0.07	0.13	0.11	-0.14; 0.35	0.41
LogMSP1		-0.04	-0.31; 0.23	0.76	0.06	-0.22; 0.33	0.70

Adjusted for; age, sex, inflammation and malaria exposure index. ID; Iron deficiency: ID was defined using two definitions: 1) Low ferritin defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation in children < 5 years or < 15µg/L in children ≥ 5 years; and 2) Transferrin saturation(TSAT) < 10%; Anemia was defined as hemoglobin < 11g/dL in children aged 0 to 4 years or hemoglobin < 11.5 g/dL in children above 4 years; IDA was defined as low ferritin and anemia: log transferrin saturation, log sTfR: log soluble transferrin receptors; Hb: hemoglobin

Table 4.5: Linear regression (adjusted and unadjusted) showing the association between AMA1 and MSP1 antibodies and iron deficiency and iron biomarkers in the RTSS cohort

		Unadjusted			Adjusted		
Iron biomarker	**n**	**Coefficient**	**[95% Conf. Interval]**	**p value**	**Coefficient**	**[95% Conf. Interval]**	**p value**
ID, Low ferritin							
LogAMA1	335	-0.10	-0.27; 0.06	0.21	-0.01	-0.19; 0.16	0.89
LogMSP1		-0.15	-0.39; 0.09	0.23	0.05	-0.20; 0.30	0.69
ID, TSAT<10%							
LogAMA1	335	-0.07	-0.23; 0.09	0.39	-0.07	-0.24; 0.11	0.45
LogMSP1		-0.003	-0.25; 0.24	0.98	-0.05	-0.30; 0.20	0.67
Log ferritin							
LogAMA1	335	0.03	-0.06; 0.11	0.52	-0.001	-0.20; 0.09	0.99
LogMSP1		0.12	-0.01; 0.24	0.07	0.03	-0.11; 0.17	0.67
Log TSAT							
LogAMA1	335	0.05	-0.10; 0.19	0.52	-0.01	-0.17; 0.15	0.87
LogMSP1		0.03	-0.19; 0.25	0.78	-0.04	-0.27; 0.19	0.74
Log hepcidin							
LogAMA1	298	-0.03	-0.10; 0.04	0.36	-0.05	-0.12; 0.03	0.25
LogMSP1		0.08	-0.02; 0.17	0.13	0.01	-0.20; 0.12	0.84
Log sTfR							
LogAMA1	339	0.22	0.01; 0.44	0.05	0.29	0.06; 0.53	0.01
LogMSP1		0.13	-0.20; 0.45	0.44	0.30	-0.04 0.63	0.08

Adjusted for; age, sex, inflammation and malaria exposure index. ID; Iron deficiency, Low ferritin defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation in children < 5 years or < 15µg/L in children > 5 years TSAT <10%: transferrin saturation <10%, log TSAT: log transferrin saturation, log sTfR: log soluble transferrin receptors.

4.1.3 Replication cohort

Iron deficient children had significantly lower geometric means of AMA1 (w2mef, hb3 and 3d7), MSP2 (3d7 and fc27) and schizont extract antibodies compared to iron replete in the replication cohort (Table 4.6).

Table 4.6: Geometric means of AMA1 alleles, MSP2 alleles and schizonts by low ferritin, IDA and anemia in Ngerenya cohort

Antibody	Iron status	N	Geometric mean	[95% Conf. Interval]	p value (t test)
ID, low ferritin					
AMA1 (W2mef)	Replete	102	1.91	1.62; 2.24	0.01
	Deficient	134	1.48	1.32; 1.65	
AMA1 (HB3)	Replete	102	1.94	1.65; 2.27	0.01
	Deficient	134	1.51	1.35; 1.69	
AMA1 (3D7)	Replete	102	1.79	1.54; 2.09	0.01
	Deficient	134	1.42	1.28; 1.57	
MSP2 (3D7)	Replete	102	2.07	1.76; 2.44	0.002
	Deficient	134	1.54	1.38; 1.70	
MSP2 (FC27)	Replete	102	1.81	1.56; 2.10	0.01
	Deficient	134	1.41	1.28; 1.56	
Schizont	Replete	102	2.05	1.85; 2.27	<0.001
	Deficient	131	1.61	1.49; 1.74	
IDA					
AMA1 (W2mef)	Replete	56	2.00	1.61; 2.48	0.0001
	Deficient	25	1.05	1.00; 1.09	
AMA1 (HB3)	Replete	56	2.00	1.63; 2.46	0.0001
	Deficient	25	1.07	0.98; 1.15	
AMA1 (3D7)	Replete	56	1.84	1.51; 2.24	0.0003
	Deficient	25	1.04	1.00; 1.08	
MSP2 (3D7)	Replete	56	2.06	1.64; 2.59	0.01
	Deficient	25	1.26	1.10; 1.43	
MSP2 (FC27)	Replete	56	1.68	1.40; 2.01	0.004
	Deficient	25	1.10	1.02; 1.19	
Schizont	Replete	56	2.02	1.77; 2.30	<0.0001
	Deficient	25	1.30	1.20; 1.42	
Anemia					
AMA1 (W2mef)	Replete	45	1.93	1.53; 2.44	0.02
	Deficient	36	1.33	1.08; 1.63	
AMA1 (HB3)	Replete	45	1.95	1.56; 2.43	0.02
	Deficient	36	1.33	1.08; 1.65	
AMA1 (3D7)	Replete	45	1.77	1.43; 2.19	0.03
	Deficient	36	1.30	1.06; 1.58	
MSP2 (3D7)	Replete	45	2.12	1.64; 2.73	0.02
	Deficient	36	1.42	1.16; 1.73	
MSP2 (FC27)	Replete	45	1.68	1.37; 2.06	0.03
	Deficient	36	1.25	1.07; 1.46	
Schizont	Replete	45	2.05	1.78; 2.36	0.001

Deficient	36	1.47	1.29; 1.67

ID; Iron deficiency: ID was defined as Low ferritin defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation in children < 5 years or < 15µg/L in children ≥ 5 years; Anemia was defined as hemoglobin < 11g/dL in children aged 0 to 4 years or hemoglobin < 11.5 g/dL in children above 4 years; IDA was defined as low ferritin and anemia: log transferrin saturation, log sTfR: log soluble transferrin receptors. P values calculated using t test

In an unadjusted model ID, IDA and anaemia were significantly associated with lower antibodies to AMA1 alleles (w2mef, hb3 and 3d7), MSP2 alleles (3d7 and fc27) and schizont extract. Ferritin and sTfR were also positively associated with AMA1 alleles, MSP2 alleles and schizont extract antibodies (Table 4.6). After adjusting for confounders (age, sex, inflammation and malaria exposure index), IDA was significantly associated with lower antibodies to AMA1w2mef (β -0.41, 95% CI -0.79; -0.03, p = 0.034), AMA1 hb3 (β -0.44, 95% CI -0.83; -0.07, p = 0.022) and AMA13d7 (β -0.38, 95% CI -0.74; 0.02, p = 0.039) and schizont extract (β -0.27, 95% CI -0.52; -0.03, p = 0.029) compared to children without IDA. ID and anemia were also associated with lower malaria antibodies, but this was not significant (Table 4.7).

Table 4.7: Linear regression (adjusted and unadjusted) showing the association between AMA1 alleles, MSP 2 alleles and schizont antibodies and iron deficiency and iron biomarkers in the Ngerenya cohort

		Unadjusted			Adjusted		
Iron biomarker	**n**	**Coefficient**	**[95% Conf. Interval]**	**P value**	**Coefficient**	**[95% Conf. Interval]**	**P value**
ID, Low ferritin							
LogAMA1 (W2mef)	236	-0.26	-0.44, -0.07	0.01	-0.15	-0.35; 0.05	0.14
LogAMA1(HB3)	236	-0.25	-0.44, -0.06	0.01	-0.13	-0.33; 0.07	0.20
LogAMA1 (3D7)	236	-0.24	-0.41, -0.06	0.01	-0.15	-0.33; 0.04	0.13
LogMSP2 (3D7)	236	-0.30	-0.48, -0.11	0.002	-0.14	-0.34; 0.06	0.17
LogMSP2 (FC27)	236	-0.25	-0.42, -0.08	0.01	-0.09	-0.28; 0.10	0.36
Logschizont	233	-0.24	-0.36, -0.12	<0.001	-0.12	-0.25; 0.01	0.06
IDA							
LogAMA1 (W2mef)	81	-0.65	-0.97; -0.33	<0.001	-0.41	-0.79; -0.03	0.03
LogAMA1(HB3)	81	-0.63	-0.94; 0.32	<0.001	-0.44	-0.83; -0.07	0.02
LogAMA1 (3D7)	81	-0.57	-0.87; -0.27	<0.001	-0.38	-0.74; -0.02	0.04
LogMSP2 (3D7)	81	-0.50	-0.85; -0.15	0.01	-0.17	-0.60; -0.26	0.44
LogMSP2 (FC27)	81	-0.42	-0.70; -0.14	0.003	-0.32	-0.67; 0.03	0.08
Logschizont	81	-0.44	-0.64; -0.23	<0.001	-0.27	-0.52; -0.03	0.03
Anaemia							
LogAMA1 (W2mef)	81	-0.37	-0.69; -0.06	0.02	-0.11	-0.45; 0.22	0.51
LogAMA1(HB3)	81	-0.38	-0.69; -0.07	0.02	-0.15	-0.49; 0.18	0.36
LogAMA1 (3D7)	81	-0.31	-0.61; -0.02	0.04	-0.09	-0.41; 0.23	0.58
LogMSP2 (3D7)	81	-0.40	-0.73; -0.07	0.02	-0.11	-0.48; 0.26	0.55
LogMSP2 (FC27)	81	-0.29	-0.56; -0.03	0.03	-0.16	-0.47; 0.15	0.30
Logschizont	81	-0.34	-0.53; -0.14	0.001	-0.18	-0.40; 0.03	0.09
Log ferritin							
LogAMA1 (W2mef)	236	0.05	-0.04, 0.16	0.21	0.06	-0.04; 0.15	0.23
LogAMA1(HB3)	236	0.06	-0.04, 0.16	0.21	0.05	-0.04; 0.15	0.27
LogAMA1 (3D7)	236	0.05	-0.04, 0.14	0.26	0.05	-0.04; 0.14	0.25
LogMSP2 (3D7)	236	0.15	0.05, 0.24	0.002	0.12	0.03; 0.21	0.01
LogMSP2 (FC27)	236	0.12	0.04, 0.21	0.01	0.08	-0.01; 0.17	0.08
Logschizont	227	0.09	0.03, 0.15	0.01	0.07	0.01; 0.13	0.03
Log sTfR							
LogAMA1 (W2mef)	230	0.21	-0.05, 0.47	0.11	0.20	-0.05; 0.44	0.12
LogAMA1(HB3)	230	0.25	-0.01, 0.51	0.06	0.23	-0.01; 0.48	0.06
LogAMA1 (3D7)	230	0.19	-0.05, 0.44	0.12	0.17	-0.06; 0.40	0.14
LogMSP2 (3D7)	230	0.20	-0.06, 0.46	0.13	0.21	-0.04; 0.46	0.09
LogMSP2 (FC27)	230	0.13	-0.11, 0.37	0.27	0.17	-0.07; 0.40	0.16
Logschizont	227	0.13	-0.04, 0.31	0.14	0.12	-0.04; 0.29	0.14
Log hepcidin							
LogAMA1 (W2mef)	236	-0.03	-0.09, 0.02	0.23	-0.01	-0.07; 0.04	0.65
LogAMA1(HB3)	236	-0.03	-0.09, 0.03	0.29	-0.01	-0.06; 0.05	0.78
LogAMA1 (3D7)	236	-0.04	-0.09, 0.02	0.16	-0.02	-0.07; 0.04	0.56
LogMSP2 (3D7)	236	-0.05	-0.10, 0.01	0.09	-0.03	-0.09; 0.02	0.24

