Nutritional zinc deficiency, immune capacity and malaria

A study on mediators of immunity to malaria caused by *Plasmodium falciparum* in African children

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This research was conducted under the auspices of the Graduate School of the Wageningen Institute of Animal Sciences

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Thesis
Submitted in partial fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M. J. Kropff,
in the presence of the
Thesis Committee appointed by the Doctorate Board
to be defended in public
on Monday 14 December 2009
at 11.00 AM in the Aula

Erasto V. Mbugi

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PhD Thesis, Wageningen University,
With references, with summaries in Dutch and English

ISBN: 978-90-8585-531-6

This Thesis is dedicated to my wife, Romana and children, Leonia-Faraja and Pelagrina

Contents

CHAPTER

1

General Introduction

Nutritional zinc and other micronutrients: effects on indicators of immune response to *Plasmodium falciparum* malaria in children of malaria-endemic countries

Introduction

Nutrition and immunity to infectious diseases are inseparable. One reason why infectious diseases such as malaria are more common in poor communities than in rich communities is that the former are more vulnerable to malnutrition. The diet of poor people in sub-Saharan Africa is based on cereal-based foods that have low contents of micronutrients (for example provitamin A) or that contain compounds that inhibit the intestinal uptake of trace metals such as zinc and iron. The aim of this first chapter is to review the effect of micronutrient deficiencies on the immune response to malaria, particularly with regards to their effect on cellular immunity. Although the focus of this review is on zinc, we will also consider other micronutrients because zinc deficiency in developing countries is commonly found in association with deficiencies in other micronutrients.

1. Zinc nutrition

1.1 Biological functions of zinc: Zinc is important for human well being because of its manifold biological functions in the body. It is a cofactor in many metalloenzymatic metabolic pathways and is required for the structure and activity of nearly 200-300 metalloenzymes, not only in humans but also in other organisms [1, 2]. It has an antioxidant and cell membrane stabilisation activity that keeps the cells intact and functioning normally. Zinc is also known to play a critical role in body's defence against infectious agents: it affects multiple aspects of the immune system, from the skin barrier to gene regulation in the lymphocytes [3]. As a result, zinc-deficient individuals are relatively vulnerable to a variety of pathogens. Zinc is fundamental in normal development and function of cells mediating both innate and acquired (specific) immunity. Zinc-deficient persons have disturbed functions of neutrophils and natural killer cells (non-specific immunity), and disturbed outgrowth and functions of cells of the adaptive immune response, affecting T lymphocyte numbers and activation, T_h1 cytokine production, and B lymphocytes and antibody production, particularly immunoglobulin G [4]. Zinc plays an important role in maintaining the proper balance between cell-mediated and humoral immunity by regulating patterns of cytokine secretion. These putative effects of zinc, together with its effects on the basic cellular functions such as DNA replication, RNA transcription, cell division, and cell activation makes its deficiency to be an issue of concern. Zinc is central in the formation of 'zinc fingers', i.e. loops within DNA-binding proteins that are required for recognizing and binding of these proteins to specific DNA regulatory sequences. Because many of these proteins act as transcription factors, zinc is required for DNA transcription. This role is particularly important in macrophages and other cells of the immune system that must divide rapidly in response to antigenic stimulation [4-6]. Zinc and iron are particularly required for the synthesis of deoxyri-

bonucleotide precursors by ribonucleotide reductase and for the various nucleotidyl transferases. Apoptosis and thymic atrophy are also potentiated by zinc deficiency predisposing to probable impaired T-cell development and decreased T-cell counts with consequent $CD4^+$ T-cell depletion.

Micronutrients other than zinc are also critical for normal immune functioning [7]. Both the innate [8] and adaptive immune responses (cytokines and antibodies) are highly affected by deficiencies of micronutrients especially vitamin A, magnesium, copper, iron and vitamin E [7]. Erickson et al [8] have stressed that these deficiencies particularly affect the functioning of macrophages, NK-cells and the polymorphonuclear cells, so that innate immune response is impaired. Because these innate response also determine the nature of the adaptive immune response, and particularly the balance between pro-inflammatory and regulatory immune responses, such deficiencies may be critical for the outcome of malarial infection. Thus an increased intake of such micronutrients may complement other interventions to control infectious diseases. **Table 1** shows the nutritional markers used in this thesis.

Table 1: Selected nutritional markers used in this thesis.

Status	**Serum/plasma marker** [1]	**Reference**
Zinc deficiency	Zinc concentration <9.9 mmol/L (children aged <10 years)	Hotz and Brown [9]
Low zinc status	Zinc concentration <10.7 μmol/L	Pilch and Senti [10]
Iron deficiency	Ferritin concentration <12 μg/L	WHO [11]
Iron deficiency anaemia	Iron deficiency accompanied by anaemia (for children <5 y: haemoglobin concentration <110 g/L)	WHO [11]
Low magnesium status	Magnesium concentration < 763 μmol/L, <777 μmol/L, <741 μmol/L, <740 μmol/L and <745 μmol/L for children aged 6-12 mo, 13-24 mo 25-36 mo, 37-48 mo and >48 mo, respectively	Adapted from Lowenstein and Stanton [12]
Vitamin A deficiency	Retinol concentration <0.70 μmol/L	Sommer and Davidson [13]

[1] In the absence of inflammation

1.2 Zinc absorption and metabolism: Dietary zinc is taken up from the intestinal lumen by mucosal cells, where it is either integrated into intracellular zinc pools or rapidly transported across the basolateral membrane to the plasma compartment. In the plasma, the newly absorbed zinc is transported in portal systemic transport by albumin as the major carrier protein in a form of albumin-zinc complex [14].

1.3 Causes and health effects of zinc deficiency: Plant foods contain phytic acid and phenolic compounds, including some tannins from legumes, that interfere with the absorption of nutritional zinc. These compounds are collectively known as antinutrients. In addition, dietary calcium can decrease the absorption of zinc and iron [15]. Plant foods grown in zinc-deficient soils are low in zinc content thus leading to dietary zinc deficiency. The varying grain zinc according to their source and not solely due to their genotype, indicates that the seed zinc content is controlled additively by both genotype and the environment of the mother plant, hence its availability from the food source when consumed by man. Balanced diets from variety of source that contain different nutrients at different levels will help to reduce risks of zinc deficiency.

Overt zinc deficiency occurs in acrodermatitis enteropathica, a rare, autosomal recessive disorder, usually occurring in the first year of life [2, 16, 17] that results in severe zinc deficiency as a consequence of impaired intestinal zinc absorption. This is associated with dermatitis, poor wound healing, retarded growth and sexual development, and reduced taste acuity, abdominal pain, diarrhea, skin rash, and loss of appetite [18]. Recent studies have also identified a genetic mutation in women with low milk zinc concentration that results in transient neonatal zinc deficiency [19]. More frequently, however, individuals with zinc deficiency have no signs or symptoms even if it is associated with an impaired immune function and an increased burden of infectious diseases. Thus zinc supplementation has been shown to prevent diarrhoea and pneumonia in young children, whilst zinc supplementation during diarrhoea reduces the severity and duration of such illnesses [20, 21]. Its effect on child mortality remains inconclusive, although subgroup analyses suggest that it reduces mortality in children aged ≥12 months, with evidence of efficaciousness in infants aged <12 months [20]. In children in Papua New Guinea and The Gambia, zinc supplementation also led to a reduced incidence of malaria [22, 23], although there was no evidence for such an effect in children in Burkina Faso [24]. Zinc supplementation can reduce infection with *Mycobacterium tuberculosis* [25], and has been shown to greatly reduce the incidence of skin and wound infections in diverse populations including infants [26] and pregnant women

[27] .

Studies on the effect of maternal zinc supplementation on infant birth weight and on infant and maternal health outcomes have ended with inconclusive results. In infants, however, an increased zinc intake may lead to improved mental and psychomotor development [28, 29]. Other consequences of zinc deficiency include impaired growth and loss of appetite.

More trials need to be carried out to assess possible benefits and adverse effects in HIV. In the few studies that have been conducted so far in HIV-infected individuals, zinc intake was associated with decreased burden of opportunistic infections [30], an improved weight gain and increased CD4 cell counts [31]; however, these studies must be interpreted cautiously [30, 32]. Yeudall et al. [33] have studied and shown that multiple micronutrients, when included in supplementation regimen, reduce incidences of common conditions like fever, diarrhoea, upper and lower respiratory infections. This suggests that precisely selected multiple nutrients rather than single micronutrient may be the preferred approach in African settings.

1.4 Risk groups for zinc deficiency: In developing countries, the groups at highest risk for zinc deficiency are young children, and pregnant and lactating women. These groups also suffer the greatest burden of infectious diseases such as malaria. Preschool children are particularly vulnerable because of the large amounts of zinc required for rapid growth during this age. Transient neonatal zinc deficiency in breast-fed infants, which cannot be corrected through maternal zinc supplementation, has been reported as a result of low zinc concentrations in breastmilk [34-38]. However, breast milk normally has adequate zinc content to meet the requirement for infants up to 4–6 months of age [39]. Thus zinc deficiency usually starts at the age when children start receiving complementary foods, which are usually based on grains that contain antinutrients (see above).

1.5 Assessment of zinc status: Although several methods are used to determine zinc status of individuals and populations, none is entirely satisfactory. A combination of zinc deficiency characteristics (e.g. growth retardation, delayed sexual and skeletal maturity, skin lesions such as orificial and acrodermatitis, diarrhoea, alopecia, and behavioural changes) and severe hypozincemia makes the detection of severe human zinc deficiency relatively easy [40]. In severely zinc-deficient individuals, concentrations of zinc in the plasma are usually < 0.4 mg/L and often < 0.2 mg/L

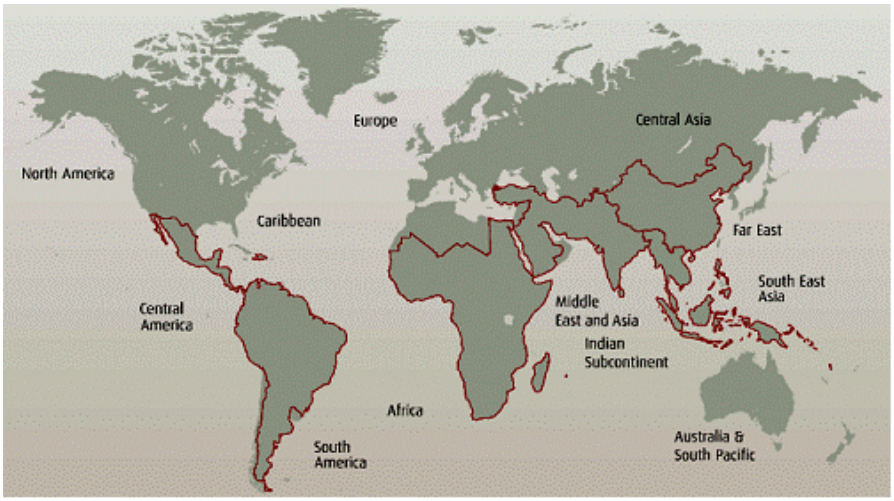

Figure 1: *Distribution of malaria worldwide; highlighted areas indicates regions worst affected by the disease. Source: WHO*

In individuals with moderate zinc-deficiency, however, there are usually no manifestations; even if features exist, they are usually non-specific. Plasma zinc concentration is the most frequently used and best available method to determine zinc status, but it can be affected by a variety of other factors. Cut-off values defining deficiency are dependent on age, sex, pregnancy status and whether plasma samples were collected in a fasted state, either in the morning or in the afternoon/evening [9]. Infection-induced inflammation can reduce plasma zinc concentration; however, mean plasma zinc concentration may be a useful indicator of population zinc status for children in low-income nations despite the high prevalence of common childhood infections [41].

The zinc status of populations can also be assessed by determining the dietary intake of zinc and phytate. This can serve to estimate the dietary intake of zinc that is available for absorption, which can be compared with required amounts to assess the adequacy of intakes [9].

Zinc deficiency at population level can also be shown by determining the response to zinc interventions of growth or functional outcomes in randomised controlled trials [42]. Individuals with adequate zinc status down-regulate their absorption of zinc from food and may not respond to zinc-enriched staple foods to the extent shown by zinc-deficient individuals [43]. Serum extracellular superoxide dismutase can possibly be used as a biochemical indi-

cator of marginal zinc deficiency in humans [44] although further testing and validation is still needed to determine the reliability of this indicator. Zinc clearance tests have also been used as diagnostic tests for marginal zinc deficiency in children of small stature [45].

1.6 Public health interventions to increase zinc intake: Despite the evidence of the health benefits of zinc supplementation, it has been difficult to translate these findings into health policies for African children. When planning the studies described in this thesis, the trials that had been conducted had been done almost exclusively in Asia, Latin America, the Pacific, and developed countries. By contrast, the response to supplementation may be different in Africa, where children's health is challenged by different environmental factors. Malaria is the prime example, with more than 90% of global deaths occurring in African children, and up to 90% of African toddlers in many areas being infected but symptom-free. Few studies have been conducted on the protective effect of zinc against malaria, and their results are still contradictory and conflicting (see above).

A group of leading economists recently recommended zinc supplementation as one of the most cost-effective interventions for reducing malnutrition and improving human welfare [46]. Nonetheless, adequate delivery of zinc supplements to populations in need is probably difficult to achieve. Preventive supplementation requires frequent contact with target groups that can possibly be achieved through integration with twice-yearly vitamin A supplementation programmes, immunization and growth monitoring programmes, or through social marketing. Single-nutrient interventions are costly and in the long term possibly unsustainable [33]; this may be overcome at least in part by supplementation strategies that simultaneously address multiple micronutrient deficiencies. Another difficulties is the lack of suitable supplementation vehicles for infants and toddlers, who have problems of swallowing tablets or capsules. There are several efforts currently being undertaken to develop and test low-cost supplementation vehicles in the form of chewable tablet-cookies and a nutrient-dense spread suitable for use in tropical environments [47-50].

Zinc supplements are now also recommended by the World Health Organization as part of a simple treatment for diarrhoea, in conjunction with oral dehydration salts. This use of zinc is safe and efficacious and may decrease the unnecessary use of antibiotics for non-dysentery diarrhoea [18, 51] and pneumonia. Short-term supplementation has the added benefit of improving health and overall immune status some weeks following treatment, and this strategy may also boost the body's immunological defence against other infections such as ma-

laria and may be an effective way of decreasing childhood morbidity and mortality [18, 52]. Acceptance and affordability of the planned programs to the target populations is important; therefore the intervention practices should not differ much from the socio-cultural ways the target population is accustomed in terms of food practices.

Several other innovative and complementary approaches are currently being developed. One is to breed and disseminate new staple crop varieties with high concentrations of micronutrients. This can be achieved through selection and crossing of promising lines as part of a classical breeding programme; however, plants may also be genetically engineered to increase the concentration of bioavailable zinc [53]. The approach has the potential to create an entirely new, safe, low-cost and self-sustaining approach to deliver zinc to poor farmer families [54]. Although the development and dissemination of such 'biofortified' crops requires large initial research investments and sustained support by national and international policy makers, these interventions have low long-term recurrent costs. Because of technical constraints, and contrary to other methods of delivery such as supplementation or fortification, the biofortification approach must focus on single nutrients or at most a few nutrients. This imposes a need to identify micronutrients that are critical to health and probably consider area or regional requirements. Efforts to develop typically 'African' crops rich in zinc have so far been limited, and require increased support that is probably best obtained when showing compelling evidence of the health benefits of increasing zinc intake.

Another option for increasing daily zinc intake is the mass fortification of centrally processed staple foods [55]. Zinc fortification has the potential to increase zinc intake and total zinc absorption, but there are no data to demonstrate the efficacy and effectiveness of this approach in improving the zinc status of young children [56]. Both the selection of appropriate food vehicles and cost considerations should be taken into account in the formulation of fortification programmes [57].

Lastly, zinc nutrition can be improved by changing food preparation and processing methods to increase zinc bioavailability [52]. For example, soaking and fermentation can reduce the content of [31, 58] and can increase the bioavailability of zinc

2. Malaria

2.1 Burden of disease: Malaria has been and still causes much morbidity and mortality in endemic areas. The disease occurs in hundred of countries and represents between 300 and 500 million clinical cases per year. More than 40% of the world's population is at risk of malaria infection with 90% of cases and more than 95% of global deaths occurring in tropical countries, particularly sub-Saharan Africa [59]. Up to 90% of African toddlers in many areas are malaria-infected but symptom-free. In malaria-endemic regions of sub-Saharan Africa, the groups at greatest risk of malaria-associated mortality are children younger than five-years of age (one child dying of malaria in every 30 seconds) and pregnant women. However, in African children it has been found that the incidence of severe malaria declines markedly after the age of four years [60]. Malaria epidemics have devastated large populations and, together with HIV/AIDS and tuberculosis, is one of major public health challenges and a serious barrier to economic progress in many developing countries.

2.3 Pathogenesis and manifestations: Illness in malaria is caused by the erythrocytic stage of the parasite. There are no symptoms associated with sporozoites, the developing liver stage of the parasite, the release of merozoites from the liver, or gametocytes. The first symptoms and signs of malaria are associated with the rupture of erythrocytes when erythrocytic stage schizonts mature. This release of parasite materials like, glycosylphosphatidylinositol (GPI) moieties [63], malaria pigment [64] and plasmodium-derived nitric oxide synthase (NOS)-inducing soluble factors [65] apparently trigger a host immune response. The simultaneous rupture of the infected erythrocytes and the concomitant release of antigens and waste products accounts for the intermittent fever paroxysms associated with malaria. These by-products stimulate human mononuclear cells to release pro-inflammatory cytokines like tumour necrosis factor-alpha (TNF-α) and other pyrogenic cytokines [66-70]. Pro-inflammatory cytokines such as TNF-α suppress haematopoiesis, thus contributing to anaemia. The spleen and liver enlarge over time especially in febrile children with malaria [60].

As stated earlier, cytokines, reactive oxygen intermediates, and other cellular products released during the immune response play a prominent role in pathogenesis, and are considered responsible for the fever, chills, sweats, weakness, and other systemic symptoms associated with malaria. *P. falciparum* has additional unique characteristics that help to explain its distinct potential to cause severe or fatal disease. The parasite is very fast and prolific in replication and in higher numbers of parasites than other *Plasmodium* species. As *P. falcipa-*

Box 1. Human malaria parasites

Four species of protozoan parasites within the genus *Plasmodium* are known to cause malaria. These include *P. falciparum*, *P. vivax, P. ovale* and *P. malariae*. In Africa, the vast majority, widespread and most deadly malaria cases are due to *P. falciparum.*

The malaria parasite, like other members of apicomplexa has a complex life cycle, pigeonholed by three distinct processes: sporogony, merogony and gametogony. The life cycle of *P. falciparum* involves an insect vector (mosquito) and a vertebrate host (human). The stage infective for humans is the uni-nucleate, lancet-shaped sporozoite (approximately 1×7 μm). Sporozoites are produced by sexual reproduction in the midgut of vector anopheline mosquitoes and migrate to the salivary gland. When an infected *Anopheles* mosquito bites a human, she may inject sporozoites along with saliva into small blood vessels (Figure 2). Sporozoites are thought to enter liver parenchymal cells within 30–60 minutes of inoculation. In the liver cell, the parasite develops into a spherical, multinucleate liver-stage schizont which contains 2,000 to 40,000 uninucleate merozoites. This process of enormous amplification is called exo-erythrocytic schizogony. This exo-erythrocytic or liver phase of the disease usually takes between 5 and 21 days, depending on the species of *Plasmodium*. However, in *P. vivax* and *P. ovale* infections, maturation of liver-stage schizonts may be delayed for as long as 1 to 2 years. These quiescent liver-phase parasites are called hypnozoites. Because *P. vivax* and *P. ovale* are relatively rare in Africa, these species will not be considered in the remainder of this review.

Mature schizonts eventually rupture, releasing thousands of merozoites into the bloodstream. Each merozoite can infect a red blood cell by entering through receptor-mediated endocytosis [61]. Inside erythrocytes a series of asexual events (3–5 cycles) takes place involving erythrocytic schizogony with differentiation of late schizonts to individual merozoites. The merozoite develops to form either an erythrocytic stage (blood-stage) schizont (erythrocytic schizogony) or a spherical or banana-shaped, uninucleate gametocyte.

The mature erythrocytic stage schizont contains 8 to 36 merozoites, each 5 to 10 μm long, which are released into the blood when the schizont ruptures. These merozoites proceed to infect another generation of erythrocytes.

The time required for erythrocytic schizogony which determines the interval between the release of successive generations of merozoites varies with the species of *plasmodium* and is responsible for the classic periodicity of fever in malaria. The gametocyte, which is the sexual stage of the *Plasmodium*, is infectious for mosquitoes that ingest it while feeding on blood meal. Within the mosquito, the parasites develop over 2 to 3 weeks to mature ookinetes that traverse the gut epithelium to the salivary glands where they form an oocyst which then ruptures to release sporozoites that can infect humans. Only a fraction of the released sporozoites end up in the salivary glands [62], the rest can be introduced into uninfected individual during mosquito bite to initiate a new cycle.

rum parasites mature within red blood cells, they induce the formation of sticky knob-like protrusions on the surface of infected erythrocytes [71]. These knobs, bind to receptors on endothelial cells in capillaries and venules in multiple vital organs in the body [72] leading to cytoadherence and sequester formation. It is important to note that erythrocytes parasitized by *P. vivax* do not readily bind to endothelium. Thus, despite very high plasma concentrations of TNF-a that may occur in vivax malaria, this infection does not lead to cerebral disease [73].

The cytoadherence and sequestration of red cells within small vessels leads to microvascular pathology and obstruction to blood flow. Infected red cells also stick to uninfected red cells and form rosettes that clog the microcirculation [74]. Ultimately, secondary organ dysfunction and severe complications in the host can occur. In the brain this causes cerebral malaria; in the kidneys it may cause acute tubular necrosis and renal failure; and in the intestines it can cause ischemia and ulceration, leading to gastrointestinal bleeding and to bacteraemia secondary to the entry of intestinal bacteria into the systemic circulation.

The severity of malaria associated anaemia tends to be related to the degree of parasitaemia. The pathogenesis of this anaemia appears to be multifactorial. Haemolysis or phagocytosis of parasitized erythrocytes and ineffective erythropoiesis are the most important factors, and phagocytosis of uninfected erythrocytes and an autoimmune haemolytic anaemia have also been implicated [72]. Consequently, children die from malaria essentially because of cerebral malaria, respiratory distress and anaemia/acidosis as prevailing syndromes [60]. Riley [73] has pointed out that cerebral malaria is predominantly an immune-mediated disease, and that immunological priming occurs during first infection, leading to immunopathology on re-infection. Massive intravascular haemolysis leading to haemoglobinuria and renal failure, frequently described in the past as blackwater fever, is also not uncommon. However, care should be taken not to confuse with haemolysis that may also occur after the use of certain antimalarial drugs (especially primaquine) in patients with glucose-6-phosphate dehydrogenase deficiency [75].

