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RELATION OF NUTRITIONAL STATUS, IMMUNITY, HEMOGLOBINOPATHY AND *FALCIPARUM* MALARIA INFECTION

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SUMMARY

The interaction between nutritional status and malaria disease is complex and often controversial. Nutritional deficiencies (macro- or micro-nutrient) are thought to lead to malnutrition with subsequent susceptibility to malaria infection. On the other hand severe malaria or repeated malaria infections lead to malnutrition. While the cause and effect are difficult to attribute, micronutrient deficiencies such as iron deficiency and malaria infection often co-exist and show complex interactions leading to mutually reinforced detrimental clinical effects.

That iron deficiency has adverse effects on human health is widely recognized. Iron plays a crucial role in processes of growth and cell division and in the transport of oxygen throughout the body. It is also important for the proliferation of cells of the immune system as well as for microorganisms including the malaria parasite. Iron deficiency results in a decrease in hemoglobin concentrations and subsequent anemia. However, the etiology of anemia is multi-factorial and may be affected, in addition, by several factors including malaria and host factors, especially hemoglobinopathies such as alpha-thalassemia and sickle cell trait. These hemoglobinopathies are also common in malaria endemic areas.

In this thesis, we have investigated the relationship between nutritional status, immunity, hemoglobinopathies and *falciparum* malaria in a cohort of children less than 8 years old living on the coast of Kenya. We have found that malaria was associated with malnutrition in an age-dependent fashion. Malaria was associated with subsequent underweight or stunting in children under the age of 2 years, but this effect was not there in older children. Also, we observed that iron deficiency was associated with protection of children against clinical malaria. Children who were iron deficient had a lower incidence of malaria episodes as compared to those who were iron replete.

While studies on the effects of single micronutrient deficiencies on components of the immune system are difficult to design and interpret, there is ample evidence that micronutrient deficiencies, in general, affect all components of immunity. In line with this, we found that nutritional iron status was associated with certain malaria-specific immunoglobulins and interleukin-4 mRNA levels. Iron deficient children had lower levels of malaria-specific IgG2 and IgG4 but higher expression levels of IL-4 mRNA as compared to the iron replete children. Finally, we observed a tendency towards a higher prevalence of iron deficiency in children carrying either alpha-thalassemia or sickle cell trait.

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"If you fix your eyes at places you cannot reach, you will miss the riches below".

To my Dad, My Hero, Nyakeriga Moya and to my mum, my Heroine & my role model, Bosibori Nyakeriga

"*I could not speak intelligibly, and had not strength to write, or even to turn over in my hammock…..I took quinine for sometime without any apparent effect, till, after nearly a forth night, the fits ceased, and I only suffered from extreme emaciation and weakness"*

A narrative of travels on the Amazon and Rio Negro, Alfred Wallace, 1953.

Original Papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I **Nyakeriga M. Alice,** Marita Troye-Blomberg, Alex K. Chemtai, Kevin Marsh, Thomas N. Williams (2004). Malaria and nutritional status in children living on the Coast of Kenya. *Amer. J. Clin. Nutr.,* **80:** 1604-10

II **Nyakeriga M. Alice,** Marita Troye-Blomberg, Jeffrey R. Dorfman, Neal D. Alexander, Rune Bäck, Moses Kortok, Alex K. Chemtai, Kevin Marsh, Thomas N. Williams (2004). Iron deficiency and malaria in children living on the Coast of Kenya. *J. Infect. Dis.,* **190:** 439-447.

III **Nyakeriga, M. Alice**, Thomas N. Williams, Marsh Kevin, Sammy Wambua, Hedvig Perlmann, Peter Perlmann Alf Grandien, Marita Troye-Blomberg. Cytokine mRNA expression and iron status in children living in a malaria endemic area. *Scand. J. Immunol. (in Press).*

IV **Nyakeriga M. Alice**, Marita Troye-Blomberg, Sammy Wambua, Jedidah K. Mwacharo, Kevin Marsh, Thomas N. Williams. Nutritional iron status in children with α^{+}thalassemia and the sickle cell trait in a malaria endemic area on the coast of Kenya. *Haematologica (Accepted for publication).*

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ABBREVIATIONS

APC:	Antigen presenting cell
CD:	Cluster of differentiation
CMI:	Cell-mediated immunity
CR:	Complement receptor
CRP:	C- reactive protein
CSA:	Chondroitin sulphate A
DC:	Dendritic cell
DFO:	Desferrioxamine
Fc:	Fraction crystallizable
G6PD:	Glucose-6-phosphate dehydrogenase
GPI:	Glycosyl phosphotidyl inositol anchor
Hb:	Hemoglobin
ICAM:	Intracellular adhesion molecule
ID	Iron deficient
IFN:	Interferon
Ig:	Immunoglobulin
IL:	Interleukin
iNOS:	Inducible nitric oxide synthase
IR	Iron replete
LBW:	Low birth weight
LPS:	Lipopolysaccharide
LSA:	Liver stage antigen
MHC:	Major histocompatibility
MCH:	Mean corpuscular hemoglobin
MCHC:	Mean corpuscular hemoglobin concentration
MCV:	Mean corpuscular volume
NF:	Nuclear factor
NK:	Natural killer
NO:	Nitric oxide
*Pf*EMP1:	*Plasmodium falciparum* erythrocyte membrane protein-1
PMN:	Polymorphonuclear
SSA:	Sub-Saharan Africa
sTfR:	Soluble transferrin receptor
TcR:	T cell receptor
TGF:	Transforming growth factor
TLR:	Toll-like receptor
TNF:	Tumour necrosis factor
VCAM:	Vascular cell adhesion molecule

INTRODUCTION

This thesis describes a "multi-disciplinary approach" with an attempt to elucidate a complex web of interactions between nutritional status and host factors, immunity or malaria infection. It is made up of various sections comprising mainly: malaria, the general outline of global impact and clinical manifestations of the disease, control of and immunity to malaria infections and a section on related background with reference to nutritional status and malaria infection, in particular iron deficiency and *falciparum* malaria infection. Finally, our original research findings consisting of four papers are summarized under the "results and discussion" section. The first paper describes the interaction between anthropometric indexes of nutritional status and malaria followed by the second paper, which focuses on iron deficiency and malaria infection. In the third paper, an attempt is made to relate iron status and cytokine mRNA levels and the fourth paper is aimed at answering the question as to whether alpha-thalassemia or sickle cell trait protect an individual against becoming iron deficient. Last but not the least is the appendix consisting of copies of each individual paper/manuscript.

Since the immune system, which forms the key to the body's defense against infectious pathogens, may also be affected by various factors including the host nutrition as well as genetic factors, this thesis opens up with a short prelude to basic concepts of the immune system as outlined below. More details of specific components of the immune system will be revisited again in a more detailed manner in the section addressing immune responses to malaria.

The Immune System

The development of immunology began with studies of resistance or immunity against infection. The immune system is broadly subdivided into innate (natural) and adaptive (specific) immunity. However, there is no clear demarcation as these two overlap in their immune effector functions/cells and act in concert with each other to bring about body's defense. Immunity to infections involves various factors/molecules and cells of the immune system. The immune cells which are generally referred to as leukocytes include mononuclear cells, monocytes and macrophages; granulocytes, namely eosinophils, neutrophils and basophils;

lymphocytes, T cells, B cells and NK cells and accessory cells, which are known as antigen presenting cells (APC) particularly the dendritic cells and Langerhans cells.

Innate immunity: This consists of host factors that prevent infection or development of diseases. Many of the molecules/or factors involved in innate immunity are characterized by pattern recognition ability, which enables them to distinguish between microbes and the host. Innate immunity, also involves various factors including physical barriers such as the skin and the mucous membranes, physiological features, e.g. the pH and oxygen tension, protective enzymes such as lysozyme, soluble factors such as the complement components (plasma proteins that are activated in an enzymatic cascade manner, on the surface of the pathogen, leading to the generation of active components with various effector functions), interferons and C-reactive proteins (CRP). Beyond physical barriers, phagocytic cells such as macrophages and neutrophils provide the first line of defense. Other molecules/processes such as cell-associated receptors e.g. toll-like receptors (TLR) and inflammatory processes also do play important roles in innate immunity.

Adaptive immunity: This is the second level of defense, which is characterized by fine specificity and acquisition of memory (recall) following an encounter with a foreign substance, in this context called antigen. Immunological memory is a hallmark of adaptive immunity. Adaptive immunity can be subdivided into 1) humoral immunity – mainly carried out by B lymphocytes 2) cellular immunity – which mainly involves T lymphocytes and immunological mediators/messengers known as cytokines. The specificity of adaptive immunity is an attribute conferred upon lymphocytes by the expression of cell surface receptors and is sustained through clonal selection. The surface receptor for B cells is an immunoglogulin (Ig) molecule, which can be secreted as an antibody while that of the T cells is known as T cell receptor (TcR). The TcR is structurally different from the Ig receptor and can either be αβ-TcR or γδ-TcR. Owing to a large repertoire of lymphocyte receptors, whose diversity is generated mainly by somatic gene rearrangement, the immune system is armed to respond to practically all the potential antigens that exist or nature can device.

B lymphocytes: The B cells arise and develop in the bone marrow in mammals. They are involved in antibody production upon activation by an antigen. B cells undergo maturation and differentiation following an antigen encounter to become memory B cells or plasma cells, which produce antibodies. Five main classes of antibodies are produced, namely IgM, IgD, IgG, IgA and IgE. Antibodies participate in host defense in three main ways: neutralization, opsonization and complement activation, but they may also have an immune-regulatory function through the Fc receptors.

T lymphocytes: T lymphocytes arise in the bone marrow but mature in the thymus. The TcR recognizes peptide fragments of antigens complexed with cell surface glycoproteins, the major histocompatibility complex molecules (MHC), class I and II, on the surface of the antigen presenting cells. The MHC binds and presents fragments of antigens following antigen processing. MHC I molecules present antigens derived from proteins in the cytosol while MHC II present antigens processed in intracellular vesicles. On priming with an antigen, a naïve T cell divides and differentiates to become either short-lived effector T cells or long-lived memory T cells. T lymphocytes are subdivided into two main groups, which are distinguished by expression of complex arrays of surface proteins, cluster of differentiation (CD) proteins: $CD4^+$ T cells and $CD8^+$ T cells. The $CD4^+$ T cells or helper cells are MHC II restricted, help B cells and produce cytokines. These cells can further be grouped into Th1 and Th2 cells depending on which cytokines they produce. The $CD8^+$ T cells or the cytotoxic T cells are MHC class I restricted, produce cytokines and can kill infected cells or tumor cells. Thus, T cells are important both for humoral and cell mediated immune responses.

*Cytokines***:** Cytokines are small proteins secreted by one cell and can alter the activity of the cell itself or on another i.e. they may act locally or systemically. A cytokine produced by T cells is given the name interleukin (IL) followed by a number. Th1 and Th2 cells produce different but overlapping sets of cytokines. Th1 cells mainly produces IFN gamma while Th2 cells are characterized by IL-4, IL-5 and IL-10 production.

Immunity and infection: All components of the immune system are important in controlling infection. However, different types of immune responses more effectively control particular infections. The eventual outcome may depend on the biology of the parasite and the characteristic of the specific type of immune response. For example, intracellular pathogens such as viral infections are cleared by cellular immunity, especially cytotoxic T cells and NK cells, while extracellular pathogens are cleared mainly by humoral reponses. Nevertheless, larger microorganisms such as parasitic infections present the immune system with elaborate life cycle associated with antigenic variation and may thus, involve various components of the immune system. Indeed, immunity to parasites such as *Plasmodium* during malaria infection, provides a clear account of how host immune responses operate and how parasites can subvert immunity. Immunity to *Plasmodium* infection involves various components of the immune system, including polymorphonuclear cells, lymphocytes and cytokines, yet this immunity is not sterile but may offer partial protection. At the same time, the immune system is faced with parasite evasive mechanisms, such as the complex life cycle with various developmental stages of the parasite, antigenic variation and intracellular infection (RBC infection), which reduce the degree of immune activation.

Other factors that may aggravate the challenges faced by the immune system during an infection, include host genetic factors and nutritional status. During malarial infection, for example, sickle cell trait is known to protect against disease. Many studies have shown that immune functions are influenced by nutrients from dietary foods (review in (Chandra, 2002)) and that immune dysfunction is associated with malnutrition. Various micronutrients have been shown to play either a protective or an adverse role in *Plasmodium* infection (Shankar, 2000). Nevertheless, the regulation of the immune responses to suppress them when unwanted or to stimulate them in the prevention of infectious diseases remains the hub of research in immunology.