LogMSP2 (FC27)	236	-0.02	-0.07, 0.03	0.50	-0.02	-0.07; 0.03	0.45
Logschizont	230	-0.02	-0.06, 0.01	0.24	-0.01	-0.05; 0.03	0.61

ID; Iron deficiency: ID was defined as Low ferritin defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation in children < 5 years or < 15µg/L in children ≥ 5 years; Anemia was defined as hemoglobin < 11g/dL in children aged 0 to 4 years or hemoglobin < 11.5 g/dL in children above 4 years; IDA was defined as low ferritin and anemia: log transferrin saturation, log sTfR: log soluble transferrin receptors

4.2 **Effect of ID on malaria vaccine responses**

4.2.1 Participant's characteristics

In the RTSS vaccine cohort, 115 children were included in the study. The median age was 10.6 (IQR 7.6-14.4) months. Malaria exposure index was low with a median of 0.072 (IQR 0.00-0.26), however malaria parasitemia data was not available for the children. ID as defined by low ferritin was 44% (50/115), and using a TSAT <10% definition, 62% (71/115). Anemia and IDA data was not available for the RTSS cohort since hemoglobin data was not available.

In the ME-TRAP cohort 91 children were included in the study. The median age for the children was 41.5 (range 26.5-56.2) months. 65% of the children had malaria parasitemia and the malaria exposure index median was 0.33 (range 1.49^{-11}-0.67). Using a low ferritin definition 8% (7/84) (for 7 children ferritin data was not available) were iron deficient while using TSAT <10% 35% (32/91) was iron deficient. 73% (66/90) of the children were anemic while IDA was at 8%. Gender was evenly distributed among the study participants in the two cohorts. Table 4.8 shows a summary of the study participants in the vaccine cohorts.

Table 4.8: Participant's characteristics

	RTSS	ME-TRAP
	no./total %	**no./total %**
Median age months (IQR)	10.6 (7.6-14.4)	41.5 (26.5-56.2)
Sex: Female	55/115 (47.8)	42/91 (46.2)
Inflammation	37/114 (32.5)	34/90 (37.8)
Malaria parasitemia	Na	59/91 (64.8)
Exposure Index (IQR)	0.072 (0.00-0.26)	0.33 (1.49^{-11}-0.67)
Iron deficiency		
Low ferritin	50/115 (43.5)	7/84 (8.3)
TSAT<10%	71/115 (61.7)	32/91 (35.2)
Anemia	na	66/90 (73.3)
IDA	na	7/83 (8.4)
Iron biomarkers	**Median (IQR)**	**Median (IQR)**
Ferritin, µg/L	18.7 (8.8-38.5)	56.2 (35.9-93.8)
Hepcidin, µg/L	8.8 (3.1-19.1)	9.7 (4.3-17.9)
sTfR, mg/L	17.7 (14.7-21.8)	18.3 (14.2-24.9)
Transferrin saturation (%)	8.9 (6.0-13.6)	12.3 (8.8-17.8)
Hb	na	10.5 (9.4-11.2)

Iron deficiency (ID) was defined using two definitions: 1) Low ferritin defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation in children < 5 years or < 15µg/L in children ≥ 5 years; and 2) Transferrin saturation < 10%; Anemia was defined as hemoglobin < 11g/dL in children aged 0 to 4 years or hemoglobin < 11.5 g/dL in children above 4 years; IDA was defined as low ferritin and anemia; transferrin saturation <10%, log TSAT; log transferrin saturation, log sTfR; log soluble transferrin receptors; Hb; hemoglobin, IQR: interquartile range, na: not available

4.2.2 RTSS vaccine cohort

The circumsporozoite antibodies induced by the RTS,S/AS01E vaccine were non significantly lower in the iron deficient children compared to IR except at 3 months after vaccination using both low ferritin and low transferrin saturation definition shown in Table 4.9

Table 4.9: Geometric means of anti-circumsporozoite antibodies by iron deficiency as defined by low ferritin and TSAT<10%.

Timepoint	Iron status	n	Geometric mean	[95% Conf. Interval]	P value (t test)
ID, low ferritin					
Screening	Replete	28	0.57	0.53; 0.65	0.32
	Deficient	29	0.56	0.54; 0.58	
Month 3	Replete	28	14.95	11.40; 19.62	0.93
	Deficient	29	15.13	13.62; 16.81	
Month 6	Replete	26	6.78	5.78; 7.96	0.43
	Deficient	28	6.23	5.29; 7.21	
Month 14	Replete	26	5.59	4.74; 6.60	0.09
	Deficient	28	4.63	3.98; 5.40	
ID, low TSAT					
Screening	Replete	44	0.572	0.54; 0.61	0.61
	Deficient	70	0.562	0.55; 0.58	
Month 3	Replete	44	14.869	12.41; 17.82	0.65
	Deficient	71	15.536	14.63; 16.50	
Month 6	Replete	43	6.682	5.88; 7.59	0.43
	Deficient	67	6.279	5.71; 6.90	
Month 14	Replete	41	5.335	4.64; 6.13	0.30
	Deficient	67	4.877	4.39; 5.42	

Iron deficiency (ID) was defined using two definitions: 1) Low ferritin defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation in children < 5 years or < 15µg/L in children ≥ 5 years; and 2) Transferrin saturation < 10%, TSAT; transferrin saturation

In the univariate model, at screening, 6 and 14 months after vaccination, ID as defined by low ferritin was associated with lower CS antibodies in iron deficient children compared to IR and this was significant 14 months after vaccination (β -0.17, 95% CI -0.34: -0.01, p = 0.04) (Table 4.8). Similarly, low TSAT was associated with lower CS antibodies, but this was not significant. At screening timepoint an increase in ferritin and TSAT levels was significantly associated with increased CS antibodies. However, since this was before the vaccine was administered, this reaction could be due to cross reaction of the naturally acquired CS antibodies in malaria exposed individuals and doesn't represent the vaccine response (Bejon et al., 2008).After adjusting for age, sex, inflammation and malaria exposure index there was no association between the CS antibodies iron deficiency or iron biomarker observed as shown in Table 4.9.

Table 4.10: Linear regression (unadjusted and adjusted) showing the association between circumsporozoite antibodies and iron deficiency and iron biomarkers

		Unadjusted			Adjusted		
Timepoint	**N**	**Coefficient**	**[95% Conf. Interval]**	**p value**	**Coefficient**	**[95% Conf. Interval]**	**p value**
ID, Low ferritin							
Screening	114	-0.02	-0.08; 0.04	0.45	-0.02	-0.09; 0.05	0.53
Month 3	115	0.05	-0.11; 0.20	0.56	0.06	-0.12; 0.23	0.51
Month 6	110	-0.10	-0.25; 0.05	0.21	-0.10	-0.26; 0.06	0.23
Month 14	108	-0.17	-0.34; -0.01	0.04	-0.15	-0.33; 0.02	0.08
ID, TSAT<10%							
Screening	114	-0.02	-0.08;0.04	0.57	-0.01	-0.08; 0.06	0.81
Month 3	115	0.04	-0.12; 0.20	0.59	0.07	-0.10; 0.25	0.42
Month 6	110	-0.06	-0.22; 0.09	0.43	-0.09	-0.25; 0.08	0.29
Month 14	108	-0.09	-0.26; 0.08	0.30	-0.11	-0.29; 0.07	0.23
Log ferritin							
Screening	114	0.03	0.01; 0.06	0.02	0.03	-0.01; 0.06	0.10
Month 3	115	-0.03	-0.10; 0.04	0.41	-0.04	-0.13; 0.05	0.33
Month 6	110	0.02	-0.05; 0.10	0.51	0.02	-0.06; 0.11	0.58
Month 14	108	0.06	-0.02; 0.14	0.13	0.06	-0.03; 0.15	0.20
Log TSAT							
Screening	114	0.06	0.001; 0.11	0.05	0.05	-0.01; 0.11	0.08
Month 3	115	-0.04	-0.18; 0.11	0.62	-0.06	-0.23; 0.10	0.44
Month 6	110	0.01	-0.13; 0.15	0.92	0.04	-0.12; 0.19	0.63
Month 14	108	0.04	-0.12; 0.20	0.61	0.06	-0.11; 0.23	0.47
Log hepcidin							
Screening	92	-0.001	-0.03; 0.03	0.95	0.0001	-0.03; 0.03	1.00
Month 3	93	-0.05	-0.12; 0.03	0.21	-0.06	-0.14; 0.03	0.20
Month 6	88	-0.01	-0.08;0.06	0.71	-0.02	-0.10; 0.06	0.64
Month 14	86	-0.01	-0.09; 0.07	0.78	-0.01	-0.10; 0.07	0.81
Log sTfR							
Screening	114	0.02	-0.05; 0.10	0.53	0.05	-0.03; 0.13	0.21
Month 3	115	-0.04	-0.23; 0.15	0.67	-0.06	-0.28; 0.17	0.62
Month 6	110	-0.06	-0.24; 0.13	0.56	-0.13	-0.33; 0.07	0.21
Month 14	108	-0.14	-0.34; 0.06	0.17	-0.22	-0.44; 0.002	0.05

