IMPACT OF MALNUTRITION ON Plasmodium falciparum SPECIFIC IgG ANTIBODY AND T CELL RESPONSES IN MALARIA INFECTED CHILDREN IN KILIFI COUNTY, KENYA

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A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science (Biochemistry) in the School of Pure and Applied Sciences of Kenyatta University.

October 2016
DECLARATION

I Albina Thira Waithaka, duly declare that this thesis is my original work and has not been presented for a degree in any other university or for any other award.

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To Mary Wambui Waithaka for your limitless love, care and support that only a mother can provide.
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ABBREVIATIONS AND ACRONYMS

AMA 1   Apical membrane antigen 1
ELISA   Enzyme linked immunoassay
FACS    Florescent activated cell sorting
FCS     fetal calf serum
g       Grams
HAZ     Height for age Z score
IgG     Immunoglobulin G
LC/MS   Liquid chromatography/mass spectrometry
ml      Milliliter
MSP2    Merozoite surface protein antigen 2
MSP3    Merozoite surface protein antigen 3
MUAC    Mid upper arm circumference
Nm      Nanometer
PBMCs   Peripheral blood mononuclear cells
PBS     Phosphate Buffered Saline
rpm     Revolutions per minute
SDS     Sodium dodecyl sulphate
SDS-PAGE Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
v/v %   Volume/volume percent
w/v %   Weight/volume percent
WAZ     Weight for age Z score
WHO     World Health Organization
WHZ     Weight for height Z scores
Xg      Times earth's gravitational force
mg      Micrograms
ml      Microliter
ABSTRACT

In Africa, overlap between malaria and under-nutrition results in high child mortality and morbidity and this is potentially due to immune dysfunction. Antibody and T cell responses play a major role in malaria immunity, however the impact of malnutrition on these responses is poorly understood. The objective of this study was to evaluate the effect of malnutrition on *Plasmodium falciparum* specific antibody and T cell responses in malaria infected children in Kilifi County, Kenya. The study was conducted in Kilifi County where malnutrition and malaria co-morbidity is prevalent. The study used 240 archived plasma samples collected from children admitted at Kilifi County Hospital with malaria. IgG responses to malaria antigens (AMA1, MSP2, MSP3 and schizont extract) were compared between 120 malnourished malaria infected and 120 age-matched well-nourished malaria infected children. To test if soluble proteins present in malnourished children plasma alter T cell function, peripheral blood mononuclear cells from a healthy adult were cultured using either plasma from malnourished malaria-infected children or well-nourished malaria infected children. Proteins in the plasma used in the T cell culture experiment were identified using liquid chromatography tandem mass spectrometry. Results obtained showed that except for AMA1 (p > 0.05) the proportion of IgG responders to MSP2, MSP3 and schizont extract was significantly higher in malnourished children (p < 0.05). The levels of antimalarial IgG for all the antigens were comparable (p > 0.05). T cells cultured using plasma from malnourished children had higher proliferative capacity than T cells cultured using plasma from well-nourished children (p < 0.05). Plasma proteomic profiling also demonstrated that malnourished children had differentially expressed proteins in comparison to well-nourished children, with notable up regulation of the inflammatory proteins; ficolin 1, CIq and lipopolysaccharide binding protein. In conclusion, malnourished malaria infected children make adequate responses against malaria antigens, however this may be dependent on the target *P. falciparum* antigen. Malnutrition leads to differential expression of various plasma proteins. Some of these proteins are involved in inflammation and could be responsible for promoting non-specific T cell proliferation. Given the presence of CIq, ficolin and lipopolysaccharide binding protein in malnourished children, the role of innate immunity in malaria and malnutrition should be considered. The relationship between various micronutrient deficiencies and protective antibody responses and T cell function should also be determined.
CHAPTER ONE

INTRODUCTION

1.1 Background information

Infectious diseases are a major cause of death among African children and malnutrition is reported to be an underlying risk factor for these deaths (Benguigui & Stein, 2006; Black et al., 2013). It is thought that malnutrition impairs the immune system, rendering undernourished children more susceptible to infections and death (Rytter et al., 2014).

Several mechanisms that drive malnutrition-induced immunodeficiency have been proposed. For instance, impaired antibody production, phagocytosis and complement function have been associated with respiratory infection, diarrheal diseases and malaria (Guerrant et al., 1992; Shankar, 2000; Chisti et al., 2010; Leonor et al., 2011). Imbalance in pro-inflammatory and anti-inflammatory cytokines has also been reported in individuals with vitamin A, Zinc and Iron deficiencies (Hughes & Kelly, 2006; Mbugi et al., 2010). The thymus is also severely affected by malnutrition leading to T cell deficits and an increase in immature T cells in the systemic circulation (Savino, 2002; Rytter et al., 2014). Previous studies have further demonstrated lower levels of memory T and B cells in malnourished children with bacterial infections in comparison to well-nourished infected children. Additionally, it has been postulated that malnutrition impairs T cell responses through altering the extra cellular environment required for T cell development (Saucillo et al., 2014). As a consequence of these immune deficiencies, malnourished children tend to be at
a higher risk of getting infections and dying than their well-nourished counterparts.

In Sub-Saharan Africa, children below five years live in conditions that predispose them to both under nutrition and malaria. The congruence of malaria and malnutrition is a major public health concern in Africa as they both contribute to high morbidity and mortality. In 2015 alone, malaria was responsible for 306,000 child deaths in Africa (World Health Organisation, 2015) whereas acute malnutrition was responsible for one million child deaths yearly (UNICEF et al., 2012).

It is suggested that malnutrition contributes to more that 50% of malaria associated deaths in children (Caulfield et al., 2004). Several studies in Africa have shown that undernourished children are at a higher risk of getting clinical malaria, severe malaria, higher parasite density and death compared to their well nourished counterparts (Rice et al., 2000; Muller et al., 2003; Nyakeriga et al., 2004; Friedman et al., 2005; Ehrhardt et al., 2006). Based on previous reports on malnutrition-induced immunodeficiency, an undernourished child may be unable to mount an appropriate immune response to the malaria parasite leading to poor outcome (Bourke et al., 2016).

Given that immunity against malaria is mediated by antibody, CD4 T cell and CD8 T cell responses (Crompтон et al., 2014), it is plausible that malnutrition may impair antibody and T cell responses leading to poor malaria outcome. However, data on the influence of malnutrition on antibody and T cell responses to malaria is limited and the findings have been inconclusive. For instance, IgG antibody responses to Plasmodium falciparum schizont extract in
Senegalese acutely malnourished children were similar to those of well-nourished children (Fillol et al., 2009). In contrast, Genton et al (1998) reported that acutely malnourished children had the lowest antibodies responses to malaria specific antigens. The discrepancies in these studies may result from differences in study design, age of children, type of malaria antigen targeted and local malaria morbidity. Furthermore, the small number of study participants used in these studies makes it challenging to draw any definite conclusions.

Genton et al. (1998) further evaluated the association between cell mediated immune responses and nutritional status. The results showed that the prevalence of lympho-proliferative responders in well-nourished children was similar to that of malnourished children. In contrast, there was a higher prevalence of cytokine producers in undernourished children than in well-nourished children. However, this study did not define the type of lymphocytes assessed.

While epidemiological evidence alludes to a relationship between malnutrition and increased risk of malaria and death, the effects of malnutrition on immune responses are not fully understood (Shankar, 2000). Therefore, this study aimed at evaluating the effect of malnutrition on antibody and T cell responses in malaria-infected children.
1.2 Problem statement
In Kilifi County, malaria and malnutrition are highly prevalent among children under the age of five and contribute to high child morbidity and mortality (Mbogo et al., 2003; Abubakar et al., 2011). The observed high mortality may be linked to defective immune response against malaria infection. However, there is poor understanding of the effect of malnutrition on antibody and T cell immune responses in children with malaria. Therefore, assessing the *P. falciparum* IgG antibody and T cell responses in malnourished malaria infected children would aid in understanding the interaction between malnutrition and adverse malaria outcomes.

1.3 Significance of study
The coexistence of malaria and malnutrition is a major public health concern in Africa as they both contribute to high mortality and morbidity among children under the age of five years. Kilifi County in Kenya provides a model location for studying the role of malnutrition in malaria immunity because of the high prevalence of both malaria and malnutrition.

Increase in malaria incidence, severity and mortality in malnourished children is thought to be due to weakening of the immune system. Given the crucial role of antibodies and T cells in malaria immunity, malnutrition is likely to compromise these immune components, consequently leading to poor outcome. In malaria immunity, IgG antibody levels have been found to be associated with protection against malaria, and are often used to measure acquisition of malaria immunity. T cells also play a critical role in immunity against malaria and their *in vitro* capacity to proliferate has been used to evaluate T cell response. The environment in which T cell develop also
influences T cell response, and profiling plasma proteins provides an ideal approach to study the T cell microenvironment.

The findings from this study contribute to the existing knowledge of the interaction between malnutrition and outcomes of malaria. The findings reveal that malnutrition alters antimalarial antibody levels and this is dependent the *P. falciparum* antigen targeted. This finding informs malaria vaccines developers of the need to consider malnutrition in evaluating the effectiveness of *P. falciparum* antigen-based malaria vaccines. The study also contributes to nutritional immunology research, by providing knowledge for the design of therapeutic options for malaria through improved nutrition to boost malaria immunity.

1.4 Research questions

i) What is the effect of malnutrition on the levels of malaria specific IgG antibodies in children below five years in Kilifi County, Kenya?

ii) Does plasma from malnourished malaria-infected under five-year-old children in Kilifi County alter *in vitro* T cell proliferation?

iii) Does malnutrition and *P. falciparum* infection result in differentially expressed plasma proteins in children below five years in Kilifi County, Kenya?
1.5 Objectives

1.5.1 General objective

To determine the impact of child malnutrition on antibody and T cell responses in malaria infected children in Kilifi County, Kenya

1.5.2 Specific objectives

i) To determine malaria specific IgG levels in malnourished malaria infected and well-nourished malaria infected children in Kilifi County, Kenya.

ii) To determine the proliferation capacity of T cells cultured with plasma from malnourished malaria-infected and well-nourished malaria infected children in Kilifi County, Kenya.

iii) To determine differentially expressed proteins in plasma from malnourished malaria-infected children in Kilifi County, Kenya.
CHAPTER TWO

LITERATURE REVIEW

2.1 Malnutrition

2.1.1 Definition of malnutrition

Malnutrition is primarily caused by eating suboptimal amounts of food leading to deficiencies in proteins, carbohydrates, fats, as well as micronutrients (Franca et al., 2009). Measuring nutrition status particularly in children below 5 years is important, as these children are the most vulnerable to malnutrition in comparison to other age groups. Additionally, the signs of malnutrition tend to be more pronounced in this age category. Nutrition status is commonly assessed by use of anthropometric measurements where attainment of growth is assessed by measuring the height, weight or mid upper arm circumference (MUAC) with reference to age and sex (World Health Organisation, 1995). Based on these anthropometric indices, malnutrition may present in three ways: stunting, wasting or underweight.

Stunting represents linear growth failure and is assessed by height for age index. It results from failure to receive proper nutrition over a long period of time and may also be caused by recurrent and chronic illness. Children are considered stunted if their height for age Z (HAZ) score falls two standard deviations below the World Health Organization (WHO) standards (World Health Organisation, 2006). Children whose HAZ-score falls below three standard deviations are considered severely stunted (World Health Organisation, 2006).
Wasting on the other hand is a depiction of acute malnutrition and represents recent failure to receive adequate nutrition or recent illness leading to malnutrition. Wasting is assessed by weight for height index (WHZ) Z scores or MUAC (World Health Organisation, 2009). Children whose WHZ score falls two standard deviations below the WHO standards are considered wasted, while those below three standard deviations are severely wasted. When using MUAC as an indicator of wasting, children with a MUAC of less than 13.5cm are considered malnourished while those below 11.5cm are severely malnourished (World Health Organisation, 2009). MUAC is preferred over WHZ index as it is considered more superior at identifying children at highest risk of mortality. Additionally, it is cheaper, easier to use and less prone to errors (Berkley et al., 2005; Myatt et al., 2006; Breind et al., 2011).

Underweight is another form of malnutrition and children who are underweight have low weight for age index (WAZ). Underweight can reflect acute malnutrition (wasting), stunting or both (World Health Organisation, 2010). It is thus a composite indicator of malnutrition and this makes it difficult to interpret. Underweight is defined as WAZ score of less than two standard deviations below the WHO standards, or 3-standard deviations in the case of severe underweight (World Health Organisation, 2006).

2.1.2 Epidemiology of malnutrition

Over a third of the children below five years in developing countries are malnourished (Black et al., 2013). According to the United Nations estimates, in 2011, among the under five year old children, 25% were stunted, 8% were wasted while 16% were underweight (UNICEF et al., 2012). In the same report, the estimated prevalence for African stunted, wasted and underweight
children was 36%, 9% and 18% respectively. Malnutrition is a common problem in Kenya and as per The Kenya Demographic Health Survey (Kenya National Bureau of Statistics (KNBS) & Macro, 2010) 35% of the under five children are stunted, 7% are wasted while 16 % are underweight.