MALARIA

Global Incidence of Malaria

Malaria remains a major global health problem with transmission occurring throughout Africa, Oceania, Asia and Latin America, covering over 100 countries. More than 40% of the world's populations live in malaria endemic areas of the world (W.H.O, 2002). Approximately 300-500 million clinical cases of malaria occur every year; of these, 200 million were estimated to be due to *P. falciparum*, resulting in more than 2 million fatalities mostly in children under the age of 5 years with these children experiencing an estimated six episodes of malaria per year (Breman, 2001; Murphy and Breman, 2001; W.H.O, 1996). In Africa alone, Snow and others (Snow et al., 1999) estimated that over 200 million clinical attacks of malaria and approximately 1 million fatalities occur every year among people resident under stable endemic conditions. In such malaria endemic areas, WHO estimates that 1/20 children die before the age of 5 years (W.H.O, 2002), with an average of one child dying in every 30 seconds in Africa (W.H.O, 2000). A recent report has indicated that, in Kenya alone, 20-30 percent of infant mortality is attributed to malaria infections (http://www.rbm.who.int/amd2003/amr2003/ch1.htm, (Njoroge, 2002)). Severe hypoglycemia and anemia affect African children and pregnant women in holoendemic areas (Marsh, 1992). Guyatt and Snow (Guyatt and Snow, 2001) suggested that approximately 400,000 pregnant women develop moderate or severe anemia each year in sub-Saharan Africa (SSA) as a result of malaria infection.

Human Malaria Parasites

Protozoan parasites of the genus *Plasmodium* cause malaria infections. The Plasmodiidae family includes about 120 known species with at least 22 species being infective to primates. The etiological agents of human malaria are recognized as four distinct species of *Plasmodium*: *P. falciparum, P. ovale, P. malariae and P. vivax.* All the four human malaria parasites have a complex life cycle (Figure 1), involving development in female anopheline mosquitoes and in humans that presents a major obstacle in the design of control measures. To initiate the life cycle, sporozoites inoculated by female anopheline mosquitoes into the human blood stream, invade hepatic cells to establish the liver stage. This stage, commonly called exoerythrocytic schizogony results in the release of merozoites that invade red cells and establish the

erythrocytic stage, responsible for the disease and malaria related deaths. Induction of gametogenesis produces sexual forms, which are taken up by mosquito to ensure the survival of the species.

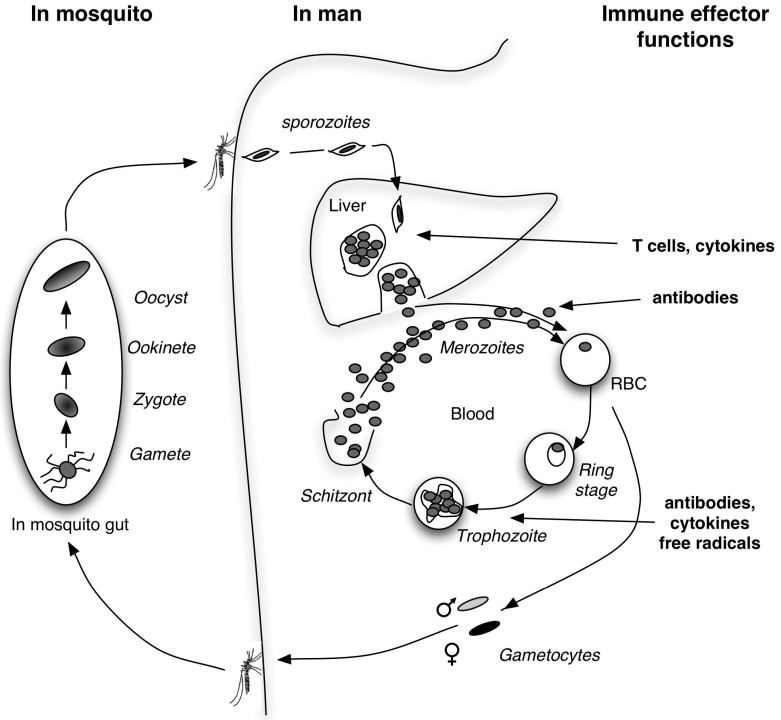

Figure 1: Life cycle of malaria parasite- highlights the immune mechanisms to various stages. *Adapted from Hoffman and Miller 1996*

P. falciparum is the most common and the primary cause of severe malaria in humans. *P. falciparum* and *P. malariae* are associated with recrudescence, while *P. ovale* and *P. vivax* cause relapses. In fact, *P. falciparum* accounts for more than 90% of worldwide malaria-associated mortality (Marsh, 1992). All the four human malaria parasites are found in Kenya with *P. falciparum* causing more than 98% of the infections in malaria endemic areas (Kenya, 1998).

Pathology and Clinical Manifestations of Malaria

Clinical manifestations of malaria range from mild uncomplicated to severe disease. Uncomplicated malaria is a febrile illness characterized by fever, malaise, cough, nausea and diarrhea. The pathogenesis of malaria fever is thought to be due to the rupturing of schizonts which stimulate human mononuclear cells to release tumor necrosis factor (TNF) and other pyrogenic cytokines (Kwiatkowski et al., 1989). The

untreated disease continues for weeks or months in non-immune patients, but only *P. falciparum* produces fulminant disease.

Certain features of severe malaria are common to all infected species, with consequences of hypoglycemia and lactic acidosis occurring in the terminal phase of avian, rodent, simian and human malarias (White, 1985). Although it appears reasonable to extrapolate observations seen in the animal model to the disease in humans (Ehrich et al., 1984), there is no good animal model for cerebral malaria (Yoeli, 1976). In humans, the manifestations of severe and complicated malaria vary geographically but are associated almost exclusively with *P. falciparum* infection. The majority of *P. falciparum* infections do not progress to life-threatening forms. However, in the minority that do, the spectrum of symptoms include, severe anemia, acidosis, respiratory distress, and shock, disseminated intravascular coagulation, renal failure, pulmonary edema and cerebral pathology with convulsions and coma (http://rbm.who.int/docs/hbsm.pdf, (English and Newton, 2002)).

Malaria during pregnancy is associated with low birth weights (LBW), high rates of abortion and stillbirth and hypoglycemia with consequent materno-fetal and infant mortality (Steketee et al., 2001). There is an approximate mean reduction in weight of 170g in babies born to primigravidae (Brabin, 1983). Indeed, previous studies have shown that approximately 12% of LBW deliveries in SSA were related with subsequent fatality rates of 37.5% in newborns (Murphy and Breman, 2001). There may be trophoblastic thickening (Walter et al., 1982), macrophage infiltration and perivilion fibrin deposition (Anagnos et al., 1986), which presumably interfere with transplacental exchange of substrates and metabolites that cause a reduction in fetal growth. There is also evidence of malaria antigen-specific cellular unresponsiveness during pregnancy (Riley et al., 1989).

Cerebral malaria is a syndrome of unrousable coma, often associated with fits and other neurological abnormalities, such as seizures, increased intramuscular muscle tone, and elevated intracranial pressure (Kwiatkowski, 1994). In areas where transmission is low, uneven or highly seasonal, symptomatic disease occurs at all ages with the principal manifestation of severe malaria being cerebral malaria (Brewster et

al., 1990). This contrasts with areas of intense and stable malaria transmission, where anemia in young children is more prominent. The etiology of cerebral malaria has been associated with rosette formation in the cerebral microvasculature (Carlson, 1993). A distinctive feature of cerebral malaria is sequestration, the attachment of the infected erythrocytes to endothelial cells lining the post-capillary venules (Land et al., 1995). Several adhesive ligands have been identified on the surface of the host endothelial cells: thrombospondin, glycoprotein IV (CD36), intercellular-adhesion molecular 1 (ICAM-1), E-selectin and vascular cell adhesion molecule 1 (VCAM-1), $\alpha_v\beta_3$ integrin receptor, chondroitin sulphate A (CSA) and Fc-receptors in the placenta. All of these molecules bind to various parts of the *P. falciparum* adhesion receptor, *Pf*EMP1 (Baruch et al., 2002). However, it is not clear whether any or all of these proteins are involved in the sequestration (Berendt et al., 1989; Ockenhouse et al., 1989; Ockenhouse et al., 1991).

In holo- or hyper-endemic areas, there is an increased incidence of maternal anemia associated with *falciparum* malaria (Greenwood et al., 1989), but most women are asymptomatic. Anemia is a common feature of complicated disease in African children and pregnant women and in adults in Asia and South America. Severe anemia is often a complication of acute febrile malaria in children, but may also occur in people with asymptomatic *P. falciparum* infection (Anstey et al., 1999; Kurtzhals et al., 1999; Premji et al., 1995). In pregnancy, this is often associated with LBW (Steketee et al., 1996). Anemia is an inevitable consequence of malaria, resulting from a combination of parasitized erythrocyte destruction at merogony, accelerated removal of unparasitized erythrocytes and ineffective erythropoeisis (Weatherall and Abdalla, 1982). The various pathological mechanisms involved in anemia are complex, although evidence suggests that TNF is involved in the bone marrow depression and erythrophagocytosis seen during the terminal *P. vinckei* infections in mice (Rickman et al., 1991).

Control of Malaria

Strategies against malaria infection and disease require an integrated approach, such as control of the mosquito vector, use of chemotherapeutic agents and control using insecticides, use of chemoprophylaxis and prompt, appropriate case management

(Jones et al., 2003; Wyler, 1983a; Wyler, 1983b). The use of chemicals has not been successful across Africa and Latin America (Bloland, 2001), due to the emergence of mosquito strains resistant to the commonly used insecticides. Furthermore, insecticides have detrimental side effects to the environment, thus there is a pressing need to seek other alternatives. Due to the increased mosquito resistance to insecticides, the global malaria control strategy adopted by governments and W.H.O. (W.H.O, 2001) emphasized the need for early diagnosis, appropriate treatment with anti-malarial drugs, and selected use of preventive measures, including mosquito control where it is effective and can lead to sustainable impact.

Chemoprophylactic measures, which have been employed as means of malaria control in conjunction with insecticide spraying, include the cheap and affordable chloroquine and sulfadoxine-pyrimethamine. However, the emergence and spread of drug resistance by the parasite poses a serious threat to effective chemoprophylaxis and treatment of *P. falciparum* malaria. *P. falciparum* has developed resistance to nearly all antimalarial drugs in current use, although the geographical distribution of resistance to any single antimalarial drug varies greatly. Chloroquine resistance has been described in almost all malaria endemic areas except for Central America, the island of Hispaniola and limited areas of East and Central Asia (Bloland, 2001). Complete or partial resistance to chloroquine and sulfadoxine-pyrimethamine has now spread to all endemic areas including Africa (Mendis and Carter, 1995). New drugs have been introduced, but resistance even to these is developing at an alarming rate. While expensive and unaffordable to many, the increasing resistance to available monotherapy has necessitated the implementation of combined therapies (W.H.O, 2001).

Vaccines

Considering the fact that traditional methods of controlling malaria have been neither practical nor cost effective across much of Africa, in regions of Latin America and Asia, and that the same methods have been thwarted by problems such as parasite drug resistance and resistance of vectors to insecticides, there is a pressing need for an affordable and effective malaria vaccine. Previous studies have shown that anti-sporozoite vaccines based on irradiated sporozoites are protective in animal models

(Nussenzweig et al., 1967), and that this provided long term protection in humans (Edelman et al., 1993), but their use is not feasible on large-scale immunization. Besides, the development of a malaria vaccine is complicated by a number of factors, including the parasite's complex life cycle, antigenic variation and poor host immunological responses to critical antigens (Good, 1995). Moreover, despite efforts made towards vaccine development, malaria remains uncontrolled because we have little understanding of the natural protective immunity against the disease. Also, nutritional deficiencies and associated "global" malnutrition, which affect the host immune responses, hence susceptibility/resistance to infection, are common in malaria endemic areas and seem to be silently active in the background.

Immunity to Malaria

The immune status of the individual and the population plays an important role in the clinical response to infection and transmission. Whereas malaria symptoms generally correlate with the level of parasitemia, the spectrum of disease is variable and can be unpredictable (Weatherall et al., 1983). Individuals with similar levels of blood stage parasitemia may remain asymptomatic or suffer morbidity and death (Playfair et al., 1990). Thus, the immune status of an individual plays a vital role in the clinical consequences of malaria infections. This may be on the level of innate or acquired immunity.