Adjusted for; age, sex, inflammation and malaria exposure index, iron deficiency (ID) was defined using two definitions: 1) Low ferritin defined as plasma ferritin < 12μg/L or < 30μg/L in the presence of inflammation in children < 5 years or < 15μg/L in children ≥ 5 years; and 2) Transferrin saturation < 10%, log TSAT; log transferrin saturation, log sTfR; log soluble transferrin receptors

4.2.3 ME-TRAP vaccine cohort

Geometric means of interferon gamma (IFN γ) producing PBMCs as assayed by culture assay were non-significantly lower in the iron deficient (as defined TSAT < 10%) children compared to the IR children (Figure 4.6). In the *ex vivo* assay the iron deficient children had non-significantly higher IFN γ producing PBMCs compared to IR children and at 6 months after vaccination this difference was significant as shown in Figure 4.6. However, geometric means of IFN γ producing PBMCs assayed by *ex vivo* assay were non-significantly lower in the anemic compared to the non-anemic children Figure 4.7. Analyses using low ferritin definition and IDA were not done since only 8 children were iron deficient using these definitions.

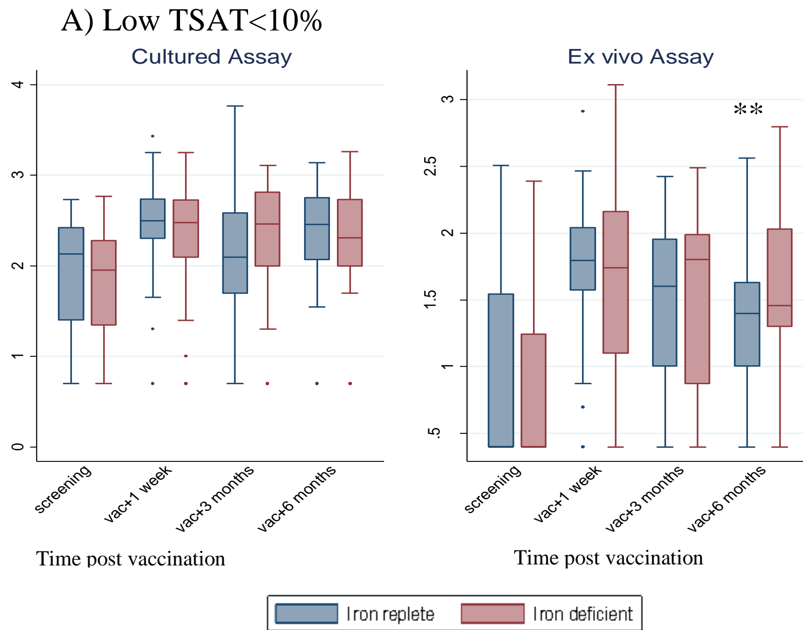

Figure 4.6: Box plot showing the geometric mean Interferon gamma producing PBMCs assayed by culture and ex vivo assays iron status defined by low TSAT (TSAT<10%).

Iron deficiency (ID) was defined as transferrin saturation (TSAT) < 10%; Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 4 years or haemoglobin < 11.5 g/dL in children above 4 years; ** p = 0.03

B) Anemia

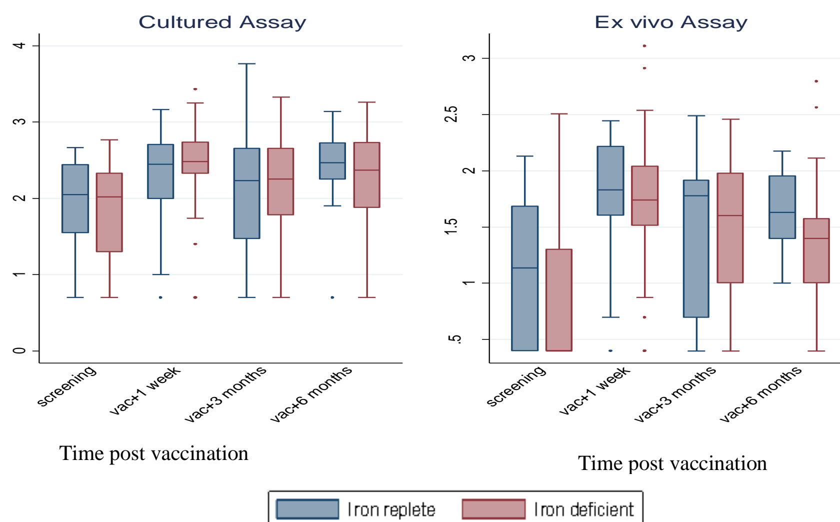

Figure 4.7:Box plot showing the geometric mean Interferon gamma producing PBMCs assayed by culture and ex vivo assays anemia.

Iron deficiency (ID) was defined as transferrin saturation (TSAT) < 10%; Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 4 years or haemoglobin < 11.5 g/dL in children above 4 years.

Univariable and multivariable analysis of culture IFN γ producing PBMCs showed that there was no association between the IFN γ producing PBMCs and iron deficiency (low transferrin) or anemia. There was also no association between the IFN γ producing PBMCs and iron biomarkers (Table 4.11)

Table 4.11: Linear regression (unadjusted and adjusted) showing the association of interferon gamma producing PBMC's (cultured) by iron deficiency and iron biomarkers

		Unadjusted			Adjusted		
Timepoint	**N**	**Coefficient**	**[95% Conf. Interval]**	**p value**	**Coefficient**	**[95% Conf. Interval]**	**p value**
TSAT<10%							
Screening	91	-0.05	-0.34; 0.24	0.74	-0.09	-0.40; 0.21	0.54
Vac+1 week	81	-0.15	-0.40; 0.09	0.22	-0.19	-0.46; 0.07	0.16
Vac+3 months	78	-0.13	-0.22; 0.48	0.46	0.10	-1.27; 0.46	0.60
Vac+6 months	61	-0.15	-0.48; 0.17	0.35	-0.19	-0.53; 0.16	0.28
Anaemia							
Screening	90	-0.05	-0.37; 0.26	0.74	0.002	-0.34; 0.34	0.99
Vac+1 week	80	0.16	-0.11; 0.43	0.24	0.152	-0.15; 0.45	0.31
Vac+3 months	77	0.02	-0.36; 0.39	0.93	-0.01	-0.41; 0.39	0.93
Vac+6 months	60	-0.14	-0.49; 0.20	0.41	-0.15	-0.53; 0.22	0.42
Log ferritin							
Screening	84	-0.03	-0.19; 0.13	0.72	-0.031	-0.24; 0.18	0.78
Vac+1 week	76	-0.03	-0.18; 0.12	0.68	0.032	-0.16; 0.23	0.75
Vac+3 months	70	0.03	-0.17; 0.22	0.79	0.081	-0.15; 0.31	0.49
Vac+6 months	55	0.05	-0.13; 0.22	0.62	0.062	-0.16; 0.28	0.58
Log TSAT							
Screening	91	0.11	-0.15; 0.38	0.41	0.140	-0.15; 0.43	0.34
Vac+1 week	81	0.15	-0.09; 0.38	0.21	0.180	-0.08; 0.44	0.18
Vac+3 months	78	0.18	-0.13; 0.50	0.25	0.244	-0.11; 0.60	0.17
Vac+6 months	61	0.24	-0.06; 0.54	0.11	0.291	-0.05; 0.63	0.09
Log hepcidin							
Screening	84	-0.09	-0.20; 0.03	0.15	-0.086	-0.23; 0.06	0.23
Vac+1 week	76	-0.03	-0.14; 0.08	0.55	-0.031	-0.16; 0.10	0.64
Vac+3 months	71	-0.08	-0.22; 0.07	0.31	-0.079	-0.26; 0.10	0.37
Vac+6 months	56	0.05	-0.09; 0.19	0.48	0.022	-0.13; 0.18	0.78
Log sTfR							
Screening	88	-0.04	-0.36; 0.28	0.80	0.071	-0.32; 0.47	0.72
Vac+1 week	78	-0.13	-0.42; 0.17	0.40	-0.112	-0.46; 0.24	0.53
Vac+3 months	76	-0.16	-0.59; 0.28	0.47	-0.152	-0.67; 0.37	0.56
Vac+6 months	59	-0.18	-0.55; 0.19	0.33	-0.095	-0.56; 0.37	0.68
Hb							
Screening	90	-0.002	-0.07; 0.07	0.96	0.01	-0.08; 0.10	0.86
Vac+1 week	80	-0.02	-0.09; 0.05	0.56	-0.03	-0.11; 0.05	0.40
Vac+3 months	77	0.02	-0.07; 0.11	0.61	0.03	-0.08; 0.13	0.65
Vac+6 months	60	0.04	-0.05; 0.13	0.34	0.02	-0.09; 0.13	0.72

Adjusted for; age, sex, inflammation and malaria exposure index, iron deficiency (ID) was defined as transferrin saturation < 10%, Anemia was defined as hemoglobin < 11g/dL in children aged 0 to 4 years or hemoglobin < 11.5 g/dL in children above 4 years; log TSAT; log transferrin saturation, log sTfR; log soluble transferrin receptors, Hb; haemoglobin, vac; vaccination, timepoint indicate the time since the vaccination was administered.