2.4 Diagnosis and treatment: In African conditions, diagnosis of malaria in both children and adults is usually based on signs and symptoms common for the disease. Confirmatory tests for definitive diagnosis rely on observation of presence of parasites by microscopy or parasite antigens by rapid diagnostic tests (RDTs) but also by genotyping of DNA by polymerase chain reaction (PCR) [76-78]. However, in most African communities where malaria is endemic, physician evaluation, microscopy and currently RDTs are more available and

reliable tools. PCR is especially effective at detecting submicroscopic levels of parasitaemia [79, 80] but its application is usually restricted to large scale surveys rather than being used in clinical practice [81] because of the high costs of reagents, trained personnel and the laboratory facilities required.

Chemoprophylaxis is highly effective in reducing mortality and morbidity from malaria in young children and pregnant women living in endemic areas [82] although it is difficult to sustain, may lead to rebounds when stopped, and, in some studies, has impaired the development of naturally acquired immunity. For decades, chloroquine and other quinoline derivatives have been the drugs of choice for treatment of falciparum malaria in endemic countries. Because of the spread and intensification of drug resistance, the mainland of Tanzania was forced to change its policy in 2001 from chloroquine to sulphadoxine-pyrimethamine (SP) as the first-line drug for treatment of uncomplicated malaria in children, and subsequently, in 2006, to artemether/lumefantrine (AL). Artemisinin-based combination therapies (ACTs) such as AL are now recommended by the World Health Organization (WHO) in all countries experiencing resistance to conventional monotherapies (chloroquine, amodiaquine or sulfadoxine–pyrimethamine) [83]. The recent finding that intrarectal artesunate administration is efficacious and safe as an initial treatment for uncomplicated malaria in children [84] is helpful in individuals who cannot swallow drugs.

2.5 Public health interventions to control malaria: Annual economic growth in countries with high malaria transmission has historically been lower than in countries without malaria. The disease may account for as much as 40% of public health expenditure, 30% to 50% of inpatient admissions, and up to 50% of outpatient visits [59] signifying that public health interventions against the disease are highly needed. Intervention programs currently implemented target the vector mosquito (insecticides), the parasite (vaccines) and cure of patient (antimalarials) to ensure effective recovery from the disease. The Malaria Vaccine Technology Roadmap [85] anticipates the development and licensing of a first-generation malaria vaccine that has a protective efficacy of more than 50% against severe disease and death by 2015; as foreseen, a malaria vaccine with protective efficacy of more than 80% against clinical disease and lasting longer than 4 years will have been developed and licensed by 2025. Whilst this strategy may serve to contribute to malaria control within 10-20 years, insecticide-treated nets, intermittent preventive treatment in pregnancy and antimalarial drug combination therapy remain the key interventions for prevention and treatment of malaria. Intermittent preventive treatment, in which full therapeutic doses of a drug are given at defined

intervals [82], has the potential to provide some of the benefits of sustained chemoprophylaxis in pregnant women (IPTp) and young children (IPTi) and is a promising new approach to malaria control. Schellenberg et al. [86] have demonstrated a reduction of episodes of clinical malaria by 60% and episodes of severe anaemia by 50% in Tanzanian infants treated with SP at 2, 3, and 9 months of age, at the time of routine immunization. However, because SP resistance has already become widespread in Tanzania, it is necessary to consider the newly WHO recommended ACTs for effective malaria control in both infants and pregnant women. Supplementation of micronutrients, if shown to protect against disease, will best work if integrated with other interventions.

3. Immune mechanisms in malaria

3.1 Overview of the role of immunity to malaria: The evolution of the immune system consists of factors that provide innate and acquired immunity, and has evolved to become more specific, complex, efficient, and regulated. One of the principal functions of the human immune system is to defend against infecting and other foreign agents by distinguishing self from non-self (foreign antigens) and to organize other protective responses from leukocytes. A dysregulated immune system can react to self antigens resulting into autoimmune diseases or failure to defend against infections. Much of the pathology associated with parasitic infections such as malaria, is immune-mediated. The immune mediators (e.g. cytokines) of protection can also cause disease, and the outcome of infection hinges on a delicate balance between appropriate and inappropriate induction of these mediators [70, 87].

Helper $CD4^+$ T-lymphocytes (T_h-cells) play a critical role in the immune response to many human diseases, including malaria, working in close interaction with cells of innate immunity. Such cells include macrophages and dendritic cells. The outer membranes of dendritic cells (DC) contain innate receptors, the Toll-like receptors (TLRs), which sense microbes and microbial products and trigger dendritic cell maturation and cytokine production, thus effectively bridging the innate and adaptive immunity against the pathogen [88]. On the DCs, are also CD36 host receptors that recognize *P. falciparum*-infected erythrocytes binding and modulate their function. The CD36 mediates non-opsonic phagocytosis of infected erythrocytes [87, 89, 90]. Binding of pathogen associated molecular patterns (PAMPs) to relevant pathogen recognition receptors (PRR, like the TLRs or CD36) will result in activation of the NF-kB transcription factor complex and the subsequent production of pro-inflammatory cytokines such as interleukin (IL)-12. Besides activation of the innate arm of the immune system this release of IL-12 will also polarise the antigen-specifically-activated

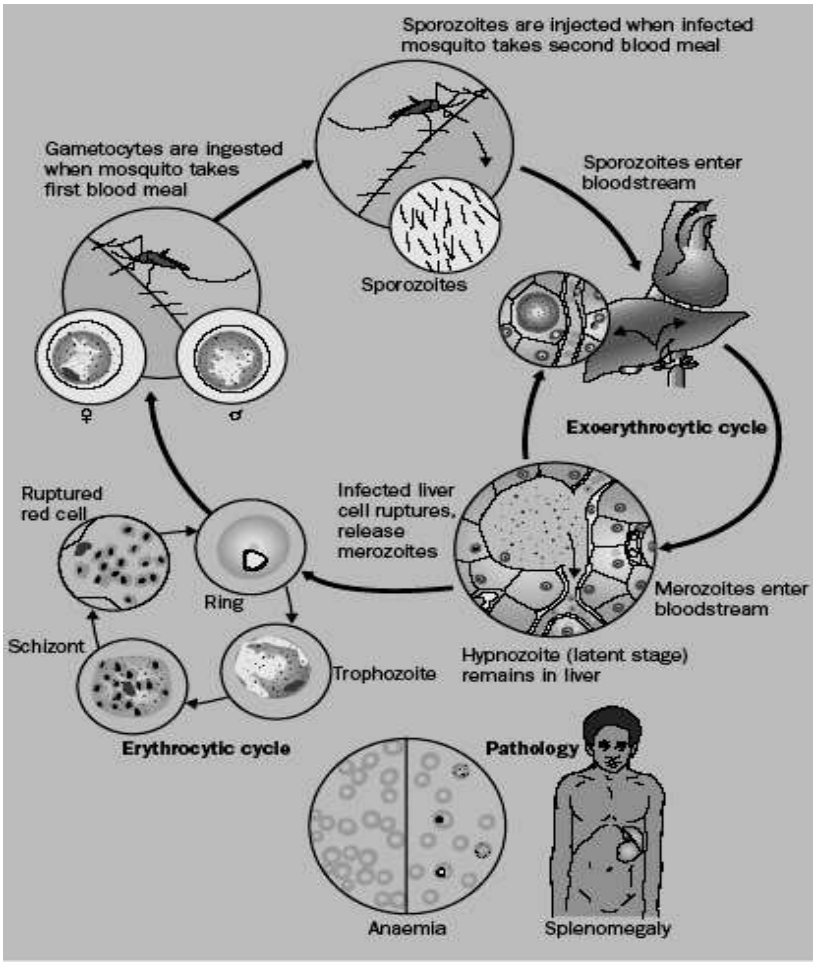

Figure 2. *Plasmodium falciparum life cycle in the vertebrate host (man) and invertebrate host (the mosquito). Source: Malaguarnera and Musumeci [61]*

T_h-cell repertoire into preferential T_h1 development. Such overactive and polarised T_h1 repertoire evokes IL-12 and IFN-γ-dependent protection against the blood-stage infection with the malaria parasite, *Plasmodium falciparum*. Regulatory T-cells, on the other hand, mediate active suppression of various immune responses [91]. These T-cells comprise classical T_h2 cells capable of inhibiting T_h1 responses, but also alternative T-cell populations. One of the primary mechanisms of tolerance induction is via secretion of immunosuppressive cytokines

such as IL-10, IL-4 and transforming growth factor-beta (TGF-β). Regulatory T-cells have been isolated from *in vitro* cultures, which appeared to produce low levels of IL-2, no IL-4, but high levels of IL-10 and TGF-β, demonstrating the importance of cytokines in regulating and dampening the immune response. Although cytokine functions are complex involving protective and pathological consequences, the analysis of a set of these soluble proteins will allow direct and complete measurement of regulatory circuits of the human immune system [92, 93].

3.2 Innate immunity to malaria: Immunity plays a critical role in the pathogenesis of malaria, as shown by two key observations [94]. First, in highly endemic, rural areas, it is commonly found that up to 90% of children are infected yet symptom-free. Secondly, hospital-based studies in such areas show that severe, acutely life-threatening malaria and malaria-associated deaths occur mostly at a very young age, when maternal antibodies obtained during pregnancy have waned out, and chronic or repeated exposure to infections has not yet resulted in protective levels of acquired immunity. Thus in highly endemic areas, severe malaria is an increasingly rare phenomenon after the age of 5 – 10 years, whilst the prevalence of parasitaemia steadily declines after the age of 2 years. These observations and others indicate that immunity against severe disease and subsequent death develops much faster than against pyrogens and parasites themselves.

With the plasmodium parasite developing within the host hepatocyte being the major target of protective immunity at the exo-erythrocytic stage [95], the body's immunologic defence mechanism develops against several liver-stage specific antigens, which along with those brought in with the invading sporozoite, are rapidly processed by the host cell and presented on the surface of infected hepatocytes in combination with MHC class I [96, 97]. Macrophages, including Kupffer cells, are antigen-presenting cells [98] and antigen presentation leads to recognition by cytotoxic T lymphocytes (CTLs) and killing of the infected cell or stimulation of NK and $CD4^+$ T cells to produce IFN-γ, which can trigger a cascade of immune reactions, eventually leading to death of intracellular parasite [96, 97]. Alternatively, the CTLs may directly be cytolytic to malaria-infected hepatocytes by releasing perforin and granzyme or by binding to apoptosis-inducing receptors on the infected cells [61, 95]. Schwenk et al [99] proposed the exo-erythrocytic opsonization of *P. falciparum* liver-stage circumsporozoite proteins (spzs) by antigen-specific antibodies in both naturally infected individuals and in persons immunized with radiation-attenuated spzs to be an important step and a possible mechanism for macrophage phagocytic activity and contribution in conferring a protective immunity against the infection. Evidence from other similar, previously

reported studies [100, 101] have revealed opsonization of spzs for uptake and destruction by monocyte/macrophages as a crucial process by which antibodies (Abs) contribute to protective immunity. All taken together, dendritic cells, NK-cells, NKT cells, macrophages and possibly γδT cells are proposed to play a great role as effectors of innate immune responses in human malaria infection. Evidence from *in vitro* studies have shown strong expansion of γδT cells during early phases of malaria infection supporting the idea that they contribute greatly in innate parasite control [102]. They can also modulate adaptive immunity through their ability to produce regulatory cytokines [87].

Although the principal defence mechanism against protozoan parasites that survive within macrophages is cell-mediated immunity, particularly macrophage activation by T_h1 cell-derived cytokines [103], immunity to malaria is said to be both cellular and humoral [104], stage-specific [105] and includes both innate and adaptive immune responses. Following inoculation, the sporozoite stage in the liver comprises the first encounter with the host immune system. The protective immunity relies on the release of pro-inflammatory cytokines such as IL-18, IL-12, IFN-γ, TNF-α, nitric oxide and reactive oxygen species by macrophages and NK cells [61, 106, 107]. Moreover, under the influence of the polarising capacity of several of these cytokines, increased activity by $CD8^+$ T-cells (CTL) will also result in an antigen-specific T_h1 and cytolytic activity [108]. These early responses will be subsequently dampened by a later anti-inflammatory response based on the release of IL-4, IL-10, and TGF-β. Omer et al [70] for example, have reported low TGF-β concentration to have a pro-inflammatory role with an ability to recruit monocytes, T-cells and neutrophils towards the inflammation site early in an infection, through modulation of endothelial cell adhesion molecule expression in murine malaria. However, at high concentration, this multifunctional TGF-β has shown an anti-inflammatory role, suppressing production of TNF-α and NO from macrophages [109], inhibiting the production of IFN-γ and TNF-α from NK cells [110] as well as antagonising an IFN-γ-stimulated upregulation of major histocompatibility complex (MHC) class II antigens [111]. These observations and results suggest that inhibition of rapid pro-inflammatory response and T_h1 outgrowth such as may occur in zinc deficiency; potentially weakens the protective immune response towards human falciparum malaria. The fate and pathophysiological consequences of malaria, as stated earlier in this chapter, is pivoted largely on the balance in the pro- and anti-inflammatory immune responses and the regulation of innate immunity might be important component of adaptive responses.

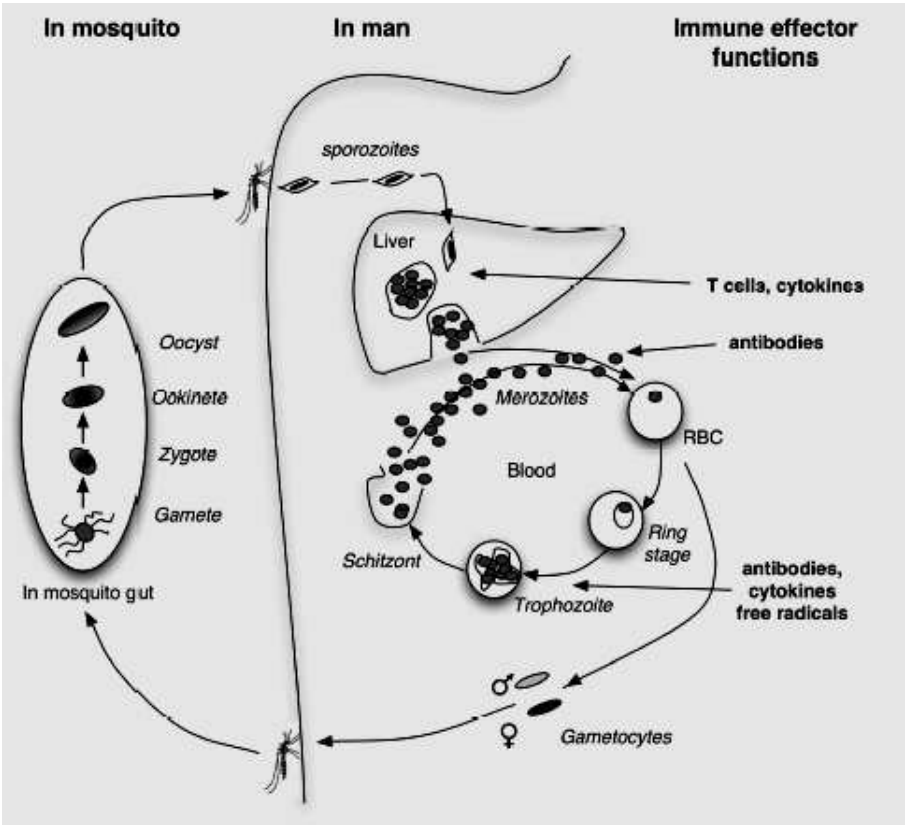

Figure 3: *Possible targets of immune mechanisms at various stages of life cycle of human malaria parasite. The immune system react by activation, differentiation of T-cells leading to cytokine production and mainly cell-mediated response against liver-stage parasites. Antibodies, cytokines and free oxygen radicals are directed towards blood-stage parasites. Some of the merozoites develop (after escaping the immune attack), by a still unknown mechanism into male and female gametocytes which mature in the mosquito gut after being ingested by a blood sucking mosquito. In the gut, they fuse and develop sporozoites that may be injected into the blood stream of the new host through bite by the infected mosquito.*

Source: *Hoffman and Miller [112]*

3.3 Acquired immunity to malaria: Both innate and acquired immunity to malaria and other infections work synergistically being bridged with soluble mediator proteins, the cytokines. Specific antibodies are also thought to be involved in protective anti-malarial immunity despite the fact that anti-malarial antibodies-induced immunity is not sterile [113]. The kind of immune response that develop against malaria is targeted towards reduction of clinical disease, lowering parasitaemia levels and is short lived unless there is repeated exposures like that occurs in areas with intense transmission. In malaria endemic areas, repeated exposure to *P. falciparum* infection induces strong humoral immune responses characterised predominantly by immunoglobulins (Ig) M and G despite other isotypes. Large proportion of these immunoglobulins are non-malaria-specific reflecting polyclonal B-cell stimulation but a small proportion (5% or more) is what is important in malaria by keeping low parasitaemia and reducing clinical disease and is species and stage-specific reacting with a wide variety of parasite antigens [114]. Various transfer experiments conducted to date have indicated the significance of humoral immunity in providing a protection against malaria. *In vitro* studies have further shown that specific antibodies can inhibit both sporozoites motility [115] and sporozoites invasion of hepatocytes [116]. Additional evidence has shown that immunity to falciparum malaria is associated with protective antibodies of different classes and subclasses [117]. Depending on the dynamics of disease transmission and on the immune status of the individual, IgG1 and IgG3 antibodies have shown to be protective, while IgG2 and IgG4 subclasses generally compete and interfere with protection [113, 118, 119]. In other words, IgG2 and IgG4 are not considered protective. In general, IgG3 is the prevailing isotype associated with protection in humans. However, this isotype has only a short half-life in circulation (7 days, as compared to 24 days for IgG1). Hence, it would be beneficial to individuals to induce specific IgG1 antibodies, which potentially provide long-term efficient protection against the pathogen. In this context, cytokines such as IL-10, IFN-γ, IL-2 and TGF-β may be of profound importance, by virtue of their potential to induce isotype switching in B-cells to the induction of antigen-specific IgG1. Importantly, the precise production of these cytokines is thought to be affected by zinc deficiency, leading to decreased levels of innate immunity, but also decreased activity of NK cells and a decreased ability to mount a protective T_h1-type immune response.

3.4 T-cell responses to falciparum infection: Antibodies contribute to protective immunity to human malaria but priming of T-cells remains to be important for the development and maintenance of this important component of immunity [120-122], and T-cell-derived gamma interferon (IFN-γ) is said to be important in mediating the cellular effector mecha-

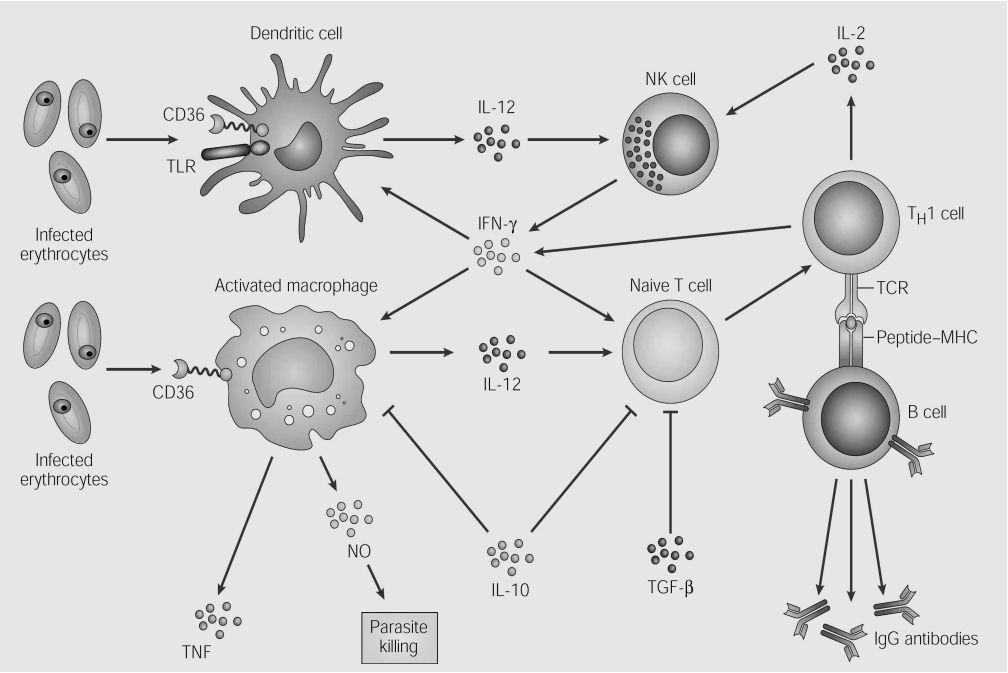

***Figure 4**: Bridging the innate and acquired immune systems. Potential regulatory mechanisms of adaptive immunity to blood-stage malaria by cytokines produced by cells of the innate immune response [87]. In response to parasite ligands recognized by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and CD36, or inflammatory cytokines, such as interferon-γ (IFN-γ), dendritic cells (DCs) mature and migrate to the spleen which is the primary site of immune responses against blood-stage Plasmodium parasites. Maturation of DCs is associated with the upregulation of expression of MHC class II molecules, CD40, CD80, CD86 and adhesion molecules and the production of cytokines including interleukin-12 (IL-12). IL-12 activates natural killer (NK) cells to produce IFN-γ and induces the differentiation of T_h1 cells. The production of cytokines, particularly IFN-γ, by NK cells results in DC maturation and enhances the effect of parasite-derived maturation stimuli, facilitating the clonal expansion of antigen-specific naive CD4+ T-cells. IL-2 produced by antigen-specific T_h1 cells further activates NK cells to produce IFN-γ, which induces DC maturation and activates macrophages, further amplifying the adaptive immune response. Cytokines such as IL-10 and TGF-β negatively regulate both innate and adaptive responses.*

***Source:** Stevenson & Riley [87]*

nisms [123]. Macrophages and dentritic cells are important in the initiation of innate immune response (**figure 4**) with capability for integrating signals from pathogens, cytokines and T-cells, leading to the generation of an adaptive immune response of the appropriate class [124]. In this way the innate immune response is translated into adaptive immune response to confer effective immune protection. Napolitani et al [125] suggest the TLRs expressed constitutively or after being induced in different cell types, to determine the innate cells capacity to detect microbial products [126-128] and discrimination to either type I or type II immune responses. This differentiation of macrophages to produce either type I or type II cytokine responses depends on activation states. The macrophages can be classically activated (caMac) to produce type I cytokines like IL-1β, TNF-α and in acute infections, IL-12 [129] that are also produced by T_h1 cells. On the other hand macrophages can be alternatively activated (aaMac) inducing differentiation of T-helper cells into T_h2 cells giving rise to production of type II cytokines like IL-4 and IL-10. While caMac expression of nitric oxide synthase (NOS) induce production of nitric oxide (NO) and polarization towards T_h1 responses, aaMac promote expression of arginase and polarization towards T_h2 responses [129, 130]. The outcome of protozoan infections is largely dependent on the timing and relative strengths between the two types of responses (type I and II) which are dictated by activation pathway that the macrophages follow [131]. In most parasitic infections including helminths [129, 132-134] the response through aaMac is dominant, polarizing immunity towards T_h2 responses. Studies by Rodriguez-Sosa et al [133] have shown chronic helminthes infections to induce aaMac expressing high levels of chemokine receptor 5 (CCR5) with low IL-12 production despite a bias towards T_h2 responses in mice. In humans, aaMac have been shown to actively inhibit proliferation of peripheral blood mononuclear cells (PBMCs) and down-regulating $CD4^+$ T-cell-mediated responses [135].