Innate immunity

Both the cells of the immune system and host genetic factors contribute to the innate immunity against malaria. The immune cells include gamma/delta (γδ) T cells, natural killer (NK cells), natural-killer-T cells (NKT), polymorphonuclear cells such as neutrophils and eosinophils, soluble factors such as interferons and complement factors (Perlmann and Troye-Blomberg, 2002). Gamma/delta T cells are more expanded than the alpha/beta T cells in the early phase of malaria infection and are believed to contribute to innate control of the parasite. Another cell type that has been shown to be associated with innate immunity to malaria is the dendritic cell (DC). While the role of DCs in malaria immunity is still under investigation, the current literature is associated with controversial reports. The pioneering human *in vitro* studies showed that *P. falciparum* infected red cells suppressed the maturation of DCs

with paralleled immune dysfunction, including impeded ability to induce antigen-specific primary and secondary T cell responses (review in (Pouniotis et al., 2004), (Urban and Roberts, 2003). However, more recent reports, involving both *in vitro* and *in vivo* studies using experimental animals, have shown that DCs from infected mice are not only fully functional APCs, but are also capable of inducing long-lasting protective immunity (review in (Pouniotis et al., 2004), (Perry et al., 2004)). Another group of molecules that may play a role in malaria immunity include germ-line encoded receptors, in particular the toll-like receptors (TLRs).

The TLRs can recognize traces of microbial components and orchestrate an early defense, largely dependent on the activation of nuclear factor kappa β (NF-kβ), which often leads to the production of pro-inflammatory cytokines and triggering of microbicidal effector mechanisms. While major advances have been made in the assignment of individual TLRs to defined roles in bacterial infections, such identification has only begun to emerge in protozoan infections. In malaria one study has suggested that an adaptor molecule shared by TLRs, the myeloid differentiation factor-88, MyD88, is associated with the pathogenesis of disease in experimental infection (Adachi et al., 2001). Similarly, an *in vitro* study using human blood and *P. falciparum* has shown that schizont extracts contain a novel and previously unknown ligand for TLR-9 suggesting that the stimulatory effects of this ligand on DCs may play a key role in immunoregulation and immunopathogenesis of human *falciparum* malaria (Pichyangkul et al., 2004). Certainly, further work is needed in this area.

Host genetic factors

In malaria endemic areas host factors, especially the genetic red cell disorders, including sickle cell trait, thalassemia, enzyme deficiencies, ovalocytosis and ABO blood groups, have been shown to confer natural protection against malaria infection, hence the term "natural immunity". All these factors affect parasite survival and provide resistance reflected by skewed distribution of their allele frequencies in malaria endemic areas the so-called "malaria hypothesis" (Haldane, 1949; Nagel et al., 1981; Weatherall, 1987). Other innate factors, such as polymorphisms in ICAM-1, the putative receptor for erythrocytes binding to the brain endothelium, and a polymorphism in the promoter region of TNF-α, appear related to the frequency of

severe disease (Fernandez-Reyes et al., 1997; McGuire et al., 1994). Also, a low frequency of the class I MHC complex molecule HLA-B53 has been associated with severe malaria in the Gambia, possibly associated with immunity to the liver stages of the parasite (Hill, 1992; Hill et al., 1991).

P. falciparum and structural hemoglobin S variant

The hemoglobin S (HbS) gene is found in SSA, the middle east, the Mediterranean countries, among certain tribes of India, and in other places of the world to which the affected population has migrated (gene flow). The geographical distribution of the HbS gene is virtually identical to areas in the world in which malaria is (or has been) endemic (Nagel, 2004). Early epidemiological studies suggested that heterozygote carriers of the β^s gene could acquire *P. falciparum* malaria but had a reduced relative risk of dying of the infection (Allison, 1954a; Allison, 1954b). Sickle cell trait (HbAS) confers more than 90% protection in African children against severe malaria symptoms: cerebral malaria and anemia (Hill et al., 1991). In Gabon, hemoglobin S has been shown to protect against severe attacks of *P. falciparum* malaria (Gendrel et al., 1992). However, the mechanism of protection is unclear. Luzzato and others (Luzzatto et al., 1970) reported that the rate of sickling in parasitized cells from sickle cell trait was higher than that of non-parasitized cells. They postulated that malaria induces sickling in circulating sickle cell trait cells that normally do not sickle, which leads to increased removal from the circulation, thus destroying the parasite in the process, "suicidal infection". Roth and others (Roth et al., 1978) have made similar observations. Other possible mechanisms by which sickle cell trait can protect from malaria include inhibition of *P. falciparum* growth in red cells, increased phagocytosis of infected red cells and release of toxic compounds during sickling.

Other studies from different parts of Africa have shown that individuals carrying HbAC and HbCC are protected from malaria (Modiano et al., 2001) and this has been supported by abnormal growth development of the parasite in red blood cells containing HbC (Fairhurst et al., 2003). Similarly, diminished growth of the parasites both in HbE trait and the HbE homozygous genotype, particularly marked in the latter, has been reported (Vernes et al., 1986).

Thalassemia and malaria

Thalassemias are disorders of hemoglobin production, falling into two categories, the α- and the β-thalassemia characterized by underproduction or deletion of the α- and the β-globin respectively (Weatherall and J.B.Clegg, 1981). There are two main types of α-thalassemia: α^+ in which one of the linked pair of a globin gene is deleted (α/-), and α^0 where both pairs of the α-globin are deleted (-/-) (Higgs et al., 1989). Depending on the size of the deletions and the underlying mechanism α^+ can be grouped into $-\alpha^{3.71}$, $-\alpha^{3.711}$, $-\alpha^{3.7111}$ and $-\alpha^{4.2}$ (Higgs et al., 1989).

Both alpha and beta thalassemias are associated with decreased susceptibility to malaria infection, where the beta thalassemia also has been reported to protect against the severe complications of *P. falciparum* infection (Flint et al., 1986). The basis for this decreased susceptibility may include several factors; beta thalassemia carriers experience a persistence of fetal hemoglobin in infancy, which can impair *P. falciparum* growth in red cells, possibly due to limitation of essential amino acids and increased susceptibility to intraerythrocytic oxidative stress (Friedman, 1979). The protection afforded by thalassemia against malaria may be related to the inability of the parasite to invade or multiply, or to cytoadhere to endothelial cells (Udomsangpetch et al., 1993).

Blood group antigen polymorphisms

The absence of certain glycophorin molecules on the surface of erythrocytes may affect parasite binding, as evident in certain West Africans population. The duffy protein plays an important role in *P. vivax* malaria infections. *P. vivax* merozoites bind to this protein to invade red cells (Hadley and Peiper, 1997). Red cells from the duffy negative phenotype are resistant to invasion by merozoites (Miller et al., 1976; Spencer et al., 1978).

Binding of parasites to erythrocytes is facilitated by the presence of other surface molecules expressed on the erythrocyte; for example *P. falciparum* binds specifically to sialic acids on erythrocyte glycophorin (Camus and Hadley, 1985). Glycophorin A is the major transmembrane sialoglycoprotein on red cells which contributes to the

expression of the MN and Wright (WrB) blood group antigens – these proteins play an important role in the invasion of human red cells by *P. falciparum.*

Other red-cell-membrane-associated proteins that have been implicated in malaria protection include complement receptors, knop-blood group and the ABO blood groups. Individuals with blood group O experienced fewer episodes of cerebral malaria compared to individuals having blood groups A or B (Hill, 1992). This might be as a result of differences in the ability of infected red blood cells to form rossettes (Carlson and Wahlgren, 1992). Another report indicated that sickle cell trait in the presence of blood group A might determine the severity of malaria outcome (Lell et al., 1999). The complement receptor-1 (CR1) is a ligand involved in the rosetting of *P. falciparum* infected red cells (Moulds et al., 2000). The most important CR1 polymorphism relevant to rosetting of malaria-infected cells appears to be the knop molecules (Roth et al., 1978). Rosetting of cells containing mature trophozoites and schizonts is believed to be an important factor in the development of cerebral malaria- a condition that is more common in individuals carrying blood group A (Nagel, 2004).

Enzyme deficiencies

In vitro studies have shown that the growth of *P. falciparum* is reduced in Glucose-6-phosphate dehydrogenase (G6PD) deficient red cells than in normal red cells (Miller et al., 1984; Roth et al., 1983). While the underyling mechanism is not clear, there is evidence that G6PD-deficient infected red cells, especially during the ring stage parasite, are phagocytosed more rapidly than those from normal individuals (Cappadoro et al., 1998). The G6PD-deficient cells lack the ability to resist sustained oxidative stress adequately and hence the free-radical-producing parasite is a challenge to such cells. This situation is thought to make the red cell more susceptible to phagocytosis. Also oxidative stress induced by the parasite, plus the normal red cell oxidative stress particularly unquenched by the enzyme deficiency, results in an environment in which normal parasite growth is limited (Nagel, 2004).

Red cells with cytoskeleton abnormalities

Invasion of the red cell by merozoites is a complex process, involving several stages including nonspecific attachment, re-orientation of the polarity of the merozoite, red

cell membrane flapping and the zipper-type introjections of the merozoite into the red cell (Miller et al., 1979). The host cytoskeleton proteins may be involved in the events that follow the invasion such as generation of the parasitophorous vacuolar membrane, without direct incorporation into the parasite membranes. Finally, cytoskeleton or membrane proteins are known to be critical for the invasion of red cells by the malaria parasites. Ovalocytosis is characterized by highly polymorphic red cells with cytoskeleton abnormality. This red cell variant confers resistance to high levels of parasitemia with *P. falciparum, P. vivax P. malariae* and has been shown to be resistant to invasion by *P. falciparum* merozoites (Kidson et al., 1981; Serjeantson et al., 1977).

Acquired immunity

Development of protective immunity is an active process involving two arms of the immune system namely, cellular and the humoral components. However, the preexisting cross-reactive T cells may skew the development of malaria specific T cells following exposure to malaria parasite through a type of "original antigenic sin" (Good and Currier, 1992). Notably no good *in vitro* correlates of acquisition of immunity in humans have so far been found. Much recent research has been conducted in animal models or with cultured *P. falciparum* parasites using human peripheral blood mononuclear cells (PBMCs).

Naturally acquired immunity to malaria may be referred to as a diminished frequency or density of *P. falciparum* parasites in adults relative to children in areas with hyper- to holo-endemic malaria (Baird, 1995). Natural immunity includes both anti- disease and anti- parasite processes. Natural immunity fails to develop where malaria is hypo-endemic, epidemic or meso-endemic. Naturally acquired immunity is, however, short-lived in the absence of repeated infections and previously immune individuals, who spent less than a year away from malarious area, have been found to be susceptible to disease (Cohen and Lambert, 1982). In areas of lower endemicity, with an unstable malaria situation, immunity in the population is generally low. Thus, in these areas clinical malaria as well as severe complications may occur in all age groups (Good, 1995). Poor acquisition of immunity may be due to the genetic diversity or variability of parasite antigens, which give rise to protective immune

responses (Troye-Blomberg and Perlmann, 1994). Furthermore, immunity acquired by constant exposure to malaria is not sterile although it appears protective.

Naturally acquired immunity is both parasite species- and stage-specific. In hyperendemic areas, infants born to immune mothers are protected against malaria during the first six months of life, reflecting the preceding transfer of protective antibodies from mother to child. Children of five years old or more living in such areas may have high numbers of parasites in their blood, but remain largely asymptomatic (Bruce-Chwatt, 1952; Lucas et al., 1969). Such apparent tolerance is generally believed to be due to "antitoxic" immunity specifically directed against soluble parasite components causing disease (Playfair et al., 1990). With progressing age, clinical symptoms are mild and rare and the level of blood parasites is generally very low, reflecting the development of antiparasite rather than antitoxic immunity (Troye-Blomberg and Perlmann, 1994).