In the *ex vivo* assay, using univariable regression model, IFN γ producing PBMCs showed no association with iron deficiency (low transferrin saturation) except at 6 months after vaccination when an association was observed. Anemia was significantly associated with lower IFN γ producing PBMCs at screening and 6 months after vaccination. Increase in ferritin was associated with lower IFN γ producing PBMCs 6 months after vaccination (Table 4.12). After adjusting for confounders (age, sex, inflammation and malaria exposure index), there was a significant association between iron deficiency (low transferrin saturation) and IFN γ producing PBMCs 6 months after vaccination. An increase in ferritin and TSAT levels was also significantly associated with lower IFN γ producing PBMCs 6 months after vaccination (Table 4.12).

Table 4.12: Linear regression (adjusted and unadjusted) showing the association between interferon gamma producing PBMC's (ex vivo) and iron deficiency and iron biomarkers

		Unadjusted			Adjusted		
Timepoint	**n**	**Coefficient**	**[95% Conf. Interval]**	**p value**	**Coefficient**	**[95% Conf. Interval]**	**p value**
TSAT<10%							
Screening	87	-0.13	-0.41; 0.15	0.35	-0.17	-0.46; 0.12	0.25
Vac+1 week	79	-0.11	-0.39; 0.17	0.44	-0.14	-0.43; 0.15	0.34
Vac+3 months	71	0.10	-0.21; 0.42	0.52	0.15	-0.15; 0.46	0.32
Vac+6 months	63	0.30	0.03; 0.58	0.03	0.38	0.10; 0.65	0.01
Anaemia							
Screening	87	-0.31	-0.60; -0.02	0.04	-0.41	-0.72; -0.11	0.01
Vac+1 week	78	-0.14	-0.45; 0.18	0.39	-0.17	-0.49; 0.15	0.30
Vac+3 months	70	-0.06	-0.40; 0.28	0.73	-0.06	-0.41; 0.28	0.72
Vac+6 months	62	-0.31	-0.60; -0.02	0.04	-0.29	-0.61; 0.03	0.07
Log ferritin							
Screening	80	-0.06	-0.23; 0.10	0.44	-0.04	-0.24; 0.16	0.68
Vac+1 week	73	-0.02	-0.196; 0.15	0.81	-0.03	-0.25; 0.18	0.75
Vac+3 months	64	-0.04	-0.24; 0.16	0.67	-0.11	-0.33; 0.10	0.29
Vac+6 months	57	-0.19	-0.35; -0.03	0.02	-0.23	-0.41; -0.04	0.02
Log TSAT							
Screening	87	0.06	-0.20; 0.31	0.67	0.13	-0.14; 0.40	0.34
Vac+1 week	79	0.05	-0.21; 0.31	0.70	0.09	-0.19; 0.38	0.51
Vac+3 months	71	-0.15	-0.43; 0.14	0.31	-0.15	-0.43; 0.14	0.32
Vac+6 months	63	-0.21	-0.47; 0.04	0.10	-0.33	-0.60; -0.06	0.02
Log hepcidin							
Screening	80	-0.03	-0.15; 0.09	0.62	0.01	-0.13; 0.15	0.92
Vac+1 week	73	0.04	-0.08; 0.16	0.54	0.003	-0.13; 0.14	0.97
Vac+3 months	65	0.01	-0.12; 0.14	0.89	-0.02	-0.17; 0.13	0.78
Vac+6 months	58	-0.002	-0.12; 0.12	0.97	0.01	-0.11; 0.14	0.83
Hb							
Screening	87	0.03	-0.04; 0.01	0.41	0.04	-0.05; 0.13	0.35
Vac+1 week	78	0.03	-0.04; 0.11	0.37	0.03	-0.06; 0.12	0.51
Vac+3 months	70	0.03	-0.06; 0.11	0.50	0.06	-0.03; 0.15	0.22
Vac+6 months	62	0.04	-0.03; 0.11	0.31	0.05	-0.03; 0.13	0.22
Log sTfR							
Screening	85	-0.15	-0.45; 0.16	0.35	-0.36	-0.71; -0.01	0.05
Vac+1 week	76	-0.09	-0.41; 0.24	0.59	-0.07	-0.45; 0.29	0.68
Vac+3 months	70	0.15	-0.23; 0.53	0.43	-0.05	-0.46; 0.37	0.82
Vac+6 months	61	0.02	-0.31; 0.35	0.91	0.01	-0.38; 0.39	0.98

Adjusted for; age, sex, inflammation and malaria exposure index, iron deficiency (ID) was defined as transferrin saturation < 10%, Anemia was defined as hemoglobin < 11g/dL in children aged 0 to 4 years or hemoglobin < 11.5 g/dL in children above 4 years; log TSAT; log transferrin saturation, log sTfR; log soluble transferrin receptors, Hb; haemoglobin, vac; vaccination, timepoint indicate the time since the vaccination was administered

4.3 **Discussion**

4.3.1 Effects of ID on acquisition of natural immunity to malaria

Malaria and ID are major public health problems in Africa and especially in Kenya. In this study AMA1 and MSP1 antibodies were significantly lower in iron deficient compared to IR children in the combined Junju and RTSS cohorts using the WHO low ferritin definition. However, using ID defined by TSAT <10% only AMA1 was significant. Similar results were obtained in individual cohorts i.e., Junju cohort and RTSS cohort, however this was statistically significant in the Junju cohort only. These results were replicated in the Ngerenya cohort where antibodies to MSP2 alleles, AMA1 alleles and schizont were significantly lower in iron deficient children compared to IR using the low ferritin definition.

ID and IDA were significantly associated with lower malaria antibodies in children in the combined cohorts. This agrees with the previous study carried out by Nyakeriga et al where they reported that concentrations of malaria-specific IgG2, IgG4, and IgE and of total IgE were significantly lower in iron deficient compared to IR children (Nyakeriga et al., 2004) However, their study did not measure specific antibodies that have been associated with natural immunity against malaria or antibodies that are being explored for use as malaria vaccines which are the focus in the current study. In addition, they had a small sample size of 195 children while the current study has over 1000 children. There is very little data on the effects of ID on malaria immunity especially on specific malaria antibodies. To support the study conducted in humans, a study conducted in a mouse model reported that total IgG levels to *Plasmodium yoelii* were much lower in mice with induced IDA compared to the control mice with no IDA (Matsuzaki-Moriya et al., 2011) In the RTSS cohort ID was found to be non-significantly associated with low malaria antibodies, which could be due to the young age of the children in this cohort, a median age of 12.6 (IQR 9.1-16.8) months. Additionally, they had low malaria

exposure with a median malaria exposure index of 0.09 (IQR 2.17^{-14}, 0.32). Data in this study show that age was strongly associated with increased malaria antibody concentrations which agrees with other studies that have reported that malaria immunity is acquired with age and exposure (Mugyenyi et al., 2017; Teo et al., 2016). Lower antibodies in iron deficient individuals have also been reported in other diseases such as pneumococcus where pneumococcal specific IgG subclasses (IgG1 and IgG2) were lower in iron deficient compared to healthy children (Feng et al., 1994). This supports our finding that ID may alter humoral response in infections.

Although there are few studies on the effects of ID on immunity to malaria, other studies have focused on the effects of ID on humoral immunity more generally. Some studies report similar findings to what we have found in this study although a few conflicts. Hassan et al., reported lower serum IgG concentrations in iron deficient children compared to healthy controls (Hassan et al., 2016). Galan et al also reported significantly lower IgG and IgA in children with severe ID compared to healthy controls but there was no significant difference between the children with mild ID and healthy controls (Galan et al., 1988). A study conducted in Algeria, showed that children with IDA had lower IgG, IgD and IgA concentrations compared to control children without IDA (Abbassia Demmouche, 2014). Studies conducted in adults have shown similar reports to that reported in children. Tang et al showed that pregnant women with IDA had lower IgG levels compared to normal pregnant women (Tang et al., 2006). A study carried out in Iran reported that ID premenopausal adult females had non-significantly lower IgG and IgA concentrations compared to heathy controls without ID (Sadeghian et al., 2010). However, a few studies have reported normal humoral immunity in ID in contrast to our results (Bagchi et al., 1980; Das et al., 2014; Thibault et al., 1993). However, these studies had small sample sizes of less than 100 participants compared to our study with over 1000 participants.

Various iron biomarkers were evaluated to investigate how they were associated with malaria antibodies. From this study higher levels of ferritin, transferrin saturation and

hepcidin were associated with higher concentrations of malaria antibodies. A study by Galan et al in 10 months children agrees with our finding showing that increased ferritin increases humoral immunity (Galan et al., 1988). Atkinson et al, also showed that plasma hepcidin concentrations were strongly positively associated with antibodies to *P. falciparum* (Atkinson et al., 2015). Sobhani et al reported a positive correlation between iron and humoral immunity in pregnant women which agrees with our findings however, they found no significant association with ferritin levels which contrasting our findings (Sobhani et al., 2011)

4.3.2 Effects of iron deficiency on malaria vaccine responses

In the RTSS vaccinated children, the CSP antibodies induced by the RTSS vaccine were non-significantly lower in iron deficient compared to IR children. This agrees with previous studies that have reported lower vaccine antibody levels in children with iron deficiency. Chandar et al, reported non significantly lower tetanus and typhoid antibodies in iron deficient children compared to IR children (Chandra & Saraya, 1975). Brussow et al reported that iron deficient children vaccinated with the diptheria-pertussis vaccine had significantly lower antibodies that were below protective levels compared to iron replete children. However, for tetanus vaccine the antibodies were non-significantly lower in iron deficient children (Brussow et al., 1995). In an animal model, ID rats immunized with influenza vaccine had significantly lower vaccine responses compared to iron replete rats (Agnès Dhur et al., 1990). However, a few studies have reported similar vaccine responses in iron deficient and IR individuals (Crogan et al., 2005; Macdougall et al., 1975), although these studies were limited by small sample sizes with some having less than 20 participants thus making interpretation hard (Crogan et al., 2005; Macdougall et al., 1975).