The domination of early responses to *Plasmodium falciparum* infection by pro-inflammatory cytokines like IL-1β, TNF-α, IFN-γ and IL-12 which sometimes results into the pathological consequences, is suggestive of a classical activation of macrophages. This type I cytokine dependent pro-inflammatory response induce classically activated macrophages during protozoan infection [131]. Investigation of the immediate cytokine response of non-immune human PBMCs following stimulation with *P. falciparum*-infected erythrocytes after rigorous exclusion of *Mycoplasma* contamination has been done *in vitro* [136]. The results suggested that, the early inflammatory response to malaria is critically dependent on lymphocyte subpopulations that are specific to *P. falciparum* antigens and that the response greatly differs from that which is directed towards bacterial endotoxins. It has been observed also that although viability of cells is slightly reduced [137] both antigen-specific

$CD4^+$ and $CD8^+$ T-cell responses are not affected in cryopreserved human PBMCs [138], signifying that T-cells are important targets for *ex vivo* measurements that reflect the magnitude and quality of *in vivo* T-cell responses. Studies on viability and functional activity of cryopreserved mononuclear cells [139] have however, indicated an association between viability and T-cell responses in HIV-based experiments. These prospective observations have not yet been well established in cryopreserved human PBMCs from exposed and non-exposed individuals; stimulated with live *P. falciparum* antigens *ex vivo*.

3.5.0 Regulation of cytokine responses in malaria: Regulation of immune responses is crucial in plasmodium infection. During malaria, pro-inflammatory cytokines are released in early response to infection and later the adaptive (anti-inflammatory) responses takes over. Cytokines play both protective and pathological roles and the outcome of the disease depends on the balance between protection and pathology. Cytokines production is elicited by schizonts rupture that result into release of merozoites and various parasite antigenic substances and products like malarial pigments (hemozoins), glycosylphosphatidyl inositol anchors (GPI) of parasite proteins and other soluble antigens. The release of both pro-inflammatory and anti-inflammatory cytokines require regulation for optimal responses and resolution of infection. Regulatory T-cells modulates parasite clearance and immune-mediated pathology during malaria infection [140, 141].

3.5.1 Regulatory T-cells; background: Regulatory T-cells (Tr cells) are subpopulation of T-cells that was known once as suppressor T-cells because they down-regulate immune responses for both foreign and self antigens. These cells have immunoregulatory properties and they actively participate in maintaining and control of various immune responses [142, 143]. Generation and differentiation of regulatory T-cells is reported to be dependent on transforming growth factor-beta (TGF-β) and that IL-6 may completely inhibit this TGF-β-induced generation of regulatory T-cells [144]. Report by Seki et al [145] points out that galactose-specific soluble lectin-9 (Gal-9) induces differentiation of naïve T-cells (Th0) to regulatory T-cells and suppresses differentiation to T_h17 cells (potentially pro-inflammatory cells, involved in autoimmune diseases such as rheumatoid arthritis) *in vitro*.

Regulatory T-cells are characterized by expression of CD4 and CD25 markers on their surfaces ($CD4^+CD25^+$) which are IL-2 receptors and are referred to as natural regulatory T-cell (nTregs) [146]. These cells are produced by the normal thymus as a functionally distinct subpopulation of T-cells and their development critically depends on a forkhead family transcription factor, Foxp3 [147] whose expression is regulated by IL-2 [148]. These cells do

not themselves produce IL-2 and act in a contact-dependent manner, antigen non-specific, prevent cell activation of other T-cells, and are dependent on exogenous IL-2 for proliferation *in vitro* and *in vivo* [149]. With broad repertoire of antigen specificities, they can recognize both self and nonself antigens and their generation is controlled at least in part, developmentally and genetically. Other subtypes of regulatory T-cells with diverse phenotypes, antigen specificity, and modes of action have been described [150, 151]. Some other antigen-induced regulatory T-cells population have been identified and referred to as adaptive regulatory (or acquired regulatory) T-cells as they are induced in the periphery after encounter with pathogens and foreign antigens [152, 153]. Regulatory T-cells type I (T_r1 cells) are regulatory T-cells that produce the immunosuppressive cytokines IL-10 and originate from naïve T-cells primed by IL-10 or from T-cells induced by tolerogenic dentritic cells expressing IL-10. T_h3 cells are also regulatory T-cells subpopulations that produce TGF-β. Regulatory T-cells play a golden suppressive role in maintaining the balance between T_h1 and T_h2 to result into disease resolution while preventing pathology. The immunosuppressive cytokines TGF-β and IL-10 therefore play important role in the balancing of immune response to a non-pathological one.

In malaria the balance between T_h1 and T_h2 has been critical and it is the lack of a balanced immune response that results into aggressive pathological outcomes. Musumeci et al [69] studies on modulation of immune response in *Plasmodium falciparum* malaria have revealed the levels of IL-12 to reliably predict the progression of the disease and they are modulated by administration of IL-18 and/or TGF-β (a T_h3 product). Oustanding reviews on malaria immunology have further stressed on the role of regulatory T-cells by pointing out that development of T_h1 response can be antagonised directly by IL-4 and TGF-β and indirectly by IL-10, which inhibit the production of pro-inflammatory cytokines [61] with IL-10, in addition inducing B-cell proliferation, which is essential for the development of protective malarial antibodies. In malaria endemic areas the population risk to severe disease decline with increasing exposure with initially T_h1/IFN-γ pro-inflammatory response switching to a predominantly anti-inflammatory response [73]. The switching between these responses is said to probably be induced by TGF-β, which has shown to be associated with reduced pathology in murine malaria [70, 154, 155]. The anti-inflammatory cytokine regulatory role of IL-10 and TGF-β produced by both the cells of innate (macrophages) and adaptive (T-cells) are the key immunoregulators [87, 141] complemented by IL-4 that play a role in control of parasitemia and alteration of severity of malaria [156]. This means that IL-4 contributes largely in conferring protection to malaria by potentially influencing adaptive immunity especially due to its role in polarization of T-cells. The TGF-β immunoregulatory

role base on its potetial property of being pro-inflammatory (at low concentration) and anti-inflammatory (at high concentration) [70] that may largely depend on the antigen stimulating its release.

3.5.2 T_h17- cells and possible contribution to malaria pathology: With advancement in science over decades, the understanding of immune aspects of regulation, pathogenesis and host defense to various infectious agents have broadened. The recent proposed T-cell subset of T_h subpopulation, T_h17, and understanding of contribution to outcome or pathology of malaria could be of particular interest. T_h17 lineage of cells expressing IL-17 cytokines have been proposed some two years ago [157-159] and have been found to develop from precursor cells independent of cytokines and transcription factors that mediate the pathways undergone by T_h1 and T_h2 cell development [157]. This unique developmental and differentiation property of IL-17 producing T_h17 provide insights into mechanisms by which signals from cells of the innate immune system guide alternative pathways of T_h1, T_h2 or T_h17 development [158].

Activation of precursor T_h cells in the presence of TGF-β and IL-6 is thought to drive differentiation of T_h17 cells in the mouse. Apart from cytokine environment, it is not clear whether any other elements of the initial activation of T_h17 cells differ from those of other T_h cell subpopulations. It has been suggested that IL-23 is involved in the expansion of already established T_h17 populations, although the cytokine alone does not induce differentiation of naïve T-cell precursors into that cell type. Cytokines produced by T_h17 cells includes IL-17, IL-21 and IL-22 and among them IL-21, has also been shown to initiate an alternative route for the activation of T_h17 populations [160]. In humans, a combination of TGF-β and IL-1β and IL-23 induces T_h17 differentiation from naive T-cells [161] with both IFNγ and IL-4 which are main stimulators of T_h1 and T_h2 differentiation respectively, negatively regulating the T_h17 differentiation [162, 163]. Studies in mice have in addition, shown the suppressive role of IL-10 on T_h17 cytokines secretion by macrophages and T-cells *in vitro* and that only IL-10 deficient macrophages can produce IL-17 [164]. Some proteins involved in the differentiation of T_h cells to T_h17 cells are signal transducer and activator of transcription 3 (STAT3) and the retinoic acid receptor-related orphan receptors alpha and gamma (ROR-α and ROR-γ) [165]. The primary role of T_h17 cells lies on its involvement in autoimmune disease. A more natural role for T_h17 cells is suggested by studies which have demonstrated preferential induction of IL-17 in cases of host infection with various bacterial and fungal species. T_h17 primarily produce two main members of the IL-17 family; IL-17A and IL-17F which are involved in the recruitment, activation, mobilization and migration of neu-

trophils and macrophages; and linking with activation of T lymphocyte subsets [166]. This is of particular attention as IL-17 produced by T_h17 cells is said to contribute in a variety of inflammatory immune-mediated diseases such as rheumatoid arthritis, psoriasis and inflammatory bowel disease [167]. The involvement of T_h17 in human malaria is still not yet elucidated although has been found to associate with inflammation in rodent malaria parasite, *Plasmodium yoelii* [141].

4. Zinc deficiency and malaria

4.1 Effects of zinc on immunity: The immuno-modulating effects of zinc supplementation can be distinguished in immune enhancing and inhibiting activities [3, 168]. As a consequence, zinc deficiency may also result in a surfeit of immune effects. Zinc deficiency rapidly diminishes antibody- and cell-mediated responses against parasites and viruses in both humans and animals. Biochemically, zinc catalyses the thymic hormone, thymulin, which stimulates differentiation, proliferation and maturation of $CD8^+$ cytotoxic T-cells, $CD4^+$ T_h1-type cells, and of suppressor T-cells that are implicated in the fight against pathogens. The T-cell system is particularly sensitive to zinc deficiency because the resulting thymic atrophy, impaired T-cell development and decreased T-cell counts with consequent $CD4^+$ T-cell depletion [169-175].

Zinc is said to be essential for proliferation and differentiation of immune cells, and for various lymphocyte functions implicated in resistance to malaria, including production of Immunoglobulin G (IgG), interferon-gamma(INF-γ) and tumour necrosis factor-alpha (TNF-α), and microbicidal activity of macrophages. The *innate* immune defence system, which precedes adaptive immune response, is said to particularly be affected by zinc deficiency, leading to impaired activity of this type of response to variety of pathogens. Zinc deficiency, may cause decreased numbers of neutrophilic granulocytes generated by the bone marrow, decreased chemotaxis by neutrophilic granulocytes, decreased phagocytosis by macrophages and neutrophils, and deficient killing by Natural Killer (NK) cells by negatively affecting KIR (killer Immunoglobulin-like receptors) binding, and impaired generation of oxidative burst. These all possible consequences from zinc deficiencies necessitates for deliberate efforts to delineate the effects of zinc on production profile and action of cytokines and possibly the impact may have on the antibody responses. This was also proposed by Keen and Gershwin [171] and it becomes worthwhile when explored together with other micronutrients in relation to more striking diseases like malaria in developing countries.

Zinc deficiency is also likely to affects the *adaptive* (antigen-specific) arm of the immune

system [176-178]. Studies in human model have shown a marked decrease in IFN-γ production but not on the IL-4, IL-6 and IL-10 in zinc deficiency [179]. Even mild zinc deficiency has been accompanied by an imbalance of T_h1 and T_h2 cell functions, resulting in dysregulated resistance to infections restored following zinc supplementation [3]. Sensitivity of the T_h1/T_h2 balance needs special attention because it has been shown in other *in vitro* studies in both human and mouse models that polarization to either T_h1 or T_h2 may result following treatment with suppressive drugs such as vitamin D3 and Dexamethasone [91]. Other studies on naïve $CD4^+$ T-cells have shown that 1α,25-dihydroxyvitamin D3 (vitamin D3) have a direct inhibition of T_h1 favouring T_h2 responses in mice models [180]. These studies have an implication that micronutrients may play a critical function in maintaining the T_h1/T_h2 balance and induction of regulatory T-cell (T_r) proliferation, differentiation and activation, particularly induction of IL-10 and TGF-β production both being implicated in providing a balanced immune protection during infections. Studying these various effects of nutrients in relation to immune response to infections in human models could be very rewarding as may complement efforts already in place to control tropical diseases like malaria in endemic areas.

An imbalance between T_h1 and T_h2 cells, decreased recruitment of T naive cells, and decreased percentage of T cytolytic cells may account for decreased cell-mediated immune functions in zinc-deficient subjects. Studies by Beck et al [179] revealed zinc to be required for regeneration of new $CD4^+$ T lymphocytes and maintenance of T cytolytic cells. By negatively affecting T-cell activation, zinc deficiency will result in decreased IL-2 production and IL-2 receptor expression. Decreased production of IL-2 in zinc deficiency may be due to decreased activation of nuclear factor kappa B (NF-kB) and subsequent decreased gene expression of IL-2 and IL-2 receptors [174]. Due to the impaired phagocytosis, intracellular killing and cytokine production, macrophages become less activated resulting in decreased levels of IL-1β production and impaired expression of MHC class II molecules, which will add to the decreased T-cell activation and decreased production of IFN-γ. The profile of decreased cytokine production by T-cells is indicative for a decreased T_h1 subset differentiation with possible functional consequences. Decreased production of T_h1 cytokines and IFN-γ by leukocytes in the healthy elderly person is correlated with low zinc serum level. Zinc induces monocytes to produce IL-1β, IL-6 and TNF-γ in peripheral blood mononuclear cells and separated monocytes. B-cell development and antibody production, particularly IgG, is compromised by zinc deficiency. In particular, B-cells are sensitive to the induction of apoptosis induced by zinc deficiency.

4.2 *Zinc deficiency and malaria; epidemiological evidence:* Evidence prevail that shows that zinc supplementation can result in dramatic reductions in morbidity and mortality from malaria [22-24] and improved growth [181, 182] in some populations of children. Two of the malaria studies have indicated zinc supplementation to reduce the malaria-attributable fevers while one showed insignificant protective effects. Additionally, several studies conducted in Asia and Latin-America have shown that preventive use of zinc by continuous supplementation leads to dramatic reductions in the incidence of diarrhoea, whereas therapeutic use of zinc for adjunctive treatment of children with either acute or persistent diarrhoea has been found to reduce the duration of illness and the risk of treatment failure [183, 184]. Combination of iron and zinc in some cases is suggested to provide protection from both malaria and diarrhoea [185].

A pooled analysis of four studies and a subsequent study, all conducted in Asia, found that zinc supplementation in children led to substantial reductions in the incidence of pneumonia [183]; however, other studies showed no or little effect on acute lower respiratory infections [41, 185], or even an increased incidence [186]. Further studies must be conducted to determine whether the discrepancies reported are possibly due to bias, or to site-specific factors that influence response to zinc supplementation, such as zinc status or deficiencies in other nutrients known to be essential for growth or immune functioning, or suspected to impair zinc absorption or utilisation. For example, findings from a study among Ethiopian infants suggest that stunting predicts the presence of zinc deficiency, and thus for the response to supplementation [187]. In the recent study carried in Tanzanian community of Pemba, Zanzibar, Sazawal et al [188], have in addition, pointed out that the response to zinc supplementation was dependent on the dose given and also that there are sex and age-related differences in the response to supplementation.

The postulated mechanism for the deleterious effects of zinc deficiency is a reduction in immuno-competence, particularly in cellular immunity [107], which may be reversible by supplementation [168, 183]. Zinc is essential for tissues with rapid cellular differentiation and turnover such as occur in the immune system and the gastrointestinal tract [183], and for various lymphocyte functions implicated in resistance to malaria [178]. Thus further studies of specific immune responses involved may provide clues about mechanisms of acquired immunity to malaria, which is essential for the development of malaria intervention programs with micronutrients inclusion to boost immune responses to the disease. This could later be used together with not yet to be discovered effective vaccines for perfect malaria control.

Despite the impact of nutrition on immune functions and contradicting findings relating zinc with immune functions, most studies relating nutrition to immune response to malaria have been done outside Africa albeit malaria-related deaths in children of this endemic area. In addition, several *in vitro* stimulation studies have used peripheral blood mononuclear cells (PBMCs) for proliferation, differentiation and cytokine production but most of them have used mouse models whose immune responses may be different from that in human beings. In addition, even where studies have used human PBMCs, freshly prepared cells have been used rather than frozen cells which could reflect African situations where the location of study sites necessitates late processing (isolation of cells from whole blood after collection) and pre-cooling cells before stimulation experiments. In this study we have used laboratory prepared live *P. falciparum*-infected human red blood cells (mimicking the real *in vivo* situation), to stimulate PBMCs *in vitro* and measured proliferation, differentiation and cytokine production of various lymphocyte subsets.

5. Aims and outline of the thesis

This study was conducted to assess the baseline nutritional status, particularly zinc, and determine its association with indicators of immune response to malaria before supplementation with zinc and other micronutrients in stunted children aged 6 - 72 months (< 5 yrs) of Handeni, Tanzania. In **chapter 1**, we provide a comprehensive theoretical background of the interaction between dietary micronutrient deficiencies, activity of the immune system and development of protection against *Plasmodium falciparum* infection and malaria disease manifestation. In the intervention study, the effect of zinc and other micronutrients on the immune response to malaria antigens will be investigated in 672 children aged 6-60 months receiving for a period of 26 weeks, a daily oral supplement with either zinc or its placebo, and additionally a daily oral supplement with either multiple micronutrients other than zinc or its placebo for group differences in cellular and serological indicators of immune response to asymptomatic malarial parasitaemia determined at the end of the intervention period; and group differences in cellular and serological indicators of the immune response to symptomatic malaria due to newly acquired infections in the course of the intervention period.

Chloroquine has been abandoned in Tanzania as a useful antimalarial drug due to parasite resistance. The antifolate drug combination, sulphadoxine-pyrimethamine (SP) is nowadays used for first-line treatment of uncomplicated malaria in most malaria endemic-African countries. Surveillance for antifolate-resistant parasites in the field was important to keep tracking the spread and intensity of drug resistance. Monitoring drug resistance through

regular exploration of gene mutations pertaining to drug resistance bears critical importance. In **chapter 2**, drug resistance to SP and detection of possible confounding factors pertaining to development of resistance in falciparum malaria was evaluated.

The best indicator of immunity against malaria is arguably the incidence of symptomatic malaria, although this does not explain the mechanisms involved. In addition, zinc deficiency has been found to result into underperformance of the immune system to combat malaria and other infections. These insights provided the basis for our hypothesis that zinc supplementation will boost the capacity of the immune system to brandish a protective response to falciparum malaria, both in a quantitative and qualitative sense. To achieve these goals, we adopted the available technical and conceptual advances to analyse the immune capacity without the need to wait for actual challenging the individuals with infectious organisms. With respect to the involvement and role of the immune system we compared the activity of the innate immune response to *Plasmodium falciparum* (**chapter 3**) and the level of T-cell response after *in vitro* stimulation with cultured *Plasmodium falciparum*-infected red blood cell preparations (**chapter 4**) and *Plasmodium falciparum* specific antibodies as serological indicators of immunity (**chapter 5**) in zinc-deficient and zinc-replete children in both parasitaemic and aparasitaemic subjects. To reflect the real field situation where facilities for sample processing may be far from the sample collection site, we performed the experiment in PBMCs kept frozen in Liquid Nitrogen (-180^0C) for a period of 6 months and assessed cells viability, differentiation and proliferation capacity.

A unique characteristic feature of infections with *Plasmodium falciparum* is the ability of infected red blood cells (IRBCs) to adhere to vascular endothelium by cytoadherence, resulting in accumulation of IRBCs in the deep microvasculature. CD36 is one of the host ligands that have been found to mediate endothelial binding of IRBCs and has been identified in most field isolates. A mutation in the gene encoding for CD36 is known to influence the malaria-associated anaemia (MA) in an endemic area. In **chapter 6**, the role of CD36 deficiency was assessed in the development of malaria-associated anaemia in children. The study specifically explored the frequency of CD36 deficiency among children in malaria endemic areas and associations between CD36 deficiency and status of malaria-associated anaemia in these children.

6.5 Ethical issues: Clearance to conduct this study was obtained from ethical review boards in The Netherlands, by the Kilimanjaro Christian Medical Centre (KCMC) and the Tanzanian National Institute for Medical Research (reference numbers for KCMC and the National Health Research Ethics Review sub-Committee: 094 and NIMR/HQ/R.8a/VolIX/540, respectively).

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CHAPTER

2

Drug resistance to sulphadoxine-pyrimethamine in *Plasmodium falciparum* malaria in Mlimba, Tanzania

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Malar J, 2006; **5**:94

Abstract

Sulphadoxine–pyrimethamine (SP) has been and is currently used for treatment of uncomplicated *Plasmodium falciparum* malaria in many African countries. Nevertheless, the response of parasites to SP treatment has shown significant variation between individuals. The genes for dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) were used as markers, to investigate parasite resistance to SP in 141 children aged less than 5 years. Parasite DNA was extracted by Chelex method from blood samples collected and preserved on filter papers. Subsequently, polymerase chain reaction (PCR) and restriction fragment length polymorphism (PCR-RFLP) were applied to detect the SP resistance-associated point mutations on *dhfr* and *dhps*. Commonly reported point mutations at codons 51, 59, 108 and 164 in the *dhfr* and codons 437, 540 and 581 in the *dhps* domains were examined. Children infected with parasites harbouring a range of single to quintuple *dhfr*/*dhps* mutations were erratically cured with SP. However, the quintuple *dhfr*/*dhps* mutant genotypes were mostly associated with treatment failures. High proportion of SP resistance-associated point mutations was detected in this study but the adequate clinical response (89.4%) observed clinically at day 14 of follow up reflects the role of semi-immunity protection and parasite clearance in the population. In monitoring drug resistance to SP, concurrent studies on possible confounding factors pertaining to development of resistance in falciparum malaria should be considered. The SP resistance potential detected in this study, cautions on its useful therapeutic life as an interim first-line drug against malaria in Tanzania and other malaria-endemic countries.

Background

Human malaria is caused by an Apicomlexan parasite of the genus *Plasmodium*. Four species are known to cause human malaria namely, *Plasmodium falciparum, Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. Nevertheless, *P. falciparum* has been found to be the most lethal of all human malaria parasites. This parasite causes epidemics in malaria-endemic countries, resulting in large numbers of deaths. Widespread chloroquine resistance has forced many countries to use alternative drugs as antimalarials against falciparum malaria, such as the combination of sulphadoxine and pyrimethamine (SP). However, the parasite has been observed to develop resistance to this drug combination associated with point mutations in the genes for the enzymes involved in the obligatory parasite-folate biosynthesis pathway. Such mutations lead to the lowering of the drug binding affinity to the parasite enzymes [1-5]. Resistance to pyrimethamine is attributed to mutations in the gene for the parasite enzyme dihydrofolate reductase (*dhfr*), whereas sulphadoxine resistance is associated with mutations in the gene for the parasite enzyme dihydropteroate synthetase (*dhps*). The increased level of resistance has been found to be associated with increased numbers of mutations in the genes for these two enzymes. Studies [6] have shown that multiple mutations in the genes for both enzymes result in exceedingly high SP treatment failure. Detection of these mutations in field isolates has been proposed as an alternative strategy for rapid screening of antifolate drug resistance [7-12].