2.1.3 Malnutrition and infection

The relationship between malnutrition and infection is bi-directional (Rytter et al., 2014). A malnourished child is more susceptible to infections and at the same time infection perpetuates malnutrition. This leads to a viscous cycle where each worsens the other.

It is estimated that malnutrition accounts for 45% of childhood deaths associated with infectious diseases globally (Pelletier et al., 1995; Black et al., 2013). In sub-Saharan Africa, more than half of the child deaths are due to pneumonia, diarrhoea and malaria and over 50% of these deaths are attributed to malnutrition (United Nations Inter Agency Group for Child Mortality Estimation, 2010). Among the major causes of death in children, 52% of deaths are as a result of pneumonia, 61% of deaths are as a result of diarrhoea, and 57% of deaths are as result of malaria are attributable to under nutrition (Caulfield et al., 2004). There is also increasing evidence that malnutrition increases the risk and severity of infection (Schaible & Kaufmann, 2007). Hence, malnutrition is an important risk factor for high childhood morbidity and mortality.

The relationship between malnutrition and infection is complicated by the effect of infection on nutrition itself (Scrimshaw et al., 1968). Infection stimulates the immune system, leading to an increased demand for nutrients,
that would otherwise be utilized in tissue synthesis and growth (Leonor et al., 2011). A sick child’s nutrition status may be worsened by diarrhoea resulting from gastro-intestinal infection leading to loss of nutrients (Brown, 2003). Intestinal parasite infections may also cause anemia and nutrient deficiencies (Schaible & Kaufmann, 2007). Lack of appetite and poor intestinal absorption also leads to nutrient losses, further injuring the immune system (Katona & Katona-Apte, 2008). Furthermore, chronic infections such as HIV have been shown to lead to nutrition deficiencies (Suttajit, 2007). The overall effect is a synergistic vicious cycle of poor nutritional status and infection.

2.1.4 Immune deficiency in malnutrition

Upon infection, the immune system is activated, leading to an increased demand for nutrients (Gerriets & Rathmell, 2012). In a malnourished individual, these nutrients are inadequate and this may adversely affect the immune system (Cunningham et al., 2005). A number of immune system abnormalities such as impaired T cell and antibody function, altered T cell subset ratios, compromised complement system, decreased phagocyte function and cytokine production have been described in malnourished individuals (Rytter et al., 2014). These immune-deficiencies have been associated with increased risk of infections and death in children.

One of the most prominent features of malnutrition is severe thymus atrophy (Savino, 2002). The thymus is a lymphoid organ where T cells undergo development; ultimately leading to migration of positively selected mature T cells to the peripheral lymphoid organs. Atrophic thymuses have reduced numbers of thymocytes resulting from massive thymocyte death as well as impaired thymocyte proliferation (Savino, 2002). A longitudinal study carried
out in Bolivian severely malnourished children showed that in comparison to well-nourished children, these children had severe atrophic thymuses associated with higher proportions of circulating immature T cells and lower proportion of mature T cells (Chevalier et al., 1998). Remarkably, after re-feeding there was full recovery of the thymus.

A decline in T cells subsets have also been reported in malnutrition (Rytter et al., 2014). Najera et al. (2001) demonstrated that infected malnourished children have decreased peripheral CD4 memory T cells to CD4 effector T cell cells ratio. Implying that malnourished children may be unable to achieve adequate memory to provide protection against infections and to provide helper activity for antigen-specific antibody production. In addition to changes in T cell subset proportions, poor nutrition impairs T cell ability to produce cytokines and causes imbalance in pro-inflammatory and anti-inflammatory cytokines (Najera et al., 2004; Rodríguez et al., 2005). Cytokines are important modulators of cellular immune functions and therefore any changes in their production may result in inadequate immune responses. For instance, unregulated production of interferon gamma in malaria infected individuals is associated with severe disease (Day et al., 1999)

Antibody-mediated immunity in malnourished children is also impaired as demonstrated in malnourished infected children studied in Mexico (Najera et al., 2004). These children had significantly lower percentages of B lymphocytes-antibody producers- than in well-nourished infected children. In the same study, there was no significant difference in the proportion of B cells between well-nourished uninfected children and malnourished infected children. These findings have been supported by Rikimaru et al (1998) and
indicate possible failure in the capacity of malnourished infected children to respond to infection. The observed B cell deficits can consequently lead to decreased antibody responses in malnutrition, which has been observed in both human and animal studies (Cripps et al., 2008; Taylor et al., 2013; Rytter et al., 2014).

Malnutrition can also lead to changes in various plasma soluble factors, affecting immune response to infection (Anderson & Anderson, 2002; Michalek & Rathmell, 2010). Cytokines and leptin are examples of immune-modulators present in plasma whose levels are altered by malnutrition. Cytokines are signaling molecules in the immune system and play a role in the activation, differentiation and proliferation of T cells (Sharma et al., 2011; Mahnke et al., 2013). It has been reported that, malnutrition decreases cytokine production, and the capacity of lymphocytes to respond appropriately to cytokines (Rytter et al., 2014).

Malnutrition also causes drastic reduction in leptin levels, leading to a reduction in T cell numbers, impaired T cell stimuli responses, and thymus atrophy (Howard et al., 1999; Faggioni et al., 2001). However, these defects can be reversed, as was observed by Howard et al. (1999) after treating starved mice with recombinant leptin. In yet another study, T cells isolated from starved mice and cultured in the presence of leptin recovered their ability to produce pro inflammatory cytokines after in vitro stimulation (Saucillo et al., 2014).

T cell function can therefore be modulated by soluble factors present in their microenvironment. It is also likely that nutrient deficiencies can alter these
factors leading to impaired T cell function and B cell antibody production. However, no comprehensive studies have been conducted to identify more soluble factors that may be altered by malnutrition of which may be important for T cell function.

2.2 Malaria

2.2.1 Aetiology

Malaria is an infectious disease caused by the plasmodium parasite, which is transmitted by infected mosquito bites. Four species of plasmodia are responsible for causing disease in humans and include *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium falciparum*. Among the four plasmodium species that transmit human malaria, *P. falciparum* is the most virulent and is responsible for the bulk of malaria associated morbidity and mortality in the world. *P. falciparum* accounts for 88% of the malaria cases and over one million deaths yearly (World Health Organisation, 2015).

2.2.2 *Plasmodium falciparum* malaria epidemiology

*Plasmodium falciparum* malaria is a major cause of morbidity and mortality worldwide. According to WHO (2015) world malaria report, in 2015 there were about 200 million cases of malaria, leading to 438 000 malaria deaths, globally. Most of these deaths (90%) occurred in Sub-Saharan Africa and 74% of the deaths occur in children under the age of five (World Health Organisation, 2015). In Kenya, there were 2,808,931 reported cases and 472 deaths in 2015 (World Health Organisation, 2015).
2.2.3 Life cycle of *Plasmodium falciparum*

With reference to figure 2.1, infection begins with a bite from an infected female anopheles mosquito releasing sporozoites into the blood stream of host. Within a few minutes, the sporozoites enter the liver cells or hepatocytes, where they go through the liver stage. The sporozoites mature, forming liver stage schizonts, which then rupture killing the infected liver cells and releasing of merozoites into the blood stream. Merozoites then quickly invade red blood cells, initiating the blood stage of the cycle. As the parasites develop in the red blood cells, they insert a variety of parasite proteins into the cell membrane of the infected red blood cells, thereby exposing them to the immune system. The merozoites develop into schizonts, which rupture, killing the infected red blood cell, releasing more merozoites that quickly invade other red blood cells. Some of the merozoites develop into female and male gametocytes that can be taken up by another mosquito during a blood meal. This stage continues the lifecycle in the mosquito after a blood meal. The clinical symptoms of malaria, the periodic fever and chills occur when infected red blood cells rupture and release merozoites (Schofield, 2007):
2.2.4 Immunity to malaria

Cellular immunity is thought to be mainly important in the liver stage of infection, where liver cells infected by the parasites are targeted by cell-mediated immunity. When sporozoites invade the liver cells, they insert their proteins on the surface of the hepatocytes in Major Histocompatibility Complex class 1 (MHC I) molecules, leading to recognition by CD8 T cells (Malaguarnera & Musumeci, 2002). CD8 effector cells produce interferon gamma (IFN-γ) that kill parasites in infected hepatocytes (Langhorne et al., 2008). With respect to CD4 T cell mediated immunity, CD4 T cells aid in B
cell activation and maturation, thereby contributing to antibody responses. In addition, CD4 T cells can kill malaria parasites through production of cytokines that activate phagocytosis and infected red blood cell killing by macrophages (Good & Engwerda, 2011).

Since red blood cells do not express major histocompatibility (MHC) complex class I or class II molecules, they cannot be targets of direct T cell attack (Wipasa et al., 2002). Based on this, it is thought that immunity to blood stage infection is driven by antibodies. This observation is supported by experiments where passive transfer of IgG antibodies from immune individuals protected children and non-immune individuals from malaria infection (Cohen et al., 1961; Sabchareon et al., 1991). Protection associated with these antibodies is further thought to depend on the breadth of the blood stage antigens that these antibodies target, as well as achievement of a critical threshold concentration (Osier et al., 2008; Murungi et al., 2013). Antibodies against the blood stage can be targeted to merozoites as they are briefly exposed to the immune system before they invade red blood cells (Wipasa et al., 2002).

2.2.5 Merozoites as potential antibody targets

Various proteins expressed on the surface of merozoites have been implicated in the erythrocyte invasion and some of these have been identified as targets for vaccine development and correlates of protection in epidemiological studies (Holder, 1994; Marsh & Kinyanjui, 2006). Antibodies to merozoite surface proteins are thought to prevent disease by inhibiting merozoite invasion of erythrocyte, inhibiting release of merozoites from schizonts, opsonizing merozoites for phagocytosis, activating compliment pathways and
inducing antibody-dependent cellular inhibition (Green et al., 1981; Joos et al., 2010; Hill et al., 2013; Boyle et al., 2015).

Several studies have demonstrated that antibodies against some of these merozoite antigens are associated with protection from malaria (Fowkes et al., 2010). Of interest are antibody responses against the merozoite antigens; apical membrane 1 (AMA1), merozoite surface protein 2 (MSP2) and merozoite surface protein 3 (MSP3), in which prospective immuno-epidemiological studies have been shown to be strongly associated with protection from clinical malaria (Osier et al., 2008; Osier et al., 2014). However, some children have reduced antibody responses to these antigens (Stanisic et al., 2015) and it is hypothesized that malnutrition may be responsible for reduced antibody responses.

2.3 Malnutrition and malaria

In malaria endemic regions such as Africa, there is an overlap between malaria and malnutrition. The number of malaria cases increase during the rainy season, a period when the acute malnutrition is also at the peak (Shankar, 2000). This co-morbidity results in a vicious cycle. Meaning that a malnourished child has a higher risk of malaria infection and will also affect how they recover from malaria. On the other hand, children sick with malaria are more likely to become malnourished.

Majority of the children in Africa live in poor and food insecure households and thus are more likely to consume inadequate and low quality diets and experience nutrient deficiencies (Lewnard et al., 2014). As a result of poor nutrition, these children may have compromised antibody and cell-mediated
immune system, increasing their risk for malaria and death. Indeed, epidemiological studies show that over 50% of the children dying from malaria have underlying malnutrition (Caulfield et al., 2004). Malnutrition has also been reported to be an independent risk factor for death in children admitted in hospital with severe malaria (Seidlein et al., 2012).

While an adequate and quality diet is necessary, in itself, it is not sufficient to ensure adequate nutrition. A child with malaria may have poor dietary intake due to loss of appetite or poor nutrient absorption due to diarrhoea. In addition, malaria induced hyper catabolism and inflammatory status may have an effect on nutritional status by promoting cachexia and anorexia (Ferreira et al., 2015).

The bidirectional relationship between malnutrition and malaria highlights the need for integrated control of malnutrition and malaria. It has been established that malaria control programs would have limited success if they do not also address malnutrition (Ehrhardt et al., 2006). Better nutrition will improve immune function, consequently reducing the malaria burden. This alongside malaria control measures could further reduce deaths from malaria in the long term.

2.3.1 Modulation of malaria infection by malnutrition

The association between malnutrition and the risk of malaria is complex, as depicted by the conflicting findings in different studies. Findings from these studies show that malnutrition is protective, deleterious or has no effect on malaria (Ferreira et al., 2015).

A prospective study in Papua New Guinea evaluated the impact of
anthropometry on getting subsequent malaria in children under 10 years (Genton et al., 1998). The study revealed that acutely malnourished children were more likely to get sick with malaria than well-nourished children. In contrast, Fillol et al. (2009) showed that acute malnutrition was associated with a 33% lower risk of getting at least one malaria episode, suggesting that malnutrition may be protective. However, previous studies conducted in the same population have shown that mothers are more aware of their children’s nutrition status and tend to be more protective of malnourished children (Simondon et al., 2001). It is therefore possible that mothers took preventative measures such as use of bed nets on malnourished children, explaining why malnutrition appeared to be protective against malaria.