In the ME-TRAP cohort, the vaccine induced protection by stimulating IFN γ producing PBMCs post vaccination. In the *ex vivo* assay ID was associated with significantly higher IFN γ producing PBMCs compared to the iron replete group at 6 months after

vaccination. In the cultured assay there was no association observed between iron deficiency and IFN γ producing PBMCs after vaccination. This is in agreement with other studies that have reported higher IFN γ levels in iron deficient individuals. Kuvibidila et al., reported higher baseline IFN γ, in the supernatant of iron deficient spleen cells compared to iron replete control cells (Kuvibidila et al., 2010). Jayson et al also reported higher induced IFN γ in iron deficient children while spontaneous IFN γ was lower in iron deficient children compared to IR children. The study also reported that the percentage of lymphocytes producing both IFN γ and tumor necrotic factor were significantly higher in iron deficient children compared to IR children (Jason et al., 2001). In contrast with these findings, Nyakeriga et al reported that mRNA levels for IFN γ were non-significantly lower in iron deficient children compared to iron replete but this was not significant (Nyakeriga et al., 2005) . In animal models, IFN γ concentrations have been reported to be significantly lower in iron deficient compared to iron replete animals (Kuvibidila et al., 2010; Kuvibidila & Warrier, 2004). The conflicting results obtained in these studies could be due to different assays used in measuring IFN γ. IFN γ was assayed using ELISPOTs in this study and in our protocol both in ex vivo and cultured assays had an induction step thus the results obtained may not be comparable to flow cytometry where ex vivo assays have no induction and ELISA where induction is not necessary.

4.3.3 Possible mechanisms via which iron deficiency might alter immunity

The results of this study showed that ID and IDA were significantly associated with low malaria specific humoral responses and lower RTS'S/AS0E vaccine responses in children. This finding indicates that ID could be affecting the immune system. We hypothesize that there may be various mechanisms through which ID could affect the immune system. Several studies have demonstrated the important role of iron in T and B cell proliferation and the generation of specific responses to infection indicating that ID alters the proliferation of these cells hence decreasing their response against infections (

Beard, 2001; Kramer et al., 2002). Thus, the low antibody concentrations observed in ID children in this study could be due to impaired B cell proliferation since for B cells to produce antibodies they have to differentiate into plasma cells and this process requires iron. T cells have also been shown to be significantly decreased in iron deficient individuals. T cells are important in B cell stimulation to antibody producing plasma cells and in the production of cytokines such as IL-2 that help in immunoglobulin class switching (Attia et al., 2009; Mullick et al., 2006). Therefore, the lower humoral response observed in iron deficient children could also be due to decreased numbers of T cells that are required in B cell stimulation to produce antibodies. Jabara et al showed that a mutation in transferrin receptors expressed in B and T cells, that helps in iron uptake, led to absence of circulating IgG due to impaired IgE class switching (Jabara et al., 2016). We hypothesize that perhaps the low malaria specific IgG in the iron deficient children could be due to insufficient iron required for immunoglobulin class switching. A fourth possible mechanism causing lower humoral responses in iron deficient children could be due to lower exposure of malaria in iron deficient children compared to IR children. This mechanism is supported by studies that have shown ID is protective against malaria infection (Muriuki et al., 2019; Nyakeriga et al., 2004). However, Feng et al reported decreased pneumococcal specific IgG subclasses (IgG1 and IgG2) in iron deficient children compared to healthy control children (Feng et al., 1994). Therefore, it is possible that the low malaria antibodies could actually be due to an effect of ID on humoral immunity.

4.4 **Challenges and strength of study**

One of the challenges faced in this study is that most iron markers are affected by inflammation. Additionally, natural immunity to malaria has been shown to develop over a period. This mean the older children that had been more exposed to malaria had built better immunity compared to the young children who are less exposed. To help curb this we adjusted for the child's age, inflammation and malaria exposure index in the linear regression models. The limitation in this study was that transferrin saturation data

for the Ngerenya was not available thus analysis was only done using low ferritin definition. In ME-TRAP vaccine cohort only 8 participants were deficient using low ferritin definition thus analysis was done using low TSAT definition. Some iron markers were also not available for all cohorts. The strength of our study is we had large sample size (n= 1,160 children) from cohorts with different malaria intensities thus giving a good representation. Two potential malaria vaccines that are current under different levels in trial phase (RTS'S/AS0E and FFP METRAP) were included in this study. This is the first study to assay effects of iron deficiency on these malaria vaccines. Additionally, we analyzed various iron markers that have not been analyzed in previous studies on iron deficiency and immunity

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1 Summary

Malaria and ID are major public health problems in Africa and especially in Kenya. In this study AMA1 and MSP1 antibodies were significantly lower in iron deficient compared to IR children in the combined Junju and RTSS cohorts using the WHO low ferritin definition. However, using ID defined by TSAT <10% only AMA1 was significant. Similar results were obtained in individual cohorts i.e., Junju cohort and RTSS cohort, however this was statistically significant in the Junju cohort only. These results were replicated in the Ngerenya cohort where antibodies to MSP2 alleles, AMA1 alleles and schizont were significantly lower in iron deficient children compared to IR using the low ferritin definition. In the RTSS vaccinated children, the CSP antibodies induced by the RTSS vaccine were non-significantly lower in iron deficient compared to IR children. In the ME-TRAP cohort, the vaccine induced protection by stimulating IFN γ producing PBMCs post vaccination. In the ex vivo assay ID was associated with significantly higher IFN γ producing PBMCs compared to the iron replete group at 6 months after vaccination. In the cultured assay there was no association observed between iron deficiency and IFN γ producing PBMCs after vaccination.

5.2 Conclusions

This study demonstrated the effects of ID and IDA were associated with low acquisition of malaria antibodies and RTSS malaria vaccine candidate which had not been previously explored. This is very key because malaria is one of the leading causes of mortality in children below 5 years. We currently do not have a licensed vaccine although various vaccines are under way at different phases of development. RTS,S was recommended for use in malaria endemic regions by the WHO in 2021.This finding is an important discovery since most vaccines focus on production of antibodies as a

mechanism of protection against malaria infection. Understanding how these vaccines are affected by children's iron status will guide policy makers and vaccine developers on the best approach to use to ensure maximum vaccine efficacy.

5.3 Recommendations and scope for future work

1. The findings in this study support the WHO recommendation to offer iron supplementation coupled with malaria treatment in malaria endemic regions since ID also affects the immune status of a child.
2. Screening children for iron deficiency before vaccination is recommended to the Ministry of health to prevent poor vaccine responses in children.
3. This observational study couldn't conclusively establish causality in the relationship between ID and natural immunity to malaria. Therefore, a more robust study such as a randomized controlled trial or Mendelian randomization is needed to establish a causal relationship between ID and immunity.
4. This study focused on quantitative levels of antibodies. This doesn't exhaustively explain how ID affects functionality of these antibodies and other immune parameters such as T cells and cytokines. A study that examines effects of ID on the function of the immune system is recommended.

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APPENDICES

Appendices I: Ethics certificate

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 April 29, 2019

TO: **DR. SARAH ATKINSON, PRINCIPAL INVESTIGATOR**

THROUGH: **THE DIRECTOR, CGMR-C, KILIFI**

Dear Madam,

RE: KEMRI/SERU/CGMR-C/046/3257 (*REQUEST FOR ANNUAL RENEWAL*): THE GENETICS OF IRON STATUS AND SUSCEPTIBILITY TO CHILDHOOD INFECTIONS

Thank you for the continuing review report for the period **May 09, 2018 to March 18, 2019**

This is to inform you that the Expedited Review Team of the KEMRI Scientific and Ethics Review Unit (SERU) was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **May 09, 2019** through to **May 08, 2020**. Please note that authorization to conduct this study will automatically expire on **May 08, 2020**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the **SERU** by **March 28, 2020.**

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

Yours faithfully,

ENOCK KEBENEI,
THE ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT.

In Search of Better Health

Appendices II: Publication

Clinical Infectious Diseases

MAJOR ARTICLE

IDSA Infectious Diseases Society of America hivma

Iron Deficiency Is Associated With Reduced Levels of *Plasmodium falciparum*-specific Antibodies in African Children

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Background. Iron deficiency (ID) and malaria are common causes of ill-health and disability among children living in sub-Saharan Africa. Although iron is critical for the acquisition of humoral immunity, little is known about the effects of ID on antibody responses to *Plasmodium falciparum* malaria.

Methods. The study included 1794 Kenyan and Ugandan children aged 0–7 years. We measured biomarkers of iron and inflammation, and antibodies to *P. falciparum* antigens including apical merozoite antigen 1 (anti-AMA-1) and merozoite surface antigen 1 (anti-MSP-1) in cross-sectional and longitudinal studies.

Results. The overall prevalence of ID was 31%. ID was associated with lower anti-AMA-1 and anti-MSP-1 antibody levels in pooled analyses adjusted for age, sex, study site, inflammation, and *P. falciparum* parasitemia (adjusted mean difference on a log-transformed scale (β) −0.46; 95 confidence interval [CI], −.66, −.25 $P < .0001$; β −0.33; 95 CI, −.50, −.16 $P < .0001$, respectively). Additional covariates for malaria exposure index, previous malaria episodes, and time since last malaria episode were available for individual cohorts. Meta-analysis was used to allow for these adjustments giving β −0.34; −0.52, −0.16 for anti-AMA-1 antibodies and β −0.26; −0.41, −0.11 for anti-MSP-1 antibodies. Low transferrin saturation was similarly associated with reduced anti-AMA-1 antibody levels. Lower AMA-1 and MSP-1-specific antibody levels persisted over time in iron-deficient children.

Conclusions. Reduced levels of *P. falciparum*-specific antibodies in iron-deficient children might reflect impaired acquisition of immunity to malaria and/or reduced malaria exposure. Strategies to prevent and treat ID may influence antibody responses to malaria for children living in sub-Saharan Africa.

Keywords. iron deficiency; immunity; children; malaria; Africa.