In Tanzania, due to high resistance that developed against the previously effective, safe and relatively cheap antimarial drug, chloroquine, SP was introduced as the first-line drug against malaria by August, 2001. Unfortunately, the change of policy to SP by the government has been challenged by the previously reported low [13] but fast spreading levels of resistant parasite strains against the drug [14]. SP resistance has been reported in variable magnitudes across the country [15-17]. Surveillance for these antifolate-resistant parasites in the field is still required to dissuade its spread over wide areas and possibly suggest effective implementation of new drug policy in Tanzania. The present study was thus carried out during the period of January 2002 to August 2004 to evaluate the frequency of point mutations in *dhfr* and *dhps* among *P. falciparum* isolates from children of Mlimba division of Kilomero district of Tanzania. This could give a picture of the level of drug pressure in the field from the time when SP was introduced as an interim first-line drug for malaria treatment in the country. Since there are currently, various drug combinations on trial for treatment of uncomplicated falciparum malaria [18,19], the anticipation was to obtain findings which would give information on the current frequencies of SP resistant *P. falciparum*

strains and probably give advice to policy makers for opting to other new effective, cheap and safe antimalarial drug combinations.

Materials and methods

Study area : The study was conducted at Ifakara Health Research and Development Center (IHRDC) situated in Ifakara Town of Kilombero District, Morogoro, Tanzania. Samples were collected during the period of January to August 2002 from Mlimba, an area about 150 km from IHRDC along Kilombero River where malaria is endemic with perennial transmission. The area is among nine sentinel sites for National Malaria Control Programme since 1997 and its human population dynamics is being closely monitored on a monthly basis by the Ifakara Centre Demographic Surveillance System (IC-DSS) since 1996. Recruitment of patients and sample collection was done by the research team at Mlimba Health Centre.

Study subjects: The ethical clearance was obtained from both National Institute for Medical Research (NIMR) and IHRDC Institutional Ethics Committee authorities. Parents or guardians of participating children accepted and gave informed consent for participation in the study. About 172 patients of both sexes with acute uncomplicated falciparum malaria and aged 6 – 60 months (< 5 years) were initially recruited in this study. However 31 (18%) of the recruited patients either were excluded from the study due to failure to comply with criteria for participating in the study or were lost during follow up.

Sample collection: Blood samples for parasite genotyping were collected on filter paper (3MM Whatman), labelled and identified, and kept in a dry clean container with desiccant for a minimum of three hours to dry. Dry filter paper blood samples were stored at room temperature until when needed for further analyses. The follow-up samples were obtained at days 3, 7 and day 14 after SP treatment. Additional follow-ups were done at any other day if the child was unwell. During all these visits, finger-prick blood was obtained for microscopy and later molecular analysis.

Extraction of parasite DNA: P. falciparum genomic DNA was extracted from blood collected on 3MM Whatmann filter paper by Chelex method as previously described [20]. The extracted DNA from each sample was used immediately for PCR and any remaining portion was stored at –20ºC in appropriately labelled storage tubes.

Genotyping of parasite genomic DNA: Sample analysis was based on the standardised polymerase chain reaction and restriction fragment length polymorphism. For amplification of the *dhfr* and *dhps* coding regions, a nested PCR protocol was adopted followed by RFLP.

The regions of the *dhfr* and *dhps* genes surrounding the polymorphisms of interest in *dhfr* 51, 59, 108 and 164 and *dhps* 437, 540 and 581 codons were analysed as described in detail elsewhere [7,21,22].

Amplification of parasite DNA by PCR: In this multiplex parasite DNA amplification of the parasite genomic DNA, two primer pairs M1/M5 and R2 + R/ were used as forward and reverse primers in primary (nest I) PCR reaction for the *dhfr* and *dhps* domains respectively. In secondary (nest II) PCR reaction M3 + F/ and F + M4 were used as forward and reverse primers to amplify the four regions on *dhfr* where the point mutation is anticipated to occur [20]. On the other hand, K +K/ and L + L/ primers were used to amplify regions on the *dhps* gene where resistance-associated mutations are said to occur [7]. The details of primer sequences, annealing temperatures and controls are shown in **table 1**. In both nest I and nest II PCR, reaction volumes ranged from 20µl to 30µl. The final concentration of each reagent was 1x PCR reaction buffer (10x PCR buffer – $MgCl_2$, Invitrogen), 1.5 mM $MgCl_2$, 125µM dNTP (Promega, Madison, WI, USA), 250nM primers (QIAGEN, Operon) and 0.02 U/µl Taq Polymerase (Invitrogen). The master mix was prepared in a 1.5ml reaction tube with Molecular biology PCR water (Sigma) as a diluent and aliquots made in PCR tubes (0.2ml size). To each PCR tube, 5µl of DNA was added in primary reaction and 2µl was re-amplified in the nested PCR reaction. The known purified genomic DNA from HB3, 3D7, W2, K1, T9/96, FCR3 and V1/S laboratory parasite clones were used as positive controls and NT (No template) was included as negative control. PCR was performed in a Programmable Thermo Controller, (PTC-100 (TM) MJ Research, Inc., Watertown, MA, USA). Samples with no detectable PCR products were re-examined at least twice starting from the DNA preparation before were declared negative.

*Restriction enzyme digestion***:** Site-specific restriction enzymes were used to digest the PCR amplicons. Seven different restriction enzymes were used in this study (**table 1**), namely *TSP509*I, *Xmn*I, *Alu*I, *Dra*I (*dhfr* domain) and *Ava*II, *Fok*I, *Bst*UI (*dhps* domain) enzymes, respectively. Essentially 8µl of PCR products were incubated with restriction enzymes (New England Biolabs, Beverly, MA, USA) according to manufacturer's protocol in 25µl final reaction volume. The *dhfr* and *dhps* variants were identified as previously described [7,21,23].

Gel electrophoresis: Nested PCR amplicons were electrophoresed on 2% agarose gels before subsequent restriction fragment length polymorphism analysis. Electrophoresis of restriction digests was done on 10% polyacrylamide gel (PAA) as described by Sambrook et

al [24] at a constant voltage of 11.25v/cm gel for 2.30 hours, stained with ethidium bromide, visualized under UV light, photographed and electronically stored..

***Statistical analysis*:** Data were analysed using the EPI Info Version 6.04 epidemiological software (Centres for Disease Control and Prevention, Atlanta, GA, USA). This made it possible to estimate the frequency of point mutations on *dhfr* and *dhps* responsible for parasite resistance against SP thus determining the prevalence of these mutations. The prevalence of each point mutation was calculated as the percentage of baseline (D0) samples containing point mutation at the particular codon on *dhfr* and *dhps*, respectively. Fragment sizes were compared with known restriction fragments (band sizes) obtained in previous studies with reference to 1Kb DNA marker [21,25].

Results

Treatment outcomes: From clinical evaluation (clinical data provided by IHRDC), a total of 172 children with acute uncomplicated malaria were recruited into the study and treated with SP. Out of these recruited patients, 141 (82%) successfully completed the study. Data from 31 (18%) patients who could not complete the 14 days follow-up were excluded from analysis. Of patients who completed the study successfully, treatment failures were depicted in 15 (10.6%) patients comprising of 6.7% early and 3.9% late treatment failures, respectively. Adequate clinical response occurred in 126 (89.4%) of patients. Consequently, molecular analysis was performed on 141 samples from patients who completed the study.

PCR amplification of dhfr and dhps: Different primers were used to amplify regions in *dhfr* and *dhps* containing various point mutations associated with resistance to SP, thus different DNA fragments with different band sizes were obtained (**figure 1**). The fragment sizes were estimated as previously described elsewhere [21]. F + M4 amplification produced DNA amplicons of about 326 bp (**figure 1a**) while DNA amplicons of approximately 522 bp band size were obtained following DNA amplification by M3 + F/ primers (**figure 1b**). PCR amplification using K + K/ (**figure 1c**) and L + L/ primers (**figure 1d**) produced fragments of 438 and 161 bp, respectively on 2% agarose gel. Of 141 samples analysed, 120 (85.1%), 136 (96.5%), 132 (93.6%) and 133 (94.3%) were successfully amplified using M3+ F/, F + M4, K + K/ and L +L/ primers, respectively.

Table 1: Polymorphic genes investigated, mutation sites, primers and primer sequences, PCR reaction conditions, restriction en enzymes and control DNA used in this study

Gene	Mutation	Primers	Primer Sequences	PCR conditions	Enzyme digest	Control DNA
DHFR		M1	5' TTTATGATGGAACAAGTCTGC3'	94°C-3min, 94 °C-1min, 50 °C-2min, 72		
Primary		M5	5' AGTATATACATCGCTAACAGA3'	°C-2min, x40, 72 °C-10min, 4 °C-hold		
DHFR Nested	N51I	M3	5'TTTATGATGGAACAAGTCTGCGACGTT3'	94°C-2min, 94 °C-1min, 45 °C-1min, 72	*Tsp509*1	(+) K1
		F/	5'AAATTCTTGATAAACAACGGAACCTttTA3'	°C-2min, x35, 72 °C-10min, 4 °C-hold		
	C59R	F	5'GAAATGTAATTCCCTAGATATGGAATATT3'	94°C-2min, 94 °C-1min, 45 °C-1min, 72	*Xmn*I	(+) T9/96
		M4	5'TTAATTTCCCAAGTAAAACTATTAGAGCTTC3'	°C-2min, x35, 72 °C-10min, 4 °C-hold		
		F	5'GAAATGTAATTCCCTAGATATGGAATATT3'	94°C-2min, 94 °C-1min, 45 °C-1min, 72	*Alu*1	(+) HB3
	S108N	M4	5'TTAATTTCCCAAGTAAAACTATTAGAGCTTC3'	°C-2min, x35, 72 °C-10min, 4 °C-hold		(+) T9/96
						(+) FCR3
	I164L	M3	5'TTTATGATGGAACAAGTCTGCGACGTT3'	94°C-2min, 94 °C-1min, 45 °C-1min, 72	*Dra*I	(+) V1/S (+)
		F/	5'AAATTCTTGATAAACAACGGAACCTTTTA3'	°C-2min, x35, 72 °C-10min, 4 °C-hold		HB3
						(+) DD2
DHPS		R2	5' AACCTAAACGTGCTGTTCAA3'	94°C-3min, 94 °C-1min, 50 °C-2min, 72		
Primary		R/	5' AATTGTGTGATTTGTCCACAA3'	°C-2min, x40, 72 °C-10min, 4 °C-hold		
DHPS Nested	A437G	K	5'TGCTAGTGTTATAGATATAGGATGAGcATC3'	94°C-2min, 94 °C-1min, 45 °C-1min, 72	*Ava*II	(+) K1
		K/	5'CTATAACGAGGTATTGCATTTAATGCAAGAA3'	°C-2min, x35, 72 °C-10min, 4 °C-hold		(+) FCR3
	K540D	K	5'TGCTAGTGTTATAGATATAGGATGAGCATC3'	94°C-2min, 94 °C-1min, 45 °C-1min, 72	*Fok*I	(+) TN-1
		K/	5'CTATAACGAGGTATTGCATTTAATGCAAGAA3'	°C-2min, x35, 72 °C-10min, 4 °C-hold		(+) VIS
						(+) W2
	A581G	L	5'ATAGGATACTATTTGATATTGGACCAGGATTCG3'	94°C-2min, 94 °C-1min, 45 °C-1min, 72	*Bst*UI	(+) K1
		L/	5'TATTACAACATTTTGATCATTCGCGCAACCGG3'	°C-2min, x35, 72 °C-10min, 4 °C-hold		(+) DD2

Source: *Duraisingh et al [21]; Foley et al. [48]*

RFLP analysis: **Figure 2** depicts representative PAA gels of the restriction digests used to detect the fragment patterns corresponding to the different variants at each codon on the *dhfr* and *dhps* domains. PCR amplification of *dhfr* with the primers F + M4 yields a PCR product sized 326 bp of the codon 108. The restriction enzyme *Alu*I was used specifically to cut fragments discriminating the two alternative forms, wild-type (180 and 118 bp) and mutant (299 plus 27 bp) at that codon. The wild type indicates presence of serine (Ser) in the amino acid sequence of the enzyme system while the mutant form of the gene indicates the substitution by asparagine (Asn). Restriction digestion of the same PCR fragment with *Xmn*I was used to distinguish wild type (189 and 137 bp) and mutant (162, 137 + 27 bp fragments) variants on codon 59 of the gene. Mutation at this codon reflects substitution of amino acid cystine (Cys, wild type) by arginine (Arg, mutant).

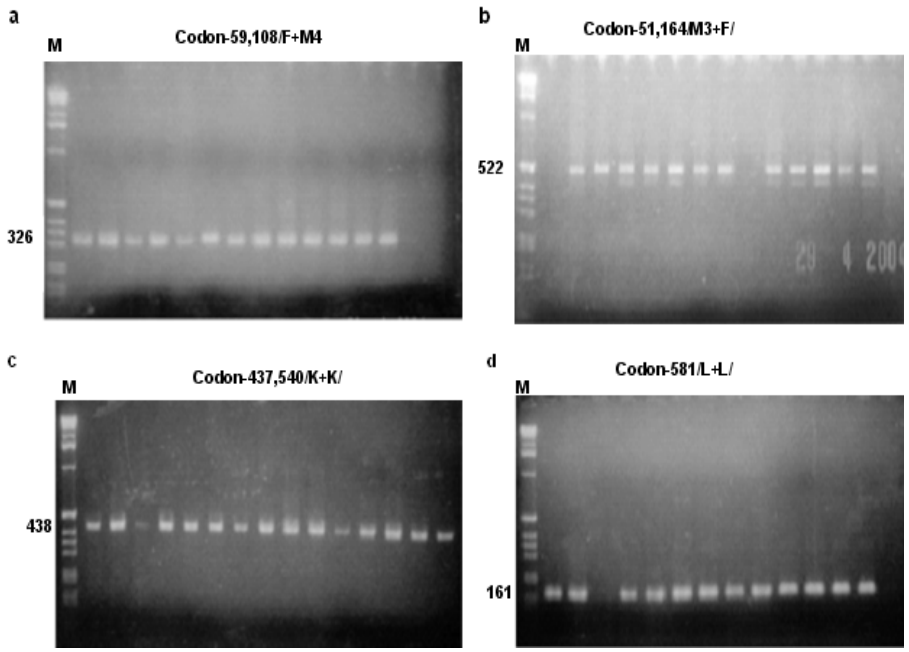

Figure 1: *Agarose gels of the PCR products ((a) F+M4; (b) M3+F/; (c) K+K/; (d) L+L/) of the tests for the polymorphisms of dhf and dhps. Fragment sizes are in bp.*

The M3 + F/ amplified PCR fragments (**figure 1b**) were digested with *TSP509*I and *Dra*I to distinguish variants at codon 51 and 164, respectively. Digestion by *TSP509*I produced fragments of sizes 153 and 120 bp indicative of presence of amino acid asparagine (wild type) and 217 and 120 bp indicating presence of amino acid isoleucine (Ile, mutant). On the other hand, digestion with *Dra*I was expected to produce fragments of sizes 245, 171 and 106 bp (wild type) and 145, 143, 106 and 28 bp (mutant) distinguishing polymorphisms at codon 164. In this context wild type denotes occurrence of isoleucine (Ile) and the mutant indicates occurrence of leucine (Le). The four restriction enzymes were used mutually to detect mutations on the *dhfr* gene ascribed to resistance to antifolate, SP by the malaria parasite.

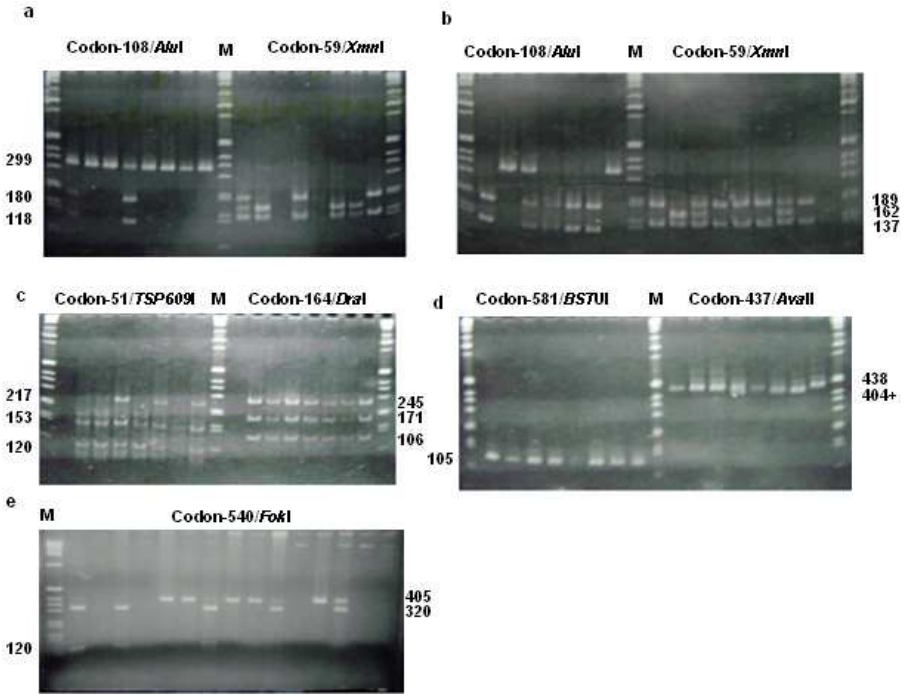

Figure 2: *Polyacrylamide gels of the restriction digest of the PCR products ((a) AluI and xmnI; (b) AluI and XmnI; (c) TSP609I and DraI; (d) BSTUI and AvaII; (e) FokI) of the tests for the polymorphisms of the dhfr and dhps genes. Fragment sizes are in bp.*

Tests for polymorphisms on *dhps* are shown in **figures 2d** and **2e**. PCR products of approximately 438 bp (**figure 1c**) were obtained from amplification with the K and K/ primers. The variants at codon 437 were discriminated by restriction digestion using the restriction enzyme *Ava*II (**figure 2d**). In this case uncut fragment (438 bp) indicated wild type while mutations at this site was shown by a cut fragment sized 404+ bp. Mutation at this codon is indicative of substitution of glycine (Gly) for alanine (Ala). The K + K/ amplified PCR products have been used to detect polymorphisms occurring at codon 540. A restriction enzyme, *Fok*I produced fragments sized 405 bp (wild type) and 320 and 85 bp (mutant) discriminating variants at that codon. The 405 bp fragment indicates presence of amino acid Lysine (Lys) while the 320 and 85 bp fragments reflect substitution of glutamate (Glu) for lysine at this codon.

Following amplification with L + L/ primers, PCR products of size 161 bp were produced (**figure 1d**). These PCR fragments have been used to describe polymorphism on the *dhps* gene occurring at codon 581 using a restriction enzyme *Bst*UI. This restriction enzyme produced restriction digests of sizes 105, 33 and 23 bp (wild type) and 138 and 23 bp (mutant) from the PCR products of size 161 bp (**figure 2d**). The former reflects presence of amino acid, alanine at this codon and the latter indicates substitution of glycine for alanine.

Mutation analysis was variably successful at each codon in both *dhfr* and *dhps* genes and an average of 120 (85.1%) of 141 patients produced fruitful outcome. Four codons (51, 59, 108 and 164) on *dhfr* and three codons (437, 540 and 581) on *dhps* were evaluated and the results are presented in **figure 2**. Mutations in the *dhfr* were highest for Asn-108 (66.9%) and progressively declined for *dhfr* Ile-51 (62.7%) and *dhfr* Arg-59 (48.8%). Mutations in *dhps* varied from *dhps* Gly-437 (43.7%), *dhps* Glu-540 (39.2%) and was lowest for *dhps* Gly-581 (0.8%) alleles. In all pre-treatment and post-treatment samples, no mutant *dhfr* Leu-164 was depicted. The triple *dhfr*, double *dhps* and the quintuple mutants (carrying the *dhfr* triple mutant and the *dhps* double mutant) were also evaluated and were considerable (**table 3**). Mixed pattern was not uncommon in many samples examined (**table 4**).

Discussion

The 141 patients represent children aged less than five years, the age most vulnerable to falciparum malaria [26]. Parasite recurrence in SP treated individuals has been linked to many factors. Such factors include overwhelmed immunity, multiple concurrent infections and drug resistance [27]. Several previous studies have investigated the association between mutations in *dhfr* and *dhps* and the parasitological and/or clinical response to SP medication

at individual level [6,28-30]. Most of these studies produced tangible results regarding the use of *dhfr* and *dhps* genotypes as resistance marker genes for SP [31]. In this study, it was observed (clinical data) that SP treatment cleared infection in 89.4% of the patients who completed the study. This treatment success is partly concordant but higher than what has previously been reported (82%) in a similar study by Aubouy et al [25] in Bakoumba village in Haut-Ogooué province of Southeast Gabon. According to that study, *dhfr* mutations that lead to high-level *in vitro* resistance to pyrimethamine plus one or two *dhps* mutations were reported to be not sufficient to induce *in vivo* failure of SP treatment in young children. Nevertheless, the semi-immune population of over 60% previously reported in Tanzania [32] is probable reminiscent for the observed high parasite clearance despite detection of high resistance-associated point mutations in this study.

Table 2: Proportions of point mutations on *dhfr* and *dhps* related to parasite resistance to antifolates

Resistance to	Gene locus	Mutations (%)	Wild type (%)	Mixed (%)	Total
Pyrimethamine	DHFR 51	74 (62.7%)	35 (29.7%)	9 (7.6%)	118
	DHFR 59	59 (48.8%)	36 (29.8%)	26 (21.5%)	121
	DHFR 108	81 (66.9%)	17 (14.1%)	23 (19.0%)	121
	DHFR 164	0 (0.0%)	104 (100.0%)	0 (0.0%)	104
Sulphadoxine	DHPS 437	56 (43.7%)	71 (55.5%)	1 (0.8%)	128
	DHPS 540	47 (39.2%)	65 (54.5%)	8 (6.3%)	120
	DHPS 581	1 (0.8%)	126 (98.4%)	1 (0.8%)	128
		318	454	68	840

Table 3: Various *dhfr*/*dhps* combinations obtained from restriction analysis to determine point mutations in genes responsible for SP resistance.

Category	Number	Percentage (%)	Percent Cum.
tDHFR	44	36.40	36.40
dDHPS	18	14.90	51.30
tDHFR/dDHPS	24	19.80	71.10
dDHFR/dDHPS	10	8.30	79.40
tDHFR/sDHPS	4	3.30	82.70
dDHFR/sDHPS	7	5.80	88.50
tDHFR/nDHPS	14	11.50	100.00
Total	121	100.0	

tDHFR = Triple mutants on dhfr gene; dDHFR = Double mutants on dhfr gene; dDHPS = Double mutants on the dhps gene; sDHPS = Single mutant on the dhps gene; nDHPS = No mutation on the dhps gene.