Humoral immunity to malaria

Although the precise role of antibodies in protective immunity against malaria is unclear, various studies have identified specific antibodies to sporozoites, intrahepatic parasites, merozoites, malaria toxins, parasite antigens on infected red cells, intraerythrocytic parasites, and the sexual stages within the mosquitoes (Hoffman and Miller, 1996). Serum immunoglobulin (Ig) levels in residents of malaria endemic areas are highly elevated (McGregor, 1972). However, only a small proportion of these Igs represent specific antiparasite antibodies, while the rest are non-specific, including autoantibodies (Freeman et al., 1980). Polyclonal B-cell activation may also lead to depressed specific responses to other pathogens or to vaccination (Perlmann and Troye-Blomberg, 2002). The level of total antimalarial antibodies increases with age and is usually taken as a measure of the length and intensity of exposure, and sometimes may indicate protection against malaria. The passive transfer of immune IgG to Gambian children gave rise to some protection (Cohen et al., 1961; McGregor, 1964). However the efficiency of antibody-mediated inhibition is usually insufficient to confer complete protection. Also, some protective mechanisms do not involve antibodies, for example, protection against sporozoite infection of liver cells is primarily mediated by T-cells (Troye-Blomberg and Perlmann, 1994). A large body

of evidence suggests that antibodies are important for clearance of parasite loads in both animal and human blood stage infections (Berzins et al., 1991; Troye-Blomberg and Perlmann, 1988). Antibodies against the sexual stages can confer transmission-blocking immunity by inhibiting the development of the parasite in the mosquito midgut (Targett, 1990). Antibodies to exo-antigens inducing TNF-α may contribute to antidisease or antitoxic immunity (tolerance), as displayed by children living in endemic areas who are relatively asymptomatic even though they carry high loads of parasites (Playfair et al., 1990; Taverne et al., 1990). Antibodies also play an important role in antibody-dependent cellular cytotoxicity and phagocytosis involving polymorphonuclear cells (PMNs), neutrophils or platelets (Ockenhouse et al., 1984; Butcher and Clancy, 1984; Kharazmi and Jepsen, 1984; Lunel and Druilhe, 1989; Bouharoun-Tayoun and Druilhe, 1992; Bolad and Berzins, 2000). Phagocytosis is generally enhanced by an antibody binding to antigens on extracellular stages of malarial parasites or infected erythrocytes, which enables recognition of the parasite by macrophages and neutrophils via Fc receptors. Brooks and Kreier (Brooks and Kreier, 1978) showed that normal rat peritoneal macrophages rapidly bound and phagocytosed antibody-coated *P. berghei* merozoites.

Immunity against the blood stage *P. falciparum* infection is associated with protective type antibodies of certain classes and sub-classes although some antibody sub/classes, have a pathogenic role. It has been postulated that acquired protective immunity to *P. falciparum* is to a large extent mediated by IgG antibodies with specificity for parasite-encoded clonally variant surface antigens (VSAs) on the surface of infected red blood cells (review in (Hviid and Staalsoe, 2004)). Indeed, passive transfer experiments have established that IgG plays a major role in premunition (Druilhe and Perignon, 1994). The protective IgG antibodies belong to a restricted panel of classes and subclasses, specifically the cytophillic antibodies of IgG1 and IgG3 isotypes, whereas IgG2 and IgG4 isotypes are considered non-protective. However, under certain conditions IgG2 antibodies can also be associated with protection (review in (Garraud et al., 2003). While IgG antibodies are generally associated with protection against malaria, the IgE antibodies seem to play a pathogenic role. Both human and experimental animal studies have shown an association between malaria pathogenesis and elevation in both total IgE and malaria specific IgE antibodies. IgE-containing

immune complexes (IC) are thought to be pathogenic via cross-linking of IgE receptors on monocytes/macrophages, with subsequent overproduction of local TNF-α, a major pathogenic factor in malaria (Perlmann and Troye-Blomberg, 2002).

Cell-mediated immune responses in malaria

Cell-mediated immunity (CMI) plays an important role in protective immunity to malaria, as shown in animal models and *in vitro*. Effector components of this CMI involve different sets of cells including cytotoxic T cells, Th1 and Th2 cells, B cells, monocytes/macrophages, NK cells and PMNs.

Role of T cells

T cells are central in the development and regulation of both humoral and cell-mediated responses. Thus, T cells are critical for B-cell activation and differentiation into plasma cells, as well as cell-mediated immunity. Depending on the type of cytokine produced, both murine and human $CD4^+$ T cells can be divided into Th1 and Th2 cells (Suss and Pink, 1992). Th1 are thought to be involved in the initial resolution of acute parasitemia through cell-mediated effector mechanisms, including clearance of parasitized red cells by phagocytes and production of Th1 cytokines (Langhorne et al., 1989a; Langhorne et al., 1989b; Stevenson et al., 1992), while Th2 are believed to lead to the eventual clearance of the parasites via T-B cell cooperation in the subsequent antibody response (Langhorne et al., 1989a; Langhorne et al., 1989b). However, a third group of cells that are still under investigation are thought to lead to immunosuppression, which consequently makes it difficult for the host to maintain long-lasting immunity, hence increased susceptibility to malaria infection. These are the T-regulatory (T-reg) cells; $CD4^+/CD25^+$ T cells. Experimental animal studies have shown that depletion of T-reg cells protects mice against lethal challenge with *P. yoelii* or *P. berghei*, and that this protection is associated with an increase in T-cell responsiveness against parasite derived antigens (Long et al., 2003). Similarly young susceptible rats have been shown to have persistent T-reg cells, while the resistant ones have high levels of $CD8^+$ T cells and NKT cells (review in (Hisaeda et al., 2004), (Adam et al., 2003)).

In animal models, $CD8^+$ T cells are critical for eliminating malaria from the liver (Kumar et al., 1988). $CD4^+$ helper T cells are required for antibody production to most malaria antigens, and also act as the effector cells with malaria antibodies to destroy parasitized red cells (review in (Mahanty et al., 2003)). Cloned lines of $CD4^+$ T cells may confer protection, and *in vivo* depletion of $CD4^+$ T cells has been associated with abrogation of immunity in mice infected with *P. Vinckei* (Kumar et al., 1989). One model suggests that parasites stimulate $CD4^+$ T cells, which then activate macrophages /monocytes and neutrophils via gamma interferon, TNF and other cytokines. The activated macrophages/monocytes in turn, kill parasites by oxygen radicals such as nitric oxide (NO), or phagocytosis of parasitized erythrocytes (Good, 1995). Thus, although T cells may be specifically activated by malaria, their effector functions may be nonspecific.

Mice depleted of $CD4^+$ cells show reduced IgM production, whereas IgM production is not affected in $CD8^+$ depleted mice (Fossati et al., 1990). T lymphocytes from human donors primed to plasmodial antigens by repeated natural infection can easily be induced *in vitro* to proliferate or produce IFN-γ by a variety of malaria antigens (Troye-Blomberg et al., 1990). On the other hand, repeated amino acid sequences, seen frequently in malaria antigens, are generally immunodominant B-cell epitopes (Anders et al., 1988), which may function as T-independent antigens. Interestingly, the parasite may use these epitopes as a mechanism of immune evasion to confer a selective advantage. In line with this, it has been shown that anti-circumsporozoite protein (CSP) antibody responses, demonstrated in individuals resident of malaria endemic areas, are relatively weak and short-lasting following immunization with antigens containing repeat sequences (Brown et al., 1988; Webster et al., 1988).

The role of cytokines in malaria

Cytokines released during malaria may play both protective and pathological roles. Cytokine production is triggered via schizont rupture leading to release of substances/molecules such as parasite antigens, merozoites, pigment, glycosyl-phosphotidyl inositol anchor (GPI), and other soluble antigens or toxins that may induce cytokine production. Various cytokines have been shown to take part in malaria protection and/or pathogenesis. These include both the pro-inflammatory

cytokines; IFN-γ, IL-1, IL-6, TNF-α, or anti-inflammatory cytokines, including IL-4, IL-10 and IL-5. The balance between pro- and anti-inflammatory cytokines may determine disease severity. An outline of various studies involving specific cytokines is described in the following section.

Interleukin 1 (IL-1)

Pied *et al.* (Pied et al., 1989) have demonstrated that IL-1, at low concentrations, inhibits the development of the hepatic stages of *P. falciparum* and *P. yoelii in vitro*. However, IL-1, has been shown to induce expression of endothelial molecules, which support parasite binding, conceivably contributing to the pathology of cerebral malaria (Berendt et al., 1989; Prada et al., 1995). In line with this, increased levels of IL-1 have been shown to have a direct correlation with disease severity in Gambian children (Kwiatkowski et al., 1990). These studies generally suggest that IL-1 is pathogenic during malaria infection.

Tumor necrosis factor (TNF)

The production of TNF in responses to malarial antigens may be essential both for protection and for the development of clinical symptoms in *P. falciparum* malaria (McGuire et al., 1994). An association between levels of TNF and parasitemia and disease severity has been reported in cerebral malaria patients and other severe complications, such as severe organ damage, hyper-parasitemia and severe anemia (Grau and Lou, 1995; Shaffer et al., 1991). Rupturing schizonts stimulate human mononuclear cells to release TNF, whose distinct peak in plasma coincides with each fever paroxysm (Karunaweera et al., 1992b). TNF-induced fever may substantially reduce the growth of erythrocytic forms of *P. falciparum* (Kwiatkowski et al., 1989). TNF is also known to increase the potential for endothelial binding of some isolates of *P. falciparum*, by upregulating ICAM-1 expression and possibly other endothelial receptor molecules (Porta et al., 1993). This may as a consequence lead to a direct damage of cells or tissues. Murine studies have shown an association between injection with *r*TNF and anemia through macrophage activation, leading to increased phagocytosis, suppression of erythropoeisis and dyserythropoiesis (Clark and Chaudhri, 1988). On the other hand, TNF elicits protective defenses by activating macrophages and neutrophils to phagocytoze parasitized erythrocytes, and by

inducing release of soluble factors, such as oxygen and nitric oxide radicals, that inhibit parasite growth (Kwiatkowski, 1994). Karunaweera and others (Karunaweera et al., 1992a) showed that TNF inhibits the growth of the sexual erythrocytic stages. Overall these studies suggest that low levels of TNF may be protective while high levels of TNF are pathogenic during malaria infection.

Interferon gamma (IFN-γ)

Systemic administration of IFN-γ partially protects mice and monkeys against *P. berghei* (Ferreira et al., 1986) and *P. cynomolgi* infections (Maheshwari et al., 1986), respectively. IFN-γ also kills *P. falciparum* in *in vitro* cultures (Mellouk et al., 1987). Other reports indicate that IFN-γ, in addition to other cytokines, induces infected hepatocytes to produce L-arginine-derived nitrogen oxides that are toxic to the intracellular parasite (Nussler et al., 1991). IFN-γ released by activated $CD4^+$ cells may activate mononuclear and polymornuclear leucocytes to either phagocytoze or lyse infected red cells (Deloron et al., 1991; Shear et al., 1989). On the other hand, Riley *et al.* (Riley et al., 1990; Riley et al., 1991) showed that IFN-γ, produced by T cells from either patients with acute malaria or children in the Gambia after stimulation by various preparations of parasite antigens, may not be effective in controlling infections. It is thought that an early acute phase may be beneficial, but sustained release of IFN-γ can contribute to anemia. Nonetheless, there is evidence that in the process of increasing immunity, antigen–specific $CD8^+$ T cells may be generated and may attenuate the response to antigen-induced IFN-γ production, thereby reducing the possible deleterious effects of sustained release of IFN-γ (Abdalla, 2004).

Interleukin-4 (IL-4)

Activated T cells of the Th2 sub-type and mast cells produce IL-4. Biemba and others (Biemba et al., 2000) reported a major reduction in the risk of developing anemia, which was associated with a rise in IL-4 production. Other studies have shown that the maintenance of protracted protection versus malaria is dependent on responses produced by memory $CD4^+$ cells to sporozoite, liver stage and the asexual stage antigens, leading to IL-4 dependent stimulation of specific $CD8^+$ (Carvalho et al.,

2002; Palmer and Krzych, 2002). Generally, IL-4 appears to have a protective role in immunity to malaria.

Interleukin-6 (IL-6)

There appears to be an agreement in most studies that IL-6 is increased in patients with acute malaria, but its role in the pathogenesis of the disease remains controversial. Many reports suggest that IL-6 plays a role in the pathogenesis of severe human malaria as levels of IL-6 correlated with TNF levels in patients with *P. falciparum* infection. In addition, the levels of these cytokines were found to parallel the levels of parasitemias and disease severity (Butcher et al., 1990). Notably, high levels of IL-6 were found in Gambian children with cerebral malaria, in adults from Thailand with complicated malaria, and in Gabonese children with severe malaria (Jakobsen et al., 1994; Kremsner et al., 1995; Saissy et al., 1994; Wenisch et al., 1998). On the other hand, IL-6 produced in response to other pro-inflammatory cytokines may represent the first step in down-regulating the initial responses to malaria. This might be followed by the formation of specific antibodies upon the production of the anti-inflammatory cytokine, IL-10 (Akanmori et al., 1996).