Iron deficiency (ID) is highly prevalent among young children living in sub-Saharan Africa [1], and iron deficiency anemia (IDA) is the leading cause of years lived with disability among African children [2] due to its negative effects on child development [3]. Malaria is also a major public health problem causing approximately 405 000 deaths in 2018, of which 85% occurred in sub-Saharan Africa, mainly among young children [4]. Children acquire immunity to malaria over time and antibodies to merozoite antigens are important mediators of naturally-acquired immunity [5, 6], in addition to other responses.

Iron is important for the development of humoral immunity and antibody production. ID impairs B-cell proliferation and antibody production [7], and a mutation in transferrin receptor 1 (TfR1), which causes insufficient cellular iron uptake, leads to defective B- and T-cell activation and combined-immunodeficiency [8]. ID is associated with reduced antibody levels in children [9–11] and in rat models [12], as well as with weakened vaccine responses [7, 13], although other studies have found little association with antibody levels [14–16] or vaccine responses [17, 18]. ID has also been associated with reduced frequencies of B and T cells and cytokines, necessary for antibody production [8–10, 19].

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Although ID is highly prevalent among African children and is known to influence immune responses little is known about the effect of ID on the acquisition of immunity to malaria. We previously observed that ID was associated with decreased total immunoglobulin G (IgG) and immunoglobulin E (IgE) levels to *P. falciparum* schizont extract [20] and that hepcidin, the master iron-hormone, was associated with increased levels of antibodies to anti-AMA-1 and anti-MSP-2 antigens [21], in small studies. In the current study, we investigated the relationship between iron status and antibody levels to specific *P. falciparum* antigens in 1794 Kenyan and Ugandan children. We evaluated antibodies to 2 major merozoite antigens, anti-AMA-1 and anti-MSP-1, which are targets of acquired immunity, and antibodies to these antigens have previously been associated with protective immunity to malaria in our study population [5, 22].

MATERIALS AND METHODS

Ethical Approval

Ethical approval was provided by the Scientific Ethics Review Unit of the Kenya Medical Research Institute (KEMRI/SERU/CGMR-C/046/3257/2983), by the Uganda Virus Research Institute (reference GC/127/12/07/32), the Uganda National Council for Science and Technology (MV625), and in the United Kingdom by the London School of Hygiene & Tropical Medicine Ethics Committee (A340) and the Oxford Tropical Research Ethics Committee (OXTREC, 39-12 and 42-14 and 37-15).

Study Population

We used data from community-based cohorts of children in Kilifi, Kenya, and Entebbe, Uganda.

Kenya: The Kenyan children included two community-based cohorts exposed to varying levels of malaria transmission, Junju and RTS,S. Junju is a surveillance cohort evaluating immunity to malaria as described elsewhere [23]. The RTS,S cohort is an extension of the RTS,S/AS01E vaccine trial against malaria conducted between 2007 and 2008 [24]. Both cohorts are under active weekly surveillance to assess for fever, and a malaria blood film is taken if the temperature is > 37.5°C. Additionally, annual cross-sectional blood samples are taken for immunology and parasitology during the dry period before the main annual malaria transmission season. Iron biomarkers and malaria antibodies were measured on the same plasma sample from a single annual cross-sectional bleed based on the availability of a sample archived at –80°C.

Uganda: The Entebbe Mother and Baby Study (EMaBS) is a prospective birth cohort that was originally designed as a randomized double-blind placebo-controlled trial to determine whether antihelmintic treatment during pregnancy and early childhood was associated with differential responses to vaccination or incidence of infections such as malaria, pneumonia and diarrhea [25]. Children had active surveillance for malaria and other infections during fortnightly home visits and quarterly clinic visits, and an annual blood sample was collected. Malaria antibodies were measured from a sample taken at 5 years of age, and iron biomarkers were measured from a single annual bleed taken between 1 and 4 years of age based on the availability of plasma samples archived at –80°C.

Laboratory Procedures

Iron and Inflammation Biomarkers

The measured biomarkers of iron status and inflammation were iron (MULTIGENT iron calorimetric assay, Abbott Architect, USA), ferritin, transferrin (chemiluminescent microparticle immunoassay [CMI], Abbott Architect), soluble transferrin receptor (sTfR, Human sTfR ELISA, BioVendor), hepcidin (DRG Hepcidin 25 [bioactive] high sensitive ELISA Kit, DRG Diagnostics), transferrin (CMI, Abbott Architect) hemoglobin (Coulter analyzer, Beckman Coulter), and C-reactive protein (CRP, MULTIGENT CRP Vario assay, Abbott Architect). In Uganda, hemoglobin concentrations were adjusted for an altitude of > 1000 m above sea level (by subtracting 0.2 g/dL) [26]. *P. falciparum* parasitemia was determined at the time of malaria antibody measurement using Giemsa-stained thick and thin blood smears.

Plasmodium falciparum Antibody Assays

Antibodies against the AMA1 3D7 sequence and $MSP1_{42}$ 3D7 sequence of *P. falciparum* antigens were measured from plasma samples by enzyme-linked immunosorbent assays (ELISAs) according to standard protocols as previously described for the RTS,S, Junju [27] and EMaBS cohorts [28]. A pool of malaria hyperimmune sera was serially diluted on each plate, and the optical densities from these dilutions were used to generate a standard curve. From this standard curve, an arbitrary unit per milliliter (AU/mL) was calculated for each sample based on the relative optical density obtained. Different pools of malaria-hyperimmune sera and ELISA antigens were used in the Kenyan and Ugandan laboratories.

Definitions

Inflammation was defined as CRP > 5 mg/L. ID was defined as plasma ferritin < 12 µg/L or < 30 µg/L in the presence of inflammation in children < 5 years or < 15 µg/L in children ≥ 5 years as defined by the World Health Organization (WHO) [29]. Low transferrin saturation (TSAT) < 10% (calculated as iron in µmol/L/(transferrin in g/L × 25.1) × 100)) [30] was considered as a secondary definition of ID. TSAT was calculated in Kenya only because Ugandan plasma samples were stored in EDTA, which chelates iron. We did not define ID by hepcidin or sTfR since there are no internationally established cutoffs. Anemia was defined as hemoglobin < 11 g/dL in children aged 0–4 years or hemoglobin < 11.5 g/dL in children >4 years. IDA

was defined as low ferritin and anemia [1]. We used malaria exposure index, which estimates a distance-weighted local prevalence of malaria infection within a kilometer radius around an index child with acute malaria, as previously described [31]. A malaria episode was defined as parasitemia and temperature > 37.5°C. Malaria incidence was calculated by dividing total malaria episodes by follow-up time. Underweight was defined as weight-for-age z-score < –2 using the WHO Growth Reference Standards [32]. Hemoglobin and anthropometric measurements were only available for the Junju and EMaBS cohorts.

Statistical Methods

Data analysis was performed using STATA 13.0 (StataCorp., College Station, TX). Non-normally distributed iron and inflammation biomarkers, and malaria antibodies were normalized by natural-log transformation. Crude differences in means of log antibody levels between iron-deficient and iron-replete children were determined using 2-tailed Student *t*-tests and univariable linear regression models. Multivariable linear regression models were used to estimate the association between ID and malaria antibody levels, pooling data from all cohorts and adjusting for study site, age, sex, inflammation, and *P. falciparum* parasitemia at the time of antibody measurement. The linear regressions were run on log transformed data; hence the beta values returned reflect changes on a log scale. To transform these to fold differences that could be applied to the linear scale, we used the formula 10^Beta. The significance of possible interactions was estimated from the Wald test. Because not all indexes of previous malaria exposure were available for all cohorts, a meta-analysis of results from multivariable models for individual cohorts was fitted, additionally adjusting for malaria exposure index in the Kenyan cohorts, malaria vaccination status in the RTS,S cohort, and prior malaria incidence, time since last malaria episode, and time between iron and antibody measurements in the EMaBS cohort. In longitudinal analyses EMaBS children were further grouped by those that had iron measurements 0–2 or 2–4 years before antibody measurement. A *P*-value of < .05 was considered significant.

RESULTS

A total of 924 Kenyan and 870 Ugandan children were included in the study. Participant characteristics and malaria exposure varied by study cohorts as shown in Table 1. Children living in Junju had the highest malaria exposure, whereas children in the RTS,S cohort had very low levels of malaria exposure. Prevalence of asymptomatic *P. falciparum* parasitemia varied by study cohort from 34.70% in Junju to 1.97% in the RTS,S cohort (Table 1).

Overall, 31% of children had ID as defined by WHO guidance [29], 47% had ID as defined by TSAT < 10%, and 13% had IDA, with prevalences varying by study cohort. Geometric means of individual iron biomarkers similarly showed that ID was highly prevalent among the cohorts. Malaria-specific antibody levels also varied by cohort. In keeping with a higher exposure to malaria, children in the Junju cohort had the highest levels of anti-AMA-1 and MSP-1 antibodies compared to other cohorts (Table 1). In univariable analyses, age, inflammation and malaria parasitemia were positively associated with both anti-AMA-1 and anti-MSP-1 antibody levels (Supplementary Table 1).

Iron Deficiency Is Associated With Reduced Malaria-specific Antibody Levels

Anti-AMA-1 and anti-MSP-1 antibodies were lower in iron-deficient compared to iron-replete children, with the largest differences in antibody levels observed in children from the Junju cohort, where malaria transmission was also the highest (Supplementary Figure 1). In multivariable regression models adjusted for age, sex, study site, inflammation, and malaria parasitemia ID was associated with decreased anti-AMA-1 and anti-MSP-1 antibody levels (adjusted mean difference on a log-transformed scale (β) –0.46; 95% CI, –.66, –.25 $P < .0001$; β –0.33; 95% CI, –.50, –.16 $P < .0001$, corresponding to –4 fold and 2-fold reductions on a linear scale, respectively) (Table 2). ID remained associated with reduced malaria-specific antibody levels after further adjustment for underweight (Supplementary Table 2).