Slightly higher values of mutations on *dhfr* (66.9% and 62.7%) were detected at codons 108 and 51, respectively as compared to those previously reported by Mshinda [33] of 50%. This is attributable to rapid, stepwise selection of mutations following use of antimalarial, SP or similar drugs in the area. This is simply because our study was carried 2 years after a similar study by Mshinda in 2000. Pharmacological case management of the disease occurring at informal level [26,34] with poor compliance with dosing schedules could be another basis for the high frequency of mutations detected in this study. In addition, sub-dosage levels of drug administration e.g. two patients sharing a single dose prescribed for a single patient (personal observation) may be a probable cause for the detected high proportion of mutations. Extensive use of different types of antifolates with mechanism of action similar to SP like trimethoprim and sulphamethoxazole (TS) combination (e.g. septrin) in treating other infections over time, probably accounts for the increased selection of mutations ascribed to SP resistance [17].

The mutation of about 66.9% observed in *dhfr* on codon 108 was higher than that reported by Jelinek et al. [35] in West Africa (54.0%) but slightly lower than that observed in Central Africa (72.4%), South Africa (68.9%) and East Africa (72.9%). This could be correlated to the high proportion of mixed genotype infections (mutant and wild type, 19.0%) detected at this codon in the present study. Pearce et al. [17] reported the increase of mixed genotypes

in codons whose mutations are associated with SP resistance in an area of high endemicity. This proportion of mixed genotypes could give rise to *dhfr* mutations of about 85.9% if at all these genotypes were additive. The *dhfr* point mutations at codons 51 and 59 were also higher than that previously reported by Jelinek et al. [35] in Africa and all together explains the significance of these mutations in causing higher SP resistance. As previously reported by Hastings et al. [36], mutations at codon 164 in *dhfr*, which is thought to confer highest SP resistance when it occurs, was not depicted in this study. Generally, mutations in the three codons in the *dhfr* domain were higher (**table 2**) than those on the *dhps* domain, suggesting that mutations on *dhfr* precede those on *dhps* in conferring parasite resistance to SP [37].

Table 4: Proportions of mixed infections detected in this study

Category	Variants detected	Total No. of variants	Percentage (%)
smDHFR	27x1	27	39.7
smDHPS	8x1	8	11.8
dmDHFR	8x2	16	23.5
dmDHPS	1x2	2	2.9
tmDHFR	5x3	15	22.1
tmDHPS	0x3	0	0.00
Total		68	100

smDHFR = Mixed variants detected at a single codon on the dhfr domain; mDHPS = Mixed variants detected at a single codon on the dhps domain; dmDHFR = Mixed variants detected at two codons on the dhfr domain; dmDHPS = Mixed variants detected at two codons on the dhps domain; tmDHFR = Mixed variants detected at three codons on the dhfr domain; tmDHPS = Mixed variants detected at three codons on the dhps domain

The point mutations in *dhps* depicted in this study are far higher than what was observed in previous studies in East and South Africa [35] and Eastern Iran [38]. This might be explained by the widespread use of septrin in Tanzania, which is said to indirectly select mutations for SP resistance [17]. Takahashi et al. [39] further reported that the use of antifolates such as co-trimoxazole for prophylaxis or medication against other infections than malaria indirectly and predominantly select double mutations at *dhps* for resistance. Nevertheless, the *dhps* mutations observed in this study were relatively lower than that observed in Central

and West Africa by Jelinek et al [35], possibly due to geographical differences [16] and differences in patterns of drug use between different areas [17]. However, Beswas [16] reported the *dhps* mutations in Tanzania ranging between 30 – 34%, which is less than values observed in our study (39.2 – 43.7%) signifying that drug pressure increasingly selects these mutant alleles with time depending on frequent use of antifolates.

Overall proportion of point mutations portrayed in this study reflects the existence of high resistance in the study area. Only 8.5% of infections carried pure wild type genotype and 4.6% of the samples showed single mutant alleles. Quintuple mutations were highest (18.5%) followed by triple (16.2%) and double mutations (13.8%). Quadruple mutations occurred in 6.9% of samples. About 41 (31.5%) of infections had at least one mixed (mutant, wild type) genotypes attributable to endemicity of the disease [40], which may result into high transmission consequently augmenting high proportion of mixed clones per infection in the population.

The co-occurrence of point mutations in both *dhfr* and *dhps* loci was also examined in this study (**table 3**) with about 44 (36.4%) of samples containing triple mutant on *dhfr*, which was relatively similar to that reported by Mshinda [33] in the same place (41%) but was almost twice to values reported by Mugittu et al [15] of 18.6%. However, the proportion could escalate if the mixed triple *dhfr* variants (22.1%) and mixed double *dhfr* variants 16 (23.53%) detected in the present study (**table 4**) were counted inclusively. Nevertheless, the proportion of these mutations was less than that reported by Pearce et al [17] in Hai district Northern Tanzania (>70%) where resistance to SP is already unprecedentedly high due to widespread use of SP and spread of resistance from nearby area, Muheza into the area. Mugittu et al [15] reported a prevalence of 80.3% in Mkuzi, an area in Muheza District where pyrimethamine was used for prophylactic and/or therapeutic trials at different periods from 1950s to 1994 [41-43]. The difference in levels of SP resistance between Tanzanian communities is also attributable to the differences in patterns of drug use between communities within the country [17]. In Muheza District Hospital, for instance, SP was implemented as first-line drug in children less than 5 years of age since 1984 [44].

The frequency of triple-*dhfr*/double-*dhps* mutants (quintuple mutation, 19.8%) depicted (**table 3**) is high as compared to previous studies in Mlimba and Idete [45] which was reported as a rare event. However, the proportion of quintuple mutation in Mlimba was less than that reported by Pearce et al [17] in Hai and Pare areas of Northern Tanzania (30 – 63%). The proportion of quintuple mutation was also less than that generally reported by

Jelinek et al [35] (42.9%) in East Africa, and that by Bwijo et al [46] in Maonga and Chimbala villages of Salima District, Malawi (78%). The lower frequency of quintuple mutations (19.8%) observed in this study as compared to other reported mutations in Tanzania and other areas of Africa could partly be attributable to the early development of resistance in those areas. In Malawi for instance, the study was carried 7 years after introduction of SP as a first-line drug while our study was carried shortly (2 years) after introduction of SP as an interim first-line drug in Tanzania, providing a relatively shorter time for selection of SP resistance mutations. In addition the low quintuple mutations can also be linked to the proportion of genotypes at similar locus possessing mixed variants observed in this study. In previous studies, the mixed variants were not reported. The random selection of these mutations might generally, be a consequent of country-wide use of SP as a second-line antimalarial drug several years before it was implemented as an interim first-line drug by August 2001.

The prevalence of double *dhps* mutation (**table 3**), which is considered to be a prerequisite for resistance to sulphonamides [7], was found to be 6x that reported by Mugittu et al [15] in the same area. This proportion (19.8%) was also higher than that reported in Kyela and Masasi areas of Tanzania but nearly equal to that reported in Butimba but less than that reported in Mkuzi areas of Tanzania by Mugittu et al This might be attributed to the effect of septrin, an antifolate extensively used in Tanzania as antibiotic agent, indirectly and preferentially selecting double mutations on the *dhps* locus [39]. Similar effects can result from septrin, which is essentially similar to co-trimoxazole in composition as previously stated. Both are antifolates basically composed of trimethoprim-sulphamethoxazole (TS) with similar effects to SP [46,47]. Observation also showed that 52.1% of infections harboured at least one mutation on the *dhps* locus (**table 3**) reflecting that probably there is high effect or prevailing use of septrin in the area. This can also be associated with long-term abuse of the drug, but also could be indicative of the differences in the generic drugs available in the country manufactured by different companies which might have different bioavailability and therapeutic values. The high proportion of double *dhps* mutations when coupled with triple mutations in *dhfr* can result into quintuple mutation to confer highest SP resistance.

Mixed genotypes in either *dhfr* or *dhps*, or both, were depicted in at least 68 DNA samples (**table 4**). Detection of mixed genotypes in *dhfr* and *dhps* is important due to its influence on the overall proportion of point mutations in baseline samples which upon drug pressure, the wild type get cleared with mutant genotypes persisting in longitudinal follow up samples. Most recrudescent infection detected in follow up cases came from pure mutant and mixed

genotypes in baseline samples as parasites with wild type genotypes are sensitive and were subsequently cleared post-medication by the drug. The variable results obtained following PCR-RFLP of follow up samples (20%) have been previously observed [25]. Infection detected during follow-up may be either an infection that previously failed to express due to presence of an abundant (detected) strain that masked the presence of minor resistant strain, thus being a recrudescent parasite. But on the other hand the detected mutant alleles during follow-up may be new infection with similar genotype as that detected pre-treatment.

Conclusions and recommendations: In conclusions, the impact of quintuple mutation on SP resistance may be weighed down by host immunity in endemic areas although may not suggest continued use of the drug for treatment of malaria. The impact of other drugs with similar mechanisms of action used as antibiotics in selecting mutations responsible for SP resistance need be studied especially for co-trimoxazole, which is currently used as a prophylaxis against opportunistic infections in HIV-infected individuals. The information obtained in the present study will be of direct and immediate relevance to current HIV and malaria control policies in Tanzania and possibly in Africa and the universe. In addition, it will add to our basic knowledge of the molecular basis of antifolate-resistant malaria. There is a need for reviewing the policy on the use of SP as a first-line drug for treating malaria in Tanzania. In addition, despite several previous studies showing SP + Amodiaquin (SP+AQ) and SP+Artesunate (SP+AS) to cause a delay in emergence of resistance and rapid gametocyte clearance, the high SP resistance potential detected in this study suggests exclusion of SP component in future planned drug combinations for treatment of malaria. Alternative combination therapies like artemisinin-based drugs (e.g. artemisinin-lumefantrine) and short-acting antimalarials such as chlorproguanil-dapsone combination (LapDap) and atovaquone-proguanil (Malarone®) may be rewarding albeit thorough clinical trials are still needed to evaluate the effectiveness and possible harmful side effects of these proposed drugs. In deployment of a new antimalarial drug for treating malaria, the effect of other drugs with similar mode of action to drug, used in treating other infections than malaria have to be considered to preclude the possibilities of early development of resistance to the target drug due to cross-resistance. The findings from this study imply that *in vivo* studies be further carried out to confirm that the high frequency of SP resistance alleles is indicative of treatment failure. Improvement of health services with adequate drugs and skilled medical staffs from village levels may reduce uncontrolled and inappropriate use of the drug, consequently reducing the chances of selecting SP resistance mutations against malaria.

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CHAPTER

3

Alterations in early cytokine-mediated immune responses to *Plasmodium falciparum* infection in Tanzanian children with mineral element deficiencies: a cross-sectional survey

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Abstract

Deficiencies in vitamins and mineral elements are important causes of morbidity in developing countries, possibly because they lead to defective immune responses to infection. Our aim was to assess the effects of mineral element deficiencies on early innate cytokine responses to *Plasmodium falciparum* malaria. Peripheral blood mononuclear cells from 304 Tanzanian children aged 6–72 months were stimulated with *P. falciparum*-infected erythrocytes obtained from *in vitro* cultures. The results showed a significant increase by 74% in TNF-α in malaria infected individuals with zinc deficiency (11% to 240%; 95% CI). Iron deficiency anaemia was associated with increased TNF-α production in infected individuals and overall with increased IL-10 production, while magnesium deficiency induced increased production of IL-10 by 46% (13% to 144%) in uninfected donors. Most donors showed a profound response towards IL-1β production, drawing special attention for its possible protective role in early innate immune responses to malaria. In view of these results, our findings show plasticity in cytokine profiles of mononuclear cells reacting to malaria infection under conditions of different micronutrient deficiencies. Our findings lay the foundations for future inclusion of a combination of precisely selected set of micronutrients rather than single nutrients as part of malaria vaccine intervention programs in endemic countries.

Introduction

In African populations, multiple micronutrient deficiencies, infections and immunodeficiencies commonly co-exist. Deficiencies in vitamins and mineral elements can impair immune responses to infectious diseases through multiple mechanisms, ranging from phagocytosis and innate immune responses to antibody formation and cell-mediated immunity. Zinc is an important micronutrient because it is essential for the development, differentiation and function of several critical types of immune cells [1, 2]. *In vitro* mitogen stimulation experiments indicate that marginal zinc deficiency can cause reduced counts of circulating leucocytes and reduced whole blood concentrations of cytokines, particularly IL-6 [3]. Zinc deficiency contributes to pneumonia, acute and chronic diarrhoea [4, 5], and possibly malaria [4-6], which together constitute the leading causes of death in African children. In addition, zinc deficiency may exacerbate the outcome of diseases such as HIV and tuberculosis that rely on macrophage killing of infected cells [7]. Deficiencies of copper [8], iron and vitamin B_{12} have been associated with impaired neutrophil functions whereas deficiencies of folic acid are not [9].

A fast-acting innate immune response, mediated by cytokines such as interleukine-1β (IL-1β), IL-12 and tumour necrosis factor-α (TNF-α), is crucial for host survival in the initial stages of *Plasmodium falciparum* infection [10]. Zinc is needed for monocytes and macrophages to produce IL-1β and for other peripheral blood mononuclear cells (PBMCs) to produce TNF-α. Zinc deficiency can lead to impaired phagocytosis and intracellular killing by macrophages and neutrophils. In addition, it can impair NK-cell function, cytokine production, the generation of an oxidative burst as well as complement activity [2, 11-14] through decreased activation of various cellular responses and low concentrations of IL-1β. In addition, innate immune responses determine the type and efficiency of subsequent adaptive immune responses [10, 15, 16] at later stages of infection.

This study was conducted to assess the impact of deficiencies of zinc and other mineral elements on early innate immune responses to *P. falciparum* infection. We assessed this by *in vitro* stimulation experiments, using PBMCs samples that were collected from Tanzanian children aged 6-72 months. We hypothesised that zinc deficiency alters the balance in cytokine production and their association in early immune responses, and that deficiencies of zinc and other mineral elements induce a decreased ability of PBMCs to produce pro-inflammatory cytokines, and the regulatory cytokine IL-10, when exposed to *P. falciparum* parasites. In addition, we investigated to what extent the magnitude of the PBMCs cytokine

response depended on the *P. falciparum* infection status of the child at the time that the blood was collected and PBMCs were isolated.

Subjects and methods

Study area and population: This study was conducted in a lowland area around Segera village (S 05º 19.447', E 38º 33.249'), Handeni District, north-eastern Tanzania, in May-July 2006. Malaria is highly endemic in this area, with virtually all infections being due to *P. falciparum*. The residents in the study population mostly comprise poor farmer families growing maize and cassava for subsistence use. Such populations are prone to deficiencies of zinc and iron because they have cereal-based diets that are rich in natural dietary constituents that inhibit the absorption of these trace metals [17]. At the time of our study, only one health centre in Segera was available to serve all of the surrounding area. The study was approved by Ethics Review Committees in The Netherlands and Tanzania (reference numbers for KCMC and the National Health Research Ethics Review sub-Committee: 094 and NIMR/HQ/R.8a/VolIX/540, respectively). Informed consent was obtained from community leaders and local government officials, and from parents or guardians.

*Sampling methods and eligibility criteria***:** A census list was made with all resident children aged 6-72 months in the study area. Using this list, 16 children were randomly selected from 19 communities, resulting in a total of 304 subjects. Further details are provided elsewhere [18].

*Field procedures***:** All children were examined by a clinical officer, who also measured axillary temperature by electronic thermometer. Subjects were eligible when they had no fever, and showed no signs of other severe disease or severe malnutrition (weight-for-height z-score below -3 SD). Both dip stick test and microscopy ware used for diagnosis providing a wide chance for detecting asymptomatic malaria case [19, 20]. Venous blood (6 mL) was collected in containers suitable for mineral element analysis with sodium heparin as anticoagulant (Becton-Dickinson, Franklin Lakes, NJ). Immediately upon collection, the cap was sprayed with ethanol and allowed to dry; approximately 1.3 mL blood was then drawn by sterile syringe. This aliquot was centrifuged and plasma samples were stored and transported to The Netherlands at -80 ºC for subsequent measurement of mineral element concentrations. The remainder of the blood sample was kept at 20-25 °C during transport the same day to the laboratory in Moshi, at approximately 300 km distance, for collection of additional plasma and PBMCs. Children were treated for common childhood infections and anaemia according to guidelines of Tanzanian Ministry of Health.

Determination of plasma concentrations of mineral elements: Plasma samples were diluted 20 times in milliQ [21], and concentrations of zinc, magnesium and copper were measured by inductively-coupled plasma atomic emission spectrometry (ICP-AES) (Vista Axial, Varian, Australia). To determine variability in outcomes, measurements were replicated five times. With mean values set at 100%, measurements varied between 97% to 102% for zinc, 99% to 102% for magnesium, and 97% and 102% for copper. Because we found no evidence for copper deficiency as assessed by plasma copper concentrations <7.1 μmol/L (unpublished data), we only report the results for zinc and magnesium in this paper.

Determination of plasma indicators of iron stores and inflammation: After arrival at the laboratory in Moshi, blood samples were immediately centrifuged (300´*g*) at ambient temperatures for 10 minutes. We collected plasma (1.2 mL) and replaced this immediately with an equal volume of Iscove's modified Dulbecco's medium (IMDM) with GlutaMAX (Invitrogen Gibco-BRL, Life Technologies, Grand Island, NY, USA) for subsequent isolation of peripheral blood mononuclear cells (see below). Plasma was stored in liquid nitrogen, and subsequently transported on dry ice to The Netherlands, where plasma concentrations of ferritin and C-reactive protein were measured as indicators of iron stores and inflammation, respectively, by using a Behring nephelometer (BN ProSpec; Dade-Behring) in The Netherlands (Meander Medical Centre).

PBMCs isolation: PBMCs were isolated by Ficoll density gradient centrifugation, cells were transferred to 10% v/v DMSO in fetal calf serum, cooled at -1^{o}C/minute in an isopropyl-loaded device (Nalgene, Rochester, NY, USA) and preserved in liquid nitrogen [22]. For a 16-h period during transport to Wageningen University, The Netherlands, the PBMCs were kept on dry ice, and immediately thereafter stored again in liquid nitrogen until stimulation experiments (see below).

Preparation of P. falciparum-parasitized and unparasitized erythrocytes: Human O and rhesus-negative erythrocytes from healthy blood donors (Sanquin, Nijmegen, The Netherlands) were cultured in medium to which live *P. falciparum* parasites (NF54 strain) produced in a continuous culture were added [23, 24]. After 2-4 days, when asexual parasitaemia reached ~8-10% of infected erythrocytes, the culture was concentrated by centrifugation at 625´*g* for 5 min; infected erythrocytes were separated on a 67% Percoll gradient as reported elsewhere [25] and washed twice in phosphate-buffered saline (PBS). Purified infected erythrocytes were preserved at a concentration of approximately 15´10^{7}/mL in 13%

glycerol/PBS in a freezing container at -80^{o}C. Glycerol (50% w/v) was added to the infected erythrocytes to avoid mechanical damage of the cells through ice formation. Unparasitized erythrocytes were processed similarly but without adding parasites to serve as a control. Both in parasitized and unparasitized erythrocyte cultures, we confirmed the absence of mycoplasma contamination by polymerase chain reaction. Both parasitized and unparasitized erythrocytes were counted by flow cytometry, and compared regarding their size and internal complexity to the counting beads and PBMCs. Aliquots were made and stored at -80^{o}C until needed for PBMCs stimulation.

PBMCs stimulation: Malaria antigens differ in their capabilities to stimulate PBMCs: intact parasitized red blood cells (pRBC) are capable of inducing more rapid and intense pro-inflammatory responses from PBMCs than freeze-thaw lysates of *P. falciparum* [26]. To simulate *in vivo* malaria-specific responses as closely as possible, we used *P. falciparum* pRBC, with an adapted protocol for stimulation of PBMCs by Jeurink et al [22]. In brief, PBMCs were cultured at 10^6 cells/well in sterile polystyrene 48-well plates with flat-bottom wells (Corning Inc, Corning, NY, USA). Based on initial optimization experiments, aliquots of pRBC were thawed and cultured with PBMCs in IMDM with glutamax containing Yssel's supplements [27] with 2% human AB serum, 1% penicillin/streptomycin and 1% fungizone (Gibco-BRL), at a PBMCs:pRBC ratio of 1:2. PBMCs were also cultured under similar conditions with unparasitized erythrocytes (uRBC) (2´10^6 cells/well) as a negative control, and with soluble antibodies to CD3 and soluble antibodies to CD28 (Cat. No.555336 and 555725, Becton-Dickinson, Alphen aan den Rijn, The Netherlands) as a positive control. Monoclonal anti-CD3 and anti-CD28 antibodies provide co-stimulatory signals and polyclonal stimulation required for maximal proliferation of T lymphocytes [22, 28]. Cell culture plates were incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2. After PBMCs culturing for 1 day, we aspirated 75 µL of the supernatant per well to measure cytokine concentrations.

*Measurement of cytokine concentrations***:** Concentrations of IL-1b, IL-10, IL-12p70 and TNF-a were determined on a FACSCanto II flow cytometer by cytometric bead array system and analysed with FCAP software (all from Becton-Dickinson).

Statistical analysis: Data were entered and analysed using SPSS for Windows (version 15.0. SPSS Inc., Chicago, IL, USA). Zinc deficiency and low zinc status were defined as plasma zinc concentrations <9.9 µmol/L and <10.7 µmol/L, respectively. Low magnesium status was defined by magnesium concentration < 750 µmol/L [29, 30]. Iron deficiency anaemia

was defined by co-existing iron deficiency (plasma ferritin concentration <12 μg/L) and anaemia (haemoglobin concentration <110 g/L). Cytokine concentrations were ln-transformed to obtain normally-distributed values. Group differences in these values were analysed assuming t-distributions. Interactions between malaria and micronutrient indicators were assessed using multiple linear regression models on log-transformed cytokine data; the resulting effect sizes were exponentiated and expressed as percentage values. Linear regression analyses were also carried out to explore the associations between IL-1β, TNF-α and IL-10, and to what extent these associations were influenced by nutrient status and malaria infection status. The association between concentrations of TNF-α and IL-10 were considered as a measure of balance between the pro-inflammatory responses and the regulatory response. We only report the analyses of the cytokine responses to pRBC. As expected, the average response to uRBC (negative control) was less than to pRBC, whereas the average response to CD3/CD28 (positive control) was higher. Correction for these responses does not change the estimates of the associations between nutrient status and cytokine responses, or between malarial infection status and cytokine responses.