Interleukin-10 (IL-10)

IL-10 overproduction in response to TNF-α may play an important role in severe malarial anemia, but not apparently through down-regulation of TNF-α production (Kurtzhals et al., 1998; Othoro et al., 1999). Overall, an appropriate production of IL-10 is required to protect against the harmful effects of TNF-α, thereby reducing the possibility of severe anemia and mortality induced by the disease. Potential protective effects of IL-10 were observed in adult Kenyan volunteers whose peripheral blood mononuclear cells, in response to the liver stage antigen 1 (LSA-1), produced increased levels of IL-10, which correlated with subsequent resistance to re-infection with *P. falciparum* (Kurtis et al., 1999).

Interleukin-12 (IL-12)

This cytokine activates cytotoxic and NK cells with subsequent production of IFN-γ. IL-12 injection prior to malaria infection was found to protect mice against challenge with *P. yoelii* sporozoites by enhanced killing of intrahepatic parasites by IFN-γ–

mediated pathways (Sedegah et al., 1994). Similar findings were described in mice infected with *P. chabaudi* (Stevenson et al., 1995) and monkeys infected with *P. cynomolgi* (Hoffman et al., 1997). Thus, IL-12 appears to play a protective role against malaria in mice, which is independent of the development of protective immunity. In humans, however, lower levels of IL-12 have been reported in children with severe malaria (Luty et al., 2000; Perkins et al., 2000), and it was postulated that heavy parasitemia and macrophage loading with hemozoin might impair the capacity to produce IL-12.

Transforming growth factor beta (TGF-β).

Experimental animal models have shown that TGF-β appears to be involved in protection against malaria infection (Omer et al., 2000). On the other hand, administration of *r*TGF-β led to increased morbidity and mortality in a strain of mice, which was otherwise resistant to lethal re-infection (Omer and Riley, 1998). The authors postulated that TGF-β may facilitate the switching between Th1 and Th2 cytokine responses and may therefore play a direct role during malaria infections. Thus, increased concentrations in TGF-β at an early stage of infection may lead to increased production of pro-inflammatory cytokines, whereas higher concentrations of the cytokine later in the course of infection, may lead to an anti-inflammatory cytokine response. In general, depending on the magnitude of TGF-β and those of other specific cytokines subsequently induced, TGF-β levels may have either protective or pathogenic role in malaria.

Nitric oxide (NO) and induced nitric oxide synthase (iNOS)

Nitric oxide is a bioactive substance acting as a neurotransmitter and vasodilator. NO is produced by various cells, including those of the endothelial system, fibroblasts, neurons, adrenal cells, hepatocytes, monocytes and macrophages. The production of NO is catalyzed by three isoforms of nitric oxide synthases: NOS1 or nNOS from endothelial cells, NOS3 or eNOS from neurons and NOS2 or iNOS from hepatocytes, monocytes and macrophages. Inducible nitric oxide synthase (iNOS) can lead to sustainable production of nitric oxide (MacMicking et al., 1997). Nitric oxide production is triggered by cytokines, mainly IFN-γ, TNF-α, IL-1, lymphotoxins and microbial products, especially lipopolysaccharides (LPS). GPI anchor from *P.*

falciparum has been reported to induce iNOS from macrophages and vascular endothelium (Abdalla, 2004). In contrast NO production *in vitro* was shown to be reduced in mouse macrophages, which had ingested malaria pigment (Prada et al., 1996).

It has been postulated that NO may play a protective role against malaria by; killing of gametocytes (Naotunne et al., 1991), induction of the crisis forms seen in certain malaria immune patients (Taylor-Robinson, 1996) and protection against blood stage parasites in mouse malaria (Stevenson et al., 1995). *In vitro* studies have reported a suppression of *P. falciparum* growth in the presence of NO or NO producers (Rockett et al., 1991; Balmer et al., 2000). In general, NO may protect against malaria, but may also be associated with severe disease in certain circumstances, especially in those associated with high TNF-α levels. It also appears that prolonged production of NO may contribute to the anemia seen in chronic malaria (Abdalla, 2004).

RELATED BACKGROUND

Malaria and Nutrition

Approximately 12 million children younger than 5 years of age die every year; most of these children live in developing countries. Recent estimates suggest that malnutrition, measured as poor anthropometric status, is associated with about 50% of all deaths among children (Pelletier, 1994; Schroeder and Brown, 1994). Although malnutrition is prevalent in developing countries, it is rarely cited as being among the leading cause of death. In such countries, young women, pregnant women, and their infants and children frequently experience a cycle where undernutrition (macronutrient and micronutrient) and repeated infections, including parasitic infections such as malaria lead to adverse consequences.

Malaria can interfere with growth of the infected individual, as shown by the faltering in growth associated with an acute clinical attack of malaria (Rowland et al., 1977). Infants born prematurely or with low birth weight (LBW) are not only at increased risk of early death, but are also at risk of poor growth and development. The impact of malaria during pregnancy varies greatly according to transmission. Severe acute

complications, including cerebral malaria or materno-foetal death, seem to be confined to areas of unstable transmission where malaria is uncommon except during epidemics. In areas of stable endemicity, the main consequences are maternal anemia and intra-uterine growth retardation. The poor growth, resulting in underweight or stunting, leaves women at reproductive age at risk in their early pregnancies of delivering premature or LBW infants. In addition, the micronutrient deficiencies, particularly iron and folate (which contribute to anemia), leave the young women at risk of anemia, leading to inadequate oxygen-carrying capacity and increased risk in pregnancy of delivering LBW infants. Thus, malnutrition assumes a cyclic form, affecting all age groups, but the outcome may depend on the prevalent parasitic infection(s) in the affected individual (review in (Steketee, 2003)).

Nutrition and infection

Micronutrient deficiency and infectious diseases often co-exist and show complex interactions, leading to mutually reinforced detrimental clinical effects. Deficiencies in micronutrients such as zinc, copper and iron are associated with suppression of numerous cellular activities of both the innate and acquired branches of the immune system; the observed alterations can either reflect decreased activities of the individual cells/and or in a reduction of the total number of effector cells. These micronutrients have been shown to affect the continuous generation of immune cells from the bone marrow, the plasma levels of hormones that regulate the development and function of the host cells, and the synthesis and secretion of cytokines and chemokines that modulate the activities of immune competent and other cells (Failla, 2003). This kind of impairment of immunity can be sufficient to increase the risk of morbidity and mortality due to viral, microbial or parasitic infections

Previous studies from Papua New Guinea, have revealed that most diets are limited in variety and low in protein and/or calories, which frequently lead to chronic malnutrition (Mueller and Smith, 1999) and micronutrient deficiencies (Gibson et al., 1991), and this can profoundly affect the outcome of malaria infection, especially in children (Shankar, 2000). Children who are underweight are thought to have increased susceptibility to malaria for a variety of reasons, most notably through a reduction in the function of the immune system; through reduced number of T cells,

impaired antibody formation, decreased complement formation, and atrophy of the thymus and other lymphoid tissues, among others (review in (Scrimshaw and SanGiovanni, 1997; Chandra, 2002)). The synergistic relation between nutrition and immunity is well recognized and nutritional interventions have been used as important approaches to reducing mortality from acute respiratory illness and diarrhea (reviewed in (Rice et al., 2000)). Elderly hospitalized patients given a supplement of vitamin A, C and E for four weeks showed increased number of $CD4^+$ and $CD8^+$ T cells and increased lymphocyte proliferation to a mitogen compared to the placebo group (Penn et al., 1991). Similarly, the administration of low-dose multi-micronutrient supplement with increased amounts of vitamin C, E and beta carotene was associated with increased numbers of T cells, enhanced lymphocyte responses to mitogen, increased IL-2 production, greater NK-cell activity and increased response to influenza virus vaccine compared to the group given placebo (Chandra, 1992).

Iron and Malaria

Between 2-5 billion people worldwide are at least mildly iron deficient, making iron deficiency the most common micronutrient deficiency in humans today (Stoltzfus, 2001). Iron deficiency is believed to be the main underlying cause of anemia (van den Broek, 2003). Anemia is frequently seen during childhood and pregnancy and may thus contribute significantly to maternal and childhood morbidity and mortality. While malaria causes a large proportion of anemia in malaria endemic areas, the contribution of underlying micronutrient malnutrition, creating a complex web of interactions with serious health repercussions cannot be oversimplified. Micronutrient deficiencies have been associated with increased morbidity and mortality from malaria, and malaria, in turn, may contribute to poor nutritional status, reflecting the classic vicious cycle of malnutrition and infection (Scrimshaw et al., 1968).

Iron status and the nutritional hypothesis

Iron status can be considered as a continuum from iron deficiency with anemia, to iron deficiency with no anemia, to normal iron status with varying amounts of stored iron, and finally iron overload, which can cause organ damage when severe. Iron deficiency is the result of long-term negative iron balance, resulting in absence of mobilizable iron stores and compromised supply of iron to tissues, including the

erythron. The more severe stages of iron deficiency are usually associated with anemia. Although iron deficiency anemia accounts for most of the anemia that occurs in SSA, several other possible causes include: hemolysis induced by malaria, G6PD deficiency, congenital hereditary defects in hemoglobin synthesis, increasing HIV/AIDS or deficits in other micronutrients such as Vit A, B12 and C and folic acid. Other blood losses such as those associated with helminthic infections (schistosomiasis and hookworm), hemorrhage in childbirth, and trauma can also result in both iron deficiency and anemia.

The treatment of iron deficiency represents one of the three priorities of the W.H.O. in the micronutrient initiative program, which include iron, iodine and vitamin A. However, correction of iron deficiency via supplementation is much debated for fear that this would lead to detrimental effects on plasmodial infections. The nutritional-immunity theory was developed from studies that observed a protective effect of iron deficiency on disease severity (Kochan, 1973). This theory suggests that depriving the parasite of essential nutrients, such as iron, creates an unfavourable internal environment, thus preventing the parasite from full proliferation.

Iron metabolism and malaria parasites

Plasmodium contains an iron-regulated protein, raising the possibility that the malaria parasite expresses transcripts that contain iron responsive elements and are iron-dependent (Loyevsky et al., 2001). About 25-75% of the hemoglobin is digested in the acid parasitophorous food vacuoles during the growth phase of the malaria parasite. Parasites require iron for essential enzymatic, respiratory and redox reactions. The possible sources of iron for plasmodia include heme iron from the breakdown of hemoglobin, plasma transferrin bound iron, intracellular ferritin, a labile intraerythrocytic pool of iron or non-heme intracellular or transit or storage iron (Rosenthal and Meshnick, 1996).

Some evidence that iron may affect the malaria parasite has come from *in vitro* studies involving use of iron chelators. Iron chelators such as desferrioxamine (DFO) inhibits the growth of malaria parasites *in vitro* (Raventos-Suarez et al., 1982), a phenomenon thought to be due to various possible mechanisms-: depletion of plasma-

bound iron (Pollack and Fleming, 1984), by a toxic effect of the iron chelator itself (Peto and Thompson, 1986), or by an intraerythrocytic labile iron pool (Loyevsky et al., 1999). Iron chelators appeared only to be effective as anti-malarials if they were capable of entering the parasite cytosol and their toxicity to the parasites appeared to be stage-specific. Thus, inhibition of parasite growth did not take place if the chelators were present at the ring stage (Whitehead and Peto, 1990; Cabantchik et al., 1996).

Experimental work using DFO on mice with *P. vinckei* (Fritsch et al., 1985) and *P. berghei* (Hershko and Peto, 1988) and in *Aotus* monkeys with *P. falciparum* showed a reduction in parasitemia. *In vitro* studies have demonstrated reduced *P. falciparum* growth in the presence of DFO (Peto and Thompson, 1986; Raventos-Suarez et al., 1982; Whitehead and Peto, 1990). However, clinical studies in humans using different iron chelators have been associated with either reduced parasitemia (Bunnag et al., 1992; Gordeuk et al., 1992) or no effect (Hershko et al., 1992; Thuma et al., 1998). The differential effects were attributed to the different iron chelators, which may possess different properties, used in the two categories of the studies.