To account more fully for the effects of previous malaria on antibody levels, we conducted a meta-analysis of individual cohorts with further adjustments for additional covariates including malaria exposure index [31], incidence of malaria prior to antibody measurement and time since last malaria episode, as available for individual cohorts. ID remained associated with decreased antibody levels to anti-AMA-1 (β –0.34; 95% CI, –.52, –.16) and anti-MSP-1 (β –0.26; 95% CI, –.41, –.11) in overall meta-analyses (Figure 1). IDA was associated with reduced anti-AMA-1 and anti-MSP-1 antibody levels in Kenyan children but not in Ugandan or in overall analyses (Supplementary Table 2 and Supplementary Figure 2).

Considering other iron biomarkers, we found that ID defined by TSAT < 10% was associated with reduced anti-AMA-1 but not anti-MSP-1 antibody levels in multivariable analyses and meta-analyses (Table 2 and Figure 1). Increased TSAT, ferritin, and sTfR levels were associated with increased anti-AMA-1 antibody levels, whereas higher ferritin and hepcidin levels were associated with increased anti-MSP-1 antibody levels in multivariable models (Supplementary Table 3).

Iron Deficiency Earlier in Life May Influence Subsequent Antibody Levels

We tested the hypothesis that ID might influence subsequent malaria antibody levels for a prolonged period of time in the EMaBS birth cohort. ID was associated with lower anti-AMA-1 and anti-MSP-1 antibody levels up to 2 years after iron status measurements and only with lower anti-AMA-1 antibody levels 2–4 years after iron measurements (Supplementary Figure 3).

Table 1. Characteristics of Study Participants

			Kenya				Uganda	
Characteristics	**Overall n = 1794**		**Junju n = 582**		**RTS,S n = 342**		**EMaBS n = 870**	
Median age months (IQR)[a]	24.0 (16.03, 34.93)		27.74 (18.03, 51.74)		12.61 (9.08, 16.79)		24.09 (23.97, 25.90)	
Sex: female no./total (%)	877/1794 (48.88)		284/582 (48.80)		168/342 (49.12)		425/870 (48.85)	
Underweight, no./total (%)[b]	169/1211 (13.71)		88/544 (15.58)		na		78/867 (9.00)	
Inflammation, no./total (%)[c]	478/1749 (27.04)		180/564 (31.91)		86/339 (26.02)		212/846 (25.06)	
Malaria parasitemia, no./total (%)[d]	282/1588 (16.50)		202/582 (34.70)		3/152 (1.97)		57/854 (6.67)	
Malaria exposure index, median (IQR)[e]	0.22 (1.5^{-11}, 0.62)		0.50 (6.16^{-7}, 0.75)		0.09 (2.17^{-14}, 0.22)		na	
Malaria incidence, gmean (95% CI)[f]	1.02 (.93, 1.10)		1.67 (1.41, 1.98)		na		.92 (.84, 1.01)	
ID, low ferritin, no./total (%)[g]	528/1683 (31.37)		111/552 (20.11)		153/335 (45.67)		264/796 (33.17)	
ID, low TSAT, no./total (%)[h]	425/889 (47.81)		220/554 (39.71)		205/335 (61.19)		n/a	
IDA, no./total (%)[i]	171/1265 (13.52)		90/488 (18.44)		n/a		80/782 (10.05)	
Anemia, no./total (%)[j]	585/1367 (42.79)		359/516 (69.57)		n/a		220/834 (26.38)	
Biomarkers and malaria antibodies	**n**	**gmean (95% CI)**	**n**	**gmean (95% CI)**	**n**	**gmean (95% CI)**	**n**	**gmean (95% CI)**
Ferritin (µg/L)	1683	22.44 (22.29, 24.64)	552	32.68 (30.0, 35.61)	335	17.40 (15.70, 19.30)	796	21.10 (19.61, 22.69)
TSAT (%)	889	10.76 (10.24, 11.16)	554	11.81 (11.20, 12.42)	335	9.21 (8.68, 9.77)		n/a
Hepcidin (ng/L)	1694	6.71 (6.24, 7.10)	546	7.01 (6.4, 7.72)	298	5.69 (4.92, 6.58)	850	6.92 (6.29, 7.48)
Iron (µg/dL)	900	7.44 (7.19, 7.69)	561	7.82 (7.62, 8.31)	298	6.68 (6.34, 7.03)		n/a
Transferrin (mg/dL)	1749	2.70 (2.68, 2.72)	568	2.70 (2.62, 2.72)	227	2.87 (2.81, 2.92)	944	2.68 (2.64, 2.72)
sTfR (mg/L)	1765	11.07 (10.68, 11.48)	573	18.22 (17.62, 18.81)	339	18.04 (17.35, 18.76)	853	6.53 (6.23, 6.85)
Hemoglobin (g/dL)	1367	10.85 (10.76, 10.94)	516	10.15 (10.02, 10.31)		n/a	834	11.30 (11.2, 11.40)
CRP (mg/L)	1749	1.77 (1.64, 1.91)	564	2.21 (2.02, 2.61)	229	2.00 (.56, .79)	946	1.41 (1.26, 1.58)
AMA-1 (AU/mL)	1678	55.84 (50.76, 61.42)	582	196.99 (164.96, 226.25)	342	28.06 (25.74, 40.51)	754	25.11 (22.15, 28.47)
MSP-1 (AU/mL)	1765	181.60 (170.0, 193.99)	582	264.04 (213.29, 400.08)	342	147.13 (132.29, 163.64)	841	124.63 (113.92, 136.38)

Abbreviations: AMA-1, apical merozoite antigen 1; CI, confidence interval; CRP, C-reactive protein; gmean, geometric mean; IDA, iron deficiency anemia; IQR, interquartile range; MSP-1, merozoite surface protein 1; MUAC, mid-upper arm circumference; n/a, not available; sTfR, soluble transferrin receptor; TSAT, transferrin saturation.

[a]Median age at time of iron measurement.

[b]Underweight was defined as weight for age z-score < −2.

[c]Inflammation, C-reactive protein > 5 mg/L.

[d]Malaria parasitemia, *Plasmodium falciparum* parasitemia at any density at the time of iron measurement.

[e]Malaria exposure index, a marker of the level of a child's exposure to malaria and was calculated as the distance-weighted prevalence of clinical malaria within 1 km radius of the child's residence.

[f]Malaria incidence, total number of malaria episodes before the time of iron measurement/follow-up time.

[g]Iron deficiency as low ferritin, plasma ferritin < 12 µg/L or < 30 µg/L in the presence of inflammation in children < 5 years or < 15 µg/L in children > 5 years or as [h]low TSAT, TSAT < 10%.

[i]IDA as ID defined by low ferritin and anemia.

[j]Anemia as hemoglobin < 11 g/dL in children aged 0 to 4 years or hemoglobin < 11.5 g/dL in children above 4 years.

DISCUSSION

We have investigated the association between iron status and anti-*P. falciparum* antibodies in 1794 Kenyan and Ugandan children. We found that ID was associated with reduced levels of anti-AMA-1 and anti-MSP-1 antibodies, even after adjustment for potential confounders including previous malaria exposure. TSAT < 10% was similarly associated with reduced levels of anti-AMA-1 antibody levels. A range of individual iron markers, including ferritin, TSAT, hepcidin, and sTfR levels were positively associated with malaria antibody levels. ID remained associated with reduced malaria antibody levels for up to 4 years.

We found that ID was associated with lower anti-AMA-1 and anti-MSP-1 malaria antibody levels, even after adjustment for potential confounders including previous malaria exposure. The relationship between malaria, iron parameters, and antibody levels differed between study sites. The effect of ID on anti-*P. falciparum* antibody levels was most marked among children with the highest malaria exposure and antibody levels, as seen in the Junju cohort, although little difference by ID was observed in children with very low levels of malaria exposure and antibody levels, as seen in the RTS,S cohort. In agreement with our findings, Nyakeriga et al reported that total IgG, IgG2, and IgE antibody levels were lower in iron-deficient compared to iron-replete Kenyan children [20]. We found that the effects of ID on anti-AMA-1 and anti-MSP-1 antibody levels persisted over time, perhaps due to continuing ID or a long-term effect of ID on immune development. IDA was similarly associated with reduced anti-AMA-1 and anti-MSP-1 antibody levels in Kenyan but not Ugandan children, perhaps because few Ugandan children had IDA or because anemia has a multifactorial etiology that may differ between countries.

We further investigated the effects of a range of iron markers on malaria-specific antibody levels. TSAT, an indicator of low levels of circulating iron, may more accurately reflect what iron status would be in the absence of malaria and inflammation compared to ferritin [33, 34]. TSAT < 10% was associated with reduced anti-AMA-1, although not anti-MSP-1, antibody levels in adjusted models. Hepcidin, the iron regulatory hormone, controls the absorption

Table 2. Association Between Iron Deficiency and AMA-1 and MSP-1 Antibody Levels in Univariable and Multivariable Regression Models