In Burkina Faso, no association was found between malnutrition and malaria morbidity. However, malnourished children had a two fold higher risk of dying from malaria than well-nourished children (Muller et al., 2003). In western Kenya, chronically malnourished preschool children were at a higher risk of getting higher parasite density and clinical malaria (Friedman et al., 2005). Similarly, in Ehrhardt et al. (2006) study, underweight increased the risk of clinical malaria by 77%.

The discrepant results in these studies may be due to the heterogeneity of the study population, definition of clinical malaria, malaria transmission, and type and severity of malnutrition. Nonetheless, these studies suggest that malnutrition may modulate the course of malaria pathology leading to poor outcome. There is however a knowledge gap in the precise mechanism in which malnutrition modulates the course of malaria, which needs to be
addressed. It is postulated that malnutrition-induced immunodeficiency may be one of the mechanisms.

2.3.2 Malnutrition and malaria immunity

Malnourished children are at a higher risk of clinical disease, higher parasite density and dying from malaria (Muller et al., 2003; Friedman et al., 2005; Ehrhardt et al., 2006; Fillol et al., 2009; Shikur et al., 2016). This suggests that underlying malnutrition may modulate the course of malaria pathology leading to poor outcome. Given the critical role of antibodies and T cells in malaria immunity, malnutrition may impair their function leading to higher morbidity and mortality.

Studies evaluating the impact of nutrition on antibody immunity against malaria are limited and have yielded conflicting results. For instance, a cross-sectional study carried out in Senegal revealed that chronically malnourished preschool children had significantly reduced IgG responses to \textit{P. falciparum} schizont extract in comparison to well-nourished children (Fillol et al., 2009). In the same study, antibody levels were lower in wasted children, however the difference was not significant. In contrast, Papua New Guinea acutely malnourished children showed decreased IgG responses to malaria specific antigens in comparison to well-nourished children (Genton et al., 1998). However, the study did not find any significant differences between chronically malnourished and well-nourished children.

Several inconsistencies between the studies could explain these conflicting results. First, the studies differ in the age range of the children: pre school children in the Senegal and 10 to 120 months in Papua New Guinea. Active
growth faltering occurs mainly during the first year of life and subsequently the effect of chronic malnutrition may be more dire in young children (Martorell et al., 1994). Additionally, acquired specific immunity to malaria is related to the age and the malaria transmission pattern (Marsh & Kinyanjui, 2006). These studies differed in malaria transmission, providing a potential explanation for the discrepancies in their findings.

A major limitation of these two studies was the small number of study participants; Fillol et al. (2009) study had 19 wasted children while the Genton et al (1998) study had 14 wasted children. Therefore, reaching a conclusion on the effect of acute malnutrition on antibody levels was quite challenging.

T cells are involved in protection against malaria through either activating of B cells to produce antibodies or production of cytokines, which activate macrophages to kill pathogens (Langhorne et al., 2008). A study conducted in Papua New Guinea (Genton et al., 1998) evaluated the association between lymphocyte proliferation capacity and nutritional status. After stimulating the lymphocytes from malnourished and well-nourished children with malaria specific antigens, cytokine release and lymphocyte proliferation was assessed. There was no significant difference in the prevalence of lympho-proliferative responders between well-nourished and malnourished children. The study in contrast demonstrated a higher prevalence of cytokine producers in malnourished children (Genton et al., 1998). It has also been shown that micronutrient deficiencies in Tanzanian malaria infected children resulted in an imbalance in pro-inflammatory and anti-inflammatory cytokine production (Mbugi et al., 2010). A balance of these cytokines is important in determining clinical outcome of malaria.
Data on the effect of malnutrition on antibody and T cell responses against malaria is however limited, necessitating further research.

2.4 Malaria and malnutrition burden in Kilifi County, Kenya

Both malaria and malnutrition are highly prevalent in children under the age of five in Kilifi County. Malaria is endemic and transmission occurs throughout the year with increases after the long rains from April to June and the short rains from October to November (O'Meara et al., 2008). Majority of the malaria infections in Kilifi are caused by *P. falciparum* with approximately one to 120 infective mosquito bites every year (Mbogo et al., 2003; Snow et al., 2015). Malnutrition poses yet another health concern in Kilifi, with 40% of children below the age of five presenting anthropometric measures of malnutrition (Abubakar et al., 2011).

There is a high incidence of malaria in children from Kilifi who are subsequently found to be either underweight or stunted (Nyakeriga et al., 2004). Moreover, 37% of the children admitted at Kilifi County hospital with severe malnutrition have malaria (Sunguya et al., 2006). Studies evaluating T cell and B cell immunity to malaria in children from Kilifi have hypothesized that defects in immune responses against malaria may be driven by malnutrition (Illingworth et al., 2013). However, there are no studies that have been conducted to verify this claim.

2.5 Plasma proteome profiling

Blood plasma proteome is a rich source of soluble factors that may help in defining normal and abnormal physiological states (Anderson & Anderson, 2002). Plasma proteins include tissue secretions, hormones, cytokines,
immunoglobulin and proteins that leak from damaged cells and tumors. These proteins play diverse roles, including shaping T cell immune responses (Pettengill et al., 2014).

In order to study plasma proteome, a proteomics approach is ideal, as it allows more than one protein to be evaluated at a time. It is a highly translatable research tool, which can facilitate examination of the changes in plasma protein expression in response to malnutrition. Identification of differentially expressed proteins in malnourished children may be important in the understanding of the molecular events that shape T cell function (Michalek & Rathmell, 2010; Saucillo et al., 2014).

Commonly used methods for identifying plasma proteins include mass spectrometry and multiplexed immunological based methods (Janzi et al., 2005; Chen & Yates, 2007; Loch et al., 2007; Cole et al., 2013). Immunological methods involve probing plasma samples with antibodies against proteins of interest. However, these assays are limited in the sense that prior knowledge of the target protein is required, and more so, antibodies targeting these proteins have to be available. It is also costly to develop high affinity antibodies and the generation of large sets of immunoassays to potential protein candidates requires substantial amount of time. Given these limitations, mass spectrometry based methods offer an attractive alternative for surveying plasma proteins with greater sensitivity and coverage (Hale, 2013).

In mass spectrometry based assays, the sample undergoes pre-fractionation steps, in order to reduce sample complexity (Whiteaker et al., 2007). This is followed by reverse phase liquid chromatography separation and the eluted
fractions are introduced through an ionizing spray interphase in the mass spectrometer. The resulting ions travel through the mass analyzer where they are separated based on mass to charge (m/z) ratio. The detector detects the ions and signals generated in the form of mass spectra showing relative abundance and m/z ratio. Raw data from the mass spectrometer is then searched against databases for protein identification.

2.5.1 Challenges of plasma proteome

Although plasma is an ideal matrix for protein biomarker discovery, it is extremely challenging to analyse due to its complexity. First, the number of proteins present is huge, with about $10^6$ different secreted proteins (Anderson, 2005). In addition to this daunting proteome size, the difference between the lowest and highest protein concentration is greater than 10 orders of magnitude. Moreover, about 99% of the total protein content consists of high abundance proteins such as albumin and immunoglobulins (Anderson & Anderson, 2002). This huge dynamic range makes it difficult to identify and quantify low abundance proteins in the presence of the high abundance proteins. The highly abundant peptides dominate the mass spectrometry analysis and mask the less abundant ones, which often originate from proteins of biological importance.

To circumvent this challenge, plasma samples need to be simplified before analysis in order to improve protein recovery. This usually involves biophysical fractionation, and immune depletion of the most abundant interfering proteins (Whiteaker et al., 2007; Tu et al., 2010; Dayon & Kussmann, 2013). Removal of albumin, the most abundant plasma protein is
often a routine step in analysis of plasma proteome. However, albumin is a carrier molecule and can bind proteins that may be important indicators of disease. This poses concerns as to how plasma proteome is changed upon removal of albumin. Several studies have reported the presence of other proteins in the albumin-enriched fraction (Mehta et al., 2004; Gundry et al., 2007; Lopez et al., 2015). Signifying that, removal of albumin removes a significant amount of other proteins that may be of biological importance. Thus, albumin fraction should be included when analyzing plasma proteome.
CHAPTER THREE
MATERIALS AND METHODS

3.1 Study site

The study was conducted at the Kenya Medical Research Institute-Wellcome Trust Research Programme, located within the Kilifi County Hospital (KCH) in Kilifi County (map in Appendix I). The research program has established a longitudinal clinical surveillance in the paediatric wards of KCH to evaluate the incidence of malaria in children (Scott et al., 2012). KCH is located in Kilifi County, 3° south of the equator and serves approximately 500,000 people living within the County (Illingworth et al., 2013).

Malaria in Kilifi County is endemic and transmission occurs during the rainy seasons from April to June and from October to November each year. *P. falciparum* is the cause of majority of the malaria infections with a prevalence of about 40% among children (Snow et al., 2015). Malnutrition is also prevalent in Kilifi County and over 40% of children less than 5 years old are malnourished (Abubakar et al., 2011). Majority of the people live below the poverty line, indicating limited access to adequate and quality food. Although majority of the people are subsistent farmers, recurrent rain failure has resulted in insufficient farm produce, compromising food access (Abubakar et al., 2011).
3.2 Study population

The target population included in this study was children below the age of five years who were admitted at KCH from the year 2003-2014. The inclusion criterion was children below five years who had parasitemia. Children over five years and who did not have data on mid upper arm circumference were excluded from the study.

Malaria was defined as a fever of greater than 37.5° C with any parasitemia. Malnutrition was defined as acute malnutrition, which is assessed by measuring the mid upper arm circumference (MUAC). Children with a MUAC of less than 13cm were considered malnourished and those above or equal to 13cm were considered well nourished.

3.3 Study design

This study was conducted using samples collected during an ongoing surveillance study (Mogeni et al., 2016). The aim of this surveillance study is to evaluate development of natural immunity to malaria in children in Kilifi County. The study recruits children admitted at Kilifi County Hospital pediatric ward. Upon admission, clinicians manage patients and collect standardized clinical and laboratory data on all pediatric admissions. After obtaining consent from the parents or guardians, a blood sample is taken from the children. Then, plasma is isolated and stored at -80 °C at the Kenya Medical Research Institute-Wellcome Trust Research Programme laboratories. These were the samples that were used in this study, which had two study groups: malnourished malaria infected children and well nourished malaria-infected children.
3.4 Sample size calculation

Sample size was determined using the formula for determining sample size of equally sized groups described by Whitley and Ball (2002).

\[ n = \frac{(p_1(1-p_1) + p_2(1-p_2))}{(p_1 - p_2)^2} \times 7.9 \]

Where

- \( n \) = The desired sample size
- \( p_1 \) = Proportion of case responders obtained from Fillol et al. (2009), where 73.2% malnourished children were seropositive
- \( p_2 \) = Proportion of control responders obtained from Fillol et al. (2009), where 88% well-nourished children were seropositive.
- 7.9 = A constant which is defined by the values chosen for \( P \) value of 0.05 and a power of 80%

\[ n = \frac{(0.732(1 - 0.732) + 0.88(1 - 0.88))}{(0.88 - 0.732)^2} \times 7.9 = 109 \]

At least 109 samples were required in each of the study groups to detect differences with a power of 80% at a \( P \) value of 0.05. Although the minimum sample size for each study groups was 109, there were 240 samples (120 malnourished malaria-infected children and 120 age matched with well-nourished malaria infected children) available for use. Since larger sample sizes give more reliable results with greater precision and that the cost of
running additional samples was reasonable, the use of 240 samples was justified.

3.5 Ethical consideration

The parents or guardians of the children gave a written informed consent for storage and future use of samples. The Kenya Medical Research Institute/Ethics Review Committee approved the study (Appendix II).

3.6 P. falciparum merozoite antigens

Recombinant AMA, MSP2, and MSP3 merozoite antigens were used in this study. The antigens were based on *P. falciparum* and were expressed in *Escherichia coli*. Full length MSP3 tagged with maltose binding protein was from 3d7 allele type (Polley *et al.*, 2007). Full-length recombinant MSP2 was from the DD2 parasite line and was expressed tagged to glutathione S-transferase (Taylor *et al.*, 1995). Recombinant AMA was his-tagged and was from the HB3 parasite line (Osier *et al.*, 2010).

3.7 Determining IgG responses to malaria specific antigens

To evaluate if antibody responses to malaria antigens are impaired in malnourished malaria infected children, the levels of IgG antibodies directed towards the schizont extract, MSP2, MSP3 and AMA1 were measured in plasma from the children using indirect ELISA as previously described by Osier *et al.* (2008)

The assay was performed in duplicate for each plasma sample against schizont extract, MSP2, MSP3 and AMA1-malaria antigens. The assay was conducted in Nunc-immuno 96 micro well plates (Sigma Aldrich). Each well was coated with 50ng of antigen per 100μl of coating buffer (preparation in appendix III).
After an overnight incubation at 4°C, the plates were washed four times in wash buffer (preparation in Appendix III). Any binding sites in the wells that were not bound by the antigen were subsequently blocked for five hours at room temperature with blocking buffer made up using the wash buffer and 1% skimmed milk at a volume of 200μl/well. After which the antigen-coated wells were washed again and incubated overnight at 4°C with test plasma at 100μl/well. A 1 in 1000 plasma dilution was used based on similar work done previously using the same antigens (Osier et al., 2008).