Further support on the negative effect of iron on the parasite has come from the mechanism of action of some anti-malarial drugs. The effects of some of the anti-malarials appear to be related to their ability to block the parasite induced iron detoxification, leading to damage of the parasite or lysis of the infected red cells. Chloroquine and other aminoquinolones are thought to act through various mechanisms, including the inhibition of heme-dependent protein synthesis and prevention of iron release from hemoglobin (Rosenthal and Meshnick, 1996). Similarly, artemesimin and other related compounds, that recently have been used in the treatment of chloroquine-resistant malaria, appear to be active through an iron-dependent mechanism (Rosenthal and Meshnick, 1996). In the view that many anti-malarials have an effect on the parasite iron metabolism, it is important to consider the possibility of reduced efficacy of these anti-malarials when iron supplementation is given at the time of treating malaria. In line with this, the use of chloroquine and desferrioxamine *in vitro* have been shown to have antagonistic effects in cultures of newly isolated *P. falciparum* (Jambou et al., 1992).

Iron deficiency- laboratory determination

The detection of iron deficiency may involve use of hematological parameters such as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) or biochemical measurements including serum iron, ferritin, transferrin and soluble transferrin receptor. The determination of iron deficiency using either hematological or biochemical parameters is difficult, particularly in malaria endemic regions, as they are both affected by infections including malaria itself as well as other infections or inflammatory processes.

Changes in red cell indexes, such as MCV, MCH and MCHC, may be attributable to malaria or to other co-existing factors such as iron deficiency itself. Nonetheless, two studies have independently shown that MCV and MCH are valid indicators of severe iron deficiency anemia in areas where malaria is endemic. While these parameters have been shown to be lower in iron deficiency anemia than in severe malarial anemia (Abdalla et al., 1980), it remains to be determined whether they can be used to distinguish between iron deficiency and iron deficiency anemia *per se*. Serum ferritin, which is the biochemical test that mostly correlates with the relative total body iron stores, is an acute phase protein and is affected by the presence of an inflammation (Dennison, 1999) and appears to be directly related to severity of malaria (Das et al., 1997). Soluble transferrin receptor (sTfR), which was initially thought to be independent of malaria and inflammation, has been shown to be affected by malaria (Williams et al., 1999; Stoltzfus et al., 2000). sTfR levels are also raised during other conditions, including erythroid hyperplasia, such as in patients with sickle cell disease (Kohgo et al., 1987), ineffective erythopoiesis (Rees et al., 1998) and congenital iron loading anemia associated with dyserythropoiesis (Cazzola et al., 1999). Thus, involvement of serum ferritin measurements in defining iron deficiency remains the parameter of choice but the presence of infection such as malaria and/or inflammation must be taken into account.

THE PRESENT INVESTIGATION

Objectives

The general aim of this thesis was to investigate the relationship between nutritional status, with special reference to iron status, and immune profile, hemoglobinopathies and malaria infections. We focused on defining the relationship between nutritional and/or iron status and malaria infection with respect to: 1) anti-malaria specific antibody responses and episodes of malaria infection 2) Th1/Th2 associated cytokines and 3) hemoglobinopathies. The specific aims of the research work presented in this thesis were:

- To elucidate the interaction between anthropometric indexes of nutrition and malaria infection
- To investigate the interaction between iron deficiency and clinical incidences of malaria
- To assess the relationship between iron status and immune status
- To investigate the relationship between iron status and haemoglobinopathies (sickle cell trait and alpha- thalassemia).

Methodology

A detailed description of the materials and methods used in this thesis is found in each paper and/or manuscript (Papers I-IV). A summary of this is outlined below:

The study area and population: This thesis comprises studies that were conducted on individuals in the two communities of Chonyi and Ngerenya, who are resident in the areas of Kilifi district on the north coast of Kenya. These communities have similar socio-demographic and ethno-linguistic characteristics. Specifically the studies covered a cohort of children that were undergoing a longitudinal study evaluating the natural history of malaria. The cohort consisted of children aged from 0–8 years old as described in detail in each paper.

Parasite cultures and antigen preparation: The F32 laboratory strain of *P. falicparum* was cultured under standard conditions using candle jars (Trager and Jensen, 1976). The cultures were maintained in culture medium; RPMI 1640 supplemented with gentamycin and L-glutamin and 10% commercially obtained albumax. The late blood stages were enriched using percoll gradient separation with subsequent sonication to obtain the parasite antigen preparations.

Human plasma and cells: Peripheral blood mononuclear cells and plasma samples used in the various studies described herein were obtained from the cohort of children described above.

Biochemical measures of iron status: C-reactive protein (CRP) and all the biochemical indexes of iron were determined using automated analyzers (papers II, III and IV)

Antibody determination: IgG, IgG1 IgG2, IgG3, IgG4 and IgE antibodies reactive with the malaria sonicate antigen and total IgE were measured using standard enzyme-linked immunosorbent assay (ELISA) methods (Papers I and II).

Hemoglobin typing: Hemoglobin S typing was done using standard cellulose acetate gel electrophoresis. Alpha-thalassemia typing was conducted using PCR based technique (Paper IV).

Cytokine determination: Cytokines were determined at the mRNA levels using real time PCR with commercially available kits and reagents (Paper III).

Definitions: Iron status: **iron deficient**: plasma ferritin <12 μg/L in combination with tranferrin saturation <10% and **iron replete**: plasma ferritin ≥12 μg/L in combination with tranferrin saturation ≥10%.

Clinical malaria episode: Measured fever (axillary temperature > 37.5˚ C) or a clinical history of fever within 48 h in conjuntion with a positive test for blood stage *P. falciparum* at any density.

RESULTS AND DISCUSSION

The results and discussions will be outlined in the following sections of this thesis, but the detailed discussions are found in the corresponding papers/manuscripts. However, I will put more emphasis on trying to 1) understand the present work through a recapitulation of the current literature and 2) flag up the possible issues and challenges arising from this thesis.

Relation between malaria and anthropometric indexes (paper I)

The relationship between protein energy malnutrition (PEM) and malaria is complex. Although it has been suspected that nutrition might influence susceptibility to infection by malaria parasites, there have been relatively few studies to examine such interactions. Among the studies, some suggest that poor nutritional status or selective nutrient deficiencies may actually be protective; others suggest exacerbation of disease due to certain deficiencies, while others have observed no effect (reviewed in (Shankar, 2000)). While much emphasis has been placed on the effects of nutritional (macronutrient or micronutrient) deficiencies on the infection and outcome of malaria morbidity and mortality, little is known concerning the effects of malaria on nutritional status. Evidence from malaria control based intervention programs, including use of chemoprophylaxis or bed-nets, indicated that a reduction in malaria infection was associated with weight gain (Davies et al., 1956; Snow et al., 1997). We therefore investigated the relationship between malaria and PEM (expressed as anthropometric indexes: low weight-for-age [underweight], low height-for-age [stunting], and low weight-for-height [wasting]) during a two-year follow-up for malaria episodes with four cross sectional surveys during which anthropometric indexes were determined. We subsequently explored our data for two possibilities; 1) that malaria might be an underlying cause of malnutrition and 2) that malnutrition might lead to susceptibility to malaria. A crude analysis of our data using Poisson regression models suggested no association between malaria and malnutrition (IRR for underweight; 0.94; 95% CI; 0.71, 1.25; p=0.67). Similarly, we arrived at the same conclusion using other secondary markers of evaluation, including use of malaria specific IgG immunoglobulins and sickle cell trait condition. IgG was shown to be associated with malaria exposure (IRR for malaria 2.90; 95% CI; 2.10, 3.99; $p< 0.0001$), so it would be expected that if malaria were the main determinant of

malnutrition, children who were malnourished would have higher IgG concentration in their plasma, but this was not the case. In line with this, there was no evidence for protection against becoming malnourished in children with sickle cell trait (HbAS), as would have been expected (if malaria was the sole contributing factor to malnutrition), since this trait had been observed to be associated with 50% protection from malaria in all age ranges, including children from our cohort (data not shown). However, further analysis based on age stratification of the cohort showed a significant association between malaria and being malnourished in the subsequent period of follow-up in children under the age of two years old (IRR for underweight; 1.65; 95% CI; 1.10,2.20; p=0.01). Similar observations were made for children who were classified as being stunted. Further examination of our data, to determine if a relationship existed between anthropometric indexes at any one survey and malaria episodes in the subsequent period of follow-up, suggested no evidence of malnutrition being a predisposing factor to succumbing to malaria infection.

Malaria or malnutrition?

From the observations described above one wonders whether malaria or malnutrition leads to the other! While early perceptions without any quantitative or methodological support held it that malnutrition can lead to greater susceptibility to malaria infection (review in (Shankar, 2000)), observational quantitative studies have suggested that malnourished individuals are less susceptible to malaria infection or morbidity and mortality (Hendrickse et al., 1971; Murray et al., 1978b). Subsequent animal studies were conducted in a bid to resolve the controversy. Most studies were in agreement with the finding that protein-deprived animals experienced less morbidity and mortality due to malaria (Ramakrishnan, 1954: Edirisinghe et al., 1981; Edirisinghe et al., 1982). Importantly, the protein-deprived animals were unable to resolve infection (Fern et al., 1984). Furthermore, a recent experimental animal study suggested that malnutrition does not protect from malaria infection or morbidity (Cardoso et al., 1996). In humans, cross-sectional studies have shown worsening malaria conditions in malnourishred children (Mbago and Namfua, 1991; Tonglet et al., 1999), while other studies have reported no association (Carswell et al., 1981; Monjour et al., 1982). More recent studies based on hospital admissions for severe malaria have indicated that malnourished patients were more likely to die or have permanent

sequelae than normally nourished patients (Olumese et al., 1997; Renaudin, 1997; Man et al., 1998). Some of the differences between the studies, and particularly between recent and early studies, might be explained by improved clinical evaluation of both malaria and nutritional status. Early studies were usually based on re-feeding of severely malnourished individuals, a condition that may lead to faster parasite recrudescence but slow recovery of both nutritional status and immune responses. Another important factor, as exemplified in our study, that might affect the interaction between malaria and nutrition is the age of the study subjects. Our study showed that the effect of malaria on nutrition became manifest only when age was introduced into the analysis. Indeed, there was an interaction between age (in tertiles) and being underweight (likelihood ratio $\chi^2 = 10.36$, $p = 0.006$) or being stunted (likelihood ratio $\chi^2 = 11.60$, $p = 0.003$).

It is worth noting that most of the studies described above based their conclusions on, only one side of the story, that is, the possibility that malnutrition might lead to increased susceptibility to malaria infection. Very few studies have investigated the possibility that malaria might be an underlying cause of the apparent malnutrition. While it is generally accepted that repeated malaria infections may lead to underweight, much evidence is based on malaria intervention programs. Malaria can interfere with growth, as shown by the faltering in anthropometric indexes associated with an acute clinical attack of malaria (Rowland et al., 1977). In addition, children living in a malaria endemic area, and who receive regular malaria chemoprophylaxis, have been reported to have higher anthropometric indexes than control children (Davies et al., 1956; Murray et al., 1978b; Ahmad et al., 1985). Similarly, studies, involving the use of (impregnated) bed-nets, have shown that children who sleep under bed-nets have better growth indexes than their control counterparts (Snow et al., 1997; ter Kuile et al., 2003). While our study did not indicate that malnutrition contributed to malaria, it was evident that malaria led to malnutrition in children less than 2 years old.

The role of age

It can hardly be overemphasized that the blunt of malaria is most significant in pregnant women and young children. Malnutrition and malnutrition-associated

adverse effects cuts across all age groups (Figure 2). However, in malaria endemic areas the greatest impact is seen in the younger children, less than the age of five years (Snow et al., 1999). Indeed, recent estimates combining prevalence data and the population attributable risk fractions, revealed that most malaria deaths were attributed to undernutrition (with 57.3 % population at risk for malaria mortality due to underweight) in children less than five years of age (Caulfield et al., 2004).