Results

General characteristics of the study population: We collected blood from 135 boys and 169 girls; these had similar age distributions. We found the following prevalence values (n): low zinc status: 63.1% (188); zinc deficiency: 48.3% (144); low magnesium status: 65.1% (194); iron deficiency anaemia: 9.4% (26); malaria: 46.1% (140). Detailed characteristics of the study population by malarial infection status are summarised in **table 1**. In addition, the associations between nutrient status and supernatant cytokine concentrations, and between malaria infection status of the child at the time of blood collection and supernatant cytokine concentrations, following 24h of PBMCs stimulation with *Plasmodium falciparum*-infected erythrocytes are also summarized (**figure 1**). Adjustment for age class, sex and/or magnesium deficiency did not lead to marked changes in the associations between zinc deficiency and supernatant cytokine concentrations shown in **figure 1**; conversely, adjustment for age class, sex and/or zinc deficiency did not lead to marked changes in the associations between magnesium deficiency and those supernatant cytokine concentrations.

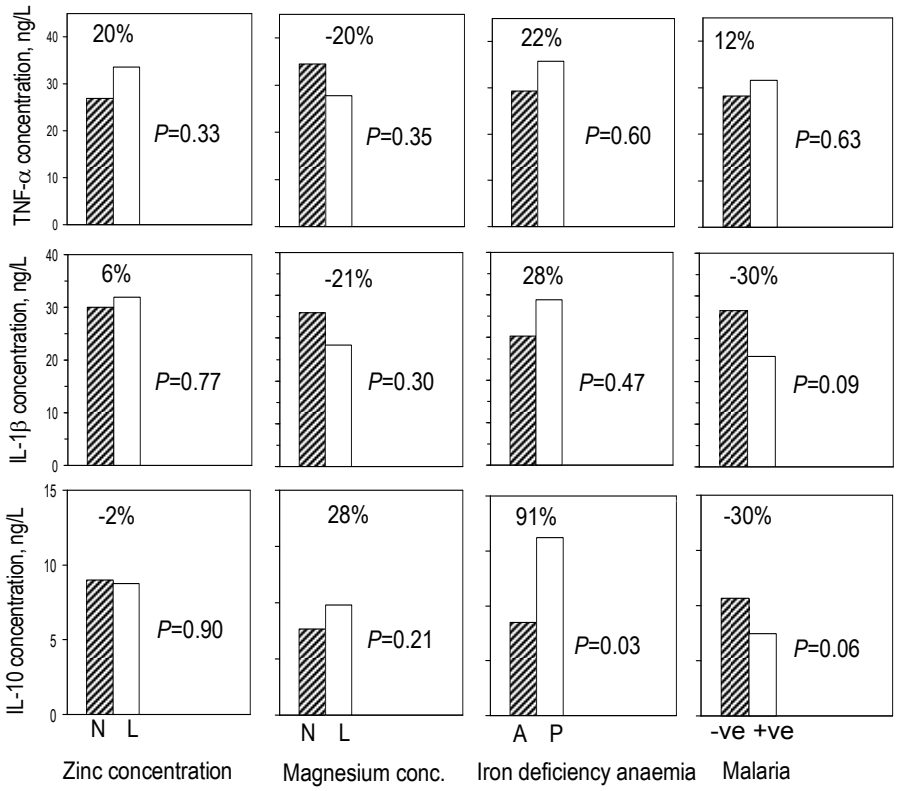

Figure 1: *Associations between nutrient status and supernatant cytokine concentrations, and between malaria infection status of the child at the time of blood collection and supernatant cytokine concentrations, following 24h of PBMC stimulation with Plasmodium falciparum-infected erythrocytes.*

N: normal concentrations; L: low concentrations (plasma concentrations of zinc and magnesium <9.9 µmol/L and <750 µmol/L, respectively); A: absence of iron deficiency anaemia; P: presence of iron deficiency anaemia (co-existing iron deficiency; plasma ferritin concentration <12 µg/L and anaemia; haemoglobin concentration <110 g/L). –ve: malaria negative; +ve: malaria positive. Percentages indicate paired group differences in supernatant cytokine concentrations, expressed as percentages relative to values observed in groups with normal plasma zinc or magnesium concentrations. P-values were obtained by assessing by multivariate analysis to what extent the proportional change in cytokine concentration that is associated with nutrient or malaria status.

Association between nutrient indicators and in vitro innate cytokine production, by malaria infection status at the time of blood collection: When analysing IL-10 concentrations, we excluded all individuals with IL-10 concentrations below the detection limit. In some instances, differences in cytokine concentrations between nutrient replete and deficient children (**figure 2**) seemed to depend on malarial infection status at the time of blood collection (**table 2**). The profile of supernatant cytokine concentration appeared different between subjects with deficiencies in zinc, magnesium and with iron deficiency anaemia. In the absence of malaria infection at the time of blood collection, zinc deficiency was associated with marginal reductions in concentrations of TNF-α, IL-1β and IL-10. Amongst donors with malaria infection at the time of blood collection, zinc status was not associated with altered concentrations of IL-1β or IL-10, but low plasma zinc concentrations were associated with an increase in TNF-α concentration by 74% (11% to 240%, 95% CI). Malaria infection at the time of blood collection seemed to determine the magnitude of the association between low plasma zinc concentration and TNF-α concentration (9% reduction in children without malaria, as compared to 74% increase in their peers with malaria; although the statistical evidence for this difference was weak (P=0.15).

Magnesium deficiency, on the other hand, was associated with increased concentrations of IL-10; this increase was 46% in children without malaria, as compared to only 6% in their peers with malaria (**figure 2**). Low magnesium concentrations seemed associated with reduced concentrations of TNF-α and IL-1β by -25% (95% CI: -64% to 55%; P = 0.79) and -44% (-70% to 6%; P = 0.13), respectively, in children with malaria infection at the time of blood collection, although these differences may have been due to chance. These results are a reverse of the situation in zinc deficiency. The numbers of individuals with both iron deficiency anaemia and malaria (**table 1**) were too low to compare groups meaningfully.

Influence of malaria and nutrient indicators on associations between cytokine concentrations: We found no evidence that the associations between concentrations of TNF-α and IL-10 depended on zinc, magnesium or malaria status at time of blood collection, as indicated by differences in slopes of 17% (-44% to 147%; 95% CI, P=0.67) for zinc status, 10% (-47% to 127%; 95% CI, P=0.80) for magnesium status, or 3% (-24% to 39%; 95% CI, P=84) for malaria (**figure 3**). There was some evidence, however, that iron deficiency anaemia (IDA) influenced the relationship between concentrations of TNF-α and IL-10, as indicated by the difference between slopes of 119% (35% to 637%; 95% CI, P=0.20).

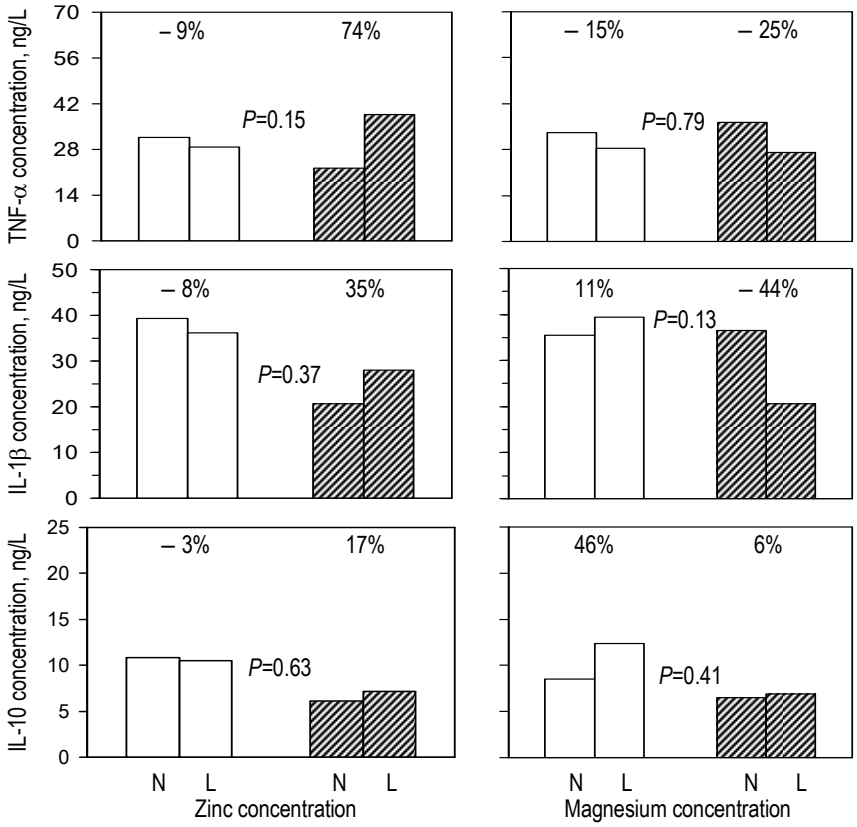

Figure 2: *Associations between nutrient status and supernatant cytokine concentrations following 24h of PBMC stimulation with Plasmodium falciparum-infected erythrocytes, by malaria infection status of the child at the time of blood collection.*

N: Normal concentrations; L: low concentrations (plasma concentrations of zinc and magnesium <9.9 µmol/L and <750 µmol/L, respectively). Data from children without and with malaria infection at the time of blood collection are indicated with open and shaded columns, respectively. Percentages indicate group differences in supernatant cytokine concentrations, expressed as percentages relative to values observed in groups with normal plasma zinc or magnesium concentrations. P-values were obtained by assessing by multivariate analysis to what extent the proportional change in cytokine concentration that is associated with nutrient status is different between children with and without malarial infection. The number of individuals with iron deficiency and malaria (table 1) was too small to meaningfully compare among groups.

Additional linear regression analyses (**figure 4**) showed evidence that zinc status influenced the association between concentrations of IL-1β and IL-10, as indicated by differences in slopes of 118% (4% to 359%; 95% CI, *P*=0.04). There was no evidence that malaria infection influenced the association between concentrations of TNF-α and IL-1β (**figure 4**, malaria panel), as indicated by a difference in the slopes of regression lines of 9% (-13% to 35%; *P*=0.47). In summary, these results show no evidence of influence on associations among innate cytokines, by the various conditions of micronutrient malaria status at time of blood collection except for zinc status, for which there was some evidence that it influenced the association between IL-1β and IL-10. We found no evidence of influence of micronutrient status and malaria on associations in other relationships. There were insufficient cases in all groups to explore and meaningfully compare the associations between IL-12 and other cytokines.

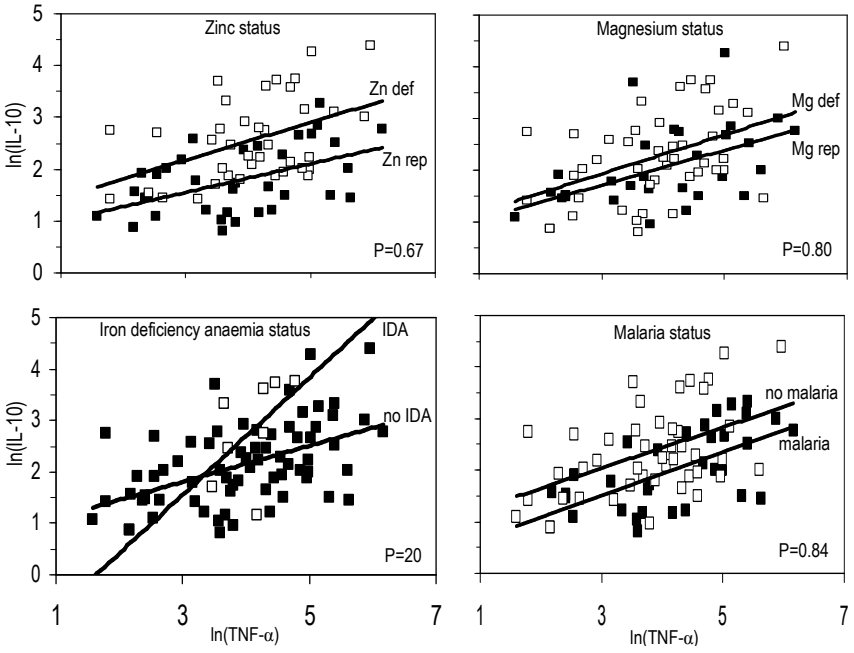

Figure 3: *Associations between supernatant concentrations of TNF-α and IL-10 following 24h stimulation of peripheral blood mononuclear cells with Plasmodium falciparum-infected erythrocytes, by micronutrient and malaria status at the time of blood collection,*

Black blocks = zinc or magnesium replete, no iron deficiency anaemia or no malaria; open blocks = zinc and magnesium deficiency, iron deficiency anaemia or positive results for

malaria tests at time of blood collection. P-values indicate probabilities of obtaining differences in associations between cytokine concentrations (as indicated by the slopes of the lines) as least as extreme as observed, assuming no differences.

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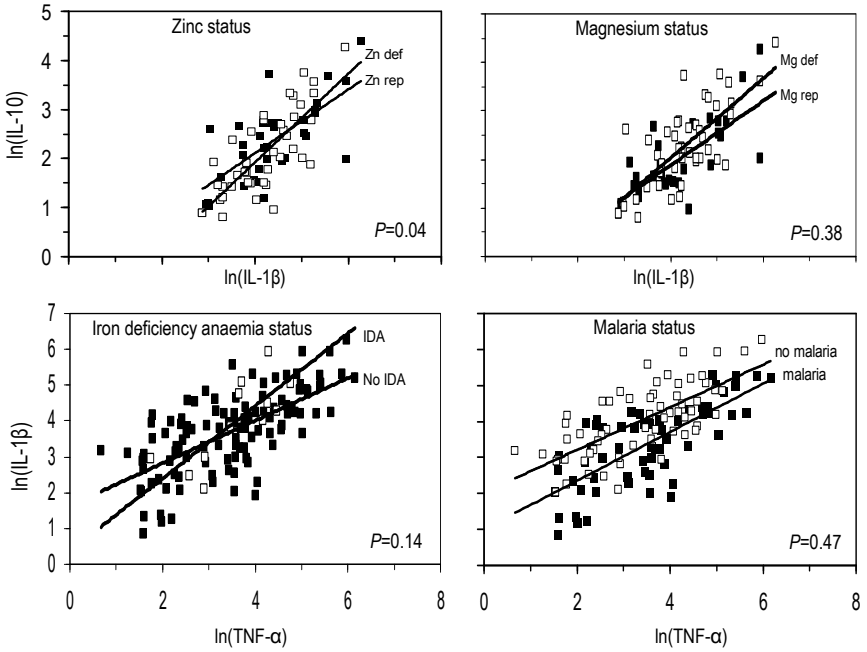

Figure 4: *Relationships between supernatant concentrations of TNF-α, IL-1β and IL-10 following 24h stimulation of PBMCs with Plasmodium falciparum-infected erythrocytes, under different conditions of micronutrient and malaria status at the time of blood collection.*

Black blocks = zinc replete, magnesium replete, or no malaria; open blocks = zinc deficiency or positive results for malaria test at time of blood collection.

Discussion

Effects of plasma concentrations of mineral elements on in vitro cytokine responses by PBMCs: Our biochemical data showed that most children in this study had nutrient deficiencies, particularly in zinc and magnesium and to a lesser extent iron deficiency anaemia. Zinc deficiency was associated with increased TNF-α responses in children with malaria infection at the time of blood collection but not in those without infection. TNF-α is a pro-

inflammatory cytokine resulting in malaria pathology if not properly regulated. We also found that, in children with malarial infection, zinc deficiency is associated with increased production of IL-1β and IL-10, even if this increase did not bring the levels to those reached by individuals in the non-infected group. This is important because IL-10 is required to limit the production of pro-inflammatory cytokines, so they do not lead to pathological consequences [31]. The low production of IL-10, however, could be due to the fact that the cytokine is said to be produced late (*in vivo*) following infection relatively to the innate cytokines. The initial production of TNF-α could also be the triggering factor by feedback mechanisms for production of IL-10 although Ramharter et al [32] reported increased responsiveness of *in vivo* primed cells as compared to malaria-naïve cells, with a tendency towards increased production of TNF-α. This can possibly explain the difference between subjects who were exposed or non-exposed at the time of blood collection, in response to *in vitro* stimulation in our study. These results show possible alterations in innate cytokine production particularly TNF-α and IL1-β due to the reported impaired macrophage functions and NK-cells activity in zinc deficiency [1, 2, 13, 33]. Interaction between these cells leads to the production of innate cytokines in the early stages of infections.

The relatively higher cytokines levels in individuals with malarial infection as compared to their uninfected peers (**figure 2**), however, can be explained by the priming of the immune system by malaria. Exposure of T cells to malaria leads to priming, so that these cells produce greatly increased amounts of IFN-γ when subsequently exposed. This cytokine is necessary for up-regulation of production of TNF-α and other pro-inflammatory cytokines in malaria infection [34, 35]. In this context, malaria-positive subjects are said to have γδT-cells primed with the capacity for immunological memory and highly contributing to rapid and early pro-inflammatory cytokine production following re-infection [35]. The increase in innate cytokine production in zinc-deficient individuals with malarial infection can be the result of a shift towards a pro-inflammatory immune response due to zinc deficiency in combination with prior priming of these cells due to previous exposure to malaria. The initial contact with the pathogen directs towards production of pro-inflammatory cytokines to limit infection. Loharungsikul et al [36] proposed Toll-like receptors (TLRs) to play a role in innate immune recognition in which the differential expression of TLRs on antigen presenting cells (APCs) could be regulated by the *P. falciparum* parasite. This could account for the increase in levels of TNF-α in malaria-positive individuals regardless of micronutrient status (**figure 1**). Studies from murine malaria [37] have revealed glycosylphosphatidyl inositols (GPIs) that anchor *P. falciparum* merozoite surface protein 1 (MSP1) and merozoite surface protein 2 (MSP2) to be the pathogen associated molecular patterns (PAMPs)

preferentially recognised by TLR-2 and TLR-4. The recognition and the interaction between these molecular patterns signal the induction of pro-inflammatory cytokine production. In addition, it is possible that parasite DNA attached to malarial pigment (hemozoin) produced in the course of infection further activates the innate immune response through TLR-9 engagement [38]. The expression of TLRs has been found to differ between malaria-infected and uninfected individuals, with higher expression being observed in infected patients [24, 39]. These recent studies have further indicated TLR-2 to be highly expressed in mononuclear cells, particularly monocytes of *P. falciparum*-infected children and that TLR-2 are well responsive following stimulation with pRBCs resulting into stronger signals with consequential change in cytokine production profiles.

The interesting result in this study is that the impact of magnesium deficiency on early cytokine responses followed a different profile from that observed with zinc status. Magnesium deficiency seemed to be associated overall with low TNF-α concentrations, low concentrations of IL-1β and higher concentrations of IL-10 in uninfected but not infected donors (**figure 2**). Low levels of pro-inflammatory cytokines in malaria are critical because they reduce the ability of the initial innate immune response to limit infection. Our results imply that magnesium deficiency directs early cytokine responses towards anti-inflammatory rather than pro-inflammatory cytokine responses, although further studies are still needed to confirm this hypothesis. The significantly increased IL-10 and variable alteration in levels of TNF-α and IL-1β in both malaria-negative and malaria-positive subjects with magnesium deficiency may explain the imbalance in cytokine production as a result of magnesium deficiency modulated by malaria status.

Methodological differences may explain contradictions between our findings and those from previous studies [5, 7, 9, 40]. We used parasitized erythrocytes to simulate the *in vivo* infection, whereas others used mitogens, lipopolysaccharides (LPS), phytohemagglutinin (PHA) and polyclonal stimulation. In addition, we used Ficoll-isolated PBMCs that had been stored for several months under frozen conditions, whereas whole blood stimulated within 15 minutes of collection was also used. McCall et al [24] stimulated freshly prepared PBMCs from adult naïve volunteers *ex vivo* with *P. falciparum* antigens. Our findings suggest that zinc and other micronutrients can protect against malaria infection by a different means such as targeting specific pathogenic processes of infection *in vivo* [41]. Nevertheless, the idea that zinc can also reduce production of pro-inflammatory cytokines by inhibiting signal transduction in monocytes in healthy human subjects [42], particularly IL-1β and TNF-α [2, 43, 424], should be further explored. The latter idea is also supported by *in vitro* studies [45, 46]

in other conditions than malaria.

In our study and those conducted by others [47, 48], IL-12 concentrations were below the detection limits. The most probable reason is the time required for maximal priming of pathogen recognition receptors (e.g. TLRs) on PBMCs by *P. falciparum*-parasitized erythrocytes. McCall et al [24] have shown that pro-inflammatory priming effects of *P. falciparum* require up to 48 hours to develop maximally, whereas we measured cytokines after 24 hours of stimulation. This priming is lacking in our culture system despite the reported poor *in vitro* induction of IL-12 by *P. falciparum* [49]. There may be a possibility that the levels were below the detection limits as a result of IL-12B gene promoter polymorphisms that is reported to be associated with low IL-12 production and increased malaria mortalities in children [50]. This gene encodes for the IL-12p40 cytokine and has been determined in Tanzania. Early IL-12 activity is also liable to suppression by transforming growth factor (TGF)-β [51, 52] that has been reported to variably influence and result in weak IL-12 activation and production, at least *in vivo*. Most of our donors responded towards production of IL-1β rather than TNF-α and IL-10. This is interesting since although different arguments reveal the pathological effect of IL-1β on cerebral malaria and severity of the disease in children [53], IL-1β together with other pro-inflammatory cytokines like IFN-γ and IL-6 is said to be protective against malaria by inducing parasite killing by monocytes, macrophages and neutrophils [54]. Production of IL-1β is induced by direct interaction between zinc and monocytes through activation of interleukine-1 receptor associated kinase (IRAK) which is dose-dependent [44]. Lower *in vivo* zinc levels, partially inhibit IRAK leading to diminished but not completely inhibited normal T-cells IL-β response. Results from this study may also reflect that stimulation of cryopreserved PBMCs by pRBCs results in a gradual production of innate cytokines preceded by IL-1β from the monocytes.

Table 1. Characteristics of the study population, by malarial infection status

	Plasmodium-infected	*Plasmodium*-uninfected	P-value
Sex			0.56
Male	65	70	
Female	75	94	
Age class			0.03
6-12 months	7	19	
12-24 months	18	31	
24-48 months	61	58	
48-72 months	54	55	
Zinc deficient [1]			0.49
Yes	63	81	
No	74	80	
Magnesium deficient [2]			0.63
Yes	87	107	
No	50	54	
Iron deficiency anaemia [3]			<0.001
Yes	2	24	
No	138	140	

[1] Plasma zinc concentration <9.9 µmol/L; [2] plasma magnesium concentration <750 µmol/L; [3] anaemia (haemoglobin concentration <110 g/L) and iron deficient (plasma ferritin concentration <12 µg/L.

Association between innate cytokines under different conditions of micronutrients and malaria status: The association between IL-1β and IL-10 was found to be influenced by zinc status (**figure 4**). The two innate cytokines TNF-α and IL-1β are a prerequisite in early responses to malaria infection and IL-10 is an important regulatory cytokine affected by nutrient deficiencies and malaria infection status. This is critical under tropical situations where both micronutrients deficiencies and malaria prevail, posing a challenge to the early immune response to infections.