After the overnight incubation, unbound antibodies were washed off four times and 100μl/well of secondary antibody (Horse Radish Peroxidase (HRP)-conjugated rabbit anti-human IgG) diluted to 1/5000 in blocking buffer was added and incubated for 3 hours. Excess secondary antibody was washed off and the detection solution containing O-phenylenediamine (OPD) peroxidase substrate was added and incubated for 15 minutes. The reaction was stopped with 25μl of 2M H₂SO₄ per well. Absorbance was read at a wavelength of 492nm using an ELISA reader. A pool of sera from malaria-exposed adults was included on every plate as a positive control and sera from non-malaria exposed adults served as negative controls.

3.8 Determining T cell proliferation

To determine whether plasma from malnourished children alters in vitro T cell proliferation, peripheral blood mononuclear cells (PBMCs) from a healthy donor were stimulated and cultured with plasma from malnourished children, well-nourished children, autologous plasma (PBMC donor) and fetal calf serum (FCS).
3.8.1 Isolation of fresh peripheral blood mononuclear cells

Approximately 30ml of blood was drawn from a healthy donor and collected in heparinized blood collection tubes. Immediately after collection, the heparinized blood was centrifuged at 1800 revolutions per minute to separate the cells from plasma. Plasma was aspirated out and stored at 4° C awaiting analysis. The packed cells were re-suspended with pre-warmed (37° C) culture media (protocol in Appendix III). To obtain PBMCs, the cells were gently layered and isolated by density centrifugation on lymphoprep gradient media (Axis-Shield) and centrifuged at 450g for 20 minutes. The PBMCs layer was collected, re-suspended in culture media and the cell count determined using a hemocytometer (Sigma Aldrich).

3.8.2 Stimulation of peripheral blood mononuclear cells

According to Quah et al. (2007) protocol, the isolated PBMCs were stained with carboxyfluorescin succinimidyl ester (Molecular Probes) at a concentration of 5μM for five minutes at room temperature. After which, the cells were washed twice with 1ml of phosphate buffered saline (PBS) containing 5% fetal calf serum (FCS) then sediment by centrifugation at 300g for five minutes at room temperature. The cells count was adjusted to 200,000 cells per 100μl in culture media (preparation in Appendix III) and distributed in 96 well sterile culture plates (BD Falcon). Plasma from five severely malnourished children, five well-nourished children, autologous plasma (PBMC donor) and FCS was added to the wells such that each well contained 10% (v/v) serum/plasma concentration. The plates were then incubated for three hours in a humidified 37° C, 5% CO₂ incubator.
Following this incubation, the cells were transferred in duplicates to a new 96 well sterile culture plate that had already been coated two hours prior with 5μg/ml of anti human CD3 (BD Pharmigen). Thereafter, 3 μg/mL of soluble anti-human CD28 (BD Pharmigen) was added to the cells. Negative controls (un-stimulated cells) were also run concurrently. The cells were incubated in a humidified 37° C, 5% CO₂ incubator for six days. On the sixth day, the cells were harvested and transferred to individual polypropylene Fluorescence-Activated Cell Sorting (FACS) tubes for staining.

**Table 3.1:** Monoclonal antibodies used to phenotype T cell lineage and their conjugated fluorochromes

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Fluorochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti human CD3</td>
<td>Peridin-chlorophyll protein Cy5.5 (PerCP Cy5.5)</td>
</tr>
<tr>
<td>Anti human CD4</td>
<td>Allophycocyanin (APC)</td>
</tr>
<tr>
<td>Antihuman CD8</td>
<td>Allophycocyanin -H7 (APC-H7)</td>
</tr>
</tbody>
</table>

### 3.8.3 T cell staining

One well of un-stimulated cells that were cultured with FCS was separated. These cells were used to set the forward scatter and side scatter voltages on the flow cytometer (CyAn™ ADP 9 color Analyzer, Beckman Coulter) and to set the live/dead cell compensation. To distinguish between live and dead cells, the cells were stained using LIVE/DEAD fixable stain (Invitrogen) for ten minutes. After which, T cell lineage was evaluated by staining for 30 minutes at 4° C in the dark with a combination of fluorophore-conjugated antibodies against CD3 (T cell lymphocytes), CD4 (T helper cells) and CD8 (cytotoxic T
cells). Table 3.1 shows the antibodies used to phenotype the T cells and their respective conjugated fluorochromes.

Any unbound antibodies were removed by washing the cells twice with 200ul FACS buffer (preparation in appendix III). After the final wash, the cells were re-suspended in 300ul of FACS buffer. Single stained cells were processed similarly and acquired in parallel with the samples so as to set post acquisition compensation. Cells cultured under similar conditions in media alone, without any stimulation acted as negative controls. Lymphocytes were acquired on a CyAn™ ADP Analyzer (Beckman Coulter).

3.9 Plasma protein profiling

To determine if malnutrition alters plasma protein levels that may be important for T cell proliferation, plasma proteins in the well-nourished and malnourished children included in the T cell culture experiment were identified using liquid chromatography tandem mass spectrometry.

3.9.1 IgG fractionation

To purify IgG antibodies in the plasma, Nab™ Protein A/G 0.2 ml spin kit was used (kit contents in appendix III). The samples were processed as per the manufacturer’s (Thermo Fisher Scientific) instructions. A plasma volume of 10μl in 90μl of binding buffer was added to the Protein A/G spin columns and incubated for ten minutes with end over end mixing, to allow IgG present in the plasma to bind the protein A/G resin. After which, the columns were centrifuged at 5000xg for one minute to remove unbound proteins. To enhance maximal binding, the flow through was transferred back to the column and centrifuged at 5000xg for one minute. The columns were then washed with
400μl binding buffer and first wash was combined with the flow through, bringing the volume to 500μl. IgG bound to the resin was eluted using the elution buffer and collected in a collection tube containing neutralization buffer. This procedure yielded two fractions, the IgG fraction and the flow through fraction comprising of non-IgG proteins.

### 3.9.2 Albumin fractionation

The flow through fraction was further processed to isolate albumin using 95% ethanol precipitation method (Kistler & Nitschmann, 1962). In this assay, 396.4μl of 95% cold ethanol was added to the 500μl flow through fraction and incubated for one hour on a rotator mixer at 4°C. After which, the samples were centrifuged at 16000xg for one hour at 4°C. The supernatant that consisted of albumin and related proteins (albuminome) was carefully transferred to a collection tube without dislodging the pellet. To dissolve the pellet, 100μl of 8M urea was added and sonicated twice for 45 minutes until the pellet dissolved. This pellet fraction contained non albuminome and non IgG proteins.

### 3.9.3 Processing the albuminome and pellet fractions

Albuminome fractions were buffer exchanged into 8 M urea to a final volume of 100μl. Urea facilitated efficient enzymatic digestion of proteins. The pellet and albuminome fractions were then reduced using 10μl of dithiothreitol (DTT) and incubated for one hour at room temperature with shaking. Following which, 40μl of Iodoacetamide was added to alkylate the proteins and samples were incubated for one hour at room temperature with shaking. To stop the reaction, the samples were incubated in 40μl of Dithiothreitol for
one hour at room temperature with shaking. Thereafter, the protein content was determined using Bradford protein assay.

3.9.4 Bradford protein assay

The protein concentration in the pellet and albuminome fractions was determined using the Quick Start Bradford kit (Biorad), kit contents in Appendix III. The Bradford dye reagent was equilibrated to room temperature. Following which, 5μl of the assay standards and samples were placed in a flat-bottomed 96 well micro plate. Afterwards, 250μl of the Bradford reagent dye was added and the sample and reagent were mixed gently by depressing and releasing the pipette’s plunger. After a 10-minute incubation, the absorbance was read by a spectrophotometer at 595nm. A standard curve was created and used to determine the protein concentration of unknown samples.

3.9.5 Trypsin digestion

A volume of sample having an equivalent of 20μg protein concentration was digested with 5μl of proteomics sequencing grade trypsin (Sigma Aldrich). After one-hour incubation at 37°C in an incubator shaker, 5μl of trypsin was added and the samples incubated overnight for 15 hours in incubator shaker at 37°C.

3.9.6 Purification of samples

After the overnight incubation, 1μl formic acid was added to the samples to stop the tryptic digestion. The protein digests were purified and concentrated using Pierce C18 spin columns (Thermo Fisher scientific) as per the manufacturer’s instructions. All reagents used were LC/MS grade. For each
column three parts sample to one part sample buffer (2% Trifluoroacetic acid, 20% acetonitrile) was loaded to allow binding of samples. Unbound compounds were removed by centrifuging at 1500xg for one minute. The columns were washed thrice using wash solution (0.5% Trifluoroacetic acid, 5% acetonitrile) and the bound peptides were recovered using elution buffer (70% acetonitrile). The eluted samples were dried in a vacuum evaporator and after drying; the samples were re-suspended in 14μl loading buffer (98% LC/MS grade water, 2% acetonitrile, 0.05% formic acid). A volume of 7μl of the protein digests was separated on a 12% Sodium dodecylsulphate - polyacrylamide gel to assess the efficiency of trypsin digestion.

3.9.7 Sodium dodecylsulphate - polyacrylamide gel electrophoresis

Gel casting frames were set up on the casting stands. The separating gel solution was set up as described in appendix III and 4 ml was pipetted into the casting frames. Isopropanol was added until it overflowed. After gelating, the isopropanol was drained off and the stacking gel (appendix III) was added. Subsequently, a well forming comb was inserted and after complete gelation, the comb was carefully removed. The casting glasses were then removed from the casting frame and set them on an electrophoresis tank. The running buffer (appendix III) was added into the inner chamber and allowed to overflow till it reached the required level in the outer chamber.

A volume of 7μl of sample buffer was mixed with 7μl of purified protein digests in eppendorf tubes. The samples plus sample buffer mixture were loaded on SDS PAGE gels and run at 130V for 2 hours. Following which, the gels were stained using SDS PAGE coomassie staining solution (appendix III)
over night and de-stained the following day using destaining solution (appendix III).

3.9.8 Peptides processing for mass spectrometry analysis

All proteins in the albuminome and pellet fractions had undergone complete digestion as proven by gels with no visible protein bands and therefore qualified for analysis by mass spectrometry (Gel image in Appendix IV). The remaining 7μl of eluted sample was topped up to 100μl with loading solvent (Appendix III). Similar fractions (albuminome and pellet) were pooled to make a representative pool by taking 5μl of each sample, making a total of 50μl per pool. 5μl of sample was injected into a Dionex Ultimate 3000 RSLCnano UHPLC focused system by an auto sampler and separated on a reverse phase LC Acclaim PepMap 100 C18 column (75μm internal diameter, 25cm length, 100Å pore size and 3μl particle size), using a binary linear gradient made of buffer A: (0.1% TFA in 100% H2O and buffer B: (0.1% TFA in 100% acetonitrile). For quality control 5μl of the pool sample was injected, and HeLa protein digest standard were injected in the system after every five samples. The separation was run for 155 minutes at a flow rate of 0.3μl /min using the following acetonitrile gradient: 2%-30% for 0-80min, 30-50% from 80-120 min, 50-90% from 120-120.1 min, 90-95% from 120.1-140 min, 95-4% from 140-140.1 min and 4% from 140.1-155min. The eluted peptides were automatically electro-sprayed in the Q-Exactive mass spectrometer for mass spectrometric analysis.
3.9.9 Database searches

Raw files containing peptide mass spectra data from the mass spectrometer were loaded into the Progenesis QI for proteomics software (Nonlinear Dynamics). The raw files were converted to peak lists, which contained peptide masses (m/z), intensity and abundances. In order to correct for retention time drifts from one sample run to another, the pool reference sample from the controls as well as the cases was selected and the retention time of all other sample runs were aligned to this reference. This ensured that the retention times were equivalent in all the runs, thus permitting accurate comparison of peptides across runs.

To identify the peptides, the MS/MS data was exported to Mascot search engine (Matrix Science Ltd.), which matched these spectra to known peptides within the Swiss prot Homo sapiens protein database. In Mascot searches, tolerances of 5 ppm (parts per million) for peptide masses and 0.05Da for fragment ions were specified. Carbamidomethylation modification of cysteine residues was selected as fixed modifications and oxidation of methionine was selected as variable modification. A decoy search was performed automatically in order to validate the peptide identification. The assigned peptides were re-imported to Progenesis QI software as XML files for statistical analysis.

Protein function was determined using the Protein Information Resource (PIR) ID mapping tool and catalogued according to their gene ontology (GO) number. The software allowed identification of the molecular function and pathways of the proteins.
3.10 Data management and statistical analysis

The data was analyzed with STATA version 11.2 (Stata Corporation, TX, USA) and GraphPadPrism (GraphPad software Inc). P values $\leq 0.05$ were considered significance.