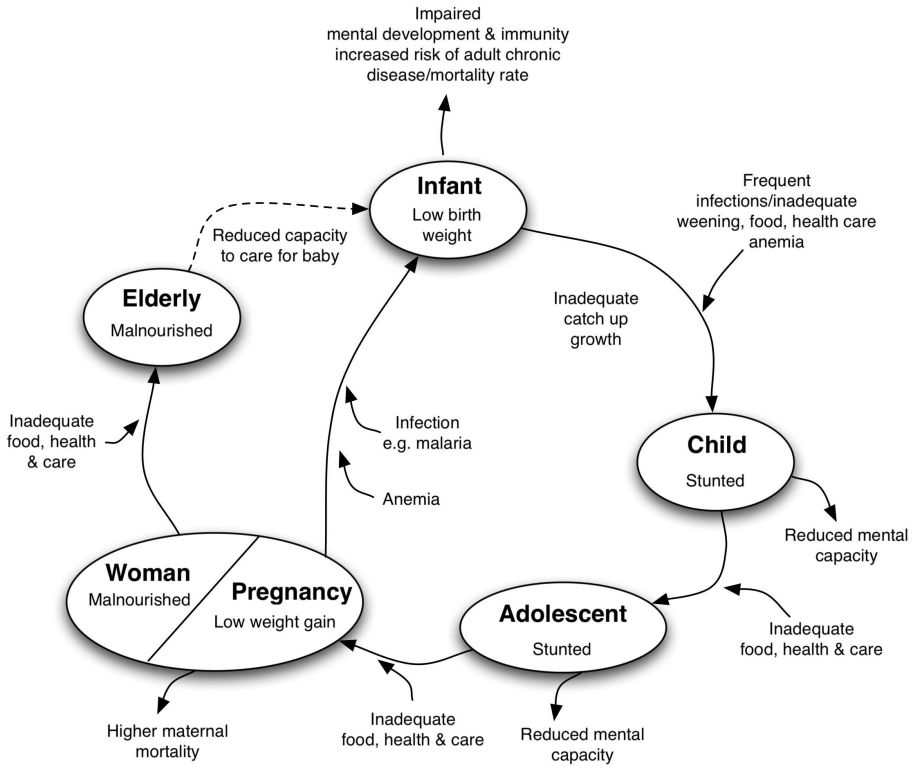

Figure 2: Vicious cycle of malnutrition and infection. *Adapted from Steketee et al 2003*

Severe malarial anemia, is more frequent in young children (≤ 3years) (Snow et al., 1999). In contrast, older children (>3yrs) frequently encounter cerebral malaria. Likewise, various indicators of nutritional status have an age-dependent distribution. Wasting and underweight are seen more frequently in young children than in older children for whom stunting is generally prevalent. If the age-associated nutritional indexes are associated with infection in individual children, maternal-related health/nutritional status or simply diet or a combination of these conditions remain unclear. Nevertheless, our data demonstrated an age-dependent association between malaria and subsequent malnutrition. We observed a higher incidence of malaria in

the malnourished children below the age of 2 years (IRR for underweight; 1.3 for the 0-1 yr old children and 1.7 for those who were aged >1-2 years), followed by a lower incidence in the older children (IRR for underweight; <1). Similar trends were observed in stunted children. The observation that older children had lower incidences of malaria is consistent with what has been observed in malaria control-based studies (Davies et al., 1956; Friedman et al., 2003) and may be explained by the acquisition of immunity with age. However, our study failed to explain the lack of association between malaria and malnutrition in children between the ages 2 and 4 years, when the annual incidence of malaria was highest. That is, if malaria would have been the main underlying cause of malnutrition, this age group would have had the worst nutritional conditions, but this was not the case. Thus, although it was evident that malaria contributed to subsequent undernutrition, this implies that there are also other factors that underlie the age-specific association observed in our study. More studies will be required to investigate the underlying mechanisms to the age-specific-association between malaria and malnutrition.

Nutritional iron status and malaria (paper II)

Based on a number of reasons, there was a need to elucidate the relationship between malaria, immune response to malaria and nutritional iron status, using our cohort of children. These included; 1) our observation that malaria was associated with malnutrition (paper I), 2) a recent meta-analysis on placebo-controlled iron supplementation trials, in human, which showed an overall significant increased risk of infection (Shankar, 2000) and 3) the fact that much evidence on the relationship between malaria and malnutrition is derived from ancient observational studies or iron intervention studies. To address this issue, biochemical markers of iron status and malaria-specific- IgG, IgG1-4, IgE and total IgE immunoglobulin concentrations in plasma samples obtained from each child were subsequently measured during two cross-sectional surveys (in May and October 2002). In addition, clinical incidences of malaria were recorded for each child during an 18-month period of a longitudinal follow-up. In accord with previous studies (Shankar, 2000), our findings suggested that iron deficiency was associated with protection against malaria, as revealed by: 1) a lower incidence of malaria episodes in the iron deficient children as compared to the iron replete children (IRR 0.70; 95% CI, 0.51-0.99; $p<0.05$); 2) a significant positive

association between ferritin concentrations, an indicator of iron stores, and the incidence of malaria, i.e. the higher the level of ferritin the higher the number of malaria episodes; 3) consistently lower malaria specific immunoglobulins in the iron deficient children as compared to the iron replete children during the two surveys (Figure 3).

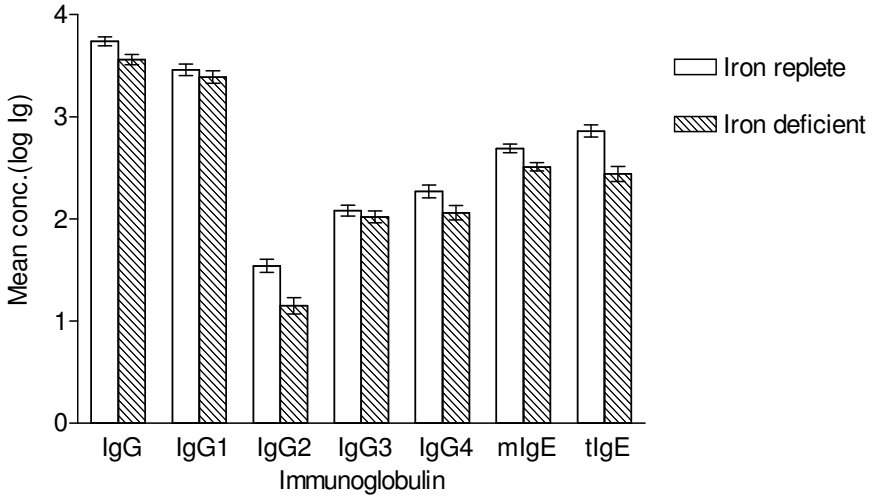

Figure 3: Immunoglobulin concentration by iron status in May 2002.

Similar observations were made in October 2002. Ig: immunoglobulin, m: malaria-specific, t: total

The latter observation could be interpreted in two possible ways: first, that iron deficiency leads to a defect in the production of certain immunoglobulins, whose reduced production is associated with protection against malaria (indeed, IgG2 and IgG4, and malaria specific IgE, Igs that have been associated with susceptibility to malaria, were lower in the iron deficient group). The second possibility is that the immunoglobulin concentration could be a reflection of previous exposure to malaria, that is, the higher the level of immunoglobulin concentration the more the exposure and *vice versa*. Subsequent analysis to investigate the second possibility was conducted using Poisson regression models. This showed an association between the immunoglobin concentration and incidences of malaria in the period preceding the cross-sectional survey in May 2002 ($p < 0.01$ for all the malaria-specific IgG immunoglobulin subclasses; IgG-1, 2, 3 &4). While either possibility holds, there was a stronger evidence for the second one. Thus, the iron replete children had both higher incidences of malaria and immunoglobulin concentrations. Consequently, this could imply that iron deficient children may have had less exposure, or they have a

dysfunctional immunoglobulin production in the IgG subclasses associated with susceptibilty to malaria and *vice versa*.

Previous studies have shown that the levels of anti-malarial IgG2 antibodies are associated with malaria susceptibility (Garraud et al., 2003; Ndungu et al., 2002). In support of this, we found that the iron replete children who had higher IgG2 concentration had also experienced higher incidences of clinical malaria. Similarly, we observed that malaria specific IgE was significantly higher in iron replete children and that this was associated with increased incidence of malaria during the subsequent season of follow-up. Malaria specific IgE has previously been reported to be associated with clinical malaria (Perlmann et al., 1994; Perlmann et al., 1997). Thus, it is possible that iron deficiency may protect against malaria through immunomodulatory effects on certain immunoglobulin sub/classes.

Does iron affect humoral immunity?

In vitro or experimental animal studies are in agreement with the fact that iron status affects both humoral and cell-mediated immune responses. However, data from human studies indicate that iron does not affect humoral immunity (review in (Dhur et al., 1989)). Our study, on the contrary, showed an association between iron status and certain immunoglobulin sub/classes namely malaria specific IgG2, IgG4, IgE and total IgE concentrations (Figure 3). Although all malaria specific immunoglobulins, that is, total malaria specific IgG and IgG subclasses, were strongly associated with previous exposure to malaria, only the aforementioned subclasses were associated with iron deficiency. IgG1 and IgG3 were not associated with iron status. Thus, there is a possibility that iron status may direct imunoglobulin responses towards certain sub/classes. However, whether the variation of immunoglobulin sub/classes observed in our study were due to influence of iron status, a parasite evasive associated mechanism, or a coincidental observation remains to be elucidated.

Nutritional deficiency and the malaria story- "who is who in this kingdom?"

Which nutritional deficiencies that might protect against malaria are uncertain; protein, riboflavin and iron have all been implicated. Thus, in rats, protection against *P. berghei* was achieved by feeding them on a diet selectively rich in proteins

(Edirisinghe et al., 1981). Lower levels of malaria parasitemia in children with biochemical evidence of riboflavin deficiency than children with normal levels have been reported (Thurnham et al., 1983; Das et al., 1988). While the role of iron in susceptibility or resistance to malaria remains controversial, our observation suggested that iron deficiency might protect against malaria, with children who were iron deficient having lower incidences of clinical malaria compared to those who were iron replete.

Recent intervention studies suggest that other micronutrients (than those already mentioned above) may reduce morbidity from malaria in children in endemic areas. A randomized double-blind placebo-controlled trial of vitamin A supplementation (Shankar et al., 1999) showed that the incidence of malaria episodes was reduced from 0.54 clinical malaria episodes per person at risk per year in the placebo group to 0.39 episodes per person per year in the group supplemented with vitamin A. A similar reduction in *P. falciparum* morbidity was demonstrated in a zinc supplementation trial (Shankar, 2000). Daily zinc supplementation resulted in a 38% reduction in the incidence of *P. falciparum*, the effect was strongest on infections with very high parasite densities. From these reports and our observation, it is almost impossible to attribute effects on protection from and/or susceptibility to malaria to any one particular micronutrient. Thus, more studies are required to resolve this issue.

Risks or benefits- where is the balance?

The burden of anemia in the tropics falls primarily on the young children and pregnant women, and iron supplementation of these groups is the primary means of preventing and treating anemia, especially with the advent and fast-spreading of HIV/AIDS that is threatening the use of blood transfusion in such areas (review in (Snow et al., 1999). In fact, the prevalence of iron deficiency in our cohort was rather high ranging from 33-41 % in the two cross-sectional surveys. Thus, given the adverse effects of iron deficiency (i.e. effects on cognition, physical development, hematological indexes and immune functions) one would recommend treatment with iron of those young children immediately. Indeed, iron supplementation, in a malaria endemic area of Tanzania, was shown to be associated with improved hematological indexes (Menendez et al., 1997). However, despite the efficacy of supplementary iron for the prevention and

treatment of iron deficiency and anemia, debates over the use of iron supplements in malaria endemic regions continue, because of concerns that it may increase susceptibility to malaria. The original observation by Masawe (Masawe et al., 1974) was that patients with iron deficiency were more likely to suffer from clinical malaria after treatment of iron deficiency than their corresponding anemic controls. In a study of Somali nomads attending a re-feeding camp, Murray and others (Murray et al., 1978a) found lower incidence of malaria in subjects who were iron deficient than in those who were iron replete. Further studies have attempted to evaluate the benefit of iron supplementation in malaria endemic areas (review in (Shankar, 2000; Oppenheimer, 2001). Some studies reported that iron supplementation increased the risk of developing or reactivating malarial illness (Oppenheimer et al., 1986; Smith et al., 1989), while others reported no significant adverse effects (Fleming et al., 1986; Harvey et al., 1989). The reasons for this discrepancy are not clear. Some of the differences might be attributed to the route of iron administration. Evidence suggests that treatment of iron deficiency, especially when parenteral iron preparations are used, can increase susceptibility to malaria during the post-treatment period (Byles and D'sa, 1970; Oppenheimer et al., 1986; Bates et al., 1987; Smith et al., 1989). However, others have failed to show this (Harvey et al., 1989). Overall, no consensus has been reached on the risks or benefits associated with iron supplementation and malaria morbidity. However, recent reports suggest that the alleviation of anemia through iron supplementation is likely to benefit all iron deficient populations, including those in malaria endemic areas. Whether this is true or not remains to be determined.