Table 2. Influence of nutritional indicators on innate cytokines responses after 24-h *in vitro* stimulation of PBMCs with malaria-infected erythrocytes

Nutrient status indicator	Supernatant concentration (ng/L) after 24h of stimulation					
	TNF-α		IL-1β		IL-10	
	Without malaria	With malaria	Without malaria	With malaria	Without malaria	With malaria
Plasma zinc concentration						
Normal	31.7 (32)	22.2 (27)	39.3 (37)	20.7 (27)	10.8 (25)	6.1 (12)
Low	28.8 (28)	38.7 (31)	36.2 (36)	28.0 (35)	10.5 (20)	7.2 (18)
Difference	-9% [-51% to 67%]	74% [11% to 240%]	-8% [-49% to 68%]	35% [26% to 146%]	-3% [-42% to 63%]	17% [-34% to 107%]
Plasma magnesium concentration						
Normal	33.2 (25)	36.3 (19)	35.5 (30)	36.5 (19)	8.5 (18)	6.5 (12)
Low	28.4 (35)	27.2 (39)	39.4 (43)	20.6 (43)	12.4 (27)	6.9 (18)
Difference	-15% [-54% to 59%]	-25% [-64% to 55%]	11% [-40% to 104%]	-44% [-70% to 6%]	46% [13% to 144%]	6% [-40% to 87%]
Iron deficiency anaemia						
Absent	27.1 (51)	31.6 (61)	35.3 (63)	26.1 (64)	9.3 (37)	7.6 (32)
Present	33.9 (11)	64.9 (1)	43.8 (13)	17.7 (2)	19.9 (8)	3.2 (1)
Difference	25% [-45% to 183%]	108% [84% to 2769%]	24% [-42% to 170%]	-32% [-88% to 270%]	113% [13% to 301%]	-58% [-92% to 104%]

Values indicate geometric means (n) or effect [95% CI]. Cut-off values to define low plasma concentrations of nutritional indicators: see text. When computing differences between values, the category with normal plasma concentration was used as the reference. 'With malaria' and 'without malaria' refers to infection status of children studied at the time of blood collection.

In conclusion, we have shown micronutrient deficiencies to variably influence some *in vitro* innate cytokine concentrations. Zinc deficiency in particular, was found to possibly influence the *in vitro* production of various innate cytokines that particularly are modulated by malaria status. Magnesium deficiency, on the other hand, seemed to associate with higher concentrations of IL-10 in donors uninfected at time of blood collection. These results may be speculative indicators that while zinc deficiency and possibly iron deficiency anaemia might increase pro-inflammatory cytokines such as IL-1β and TNF-α, magnesium deficiency may have greater influence on anti-inflammatory cytokines such as IL-10. With regards to early innate cytokine responses to malaria, an ideal situation should be to supplement children with a combination of a few precisely selected micronutrients rather than single nutrients, although further studies involving larger sample sizes still need to be performed. This study has indicated the effect of poor nutrition on innate immune responses in children from malaria endemic area and how malaria infection may modulate these relationships. The findings have also shown plasticity in cytokine profiles of mononuclear cells reacting to malaria infection under conditions of different micronutrient deficiencies. Our findings therefore lay the foundations for future inclusion of selected micronutrients in malaria vaccine intervention programs, particularly in developing countries, to boost immune response to malaria.

Acknowledgement: We received financial support from the Netherlands Organization for Scientific Research, NWO/WOTRO (grant numbers W93-413, WAO93-441, and WIZ93-465) and UN Children's Fund (UNICEF). HV is currently supported by the European Community's Seventh Framework Programme under grant agreement no 211484. We gratefully acknowledge the participating children, parents and the assistance given by community leaders, volunteers and colleagues in Tanzania and The Netherlands, and Marga van der Vegte, Henry Witteveen and Robert Sauerwein, Radboud University, Nijmegen, The Netherlands, for providing parasitized erythrocytes. This paper was published with support of the Executive Director of KCMC, Moshi and the Director-General of the National Institute for Medical Research, Dar es Salaam, Tanzania. Marita Troye-Blomberg and Nele Wellinghausen are acknowledged for their material support.

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CHAPTER

4

Effect of nutrient deficiencies on *in vitro* T_h1 and T_h2 cytokine response of peripheral blood mononuclear cells to *Plasmodium falciparum* infection

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Submitted

Abstract

An appropriate balance between pro-inflammatory and anti-inflammatory cytokines that mediate innate and adaptive immune responses is required for effective protection against human malaria and to avoid immunopathology. In malaria endemic countries this immunological balance may be influenced by micronutrient deficiencies. Here, we stimulated peripheral blood mononuclear cells from Tanzanian preschool children *in vitro* with *Plasmodium falciparum*-infected red blood cells to determine T-cell responses to malaria under different conditions of nutrient deficiencies and malaria status. Our data indicate that zinc deficiency is associated with an increase in TNF-α response by 37%; 95% CI: 14% to 118% and IFN-γ response by 74%; 95% CI: 24% to 297%. Magnesium deficiency, on the other hand, was associated with an increase in production of IL-13 by 80%; 95% CI: 31% to 371% and a reduction in IFN-γ production. These results reflect a shift in cytokine profile to a more type I cytokine profile and cell-cell mediated responses in zinc deficiency and a type II response in magnesium deficiency. Our data also reveal a non-specific decrease in cytokine production in children due to iron deficiency anaemia that is largely associated with an malaria infection status. The pathological sequel of malaria potentially depend more on the balance between type I and type II cytokine responses than on absolute suppression of these cytokines and this balance may be influenced by a combination of micronutrient deficiencies and malaria status. In conclusion, it should be further assessed to what extent supplementation with zinc, magnesium and iron is beneficial in children with deficiencies for these nutrients.

Introduction

Frequent or chronic exposure to *Plasmodium falciparum* infection is thought to be a key element to immune protection against malaria in endemic areas [1]. Although the human immune system can kill parasites, it can also contribute to severe disease if not regulated and controlled to optimal levels [2, 3]. In African countries, micronutrient deficiencies are common and may modulate immunity and predispose to infections. This is particularly relevant for young children who are most at risk of both malaria and micronutrient deficiencies.

Deficiencies in mineral elements and vitamins can result in suppression of innate, T-cell mediated and humoral responses [4, 5]. Coordinating these responses are the cytokines which are produced interactively by several types of immune cells [2, 4]. The immune response to malaria is specific for individual developmental stages of the parasite, and the balance in production of pro-inflammatory and anti-inflammatory cytokines seems to be critical for prognosis [5, 6]. Following presentation of malaria antigens by antigen-presenting cells including dendritic cells, macrophages and occasionally B cells, naïve T helper (T_h) cells proliferate and differentiate into specific T_h cell subsets. The pattern of T_h cell types, and the associated cytokine profile, probably depends on the type of antigen-presenting cells and their cytokine milieu, and on regulatory T-cells that suppress the proliferation and activity of B cells and T_h cells by the production of IL-10 and transforming growth factor (TGF)-β. Imbalance in these responses can result in an inefficient adaptive immune response to clear infection, and may contribute to pathological consequences. Several reports [7-15] have indicated possible roles of micronutrients on immune responses but either they have focused on other infections than malaria, or their effects have been evaluated in individuals older than 5 years, the age with the highest vulnerability to malaria.

We hypothesized that the adaptive cytokine response to *Plasmodium falciparum* is influenced by micronutrient deficiencies that result in an imbalance between T_h1 cells, with interferon (IFN)-γ as a signature cytokine, and T_h2 cells, characterized by the production of interleukin (IL)-4, IL-5 and to some extent IL-13. We isolated peripheral blood mononuclear cells (PBMCs) from Tanzanian children aged 6-72 months, and assessed *in vitro* the cytokine responses of these PBMCs upon exposure to erythrocytes parasitized by *P. falciparum*. We compared these responses between donors with and without micronutrient deficiencies and in addition, we assessed to what extent the magnitude of PBMCs cytokine responses depended on *P. falciparum* infection status of the child at the time of blood collection.

Subjects and methods

Study area and population: The field work for this study was conducted in a lowland area around Segera village (S 05º 19.447', E 38º 33.249'), Handeni District, north-eastern Tanzania, in May-July 2006. Malaria is highly endemic in this area, with virtually all infections being due to *P. falciparum*. The local population comprises mostly poor farmer families growing maize and cassava for subsistence use. The study was approved by both Ethics Review Committees in The Netherlands and Tanzania (for Tanzania ethics review bodies, the reference numbers for KCMC and National Ethics Review Committee were 094 and NIMR/HQ/R.8a/VolIX/540, respectively). Informed consent was obtained from community leaders and local government officials, and from parents or guardians.

Sampling methods, eligibility criteria and preliminary laboratory analyses: The details of sampling method, field procedures, isolation of peripheral blood mononuclear cells (PBMCs) are provided elsewhere [16, 17]. In brief, children aged 6-72 months were recruited in the study and were clinically examined before sample collection. Children were eligible to participate if they had no signs of severe febrile disease or severe malnutrition at the time of assessment. Dip stick test was used for diagnosis complimenting microscopy and providing a wide chance for detecting asymptomatic malaria infection [18, 19]. Whole blood samples from the study children were collected after overnight fasting. PBMC were isolated using Ficoll density gradient centrifugation. *P. falciparum*-parasitized and unparasitized erythrocytes were prepared as described elsewhere [20, 21], and kept under frozen conditions until the stimulation experiments.

Determination of plasma indicators of mineral element status: Plasma samples were diluted 20 times in milliQ [22], and concentrations of zinc, magnesium and copper were measured by inductively-coupled plasma atomic emission spectrometry (ICP-AES) (Vista Axial, Varian, Australia). To determine variability in outcomes, measurements were replicated five times: with mean values set at 100%, measurements varied between 97% to 102% for zinc, 99% to 102% for magnesium, and 97% and 102% for copper. Plasma concentrations of ferritin and C-reactive protein were measured as indicators of iron stores and inflammation, respectively by using a Behring nephelometer (BN ProSpec; Dade-Behring) in The Netherlands (Meander Medical Centre) and will be reported separately.

PBMCs stimulation: PBMCs were cultured at 10^6 cells/well in sterile polystyrene 48-well plates with flat-bottom wells (Corning, Cat No. 3548, NY 1483, USA) in Yssel's culture medium [23], which is a modification of Iscove's modified Dulbecco's medium (IMDM),

lacking the nutrients considered in this study. The medium is recommended for the culture of cells growing in suspension, such as human T and B cell lines and is especially recommended for the generation and long-term culture of antigen-specific T cell and NK clones [24, 25]. Aliquots of *P. falciparum*-parasitized red blood cells (pRBC) were thawed, re-suspended in Yssel's^{+} medium [17] with 1% human AB^{+} serum plus 1% penicillin-streptomycin and 1% fungizone (Gibco-BRL, Invitrogen, Grand Island NY, USA), and added to PBMCs in a ratio of 2:1 (2×10^6 pRBC to 1×10^6 PBMC). PBMC were also cultured under similar conditions with unparasitized erythrocytes (uRBC) ($2'10^6$ cells/well) as a negative control, and with soluble antibodies to CD3 and soluble antibodies to CD28 (Cat. No.555336 and 555725, Becton-Dickinson Pharmigen, Alphen aan den Rijn, The Netherlands) as a positive control. Cell culture plates were incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2. Based on previous studies [26-28] and our own preliminary experiments (Mbugi E, Meijerink M et al, unpublished data), we expected 7 days of continuous stimulation to be optimal for observing differences in PMBCs responses to exposure with non-parasitized and parasitized RBCs. Thus after 6 days of culture, monensin was added to cells to allow for accumulation and subsequent staining of intracellular cytokines. At day 7 of culture, aliquots of supernatant were collected from parallel non-monensin treated cultures; concentrations of type I cytokines (IL-1β, IL-12p70, TNF-α, ILN-γ) and type II cytokines (IL-4, IL-5, IL-10, IL-13) in culture supernatants were measured using a Cytometric Bead Array System (FACSCanto, Becton-Dickinson).

Proliferation and activity of leukocyte subsets: Proliferation assays were performed to determine the activity potential of cells and to determine whether selected individuals displayed intrinsic differences in their T-cell compartments. To distinguish PBMCs subsets, we stained cultured cells (5×10^5) for 30 min, at 4^{o}C in the dark with a combination of fluorophore-bound antibodies against CD4 (T helper cells), CD8 (cytotoxic T cells) and CD45 (all leucocytes) (Becton-Dickinson Pharmingen, Alphen aan den Rijn, The Netherlands). The cells were then centrifuged (500×*g*, 5 min, 4°C), washed, re-suspended in PBS for subsequent staining for Ki-67 protein. This protein is present during all active phases of the cell cycle, but not in resting cells [29]. After CD marker staining, cells were fixed and permeabilised by incubation (15 min, 4°C, dark condition) with BD Cytofix/Cytoperm (catalogue no. 554722, Becton-Dickinson Pharmingen). The cells were subsequently washed twice with BD Perm/Wash buffer™ (catalogue no.554723, Becton-Dickinson Pharmingen), centrifuged (300×*g*, 10 min, 4°C), re-suspended in BD Perm/Wash buffer and incubated with Ki-67 detection antibodies (catalogue no. 556026, Becton-Dickinson Pharmingen) (30 min, 4°

C, dark conditions). Thereafter, the cells were washed twice with perm/wash buffer and suspended in PBS with counting beads for subsequent flow cytometry.

Intracellular cytokine staining: Cultured cells (5×10^5) were incubated (for 30 min, at 4°C in the dark) with 20% human AB serum in PBS to block Fc receptor binding and stained with antibodies against CD4 and CD25 to detect activated Th cells, centrifuged (500×*g*, 5 min, 4° C), fixed, permeabilized and washed as described above, and re-suspended in PBS for subsequent intracellular staining for IL-10 and IL-4 using antibodies against these cytokines (BD Pharmigen, Alphen aan den Rijn, The Netherlands). After incubation with anti-IL-10 and anti-IL-4 detection antibodies, the cells were washed twice with perm/wash buffer and re-suspended in PBS for analysis by flow cytometry.

Flow cytometry: Analyses were performed on a FACSCanto II flow cytometer and analysed with FACSDiva™ software (both Becton-Dickinson Biosciences).

Statistical analysis: Data were entered and analysed using SPSS for Windows (version 15.0. SPSS Inc., Chicago, IL, USA). Zinc deficiency and low zinc status were defined as plasma zinc concentrations <9.9 μmol/L and <10.7 μmol/L, respectively; low magnesium status was defined by magnesium concentration <750 μmol/L; iron deficiency anaemia was defined by coexisting iron deficiency (plasma ferritin concentration <12μg/L) and anaemia (haemoglobin concentration <110 g/L). Because we found no evidence for copper deficiency as assessed by plasma copper concentrations (<7.1 μmol/L) (data not shown), we currently only report the results for zinc, magnesium and iron deficiency anaemia. Cytokine concentrations were log-transformed to obtain normally distributed values. Group differences were analysed assuming t-distributions, and associations between continuous variables were assessed using linear regression analysis. Effects of log-transformed data were expressed in their natural units by exponentiation, and reported as the percentage difference relative to the reference value. We only report the analyses of the cytokine responses to pRBCs. As expected, the average response to uRBCs (negative control) was less than to pRBCs, whereas the average response to CD3/CD28 (positive control) was higher. Correction for these responses does not change the estimates of the associations between nutrient status and cytokine responses, or between malarial infection status and cytokine responses.

Results

Study population and characteristics: The study population consisted of 304 children; 301 were within the eligible age range; for 3 children we found after recruitment that they were

older but these were retained in the analysis. Characteristics of the study population and crude associations between malarial infection and nutrient markers are provided elsewhere [16] and (Chapter 3). In short, the following prevalence values were found: malarial antigen as assessed by dipstick test: 45.2 %; low zinc status 63.1% (188); zinc deficiency: 48.3% (144); low magnesium status: 65.1% (194); iron deficiency anaemia: 9.4% (26); malaria: 46.1% (140). Malaria status at inclusion was found to associate with age and iron deficiency anemia, but not with zinc or magnesium deficiency (Mbugi et al, chapter 3).

Induction of cytokine production: Lower concentrations of cytokines were detected in uRBC than in pRBC indicating the differences in *in vitro* mitogenic activities on PBMC. Stimulated PBMC responded more strongly with IFN-γ production as compared to other cytokines. Production of IL-4 was lowest regardless of the micronutrient status. In general, the composition of cytokines in day 7 supernatants consisted of IFN-γ, TNF-α, IL-1β, IL-13, IL-10, IL-12p70, IL-5 and IL-4, in declining order of concentration.

Proliferation and intracellular cytokine staining: The average proportion of malaria extract-specific proliferating cytotoxic T-cells ($CD8^+$ $Ki67^+$) and proliferating T_h cells ($CD4^+$ $Ki67^+$) relative to the general proliferating leucocytes were 4% and 21%, respectively. In **figure 1** a representative example of a flow cytometric analysis of a malaria-specific CD4+ T-cell response in the PBMCs of a malaria-infected child is shown. Of the leukocytes responding to the malaria extract after 7 days of culture, on average 20% of the leukocytes were activated Th cells ($CD4^+$ $CD25^+$; 23 % in **figure 1**), part of them may be naturally occurring or probably inducible regulatory T-cells (Tr). Intracellular cytokine staining revealed that the proportion of $CD4^+/CD25^+$ cells producing both IL-4 and IL-10 (average 19%; 7.2% in **figure 1**) was higher than cells producing only IL-10 (average 4%; 2.5 % in **figure 1**) or only IL-4 (14 % in **figure 1**). This indicated that most anti-inflammatory cytokine response came from $IL\text{-}4^+IL\text{-}10^+$ double producing cells, rather than from single $IL10^+$ cells.

Table 1. Associations between nutrient deficiencies and type I cytokine responses to *in vitro* stimulation of PBMCs with malaria-parasitized red blood cells

	Supernatant concentration (ng/L) after 7 days of stimulation			
Nutrient status	IL-12	TNF-α	IFN-γ	IL-1β
Zinc				
Deficient	2.4 (19)	4.3 (35)	46.6 (42)	4.5 (33)
Replete	2.4 (13)	3.2 (28)	26.8 (37)	5.5 (27)
Effect	-1% [-29% to 38%]	37% [14% to 118%]	74% [24% to 297%]	-19% [-47% to 26%]
Magnesium				
Deficient	2.4 (23)	3.7 (43)	34.1 (56)	4.9 (41)
Replete	2.3 (9)	3.9 (20)	41.1 (23)	4.8 (19)
Effect	4% [-28% to 51%]	-6% [-43% to 57%]	-17% [-66% to 108%]	3% [-36% to 64%]
Iron deficiency anaemia				
Yes	2.88 (15)	3.82 (18)	37.50 (19)	5.18 (25)
No	2.11 (16)	3.72 (49)	39.21 (61)	4.55 (38)
Effect	37% [1% to 88%]	3% [-34% to 61%]	-4% [-58% to 120%]	14% [-25% to 73%]

Values indicate geometric means (n) or effect [95% CI]

Table 2: Associations between nutrient deficiencies and type II cytokine responses to *in vitro* stimulation of PBMCs with malaria-parasitized red blood cells

	Supernatant concentration (ng/L) after 7 days of stimulation		
Nutrient status	IL-5	IL-10	IL-13
Zinc			
Deficient	6.6 (15)	3.2 (29)	10.0 (31)
Replete	5.2 (14)	3.4 (26)	7.3 (28)
Effect	26% [-54% to 146%]	-6% [-35% to 35%]	37% [-41% to 219%]
Magnesium			
Deficient	6.5 (22)	3.4 (40)	10.0 (44)
Replete	4.3 (7)	3.2 (15)	5.6 (15)
Effect	49% [-54% to 382%]	5% [-30% to 58%]	80% [31% to 371%]
Iron deficiency anaemia			
Yes	4.63 (15)	3.51 (26)	8.30 (20)
No	7.01 (15)	3.35 (30)	9.32 (41)
Effect	-34% [-75% to 74%]	5% [-27% to 50%]	-11% [-60% to 100%]

Values indicate geometric means (n) or effect [95% CI]

Influence of nutrient deficiencies on cytokine responses to stimulation with malaria parasites: We next explored the association between nutrient deficiencies and cytokine responses assuming no interaction with malarial infection. The effect change in cytokine concentration under different conditions of micronutrients status are shown in **tables 1 and 2**, respectively. For this analysis only the data were used of individuals of which the PBMCs cultures stimulated by malaria extract yielded detectable cytokine levels that are indicative of malaria-specific responding T-cells by induced proliferation and cytokine synthesis. Thus the number of individuals differ for every individual cytokine analyzed. Overall, zinc deficiency was associated with increased supernatant concentrations of TNF-α and IFN-γ (by 37% and 74%, respectively), and seemed associated with increased concentrations of IL-5 and IL-13 **(tables 1 and 2).** Magnesium deficiency was associated with an 80% increase in IL-13 concentrations, and seemed associated with a 49% increase in IL-5 concentrations. Iron deficiency anaemia was associated with increased concentrations of IL-12 by 37%, and seemed associated with a 34% decrease in IL-5 concentrations.

Interaction between nutrient deficiencies and malarial infection on cytokine response to stimulation: In some cases, malarial infection at the time of blood collection seemed to influence the associations between nutrient deficiencies and cytokine responses to stimulation. For example, in children without malaria, zinc deficiency was associated with an increase in supernatant concentration of IFN-γ by 114%; 95% CI: 41% to 677% as compared to an increase of 40%; 95% CI: -53% to 314% in their peers with malaria infection. Similarly, in children without malarial infection, iron deficiency anaemia was associated with a decrease in IFN-γ concentration by 23% (95% CI: -78% to 177%) as compared to a 60% increase (95% CI: 45% to 368%) in their peers with malaria infection. In none of these cases, however, was such interaction supported by statistical evidence, as indicated by the high *P*-values in **figures 2 and 3.** In other words, there is no evidence that the relationships between nutrient markers and cytokines depend on malarial infection. This reflects that nutritional deficiencies association with *in vitro* cytokine responses is independent of malaria status at time of blood collection.

Table 3: Effect change in linear relationships and differences in slopes of supernatant concentrations of cytokines measured after 7 days of stimulation with malaria antigens in children with different nutritional and malaria status at time when blood was collected.