For the ELISA experiment, the optical density (OD) of the samples was obtained from the ELISA reader. To ensure that the antibody level measurements were accurate, the coefficient of variation (CV) between duplicate wells was calculated in STATA using the formula below:

$$CV = \frac{\text{Standard deviation}}{\text{mean OD of duplicate wells}} \times 100\%$$

If the CV was greater than 20% and the OD difference between duplicates was greater than $\pm 0.1$ the assay was repeated. The mean OD of the duplicate wells was used as the final read-out. MSP3 and MSP2 were conjugated to maltose binding protein (MBP) and Glutathione S-transferase (GST) respectively and therefore to correct for this, the OD of MBP and GST for each sample was subtracted from that of MSP3 and MSP2 respectively. The mean optical density plus three standard deviations of twenty non-immune sera determined the cut-off for seropositive samples. This is the approach that has been used to determine immunoresponders in previous similar studies (Muema et al., 2011; Murungi et al., 2013).

Following calculation of the cut offs for each run, day-to-day variation between plates was corrected for. This was done by obtaining a correction
factor calculated by taking the OD of a reference positive control and dividing this with OD reading taken on subsequent days. Then the sample OD was multiplied by this factor.

Additionally, eleven three-fold serial dilutions of a reference Malaria Immune Globulin (MIG) reagent were included for every antigen to generate a standard ELISA curve and which allowed for the interpolation of the relative antibody concentration. This preparation contains 50 mg/ml of 98% IgG purified from a pool of healthy Malawian adult plasma (Taylor et al., 1992). The standard curve was generated and interpolation for relative concentration was performed using GraphPad Prism version 5.0) statistical software. The interpolated concentration was multiplied by a dilution factor of 1000 to obtain concentration. The concentration was then converted to arbitrary units (AU) based on Murungi et al. (2013), where it was assumed that the 50mg/ml purified MIG contained 50 arbitrary units (AU) of antigen-specific antibodies.

Chi-square test was used to compare the proportion of immuno-responders between malnourished and well-nourished children and the data was presented in bar graphs. The proportion of immuno-responders in well-nourished, moderately and severely malnourished children was compared using Chi-square test, which was followed by post hoc analysis, where the proportion between two groups was compared using Chi-square with Bonferroni adjusted p values. Since comparisons were made among the three nutrition status groups, the Bonferroni-adjusted $P$ value was $0.05$ (p value) /3 (number of groups being compared) = 0.017.
To compare the levels of antibodies between malnourished and well-nourished children, the antibody levels were normalized by log transformation \( (1 + \log_{10} \text{antibody level}) \). Since antibody levels were skewed even after data transformation (Appendix V), the Mann Whitney U test was used to compare median antibody levels between children with and without malnutrition. The Median antibody levels were compared among well-nourished, moderately and severely malnourished children using Kruskal-Wallis test followed by \textit{pos hoc} Dunns test. The data was presented in form of dot plots. In order to assess the breadth of antibody response, the children were assigned a score of zero, one, two or three on the basis of the number of antigens to which they were seropositive. The relationship between breadth of response and nutrition status was tested using the Chi square test for trend. The data was presented in form of a stacked bar graph.

Lymphocyte data was analysed using Flowjo Africa software version 9.2 (Tree Star, Inc 1997-2008). The gating strategy that was applied is illustrated using a representative sample in Appendix VI. The initial gate was performed on lymphocytes using side scatter linear (ss lin) and forward scatter linear (fs lin). After which the selected lymphocytes were gated to select single cell events (singlets) using forward scatter area (fs area) verses forward scatter linear (fs lin). To ensure that only live viable cells were included in the analysis, a gate was set to distinguish between live and dead cells using live/dead fluorescence and fs lin parameters. Having identified live cells, CD4 and CD8 T cells were selected based on concomitant expression of CD3 and CD4 or CD8 fluorescence. Finally, within CD4 and CD8 cells the subset of proliferating cells was identified using CD3 and CFSE fluorescence.
The proportion of live proliferating CD4 T cells (CD3⁺CD4⁺CFSE⁻) and CD8 T cells (CD3⁺CD8⁺ CFSE⁻) was expressed as the percentage of live CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells, respectively. The percentage of proliferation obtained from PBMCs incubated with medium alone was subtracted from values obtained after activation of PBMCs. The unpaired t test was used to compare the mean percentage proliferation between T cells cultured with either plasma from malnourished children or well-nourished children. The data was presented in bar graphs.

For relative quantification of plasma proteins, the total cumulative abundance of each protein was calculated by summing the abundances of all peptides allocated to that particular protein. Comparison between mean protein abundance between well-nourished and malnourished children was evaluated using t test using Progenesis QI software. The data was presented in a bar graph and a heat map.

3.11 Study limitations

The cross-sectional design of this study limits the ability to make causal inferences. Such studies make comparisons at a single point in time and they do not consider what happens before or after this time point. Therefore, it cannot be known for sure if the malnourished children in this study had adequate immune responses because they previously had more malaria episodes.

Second, there was no data available on co-infections. Co-infections may induce immune modulatory mechanisms that would affect the immune responses in malnourished malaria infected children. The observed up
regulation of the LBP, c1q and ficolin could have been a response against underlying bacterial infection. On addition, HIV co-infection may affect T cell responses.

Third, the presence of highly abundant proteins in the plasma samples may have masked the less abundant proteins which could have been of clinical significance (Ahn & Khan, 2014).

The study was limited to studying acute malnutrition. However, the impact of other forms of malnutrition such as stunting, underweight and micronutrient deficiencies was not explored. Given that the different forms of malnutrition have distinctive aetiologies, it is possible that they would have differing effects on immune response.

The small sample size used in the T cell and proteomic experiment was another limitation. Nonetheless, the experiments provide evidence of a working experiment that can be scaled up to evaluate how malnutrition may modify T cell microenvironment leading to altered T cell function.
CHAPTER FOUR

RESULTS

4.1. Study profile and baseline characteristics of study participants

A total of 240 malaria-infected children were included in the study (Table 4.1) and comprised of 120 malnourished (MUAC <13cm) and 120 age-matched well-nourished children (MUAC ≥13). Case control matching was based on age categories, where each study group had 5 children below 6 months, 23 children between 6 and 12 months, 41 children between 12 and 24 months, 25 children between 24 and 36 months, 14 children between 36 and 48 months and 12 were between 46 and 65 months old. Of the malnourished children, 39 were severely malnourished (MUAC < 12cm) and 81 were moderately malnourished (MUAC ≥12cm- <13cm) (Table 4.1).

There was no significant difference in age between malnourished infected children and well-nourished infected children (p > 0.05). Malnourished children had an average MUAC of 11.7 cm and this was significantly different from the well-nourished children’s average MUAC of 14.5cm (p < 0.05). With regard to laboratory parameters, there was no significant difference in the mean parasite density, white blood cells and platelets between cases and controls. On the other hand, red blood cell, haemoglobin and haematocrit levels were significantly lower in malnourished children (Table 4.1). However, in all the children the mean values for these parameters were below the normal range of 8.2-12.7 g/dl for haemoglobin, 3.6-5.7 X 10^6 cells/ul for red blood cell count and 26.8-38.1% for haematocrit.
Table 4.1: Baseline characteristics of the study participants. n=total number of participants. P values in bold represent parameters that differed significantly between well-nourished and malnourished children. Comparison was performed by t test and significant difference detected at p ≤ 0.05. CI represents confidence interval.

<table>
<thead>
<tr>
<th></th>
<th>Malnourished (n=120)</th>
<th>Well nourished (n=120)</th>
<th>P value (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean age in months</strong></td>
<td>24.9 (2.0-64.7)</td>
<td>26.3 (2.3-65.0)</td>
<td>0.49 (-2.58-5.37)</td>
</tr>
<tr>
<td><strong>(range)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age category</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 6 months</td>
<td>n=5 4.4</td>
<td>n=5 4.8</td>
<td>0.75 (-1.95, 2.60)</td>
</tr>
<tr>
<td></td>
<td>6-12 months</td>
<td>n=23 9.3</td>
<td>0.70 (-1.31, 0.88)</td>
</tr>
<tr>
<td></td>
<td>12-36 months</td>
<td>n=41 18.08</td>
<td>0.33 (-0.81, 2.35)</td>
</tr>
<tr>
<td></td>
<td>24-36 months</td>
<td>n=25 29.8</td>
<td>0.17 (-0.55, 2.97)</td>
</tr>
<tr>
<td></td>
<td>36-48 months</td>
<td>n=14 41.5</td>
<td>0.57 (-2.23, 4.00)</td>
</tr>
<tr>
<td></td>
<td>48-65 months</td>
<td>n=12 57.2</td>
<td>0.61 (-3.71, 6.18)</td>
</tr>
<tr>
<td><strong>Mean MUAC (Range)</strong></td>
<td>11.7 (5.6-12.9)</td>
<td>14.5 (13-18)</td>
<td><strong>&lt;0.001</strong> (2.5, 3.07)</td>
</tr>
<tr>
<td><strong>Mean parasite density</strong></td>
<td>202374.2 (268-1590000)</td>
<td>254129.4 (102-1716800)</td>
<td>0.20 (-27568.76, 131079.2)</td>
</tr>
<tr>
<td><strong>(Range)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean Haemoglobin</strong></td>
<td>6.71 (1.6-17.2)</td>
<td>7.75 (2.1-12.2)</td>
<td><strong>0.006</strong> (6.84, 7.59)</td>
</tr>
<tr>
<td><strong>(Range)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean White blood cells</strong></td>
<td>15.3 (2-81.6)</td>
<td>13.7 (3.8-48.8)</td>
<td>0.25 (13.20, 15.95)</td>
</tr>
<tr>
<td><strong>(Range)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean red blood cells</strong></td>
<td>3.1 (0.4-6.2)</td>
<td>3.7 (0.8-5.8)</td>
<td><strong>0.001</strong> (3.28, 3.62)</td>
</tr>
<tr>
<td><strong>(Range)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean haematocrit</strong></td>
<td>21.1 (4.5-55)</td>
<td>24 (6.7-38.1)</td>
<td><strong>0.01</strong> (21.33, 23.64)</td>
</tr>
</tbody>
</table>

Anaemia was defined as haemoglobin level of <11g/dl. Based on this definition, 80.83% of the malnourished children and 74.17% of the well-nourished children were anaemic and logistic regression analysis demonstrated that malnutrition was significantly associated with anaemia (Odds Ratio [OR]
= 1.47, Table 4.2). The study also revealed that 14.17% of the malnourished children died and 2.5% well-nourished children died (Table 4.2). In logistic regression analysis, malnutrition was significantly associated with higher mortality (Odds Ratio [OR]=6.4363, Table 4.2).

**Table 4.2**: Morbidity and mortality of study participants.

<table>
<thead>
<tr>
<th></th>
<th>Well nourished (Prevalence)</th>
<th>Malnourished (Prevalence)</th>
<th>Odds Ratio (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaemic</td>
<td>n=89 (74.85%)</td>
<td>n=97(80.83%)</td>
<td>1.47 (0.80, 2.71)</td>
</tr>
<tr>
<td>Mortality</td>
<td>n=3(2.5%)</td>
<td>n=17(14.17%)</td>
<td>6.43 (1.833, 22.59)</td>
</tr>
</tbody>
</table>

n=number of children and CI=confidence interval.

4.2. Prevalence of immune responders according to nutrition status

Among the 120 malnourished children, 72, 48, 40 and 98 were seropositive for AMA1, MSP2, MSP3 and the schizont extract, respectively. On the other hand 66, 32, 24 and 85 well-nourished children were seropositive for AMA1, MSP2, MSP3 and the schizont extract, respectively. The proportion of well-nourished immune responders to AMA1, MSP2 and MSP3 and the schizont extract was 55%, 26.67%, 20% and 70.83%, respectively while in malnourished children the proportion was 60% for AMA1, 40% for MSP2, 33.33% for MSP3 and 81.67% for the schizont extract (Figure 4.1). For all the antigens, the proportion of responders was higher in malnourished children (Figure 4.1). However, the difference was only significant for MSP2 ($X^2=4.8$, degrees of
freedom =1, p=0.03), MSP3 ($X^2=5.5$, degrees of freedom =1, p=0.02) and schizont extract ($X^2=3.8$, degrees of freedom =1, p=0.05)

![Graph showing immune responders to AMAl, MSP2, MSP3, and schizont extract in malnourished and well-nourished malaria infected children.](image)

**Figure 4.1**: Comparison of the proportion of immune responders to AMAl, MSP2, MSP3 and the schizont extract in malnourished malaria infected (red bars) and well-nourished malaria-infected children (blue bars). Chi-square test was used to compare proportions between the two groups and significant differences were detected at p ≤ 0.05.

### 4.3. Prevalence of immune responders based on severity of malnutrition

Among the 39 severely malnourished children, 43.59%, 35.90%, 28.21% and 76.92% were seropositive for AMAl, MSP2, MSP3 and the schizont extract respectively (Figure 4.2). Of the 81 moderately malnourished children, 67.90%, 41.98%, 35.80% and 83.95% were seropositive for AMAl, MSP2, MSP3 and the schizont extract respectively (Figure 4.2). Based on the Chi-square test, the proportion of responders to MSP2 ($X^2=5.24$, degrees of
freedom =2, p=0.07) and Schizont (χ²=4.6, degrees of freedom =2, p=0.10) was comparable across all the three groups.