While our finding does not resolve the existing controversy on the relationship between iron deficiency and clinical malaria, our data support the notion that iron deficiency may be beneficial in populations living in malaria endemic areas. We found an association between iron deficiency and lower incidence of malaria. This finding points to the need for larger and well-controlled studies under different malaria transmission settings, to provide knowledge which would form the basis for informed iron supplementation and/or intervention programs to alleviate the already widespread and devastating problem with iron deficiency and anemia in malaria endemic areas, particularly SSA.

Iron status and cytokine expression (paper III)

Epidemiological observations have confirmed that infections and malnutrition aggravate each other, with either adverse effects, no effects or with moderate effects on disease outcome as discussed in papers I and II above. Although it is generally believed that nutritional deficiencies impair immune responses, little attention has been given to how various micronutrient status, such as iron, affect immune status. Given the high prevalence of iron deficiency in our cohort, we sought to investigate the relationship between nutritional iron status and cytokine mRNA expression in our cohort. We subsequently measured mRNA levels of various cytokines, including IL-2, IL-4, IL-6, and IL-10, TNF-α and IFN-γ, and of iNOS in peripheral mononuclear cells obtained from 29 ID and 30 IR children. Our study showed that certain cytokines might be affected by the iron status. In particular, IL-4 was found to be higher in the iron deficient children than in those who were iron replete. No such associations were made for all the other cytokine mRNA expression levels or with iNOS mRNA levels. Also, we found an association between malaria infection and IL-10 mRNA levels. Possible explanations for these observations are discussed in light of current knowledge in the following section.

Iron and immunity

Iron is a crucial micronutrient for immunosurveillance. It has a growth-promoting role for immune cells and interferes with cell mediated immune effector pathways including cytokines. Iron is essential for enzymes such as ribonucleotide reductase, which is involved in DNA synthesis (Leberman and Egner, 1984). It has been demonstrated that iron deficiency as well as iron overload can exert subtle effects on immune status by altering the proliferation and activation of T-, B- or NK cells. Cellular iron availability modulates the differentiation and proliferation of Th1 and Th2 cell subsets, which may partly be related to the different dependence of cells on transferrin-mediated iron uptake - Th1 clones are sensitive to treatment with anti-transferrin receptor antibodies while Th2 are not (Thorson et al., 1991). As a result, Th1-mediated effector functions are thought to be sensitive to changes in iron homeostasis *in vivo*. Iron loading of macrophages leads to the inhibition of IFN-γ immune-mediated pathways such as TNF-α production, expression of MHC II and formation of iNOS. Moreover, iron by itself is directly involved in cytotoxic immune

defense mechanism by virtue of being a central catalytic compound for the production of highly toxic hydroxyl reactions (Rosen et al., 1995).

Iron has been associated with disease aggravation in various infections, including parasitic infections such as leishmania, bacterial infections such as tuberculosis, fungal infections such as candida and viral infections such as HIV (review in (Weiss, 2002). During hepatitis C infection iron accumulation in the liver was found to impair response to IFN-γ treatment with consequent faster progression of disease (Shedlofsky, 1998). During malaria infection, the effects of iron status on disease outcome vary (see discussion in paper II). Thus, in addition to protection against malaria, iron chelation has been shown to be associated with higher levels of Th1 cytokines and NO, while serum concentration of the Th2 cytokine (IL-4) tended to be lower (Weiss et al., 1997). In a recent study, Biemba (Biemba et al., 2000) reported a major reduction in the risk of developing anemia, a condition, which was associated with a rise in IL-4 levels in serum. Similarly, we observed an association between IL-4 expression and iron status. However, there was no difference between the parasite positive and the parasite negative children (irrespective of their iron status) in IL-4 as well as in the other cytokines or in iNOS, except for IL-10 mRNA expression levels. The small numbers of parasite positive children did not allow us to examine the effect of iron on the cytokine profile seen during malaria infection. More studies will be required to elucidate this question.

In a number of infectious diseases the Th1/Th2 cytokine balance is associated with the course of disease progression. For example, endogenous IL-10 seemed crucial for counter-regulating an overshooting pro-inflammatory cytokine response, resulting in TNF-α mediated septic shock (Reed et al., 1994). Some studies have shown the importance of IFN-γ mediated immune responses in the initial defense against *Trypanosoma cruzi* (review in (Rivera et al., 2003). Similarly, during malaria infection, Th1-mediated immune responses are thought to be involved in the initial resolution of the infection, while Th2 are associated with subsequent parasite clearance (Langhorne et al., 1989a). Moreover, the Th1-Th2 cytokine balance has been shown to be associated with disease outcome (Kurtzhals et al., 1998; Othoro et al., 1999). In our study we observed an association between IL-10 mRNA expression

and malaria infection. However, we could hardly delineate this observation to either a Th1 or Th2 response, as no associations were seen between malaria infection and either IFN-γ or IL-4. Thus, it is difficult to distinguish between the influences of either malaria infection or iron status on cytokine expression, just as it is hard to attribute cause and effect between the cytokines, infection and iron status. For example, in a recent study with differentiated cultures of Caco-2 cells, (human intestinal carcinoma cell line) TGF-β and monocyte chemotactic protein-1 (MCP-1) mRNA levels were decreased in uninfected cells having elevated cellular iron stores. However, these cytokines, together with IL-8 and TNF-α mRNA, were higher in *Salmonella*-infected cells with higher iron levels than in infected cells with normal iron levels (Foster et al., 2001). This implies that infection and iron status might have both antagonistic and synergistic effects on the production of different cytokines. Overall, our study showed associations between IL-4 mRNA expression levels and iron deficiency, and also between IL-10 mRNA levels with malaria infection. However, there is need for further studies to elucidate the relationship between iron, malaria infection and cytokine balance.

Relation of iron status, hemoglobinopathies and malaria (Paper IV)

Given the high prevalence of iron deficiency in our study cohort, we wanted to test the hypothesis that the alpha thalassemia or the sickle cell trait might protect individuals from becoming iron deficient. We subsequently investigated for an association between biochemical indexes of iron status and these hemoglobinopathies.

There was a high frequency of alpha thalassemia, 0.52, and the prevalence of sickle cell trait was 14.6% in our cohort. Further, we found that iron deficiency was associated with age, being most prevalent in the youngest children, followed by a significant reduction thereafter for each year of life (odds ratio for iron deficiency 0.79; 95% CI: 0.7, 0.93; p=0.004), but this rose again at the age of six years. The increased iron deficiency in the early years of life might be explained by increased iron requirement for growth at that age, while the later rise could partly be explained by secondary infections such as helminthhic infections. Overall, our results showed that children with either hemoglobinopathy were not protected from becoming iron deficient. Instead, we observed an association between low ferritin and sickle cell

trait (β = -0.20; -0.38, -0.02; *p*= 0.037) and a tendency towards high prevalence in iron deficiency in children with either hemoglobinopathy. These observations are discussed in light of previous reports in the following sections.

Hemoglobinopathies and protection against iron deficiency or malaria

Iron deficiency and malaria are singly or in combination the most common causes of anemia. However, the etiology of anemia is multi-factorial, involving host factors such as the hemoglobinopathies, e.g. thalassemia and hemoglobin S, which are also common in malaria endemic areas. The interaction between these hemoglobinopathies, iron status and malaria is complex and rather controversial. While a large body of evidence from the literature shows that the milder forms of these hemoglobinopathies (α-thalassemia and sickle cell trait) are associated with protection against malaria, the severe forms have been shown, on one hand, to be associated with anemia and, on the other hand, protect against iron deficiency through increased iron absorption (Hershko et al., 1982; Pippard et al., 1979). Another report has also shown that serum ferritin levels are relatively increased in alpha-thalassemia (Rees et al., 1998). This group postulated that the observed phenomenon was due to increased iron absorption accompanying ineffective erythropoeisis. Based on these earlier observations, we anticipated that alpha thalassemia or sickle cell trait might protect the affected persons against becoming iron deficient through increased iron absorption. On the contrary, we found that these forms of hemoglobinopathies were associated with increased prevalence of iron deficiency, albeit this did not reach statistical significance. Thus, the interpretation of iron status in areas where such hemoglobinopthies are common should be done with caution.

Previous studies have documented that the aforementioned hemoglobinopathies are associated with protection against development of severe malaria, although the conditions may not protect an individual against infection with malaria (see in the introduction -innate immunity to malaria). Accordingly, we at the time of survey observed no difference in malaria parasite positive blood smears between the normal children and those with sickle cell trait. Also, there was a decrease in the prevalence of parasite positivity in the children with the alpha thalassemia, being lowest in the homozygous carriers, but the differences were not statistically significant.

Mechanisms of protection

There is no consensus concerning the specific mechanisms of protection against malaria infection by any of the hemoglobinopathies, but variant hemoglobin has been associated with various pathophysiological conditions. For example, alpha-thalassemia is associated with mild hemolytic state and increased erythropoeisis, HbS with auto-oxidation of hemoglobin and β-thalassemia with ineffective erythropoeisis (reviewed in (Schrier, 2002)). Nevertheless, several mechanisms have been postulated including; reduced invasion of variant red blood cells by the parasite (HbE, HbC, HbH) and/or consequent reduced growth, reduced cytoadherance of infected red blood cells, as well as reduced rosetting of uninfected red cells and increased clearance of the infected red cells via phagocytosis: directly or through immunoglobulin or complement-mediated pathways (review in, (Roberts and Williams, 2003)). Bayoumi (Bayoumi, 1987) proposed that immune responses to malaria are enhanced in individuals with sickle cell trait, in particular increased malaria specific antibody production (Marsh et al., 1989) and significantly increased lymphoproliferative responses to malaria antigens (Abu-Zeid et al., 1992).

Another possible mechanism, through which hemoglobinopathies could protect against malaria, is via iron deficiency. While the role of iron in protection against malaria remains controversial, there is some evidence that iron deficiency might protect against malaria, as described in paper I. In line with this, it has been reported that iron supplementation of pregnant women with sickle cell trait (HbAS) increased their susceptibility to malaria (Menendez et al., 1995). This implies that iron deficiency might contribute to protection against malaria seen in HbAS condition. In our study, we observed an increased risk of being iron deficient in carriers of either alpha-thalassemia or sickle cell trait. Furthermore, there was an association between low plasma ferritin and sickle cell trait. We speculate that if our observations are corroborated by future studies, iron deficiency may be yet another mechanism through which hemoglobinopathies might protect against malaria.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In conclusion the studies presented in this thesis, although like a grain of sand in the desert compared to what is available in the literature, are in support of the notion that there is rather a complex web of interactions between nutritional status, immunity, host genetic factors and malaria infection. We found that malaria might be a cause of malnutrition in children under the age of 2 years. Paradoxical to the general belief that nutritional deficiencies lead to susceptibility to infection, we found that iron deficiency might be beneficial to such individuals, that is, iron deficiency is associated with protection against malaria. While similar observations have been documented previously, our finding raises further the concerns as to whether iron supplementation programs in malaria endemic areas should be implemented without further investigations to clear the existing controversy of the effect of iron supplementation on malaria outcome. Furthermore, such studies ought to take into consideration the host immune status and the presence of host genetic factors such as hemoglobinopathies, which might affect or be affected by nutritional status as shown in this thesis. Besides such host status (genetic or immune) may act in concert with the nutritional status to determine the eventual outcome of malaria infections. For example, based on our observations in this thesis, we postulated that iron deficiency might be one of the mechanisms through which alpha-thalassemia or sickle cell trait might protect against malaria infection.

Anemia, which is mostly due to iron deficiency in many parts of the world, is a problem in the same places where low birth weight is a concern. Data from the industrialized countries illustrate the role of iron supplementation and fortified foods in improving the iron status among women of reproductive age and children. The challenge, however, is to extend this knowledge to the parts of the world particularly to malaria endemic areas considering the possibility that iron supplementation might lead to exacerbated disease. Furthermore, this is complicated by the apparent lack of established: collaboration between the public and the private sectors, monitoring and evaluation system, quality control, regulation and legislation, appropriate targeting, and inadequate regional and local expertise! No need to emphasize the lack of

understanding of precise interactions between such micronutrient deficiencies and immunity to infections.

This far, there is still need for more population–based, longitudinal studies that carefully monitor nutritional status and cause-specific mortality to 1) better quantify the risk of death associated with malnutrition and 2) investigate the possibility that infections such as malaria might be the underlying cause of malnutrition and related morbidity and mortality. Moreover, the relation between the different components of malnutrition – wasting, underweight, stunting and micronutrient deficiencies and their interaction with host immune status and the genetic factors, needs to be studied so that their contribution to malaria and malaria-related child mortality and or morbidity is better understood. Such information could help in designing intervention programs that promote the survival and better health of children.

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