Cohort	Iron Replete		Iron Deficient		Unadjusted		Adjusted	
	n	Geometric Mean (95% CI)	n	Geometric Mean (95% CI)	Coefficient (95% CI)	P-value	Coefficient (95% CI)	P-value
Log AMA-1 antibody								
ID, low ferritin								
Overall n = 1706	1094	77.27 (68.74, 87.61)	612	26.91 (23.77, 30.47)	−1.05 (−1.26, −.85)	<.0001	−.46 (−.66, −.25)	<.0001
Jinja n = 552	441	284.52 (232.90, 347.61)	111	40.77 (30.04, 55.32)	−1.94 (−2.37, −1.51)	<.0001	−.51 (−.89, −.12)	.01
EMaBS n = 696	471	29.49 (25.02, 34.76)	225	17.71 (14.42, 21.75)	−.51 (−.79, −.23)	<.0001	−.36 (−.64, −.08)	.01
RTSS n = 335	182	39.71 (36.22, 43.53)	153	36.87 (33.90, 40.10)	−.07 (−.20, .06)	.24	−.03 (−.17, .10)	.64
ID, low TSAT								
Overall n = 889	464	176.19 (145.91, 212.76)	425	58.64 (51.20, 68.47)	−1.08 (−1.32, −.83)	<.0001	−.34 (−.58, −.10)	.007
Jinja n = 552	334	318.79 (252.46, 400.97)	220	92.15 (70.04, 121.26)	−1.24 (−1.60, −.88)	<.0001	−.30 (−.61, .01)	.06
RTSS n = 335	130	38.40 (34.58, 42.66)	205	37.39 (34.59, 40.41)	−.02 (−.15, .10)	.68	.05 (−.09, .18)	.49
Log MSP-1 antibody								
ID, low ferritin								
Overall n = 1656	1137	213.51 (196.33, 232.18)	519	126.21 (113.14, 140.80)	−.53 (−.67, −.38)	<.0001	−.33 (−.50, −.17)	<.0001
Jinja n = 552	441	418.38 (364.24, 480.57)	111	166.45 (128.78, 215.12)	−.92 (−1.22, −.62)	<.0001	−.50 (−.82, −.18)	.005
EMaBS n = 768	514	132.98 (118.57, 150.12)	254	108.42 (90.99, 126.88)	−.22 (−.42, −.02)	.03	−.20 (−.40, .01)	.07
RTSS n = 335	182	155.98 (134.65, 180.70)	153	134.93 (115.01, 158.30)	−.15 (−.38, .09)	.23	−.06 (−.27, .15)	.60
ID, low TSAT								
Overall n = 889	464	305.18 (268.12, 347.35)	425	208.09 (192.78, 226.91)	−.38 (−.57, −.20)	<.0001	−.02 (−.24, .20)	.82
Jinja n = 554	334	405.29 (345.72, 475.11)	220	287.97 (234.89, 353.05)	−.50 (−.74, −.26)	<.0001	−.02 (−.28, .25)	.86
RTSS n = 335	130	147.23 (125.45, 172.79)	205	148.04 (127.20, 169.51)	−.003 (−.25, .24)	.98	.05 (−.16, .26)	.65

Overall models, including all cohorts, were adjusted for age, sex, inflammation, study site, and malaria parasitemia at time of antibody measurement. For individual cohorts we further adjusted for malaria exposure index (in Kenyan cohorts), for malaria vaccination (RTS,S cohort), and for malaria incidence, time since last malaria episode, and time between iron and antibody measurement (EMaBS cohort). Iron deficiency was defined as (a) ID, low ferritin: plasma ferritin < 12 µg/L or < 30 µg/L in the presence of inflammation in children < 5 years or < 15 µg/L in children > 5 years and (b) ID, low TSAT (TSAT < 10%).
Abbreviations: EMaBS, Entebbe Mother and Baby Study; ID, iron deficiency; TSAT, transferrin saturation.

and recycling of iron, and is regulated by iron stores, infection and erythropoietic drive [35]. We found that increased hepcidin levels were associated with increased anti-MSP-1 antibody levels in overall multivariable analyses. In a previous study of 324 Kenyan children we similarly found that hepcidin levels were positively associated with anti-AMA-1 and anti-MSP-2 antibody levels [21]. In contrast to the other iron markers, we found that increased sTfR levels, an indicator of both increased ID and erythropoietic drive, were associated with increased anti-AMA-1 antibody levels. This might be explained by the strong association between sTfR levels and malaria [36], thus increased sTfR could indicate recent malaria exposure rather than ID.

How might ID lead to reduced malaria-specific antibody levels? One explanation is that iron may play a critical role in humoral immunity and particularly in antigen-specific antibody production as suggested by recent studies [7, 8, 11]. A missense mutation in transferrin receptor 1, necessary for iron uptake by cells, was associated with defective B-cell proliferation and reduced IgG production in children and in mouse models [8]. ID is similarly associated with markedly reduced antigen-specific antibody responses, likely due to impaired iron-dependent histone 3 lysine 9 demethylation, critical for B cell proliferation [7]. There is sparse literature in humans with conflicting findings. ID has been associated with reduced IgG antibodies, including to pneumococcal antigen [8–11], although some studies report little association [14, 16]. ID has also been associated with weakened antibody responses to measles, diphtheria, whooping cough, and tetanus vaccines in some studies [7, 13] but not others [17, 18].

Reduced malaria-specific antibodies in iron deficient children may also be explained by the complex relationship between iron status and malaria. ID has some protective effect against malaria infection in children [37], and thus iron-deficient children may have fewer malaria episodes leading to reduced malaria-specific antibody levels. Another explanation is that malaria influences measures of iron status. Ferritin levels are elevated for a prolonged period after a malaria infection, even after CRP levels have normalized [34], so that children with low ferritin levels may be less likely to have had recent malaria and thus might have reduced antibody levels. Moreover, the malaria-specific antibodies, anti-AMA-1 and anti-MSP-1, are markers of malaria exposure [38, 39], as well as correlates of naturally acquired immunity against clinical malaria [5, 6, 22]. We adjusted for previous malaria exposure in meta-analyses; however, it is likely that not all previous malaria was fully accounted for.

Strengths of our study included its large sample size of 1794 children from cohorts of varying malaria intensity in Kenya and Uganda. We also measured specific malaria antibodies known to contribute to immunity to clinical malaria [6, 22], assayed a wide range of markers of iron status, and adjusted for known potential confounders in our models. There were also some important limitations to our study. First, apart from malaria parasitemia, we did not have standardized measures for malaria exposure available for

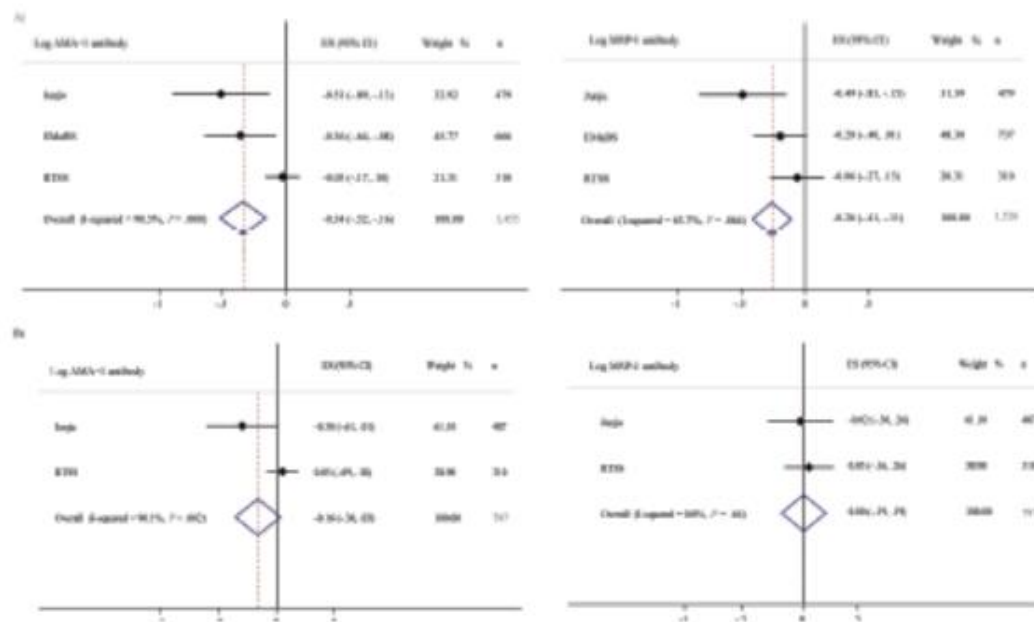

Figure 1. Meta-analysis of association of iron deficiency with AMA-1 and MSP-1 malaria antibodies. *A*, ID, low ferritin; *B*, ID, low TSAT. Regression models were adjusted for age, sex, inflammation, and malaria parasitemia in all individual cohorts. We additionally adjusted for malaria exposure index in Kenyan cohorts, malaria vaccination (RTS,S cohort), and malaria incidence, time since last malaria episode, and time between iron and antibody measurements (EMaBS cohort). ID was defined as plasma ferritin < 12 µg/L or < 30 µg/L in the presence of inflammation in children <5 years or < 15 µg/L in children ≥ 5 years. Malaria exposure index, a marker of the level of a child's exposure to malaria, was calculated as the distance-weighted prevalence of clinical malaria within 1 km radius of the child's residence. Malaria incidence was defined as total number of malaria episodes/follow-up time. Abbreviations: AMA-1, apical merozoite protein 1; CI, confidence interval; EMaBS, Entebbe Mother and Baby Study; ES, effect size; ID, iron deficiency; MSP-1, merozoite surface protein 1.

all cohorts, however, we conducted meta-analyses that accounted for measures of previous malaria, including malaria exposure index, incidence of clinical malaria, and time since a malaria episode, as available for each study site. Another limitation was that ID was defined using WHO guidance [29], which adjusts ferritin levels for inflammation (CRP > 5 mg/dl), however since ferritin levels are elevated for a prolonged period after CRP levels have normalized following malaria infection [34], lower ferritin levels could also reflect less recent malaria exposure. In addition to adjusting for recent malaria, we also defined ID using TSAT, which is less influenced by inflammation and malaria [33, 34], although this marker was not available for all cohorts. A further limitation of our study is that elevated anti-AMA-1 and anti-MSP-1 antibody levels may not be mechanically related with clinical protection against malaria. However, even as correlates of exposure the responses may still be useful indicators of the host's immunological response. Antibody levels were also measured using different pools of malaria-hyperimmune control sera in different laboratories in Kenya and Uganda, although protocols were similar between sites. Despite these differences, our findings were notably similar between the different study sites.

In summary, we found that ID was associated with lower levels of anti-AMA-1 and anti-MSP-1 malaria antibodies, known to be important in antibody-mediated immunity to clinical malaria in African children [5, 6, 22]. Our findings are supported by studies demonstrating that iron is critical for the development of humoral immunity [7, 8]. ID is highly prevalent among African children, and it is not known whether improving iron status might improve immune function and reduce disease burden. The current study supports WHO recommendations to offer iron supplementation coupled with malaria treatment in malaria endemic regions to prevent and treat iron deficiency [40]. Further research to infer causality between ID and malaria immunity, such as randomized controlled trials of the effects of iron supplementation on malaria antibody levels are needed, as well as further studies to assess associations between ID and malaria vaccine responses.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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