Conversely, there was a significant difference in the proportion of responders against AMA1 (χ²=6.98, degrees of freedom=2, p=0.03) and MSP3 (χ²=6.23, degrees of freedom =2, p=0.04). Post hoc analysis using Bonferroni correction (adjusted p=0.017) revealed a significantly higher proportion of moderately malnourished children who were seropositive for AMA1 in comparison to severely malnourished children (p=0.01). Additionally, the proportion of MSP3 responders was significantly higher in moderately malnourished children in comparison to well-nourished children (p=0.01).

Figure 4.2: Comparison of the proportion of immune responders to AMA1, MSP2 MSP3 and schizont extract by degree of malnutrition. Well nourished (blue bars), moderately malnourished (grey bars) and severely malnourished (green bars). Comparison of proportion in the three was performed using Chi-square followed by Bonferroni correction. Significant differences were detected at Bonferroni-adjusted P ≤ 0.017.
4.4. Levels of antibodies according to nutrition status

Antibody levels against AMA1, MSP2, MSP3 and the schizont extract were compared between seropositive malnourished and seropositive well-nourished children (Figure 4.3). The level of antibodies against AMA1, MSP2 and the schizont extract was higher in well-nourished children while the levels against MSP3 were higher in malnourished children. However, the observed differences were not significant (p > 0.05).

Figure 4.3: Dot plots showing comparisons between the levels of malaria specific antibodies in malnourished (red circles) and well-nourished (blue squires) seropositive children. Graph A= AMA1; graph B =MSP2; graph C= MSP3 and graph D= schizont extract. Differences in antibody levels were determined using Mann-Whitney U test and significant differences were detected at p ≤ 0.05. The horizontal bars represent median.
4.5. Levels of antibodies based on severity of malnutrition

With regard to the degree of malnutrition, Kruskal-Wallis test showed that there was no significant difference in the levels of antibodies against AMA1 ($\chi^2=0.224$, degrees of freedom =2, $p=0.89$), MSP3 ($\chi^2=0.111$ degrees of freedom =2, $p=0.95$) and the schizont extract ($\chi^2=3.92$, degrees of freedom =2, $p=0.14$) in the three groups (Figure 4.4 A, C and D, respectively). However, MSP2 antibody levels were significantly different among the three groups ($\chi^2=7.19$, degrees of freedom =2, $p = 0.03$). Further analysis by post hoc Dunns multiple test revealed that moderately malnourished children had significantly lower MSP2 antibody levels compared to severely malnourished children ($p= 0.01$) and well-nourished children ($p=0.02$, Figure 4.4 B). On the other hand, the levels of MSP2 antibodies were comparable between between severely malnourished and well-nourished children ($p=0.25$, Figure 4.4 B).
Figure 4.4: Dot plots showing comparisons between the levels of malaria specific antibodies in well nourished (blue circles), moderately malnourished (grey squares) and severely malnourished (green triangles) seropositive children. Graph A= AMA1; graph B =MSP2; graph C= MSP3 and graph D= schizont extract. Antibody levels were compared using Kruskal-wallis followed by Dunn’s multiple comparison test and significant differences were detected at \( p < 0.05 \). The horizontal bars represent median.
4.6. The breadth of antibody responses

There were 67 children seropositive for only one antigen, out of which 52.24% (35/67) were malnourished and 47.76% (32/67) were well nourished (Figure 4.5). Among the children who were seropositive for only two antigens, 49.25% (33/67) of them were well-nourished and 50.75% (34/67) were malnourished (Figure 4.5). There were 27 children seropositive for all the three antigens, with more malnourished children being seropositive (70.37% (19/27)) than well-nourished children (29.67% (8/27), Figure 4.5). There were 79 children who did not respond to any of the three antigens, out of these 59.49% (47/79) were well-nourished children and 40.51% (32/79) were malnourished children (Figure 4.5). The proportion of malnourished children increased as breadth score increased ($X^2 = 15.59$, degrees of freedom = 1, $p = 0.0001$, Figure 4.5).

![Figure 4.5: Breadth of response to AMA1, MSP2 and MSP3 among malnourished (red section) and well-nourished (blue section) children. Breadth score of 0, 1, 2, 3 represent response to: neither of the three antigens, one antigen, two antigens and all the three antigens respectively. The Chi squared test for trend was used to test the breadth of response at P ≤ 0.05](image-url)
4.7. T cell proliferation capacity

The proportion of proliferating CD4 T cells that were cultured with plasma from malnourished children was significantly higher (20.59%) than CD4 T cells cultured with plasma from well-nourished children (4.15%, p=0.04, Figure 4.6-A). Additionally, the proportion of proliferating CD4 T cells cultured with plasma from the PBMCs donor (autologous plasma) was significantly higher than those cultured in well-nourished children’s plasma, p =0.01 (Figure 4.6-A). Although, the proportion of CD4 T cell proliferation was comparable between autologous and malnourished groups, the proportion of proliferating CD4 T cells cultured with autologous plasma (37.17%) was about twice that of CD4 T cells cultured with plasma for malnourished children (20.59%).

Similarly, as shown in Figure 4.6-B, there was a significant difference in the percentage of proliferation between CD8 T cells cultured in plasma from malnourished children (16.19%) and CD8 T cells cultured using plasma from well-nourished children (2.75%, p=0.04). Autologous plasma (45.88%) promoted significantly higher CD8 T cell proliferation in comparison to malnourished (2.75%) or well-nourished (16.19%) children’s plasma.
Figure 4.6: Bar graphs showing the percentage of CD4 (A) and CD8 (B) T cell proliferation capacity, after culturing PBMCs with plasma from well-nourished children (blue bars), malnourished children (red bars), autologous plasma (green bars) and FCS (grey bars). Autologous plasma and FCS were controls. Comparison of mean was performed using by t test and significant differences determined at $p \leq 0.05$.

4.8. Changes in plasma protein expression profile

A total of 383 and 230 proteins were identified in the pellet and albuminome fractions, respectively. There were 134 proteins identified in both the pellet and albuminome fraction while 249 and 96 proteins were uniquely identified in the pellet and albuminome fraction, respectively (Figure 4.7).
In the pellet fraction, there were 239 proteins that were more abundant in malnourished children and 144 plasma proteins were less abundant in malnourished children. Out of these proteins, the abundance of 27 proteins was significantly different between well-nourished and malnourished children, with 18 proteins being more abundant and 9 proteins being less abundant in malnourished children (p < 0.05, Figure 4.8A). The heat map in Figure 4.8-B demonstrates differential expression of these 27 proteins with increasing MUAC measurements. Appendix VII shows the functions and biological pathways that these 27 proteins are involved in.
**Figure 4.8:** Graph A depicts 27 plasma pellet proteins whose abundance differed significantly between well-nourished and malnourished children. Bars above the zero mark represent proteins that were more abundant in malnourished children, while bars below the zero mark represent proteins that were significantly lower in malnourished children. Graph B is a heat map showing the expression of the 27(Y axis) proteins in 10 children (X axis). The children were arranged according to increasing MUAC where patients 1 to 5 were severely malnourished children (MUAC < 10cm) and patients 6 to 10 were well-nourished children (MUAC ≥ 15.8cm - ≤ 17.5cm).
Equally, malnutrition resulted in changes in the abundance of proteins in the albuminome fraction (Figure 4.9). In this fraction, 141 proteins were more abundant and 89 proteins less abundant in malnourished children. The abundance of 13 proteins was significantly different between malnourished and well-nourished children, with ten proteins being more expressed and three proteins being less expressed in malnourished children \((p < 0.05, \text{Figure 4.9A})\).

Figure 4.9-B, demonstrates differential expression of these 13 proteins with increasing MUAC measurements. The functions and biological pathways of the 13 albuminome proteins are illustrated in Appendix VIII.

**Figure 4.9:** Graph A illustrates 13 proteins from the albuminome fraction whose abundance differed significantly between well-nourished and malnourished children. The bars above the zero mark represent proteins that were more abundant in malnourished children, while bars below the zero mark represent proteins that were significantly lower in malnourished children. Graph B is a heat map showing the expression of the 27(Y axis) proteins in 10 children (X axis). The children were arranged according to increasing MUAC. Patients 1 to 5 were severely malnourished children (MUAC ≤ 10cm) and patients 6 to 10 were well-nourished children (MUAC ≥ 15.8cm - ≤ 17.5cm)
CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Effect of malnutrition on malaria morbidity and mortality

Consistent with previous studies, this study demonstrated that malnutrition results in higher malaria morbidity and mortality. In terms of morbidity, malnourished children had considerably lower levels of haematocrit, red blood cells and haemoglobin than well-nourished children. These blood parameters are markers of anaemia and the observed low levels in malnourished children suggest that malnutrition may worsen anaemia in malaria. Association analysis supported this hypothesis by demonstrating increased odds of anaemia among malnourished children. Literature has shown that malnutrition compromises haematopoiesis leading to a reduction in red blood cells, promoting anaemia (Cunha et al., 2013). Therefore, it is hypothesized that higher degree of anaemia in malnourished children may be due to alteration of haematopoiesis by poor nutrition.

Although haematocrit, red blood cell and haemoglobin mean levels were higher in well-nourished malaria-infected children, they were below the normal levels, meaning the well-nourished malaria infected children were also anaemic. This was expected because these children were sick with malaria and malaria in itself causes substantial proportion of anaemia. Anaemia in malaria occurs as a result of massive lysis of red blood cells, which ultimately leads to reduction of red blood cells, haemoglobin and haematocrit levels (Perkins et al., 2011). However, malnutrition appeared to worsen anaemia, indicating that it might be an even more important risk factor for anaemia than malaria. These
findings concur with studies conducted by Ehrhardt et al. (2006) and Muller et al. (2003).

Consistent with the malaria infection morbidity related with malnutrition, the study revealed that malnutrition is also associated with higher mortality, as malnutrition was associated with higher odds of a child dying of malaria. Comparably, Muller et al. (2003) showed that malnourished children had a greater than 2 fold risk of dying of malaria than well-nourished children.

Taken together, the findings from this study suggest that malnutrition increases malaria morbidity and death.

5.2 Effect of malnutrition on the prevalence of immune responders

It was postulated that malnutrition lowers the prevalence of malaria specific immune responders. To test this hypothesis, this study evaluated the proportion of children who were seropositive for AMA1, MSP2, MSP3 and the schizont extract. The results revealed that malnourished children had a higher prevalence of immune responders than well-nourished children. This implied that malnourished children more commonly had malaria specific antibodies than well-nourished children.

These findings are inconsistent with Fillol et al. (2009) study, where the prevalence of wasted immune responders to the schizont extract was similar to that of well-nourished children. However, the small sample size used in that study was a limitation and thus no definite conclusions could be made. In the present study, the sample size limitation is overcome. Therefore, it can be
concluded that malnourished children have higher prevalence of immune responders.

A possible explanation for the higher prevalence of immune responders in malnourished children is that, malnourished children may have previously had more malaria episodes than well-nourished children. It has been evidenced that in malaria endemic regions, the more exposed an individual is to malaria, the higher the level of antibodies they have (Akpogheneta et al., 2010; Ibison et al., 2012; Olotu et al., 2012). Although the current study did not have data on the children’s past malaria episodes, earlier studies have shown that the incidence of malaria is elevated in malnourished children (Genton et al., 1998; Nyakeriga et al., 2004; Ehrhardt et al., 2006). Taking these previous findings in mind, malnourished children in the current study may have had more malaria episodes, and hence had higher prevalence of immune responders.

5.3 Effect of severity of malnutrition on the prevalence of immune responders

Based on previous findings that severe malnutrition leads to higher mortality, this study tested whether severity of malnutrition affects the prevalence of malaria immune responders. It was observed that moderately malnourished children had higher prevalence of immune responders in comparison to well nourished, and the difference remained significant for MSP2, MSP3 and schizont extract. On the other hand, the prevalence of immune responders among severely malnourished children was similar to that of well-nourished malnourished children, suggesting that moderately malnourished children more commonly had antimalarial antibodies.
These findings contrast what has been observed in a similar study conducted in Senegal, where they evaluated the impact of stunting severity on the prevalence of immune responders to the schizont extract (Fillol et al., 2009). The findings revealed that the prevalence of immune responders was significantly lower in mildly stunted and severely stunted children in comparison to well-nourished children. This discrepancy can be related to the difference in type of malnutrition. Stunting reflects past malnutrition while wasting corresponds to relatively recent malnutrition (World Health Organisation, 2006). Therefore, the detrimental effects of stunting may be different from that of wasting.

Studies that focus on malnutrition and malaria immunity have not evaluated the effect severity of wasting (acute malnutrition) on the prevalence of immune responders. The present study provides evidence that moderate acute malnutrition increases the prevalence of immune responders, while severe acute malnutrition does not affect the prevalence.

5.4 **Effect of malnutrition on antibody levels**

Based on reports on immune deficiency secondary to malnutrition, one of the questions this study aimed to address was whether malnutrition alters the levels of malaria specific antibodies. This study did not demonstrate a significant difference in the levels of antibodies against the *P. falciparum* antigens: AMA1, MSP2, MSP3 and the schizont extract between well-nourished and malnourished children. Suggesting that acute malnutrition does not alter the level of antibodies against these antigens.
Similarly, earlier studies assessing the levels of antibodies against *P. falciparum* schizont extract and MSP2 in acutely malnourished children did not observe any significant differences in the level of antibodies (Genton *et al.*, 1998; Fillol *et al.*, 2009). However, the main limitation of these earlier studies was the small number of study participants (n=19 and n=14 in Fillol *et al.* (2009) and Genton *et al.* (1998), respectively) and thus may not have been powered enough to observe differences. The present study overcomes this limitation as a larger sample size was used, highlighting a major strength of the present study.

The finding that malnutrition does not alter the levels of malaria specific antibodies during malaria infection may be explained by the chronic inflammation generally experienced by malnourished children (Prendergast *et al.*, 2014). As a result of this chronic inflammation, the immune system of malnourished children is continuously activated and hence they are likely to have immune response against the malaria antigens.

### 5.5 Effect of malnutrition severity on the levels of anti-malaria antibodies

The stratification of malnutrition as moderate or severe is critical because the risk of death is directly correlated with the degree of malnutrition (World Health Organisation, 2009; Moïsi *et al.*, 2011). Thus, the undesirable influence of malnutrition on anti-malarial antibody levels would possibly be observed in severely malnourished children rather than in moderately malnourished children. Therefore, the aim of this study was to determine whether severity of malnutrition lowers the level of anti-malaria antibodies. Severity of malnutrition did not have impact on the level of antibodies against AMA1,
MSP3 and the schizont extract. However, moderate malnutrition lowered the levels of MSP2 antibodies.

Although anti-AMA1, MSP2 and MSP3 antibodies have been found to be associated with protection against malaria, antibody responses to these antigens may differ in risk ratio point estimates. In Osier et al. (2014) study, individuals with antibodies against MSP2 were at a much lower risk of getting clinical malaria compared to those with MSP3 and AMA1 antibodies. Based on Osier's findings and the finding that moderate malnutrition lowers the level of antibodies to MSP2 implies that MSP2 is an important target for malaria immunity and moderate malnutrition may adversely affect the anti MSP2 antibody responses.

Previous studies have not evaluated the impact of severe and moderate acute malnutrition on the levels of malaria specific antibodies. Although stratification of the children based on severity of malnutrition reduced the sample size, the data suggested that malaria specific antibody levels might be altered by moderate malnutrition and particularly antibody responses directed towards MSP2.

5.6 Effect of malnutrition on the breadth of antibodies

The breadth of antibodies may predict protection from clinical malaria (Osier et al., 2008). This study addressed the question whether malnutrition interferes with the breadth of antibodies against malaria antigens. It was observed that the proportion of malnourished children increased as breadth score increased. This suggested that malnutrition does not interfere with the breadth of antibody response to the three antigens. The observation provides further evidence that
malnutrition does not impair *P. falciparum* antibody responses. No studies to the best of my knowledge have evaluated the impact of malnutrition on the breadth of antibody response in malnourished children.

5.7 Effect of malnutrition on T cell proliferation

The T cell *in vitro* proliferation experiment was set up to assess if plasma from malnourished children alters T cell proliferation. The results indicated that plasma from severely malnourished children promoted CD8 and CD4 T cell proliferation in comparison to T cells cultured with plasma from well-nourished children. Therefore, it can be inferred that malnutrition results in changes in T cell microenvironment and these changes promote T cell proliferation.

One possible explanation for this observation lies in the inflammatory state of malnourished children. It is widely known that malnourished children have chronic inflammation (Prendergast *et al.*, 2014). Inflammation is characterized by an increase in soluble inflammatory factors of the innate immune system. These are regulators of the expression of co-stimulatory molecules on the surface of antigen-presenting cells (Curtsinger & Mescher, 2010). Accordingly in the presence of inflammation, antigen recognition by specific T cells is often associated with activation of T cells, accelerating their maturation and proliferation. Therefore, these results raised the testable hypothesis that malnutrition alters soluble factors in the T cell microenvironment that may be involved in promoting T cell proliferation.

5.8 Effect of malnutrition on plasma proteins

Proteomic profiling of plasma proteins using liquid chromatography tandem
mass spectrometry revealed differentially expressed proteins in malnourished children. This finding implied that malnutrition leads to changes in the expression profile of plasma proteins.

Comparable results have been demonstrated in a number of studies. For instance, Cole et al. (2013) reported plasma proteins that can be used to identify micronutrient deficiencies in undernourished children. Leptin levels in plasma have been shown to be considerably lower in malnourished children in comparison to their well-nourished counterparts (Gerriets & Rathmell, 2012). Additionally, there is evidence that malnutrition alters the level of plasma cytokines and causes imbalances in pro-inflammatory and anti-inflammatory cytokines (Gonzalez-Torres et al., 2013). These selected studies add support to the finding in this study that malnutrition leads to changes in expression levels of certain circulating proteins in blood plasma.

It is possible that malnutrition interacts and modulates molecular processes underlying the individual’s physiological functions, resulting in changes in plasma protein expression. These protein alterations could be markers of immune mechanisms underlying malnutrition and malaria. It is thus likely that the observed changes in protein expression promoted T cell proliferation. This hypothesis is supported by the observation that malnourished children had elevated levels of the acute phase proteins namely, complement factors (ficolin and complement c1q), coagulation factors and lipopolysaccharide binding proteins (LBP). These acute phase proteins are proxy indicators of inflammation (Gruys et al., 2005) and their up regulation in malnourished children is in concert with the general view that malnourished children have chronic inflammation (Bourke et al., 2016). This means that malnourished
children may not be able to down regulate immune responses, leading to continuous stimulation of the immune system, which ultimately increases T cell proliferation.

Besides being indicators of inflammation, acute phase proteins are clinically important as they modulate the immune system. LBP for example plays a role in the innate immunity and facilitates recognition of lipopolysaccharide (LPS) by monocytes (Knapp et al., 2003). The presence of LPS is usually a sign of endotoxemia, which has been reported in both malaria infection and malnutrition (Olupot-Olupot et al., 2013; Prendergast et al., 2014). Though it is not possible to tell the source of LPS in this study, the gut is a major potential source (Prendergast et al., 2014). It has been demonstrated that malnourished children guts are disrupted, allowing translocation of bacterial material such a LPS into the systemic circulation (Humphrey, 2009; Carvalho & Saad, 2013). These endotoxins result in continuous stimulation of the immune system leading to inflammation characterized by the increased production of acute phase proteins.

Clq is a molecule involved in the classical pathway of the complement system and elevated levels in this study indicate an activated complement system. Recently, it was shown that malaria specific antibodies help in fixing Clq, thereby preventing erythrocyte invasion and lysing P. falciparum merozoites (Boyle et al., 2015), ultimately preventing replication of the parasite. Therefore, high levels of Clq proteins in the present data support the finding that malnourished children make adequate antibody responses to malaria specific antigens.
Similarly, ficolins are pathogen-recognition molecules that participate in the clearance of microbes by activating complement [reviewed in (Matsushita, 2010)]. More recently, Panda et al. (2014) showed that IgG antibodies collaborate with ficolin that is pre-bound to the pathogen, resulting in effective recognition and opsonization of the invading pathogen. Therefore, up regulation of ficolin proteins in malnourished children indicates that malnourished are likely develop adequate antibody responses against malaria.

This study therefore demonstrated that malnutrition results in differentially expressed plasma proteins and these changes may be involved in promoting T cell proliferation.

5.9 Conclusions

i) There is little evidence to suggest that malnutrition alters the level of malaria specific antibodies during an acute malaria infection. However, immune responses in the context to *P. falciparum* antigens may be antigen dependent.

ii) Plasma from malnourished malaria infected children promotes T cell proliferation.

iii) Malnutrition leads to differential expression of various plasma proteins.

5.10 Recommendations

Despite the observation that malnutrition does not impair antibody responses, malnutrition was associated with higher malaria morbidity and risk of dying. Therefore, this study recommends improving nutrition in children who are vulnerable to malaria.
Given the finding the effect of malnutrition on antibody responses may be antigen dependent, this study recommends that antibodies against MSP2 may be used to assess the performance of malaria vaccines in malnourished children.

5.11 Suggestions for future work

This study proposes the assessment of antibody function in terms of their ability to kill malaria parasites. This is imperative because even though malnutrition did not seem to affect the level of malaria antibodies, it is not known whether these antibodies are functional or not.

The study also suggests using larger samples size to validate the T cell proliferation and plasma protein expression profiles findings.

The finding that malnutrition results in up regulation of proteins associated with innate immunity motivate future studies to focus on the impact of malnutrition on the innate immunity.

Additionally, the presence of higher levels of inflammatory proteins in malnourished children motivates future research to address the role of malnutrition-induced inflammation on malaria outcome.
REFERENCES


erythrocytes and are associated with protection against malaria. *Immunity*, 42(3), 580-590.


isoforms in infected malnourished and infected well-nourished children. *Clinical and Experimental Immunology*, 126(3), 461-465.


APPENDICES

APPENDIX I: MAP OF KILIFI COUNTY

Map adapted from (Etyang et al., 2016)
APPENDIX II: ETHICAL CLEARANCE

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1

TO: PROF. KEVIN MARSH (PRINCIPAL INVESTIGATOR)

THROUGH: DR. CHARLES MBOGO;
AG DIRECTOR, CGMR-C,
KILIFI

Dear Sir,

RE: SSC PROTOCOL NO. 1131 (REQUEST FOR ANNUAL RENEWAL): INTEGRATED STUDIES OF THE DEVELOPMENT OF NATURAL IMMUNITY TO MALARIA IN CHILDREN IN KILIFI DISTRICT

Thank you for the continuing review report for the period February 28th 2013 to March 12th 2014.

This is to inform that during the 226th meeting of the KEMRI/ERC meeting held on 22nd April 2014, the Committee conducted the annual review and approved the above referenced application for another year.

This approval is valid from today 22nd April 2014 through to April 21, 2015. Please note that authorization to conduct this study will automatically expire on April 21, 2015. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the ERC secretariat by March 10, 2015.

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

You may continue with the study.

Yours faithfully,

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

AUG 2014

RECIEVED
P.O. Box 28 APR 2014 233-000 KILIFI
APPENDIX III: REAGENTS AND BUFFERS

1. Elisa buffers
* All buffers were prepared and stored at room temperature

1.1 1x Phosphate Buffered Saline (PBS)
Ten PBS tablets (Sigma Aldrich) were dissolved in one liter of distilled water to make 1x PBS, which consisted of 137mM sodium chloride, 2.7mM Potassium Chloride and 10mM phosphate buffer solution.

1.2 Coating buffer (15mM Na$_2$CO$_3$, 5mM NaHCO$_3$)
To prepare the coating buffer, 1.6g Na$_2$CO$_3$ and 0.42g NaHCO$_3$ were dissolved in 1 liter of distilled water. The pH was then adjusted to adjusted to 9.3.

1.3 Wash buffer
To make 1 L solution of wash buffer, 1 ml Tween 20 was mixed with 1000ml 1x PBS.

1.4 1% Blocking Buffer
For one 96 well ELISA plate, 0.2g of skimmed milk was dissolved in 20ml wash buffer.

1.5 Detecting reagent
One o-Phenylenediamine dihydrochloride (OPD) tablet (Sigma Aldrich) and one urea hydrogen peroxide tablet (Sigma Aldrich) was dissolved in 20 ml of distilled water.

1.6 Stop solution (2M H$_2$SO$_4$)
The stop solution was prepared in a fume cupboard, where 11ml of 98% H$_2$SO$_4$ was slowly added to 150ml of distilled water with constant stirring.

2. T cell culture assay buffers

2.1 Culture media
The T cell culture media was prepared by mixing 5ml Penicillin-Streptomycin, 5 ml L-glutamate and 500 ml RPMI-1640.

2.2 FACs buffer (0.1% BSA, 0.01% Sodium azide)
To prepare FACs buffer, 0.5g Bovine Serum Albumin and 0.05g Sodium Azide were dissolved in 500 ml 1x PBS

2.3 Monoclonal antibodies

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Fluorochrome</th>
<th>Company</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human CD3</td>
<td>Peridin-chlorophyll protein Cy5.5 (PerCP Cy5.5)</td>
<td>eBioscience</td>
<td>45-0037-73</td>
</tr>
<tr>
<td>Anti-human CD4</td>
<td>Allophycocyanin (APC)</td>
<td>eBioscience</td>
<td>17-0049-42</td>
</tr>
<tr>
<td>Anti-human CD8</td>
<td>Allophycocyanin (APC-H7)</td>
<td>BD Pharmigen</td>
<td>560179</td>
</tr>
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</table>
3. Proteomics assay reagents and buffers

3.1 Kits

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Kit contents</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nab™ Protein A/G 0.2 ml spin kit</td>
<td>Spin columns, A/G binding buffer, IgG elution buffer and neutralization buffer.</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Bradford Reagent Kit</td>
<td>1x Ready to use dye reagent, BSA standards (0.125, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 mg/ml).</td>
<td>Biorad</td>
</tr>
</tbody>
</table>

3.2 Buffers

* All buffers were prepared fresh

3.2.1 95% cold ethanol
To prepare 95% cold ethanol, 47.5 ml of 100% ethanol was mixed with 2.5 ml of 98% LC/MS grade water and stored at -20°C until use.

3.2.2 50mM Ammonium bicarbonate
To prepare 50mM Ammonium bicarbonate, 0.198 g Ammonium bicarbonate was dissolved in 50 ml LC/MS grade water.

3.2.3 8M urea
This was prepared by dissolving 4.8 g Urea in 10 ml 50mM Ammonium bicarbonate.

3.2.4 Reducing reagent: 200mM dithiothreitol (DTT)
The reducing agent was prepared by dissolving 0.031 g DTT in 1 ml 50mM Ammonium bicarbonate.

3.2.5 Alkylating reagent: 200mM Iodoacetamide (IAA)
To prepare the 200mM Iodoacetamide, 0.037 g IAA was dissolved in 1 ml 50mM Ammonium bicarbonate.

3.2.6 Activation buffer: 50% acetonitrile (ACN)
The activation buffer was prepared by mixing 2650 µl ACN and 2650 µL LC/MS grade water.

3.2.7 Equilibration buffer: 0.5% Trifluoroacetic acid (TFA), 5% Acetonitrile (ACN)
To prepare the equilibration buffer, 26.5 µl TFA, 265 µl ACN and 5008.5 µl LC/MS grade water were mixed together.

3.2.8 Sample buffer: 2% Trifluoroacetic acid (TFA) in 20% Acetonitrile (ACN)
The sample buffer was prepared by mixing 8 µl TFA, 80 µl ACN and 312 µL LC/MS grade water.
3.2.9 Wash solution: 0.5% Trifluoroacetic acid (TFA) in 5% acetonitrile (ACN)
The wash solution was prepared by mixing 26.5μl TFA, 265μl ACN and 5008.5μl of LC/MS grade water.

3.2.10 Loading buffer (2% Acetonitrile (ACN), 0.05% Formic acid)
The loading buffer was prepared by mixing 20ml ACN, 0.5ml Formic acid and 979.5ml LC/MS grade water.

3.2.11 Buffer A: 0.1% Trifluoroacetic acid (TFA)
Buffer B was prepared by mixing 1ml TFA and 999ml LC/MS grade water.

3.2.12 Buffer B: 0.1% Trifluoroacetic acid (TFA)
Buffer B was prepared using 1ml TFA and 999ml of 100% acetonitrile

4. Sodium dodecylsulphate - polyacrylamide gel electrophoresis (SDS-PAGE) gel reagents and buffers

4.1 Separating gel
To make 10 ml of separating gel, 3.2ml distilled water, 4ml of 30% acrylamide and bis-acrylamide solution and 2.6ml 1.5M Tris (PH 8.8) added into a 50ml falcon tube. After which, 0.1ml of 10%(w/v) Sodium dodecylsulphate was added followed by 10μl TEMED. Then, 100μl of 10%(w/v) Ammonium persulfate was added.

4.2 5 ml Stacking gel preparation
The stacking gel was prepared by mixing 2.975ml distilled water, 1.25ml 0.5M Tris, pH 6.8, 50μl 10%(w/v) sodium dodecyl sulphate, 67μl of 30% acrylamide and bis-acrylamide solution, 50μl Ammonium persulfate and 5μl TEMED.

4.3 Sample buffer
The sample buffer was prepared using 50μl β-mercaptoethanol and 950μl Laemmli buffer.

4.4 Running Buffer: 25mM Tris, 200mM Glycine, 0.1% (w/v) SDS
To make 1x SDS-PAGE running buffer, 3.03g Tris, 15.01g glycine and 100g SDS were dissolved in 1000ml distilled water.

4.5 Staining solution: 0.25% Coomassie R-250, 45% methanol, 10% glacial acetic acid
The staining solution was prepared by dissolving 2.5g Coomassie Brilliant Blue dye in 450ml methanol, 100ml glacial acetic acid and 450ml distilled water.

4.6 Destaining solution: 30% methanol, 10% glacial acetic acid
The destaining solution was made by mixing 300ml of methanol, 100ml glacial acetic acid and 600ml of distilled water.
APPENDIX IV: TRYPIC PROTEIN DIGESTS ON SDS-PAGE

Tryptic digests on an SDS PAGE gel. P1-P5 represents pellet fraction tryptic digests while A1-A5 represents albuminome fraction tryptic digests. The absence of visible bands in these samples represented sufficiently digested proteins. Such samples could be analysed by LC/MS. Samples highlighted in red had visible bands and were interpreted as poorly digested and hence not suitable for LC/MS analysis.
APPENDIX V: HISTOGRAMS FOR TRANSFORMED ANTIBODY LEVELS DATA

- Histograms by transformation for amat\textsubscript{au}

- Histograms by transformation for msp2\textsubscript{au}

- Histograms by transformation for msp3\textsubscript{au}

- Histograms by transformation for schizont\textsubscript{au}
APPENDIX VI: T CELL GATING STRATEGY

Lymphocytes  
Singlets  
Live cells  

CD3+CD8+  
CD3+CD4+  

CD3+CD8+ CFSE-  
CD3+CD4+ CFSE-  

FS Lin=Forward scatter linear  
SS Lin=Side scatter linear
# APPENDIX VII: PROTEINS IN THE PELLET FRACTION

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Protein name</th>
<th>Function</th>
<th>Biological pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KAD7_HUMAN Adenylate kinase 7</td>
<td>-Nucleotide binding</td>
<td>-Purine metabolism</td>
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<tr>
<td></td>
<td></td>
<td>-Nucleoside binding</td>
<td>-Metabolic pathway</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Ion binding</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>-Transferase activity</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CLCF1_HUMAN Cardiotrophin-like cytokine factor 1 precursor</td>
<td>-Protein binding</td>
<td>-Cytokine-cytokine receptor interaction</td>
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<td></td>
<td></td>
<td></td>
<td>-Jak-STAT signaling pathway</td>
</tr>
<tr>
<td>3</td>
<td>CLUS_HUMAN Clusterin precursor</td>
<td>-Protein binding</td>
<td>Hemostasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Hydrolase activity</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>FA5_HUMAN Coagulation factor V precursor</td>
<td>-Ion binding</td>
<td>-Complement and coagulation cascades</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Protein binding</td>
<td>-Hemostasis</td>
</tr>
<tr>
<td>5</td>
<td>CIQA_HUMAN Complement C1q subcomponent subunit A precursor</td>
<td>-Protein binding</td>
<td>-Complement and coagulation cascades</td>
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<td></td>
<td></td>
<td></td>
<td>-Prion diseases;</td>
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<td>-Chagas disease</td>
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<td>-Staphylococcus aureus infection</td>
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<td>-Systemic lupus erythematosus</td>
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<td></td>
<td></td>
<td></td>
<td>-Immune System</td>
</tr>
<tr>
<td>6</td>
<td>FCN3_HUMAN Ficolin-3 precursor</td>
<td>-Antigen binding</td>
<td>Immune System</td>
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<tr>
<td></td>
<td></td>
<td>-Binding</td>
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<td>-Carbohydrate binding</td>
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<tr>
<td></td>
<td></td>
<td>-Protein binding</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>HBG1_HUMAN Hemoglobin subunit gamma-1</td>
<td>-Ion binding</td>
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<td>-Oxygen binding</td>
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<td>-Transporter activity</td>
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<td>-Tetrapyrrole binding</td>
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<td>HGFA_HUMAN Hepatocyte growth factor activator precursor</td>
<td>-Peptidase activity</td>
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<td></td>
<td></td>
<td>-Hydrolase activity</td>
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<td>Function</td>
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</tr>
<tr>
<td>9</td>
<td>IGHG3_HUMAN Ig gamma-3 chain C region</td>
<td>-Antigen binding</td>
<td>Immune system</td>
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<td>IGHG4_HUMAN Ig gamma-4 chain C region</td>
<td>-Antigen binding</td>
<td>Immune system</td>
</tr>
<tr>
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<td>KV206_HUMAN Ig kappa chain V-II region RPMI 6410 precursor</td>
<td>-Antigen binding</td>
<td>Binding and uptake of ligands by scavenger receptors immune system</td>
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<td>12</td>
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<td>-Antigen binding</td>
<td>Binding and uptake of ligands by scavenger receptors immune system</td>
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<td>13</td>
<td>ITIH1_HUMAN Inter-alpha-trypsin inhibitor heavy chain H1 precursor</td>
<td>-Enzyme regulator activity; -Ion binding</td>
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<td>14</td>
<td>IFT81_HUMAN Intraflagellar transport protein 81 homolog</td>
<td>-Protein binding</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>K2C6A_HUMAN Keratin, type II cytoskeletal 6A</td>
<td>-Structural molecule activity; -Protein binding</td>
<td></td>
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<tr>
<td>16</td>
<td>LMTD2_HUMAN Lamin tail domain-containing protein 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>LBP_HUMAN Lipopolysaccharide-binding protein precursor</td>
<td>-Binding; -Protein binding; -Lipid binding</td>
<td>-Toll-like receptor signaling pathway; -S. aureus infection - Immune system</td>
</tr>
<tr>
<td>18</td>
<td>MTP_HUMAN Microsomal triglyceride transfer protein large subunit precursor</td>
<td>-Transporter activity; -Lipid binding</td>
<td>-Fat digestion and absorption - Metabolism</td>
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<tr>
<td>19</td>
<td>ZNFX1_HUMAN NFX1-type zinc finger-containing protein 1</td>
<td>-Nucleic acid binding; -Ion binding</td>
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<tr>
<td>20</td>
<td>PZP_HUMAN Pregnancy zone protein precursor</td>
<td>-Enzyme regulator activity</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>DIAP1_HUMAN Protein diaphanous homolog 1</td>
<td>-Protein binding; nucleic acid binding</td>
<td>Focal adhesion -Regulation of actin cytoskeleton -Shigellois</td>
</tr>
<tr>
<td>Protein ID</td>
<td>Protein name</td>
<td>Function</td>
<td>Biological pathway</td>
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<tr>
<td>22</td>
<td>TRI75_HUMAN Putative tripartite motif-containing protein 75</td>
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<td>23</td>
<td>SHBG_HUMAN Sex hormone-binding globulin precursor</td>
<td>-Steroid binding; hormone binding -Lipid binding</td>
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<td>T3JAM_HUMAN TRAF3-interacting JNK-activating modulator</td>
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<td>TPPC8_HUMAN Trafficking protein particle complex subunit 8</td>
<td>-Protein binding</td>
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<td>27</td>
<td>TNR6C_HUMAN Trinucleotide repeat-containing gene 6C protein</td>
<td>-Nucleic acid binding -Nucleotide binding -Protein binding</td>
<td>-Cellular responses to stress -Disease -Gene expression -Immune system -Signal transduction</td>
</tr>
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The functions and biological pathways of proteins in the pellet fraction. The common protein name, function and pathway are tabulated. Blanks occur where the search was unclassified.
APPENDIX VIII: PROTEINS IN THE PELLET FRACTION.

<table>
<thead>
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<th>Accession number</th>
<th>Protein name</th>
<th>Function</th>
<th>Biological pathway</th>
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<td>HEAT1_HUMAN</td>
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<td>2</td>
<td>IGHD_HUMAN</td>
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<td>-Chromatin binding -Dioxygenase activity -Oxidoreductase activity -Ion binding -Demethylase activity</td>
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The functions and biological pathways of proteins in the albuminome fraction. The common protein name, function and pathway are tabulated. Blanks occur where the search was unclassified.
CONDITIONS

1. You must report to the County Commissioner and the County Education Officer of the area before embarking on your research. Failure to do that may lead to the cancellation of your permit.
2. Government Officers will not be interviewed without prior appointment.
3. No questionnaire will be used unless it has been approved.
4. Excavation, filming and collection of biological specimens are subject to further permission from the relevant Government Ministries.
5. You are required to submit at least two (2) hard copies and one (1) soft copy of your final report.
6. The Government of Kenya reserves the right to modify the conditions of this permit including its cancellation without notice.

THIS IS TO CERTIFY THAT:
MISS. ALBINA THIRA WAITHAKA
of KENYATTA UNIVERSITY, 844-502 Karen, has been permitted to conduct research in Kilifi County on the topic: IMPACT OF MALNUTRITION ON HUMORAL AND T CELL RESPONSES IN MALARIA INFECTED CHILDREN

for the period ending: 8th December, 2016

Applicant's Signature

Permit No: NACOSTI/P/15/99993/8298
Date Of Issue: 8th December, 2015
Fee Received: Ksh 1,000

Director General
National Commission for Science, Technology & Innovation