Predictors	Cytokine pairs	Change in slope	95% CI
Zinc	IFN-γ vs IL-5	32%	-62% to 357%
Zinc	IFN-γ vs IL-10	-12%	-43% to 35%
Zinc	IFN-γ vs IL-13	12%	-52% to 157%
Magnesium	IFN-γ vs IL-5	-38%	-89% to 256%
Magnesium	IFN-γ vs IL-10	-36%	-61% to 5%
Magnesium	IFN-γ vs IL-13	-20%	-71% to 118%
IDA	IFN-γ vs IL-5	-45%	-81% to 58%
IDA	IFN-γ vs IL-10	**-48%**	**-63% to -36%**
IDA	IFN-γ vs IL-13	-26%	-66% to 58%
Malaria	IFN-γ vs IL-5	174%	-5% to 689%
Malaria	IFN-γ vs IL-10	-23%	-17% to 83%
Malaria	IFN-γ vsIL-13	40%	-34% to 197%

IDA: Iron deficiency anaemia

Relationship between IFN-γ and type II cytokines: Lastly, we explored the influence of nutrient deficiencies on the relationships between IFN-γ and some type II cytokines (**table 3, figure 4**). Overall, there was a clearly detectable positive linear relationship between IFN-γ and IL-5, IL-10 and IL-13. There was weak evidence however, that the slopes of regression lines differ with zinc, magnesium or malaria infection status. On the other hand, there was strong evidence that the slopes of regression lines for the association between IFN-γ and IL-10 differed with iron deficiency anaemia status (P = 0.001). The change in slopes in the latter relationship was such that in iron deficiency anaemia there existed a negative linear relationship signifying that an increase in IFN-γ lead to a decrease in IL-10. There was no evidence that the slopes of regression lines for the association between IFN-γ and IL-10 differed (**figure 4**) in zinc deficiency, magnesium deficiency and malaria infection. However, the results indicate that a smaller increase in concentration of IL-10 was associated with a relatively larger increase on IFN-γ (slopes). Subsequent analysis on whether deficien-

cies and malaria infection status influence the association among T_h1 and T_h2 cytokines, apart from IFN-γ (data not shown) revealed an overlap in slopes of linear associations. There was weak evidence that magnesium deficiency, zinc deficiency and malaria infection at time of blood collection influenced these associations.

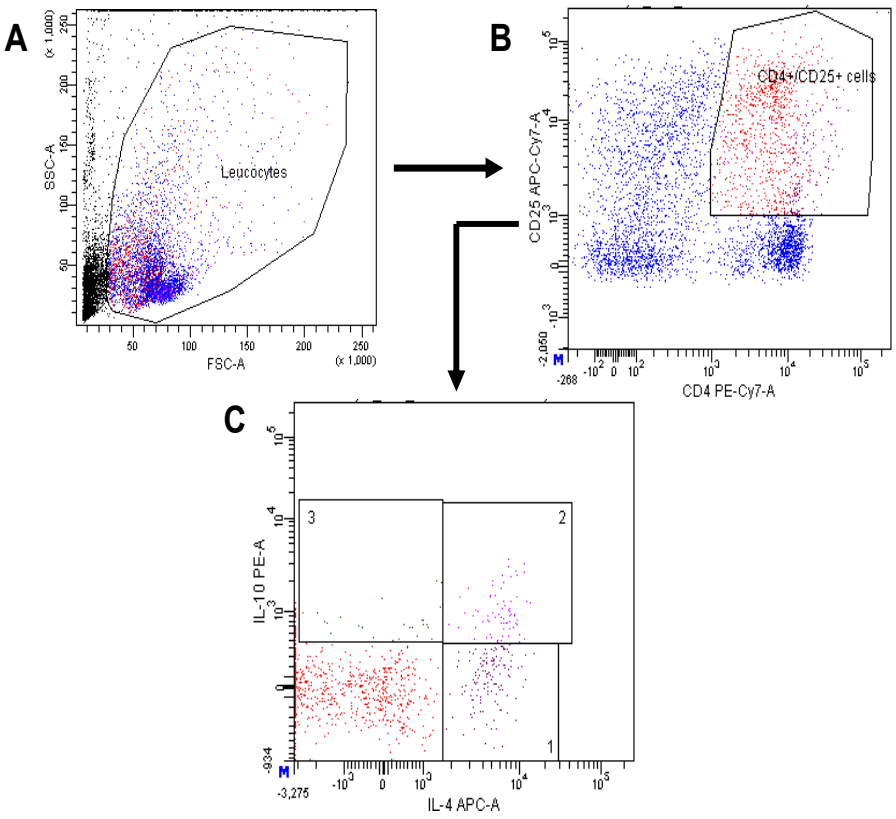

Figure 1: *Representative example of the flowcytometric analysis of the IL-10 secreting $CD4^+CD25^+$ population of PBMCs stimulated with malaria extract for 7 days.* ***A****. Leukocytes were identified by forward (FSC) and sideward (SSC) scatter.* ***B.*** *Of these leukocytes staining was performed with CD4-PE-Cy7-A and CD25-APC-Cy7-A labelled monoclonal antibodies from BD Pharmingen.* ***C.*** *The $CD4^+CD25^+$ double positive cells were stained intracellularly with IL-4 APC-A and IL-10 PE-A labelled monoclonal antibodies from BD Pharmingen. Panel 1 contains 14% IL-4 single positive $CD4^+CD25^+$ cells, panel 3 contains 2.5% IL-10 single positive $CD4^+CD25^+$ cells, and panel 2 contains 7.2% $IL\text{-}4^+IL\text{-}10^+$ double positive $CD4^+CD25^+$ cells.*

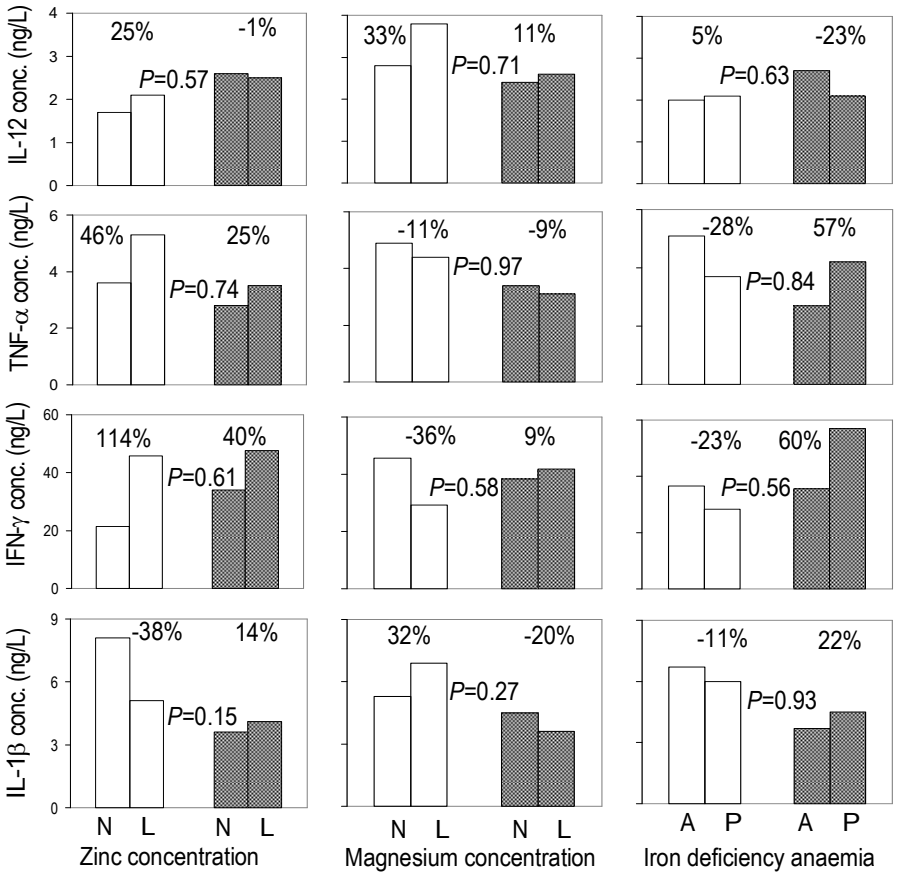

Figure 2: *Associations between micronutrient status and supernatant type I cytokine concentrations following 7 days of PBMCs stimulation with Plasmodium falciparum-infected erythrocytes, by malaria infection status of the child at the time of blood collection.*

N: Normal concentrations; L: low concentrations; A: absent; P: present. Percentages indicate paired group differences in cytokine concentrations. Data from children without and with malaria infection at the time of blood collection are indicated with open and shaded bars, respectively. P-values indicates the interaction between nutrition and malaria infection status.

Discussion

Cytokine production: The results from this study reveal that pro-inflammatory cytokine do not only initiate immune response to infections but are also responsible to make it persistent and effective. We have revealed a cytokine concentration of IFN-γ, TNF-α, IL-1β, IL-13, IL-10, IL-12, IL-5 and IL-4 in declining order following a 7 days *in vitro* stimulation of PBMCs using pRBCs (**tables 1 and 2**). The cytokine concentration in the supernatant following *in vitro* stimulation cannot be extrapolated to number of responding cells but at least hints on what could happen *in vivo* amid natural infection. The relatively low concentrations of IL-12, IL-5 and IL-4 may reflect that probably these cytokines are needed in very minutes amounts, present only very temporarily or are gradually degraded or consumed by cells earlier after response to infection. We anticipate that these cytokines are more active earlier than 7 days. Comparably, in both zinc replete and zinc deficient groups cells seemed to respond better towards production of IFN-γ, TNF-α, IL-1β, IL-13 and IL-10 than other cytokines *in vitro*. This might mean that these cytokines are crucial for continued elimination of the parasite at different stages of infection (pre-erythrocytic and erythrocytic stages) *in vivo* although this hypothesis may be unjustifiable based only on *in vitro* data.

Effects of zinc and other micronutrients on cytokine production: In malaria endemic areas, repeated exposure to infection by *P. falciparum* results into naturally acquired immunity that fails to develop in areas where malaria is hypoendemic, epidemic or mesoendemic. This means that the potential mechanisms of protection and immunological memory depend among other factors, on the degree of exposure and pattern of malaria transmission [30]. Regardless of age, immunity to malaria is generally low in populations living in areas with low or unstable transmission. In such a situation, clinical malaria and possibly severe complications can occur in both children and adults [31]. Although it appears not to be sterile, immunity to malaria is protective provided there is a constant exposure to infection and may be strengthened by good nutrition. This study provides *in vitro* results on the effect of some nutrients on the mediators of immune response to malaria in Tanzanian children. We have used intact *P. falciparum* infected erythrocytes (pRBC) to induce immuno-regulatory cytokines [26, 32] reflecting the real *in vivo* situation. Among nutrients explored in this study, zinc, magnesium and iron deficiency anaemia was associated with variable concentrations of one or more cytokines from both Th1 and Th2 groups, that mediate the immune response to malaria. Prasad [33] reported zinc deficiency to cause an imbalance between T_h1 and T_h2 functions in an experimental human model in which production of IFN-γ (product of T_h1) was decreased and that no effects were predictable in production of IL-4 (and IL-10)

(products of T_h2). Our findings contrast with previous findings by Prasad in the sense that zinc deficiency was associated with higher levels of IFN-γ, TNF-α and IL-12 (figure 2) but concur with the findings on IL-10 (figure 3). This is especially intriguing as it may imply that in zinc deficiency, the immune response to malaria shifts to more cellular-mediated immune response before tailing off. Previous results [13, 34, 35] have indicated that zinc deficiency is associated with a decreased ratio of $CD4^+$ to $CD8^+$ cells and is indicative of cytotoxic immune response. It could be that, in our study, of the activated $CD4^+$ T-helper, T_h1 cells were dominant in producing type I cytokines in cells from zinc deficient children. Our study partly agrees with available reports that zinc deficiency affects both cell-mediated immune responses and humoral responses [14] and that B cell proliferation is less dependent on zinc, albeit zinc deficiency may result in fewer naïve B cells for production of antibodies to new antigens [36].

Magnesium deficiency was associated with an increase in the concentration of IL-13 among type II cytokines. Little work has been done on the role of magnesium in immune response to malaria and our results draw attention to the role of magnesium in cytokine production in reaction to malaria infection. Report [37] indicates that IL-13 and IL-4 are major cytokines driving the polarization of the immune response towards T_h2. IL-13 is also believed to regulate immunoglobulin switching from IgG isotype to IgE, this is particularly important because it signifies that prolonged magnesium deficiency may predispose individuals to hypersensitivity reactions. Type I cytokines dominate in cellular immune responses while type II cytokine dominance implies humoral immune response [38]. Our findings may reflect that magnesium deficiency is associated with an increase in IL-12 and IL-1β but these responses become weakened in malaria infection. On the other hand, the increase in IL-12 and IL-1β concentrations in zinc deficiency further go up in malaria infection (**figure 2**) which may imply that in zinc deficiency the potential for production of pro-inflammatory cytokines following malaria infection is high, rising the risk for development to pathology. To the contrary, in malaria-infected, magnesium deficient children, the concentrations of IL-12, IL-1β (type I) and IL-5, IL-10 and IL-13 (type II cytokines) do not increase to levels attained in (**figures 2 and 3**) uninfected peers. In other words, the increase in cytokine concentrations due to magnesium deficiency in malaria infection does not compensate for that observed in uninfected. Thus although we speculate that in zinc deficiency, the immune system is more likely to use cellular responses as a weapon to fight against malaria opting to antibody responses in case of magnesium deficiency, the responses in magnesium deficiency might be weaker than in zinc deficiency.

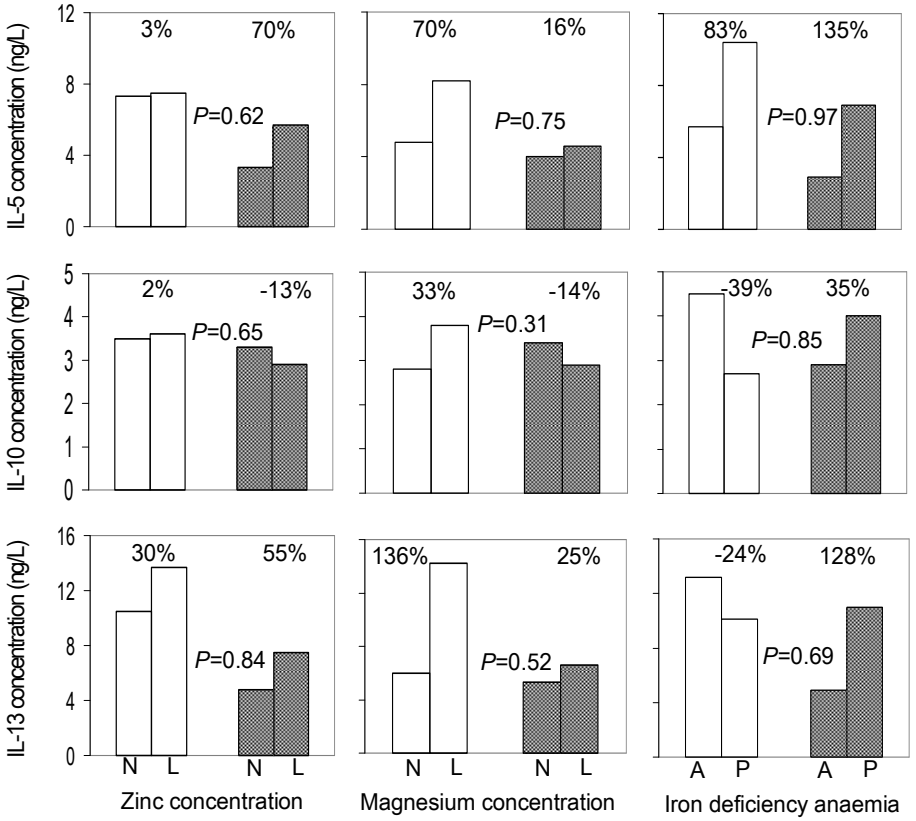

Figure 3: *Associations between micronutrient status and supernatant type II cytokine concentrations following 7 days of PBMCs stimulation with Plasmodium falciparum-infected erythrocytes, by malaria infection status of the child at the time of blood collection.*

N: Normal concentrations; L: low concentrations; A: absent; P: present. Percentages indicate paired group differences in cytokine concentrations. Data from children without and with malaria infection at the time of blood collection are indicated with open and shaded bars, respectively. P-values indicates the interaction between nutrition status and malaria infection status (nutrient vs. malaria).

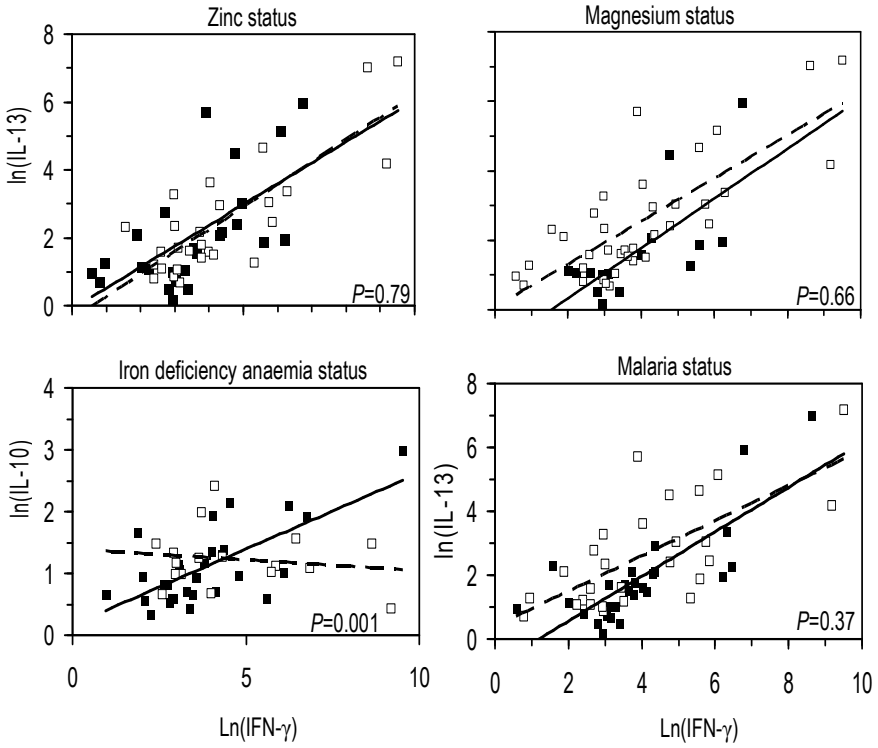

Figure 4: *Relationships between supernatant concentrations of IFN-γ and IL-10 under different conditions of micronutrient and malaria status at the time of blood collection, following 7 days stimulation of PBMCs with Plasmodium falciparum-infected erythrocytes.*

Regression lines and blocks in respective panels: closed blocks and solid lines = zinc replete, magnesium replete, no iron deficiency anaemia and no malaria; open blocks and dashed lines = zinc deficiency, magnesium deficiency, iron deficiency anaemia and positive malaria tests at time when blood was collected. The differences in slopes for other relationships are shown in Table 3. P-values have been calculated to indicates whether the interaction between nutrition status and malaria infection status have impact on the association between IFN-γ and IL-10.

The role of iron in the induction of a protective immune response is still debatable. Our findings indicate variable effects of iron deficiency anaemia on cytokine concentration. While levels of TNF-α, IFN-γ and IL-β (type I) and IL-10 and IL-13 (type II) seemed decreased in iron deficiency anaemia, the levels of IL-12 and IL-5 appeared increased (**figure 3**). These variable effects of iron deficiency on a range of both type I and type II cytokines are critical as these may lead to unstable cytokine response failing to inhibit the parasite. Reports on iron nutrition in children living in malaria endemic areas have indicated some association between IL-4 with all biochemical indices of iron [39]. In this study that was carried on the coast of Kenya, authors also report an increase in IL-10 serum mRNA expressions in malaria blood-smear positive children, results which are concordant with our *in vitro* results on iron deficiency anaemia despite the weak evidence.

Malaria infection status and the profile of cytokine production under conditions of nutrient deficiencies: Comparing within groups our findings show an increase in type I cytokines (TNF-α, IFN-γ and IL-β) in association with malaria infection in zinc deficient children as compared with zinc sufficient individuals, although this increase is less than amongst uninfected donors. However, an increase in IL-12 concentration seems to be independent of zinc deficiency (**figure 2**) signifying that malaria infection is associated with induction of increased IL-12 production independent of zinc status. The levels of IL-12 at day 7 of stimulation supports our previous results (Mbugi et al, submitted) that in malaria infection, IL-12 is produced later than 24 hrs of stimulation. With slightly decreased levels of IL-10 in association with malaria infection in zinc deficient children, the findings possibly reflect that zinc deficiency primarily results in pathological consequences of type I cytokines due the reduced regulatory role of the cytokine IL-10. In magnesium deficiency, we found malaria infection to associate with both type I and type II responses but the increase is not sufficient to compensate for the levels attained in malaria negative individuals regardless of magnesium status (**figures 3 and 4**). These results may reveal that magnesium deficiency can lead to immune incompetence in response to malaria infection.

Iron deficiency anaemia appeared to induce a similar increased trend in both types of cytokine amongst malaria infected donors. The only exception was IL-12, which was reduced in association with iron deficiency anaemia although the levels were higher than those attained in children without malaria infection. The high levels of IFN-γ in iron deficiency anaemia may be an indication that protection from clinical malaria reported in iron deficiency [39, 40] is probably through cell mediated immune responses. Interestingly, with the exception

of IL-12, this study found an increase in both type I and type II cytokines in association with iron deficiency anaemia in children with malaria infection (**figures 2 and 3**). Available report [41] have speculated about the role of iron deficiency in limiting the severity of the inflammatory response. The argument corresponds with our findings and it could be a result of increased secretion of anti-inflammatory cytokine in response to increase in levels of pro-inflammatory cytokines in malaria infection. However, the observation that the increase in cytokine production could not reach the levels in children without malaria may be due to a combined effect of nutrients deficiencies other than iron in co-existence. It is possible that in addition to the depletion of iron to the parasite that may occur in iron deficiency anaemia the host cells, including immune cells, are also depleted of iron [42] thus reducing the capacity for sufficient cytokine production The modulation of immune response by iron rests on its effects on the function of T_h1 mediated response and supply of this nutrient to the parasite [43], in particular, withdrawal of iron is said to increase T_h1 mediated immune function in vivo [42]. Our study found an association of iron deficiency anaemia with slightly reduced concentrations of IL-1β, IFN-γ and TNF-α in children without malaria but an increment in children with malaria infection. This reflects that iron deficiency anaemia may be associated with increase in concentration of Type I cytokines in malaria infection. From our study it transpires that iron deficiency anaemia is most likely associated with variable effects of both type I and type II cytokine responses (figures 2 and 3) rather than the reported discriminate effects between the two arms [44].

Linear association between type I and type II cytokines: Associations between cytokine production under different conditions of nutrients and malaria status may be predictive for disease outcome. We found relationships between type I and type II cytokines in micronutrient deficient and replete groups and they were variably influenced by the malaria status (table 3). However, the significant difference in slopes in the association between IFN-γ and IL-10 with respect to iron deficiency anaemia status, in particular the negative association seen in iron deficiency anaemia, emphasises that probably the response shifts in deficiency situations from one type of cytokine response to the other. Particularly the balance between IFN-γ and IL-10 which is said to be critical in controlling malaria infection. This underscores the notion that micronutrients may have no grossly visible effects under normal situations but they do when the body is destabilized in terms of immune protection during infections. To emphasize, there is strong evidence that the association between IFN-γ and IL-10 is influenced by iron deficiency anaemia: in children without iron deficiency anaemia, IFN-γ responses are positively associated with IL-10 responses, whereas this association seems ab-

sent or weakly negative in children with iron deficiency anaemia (lower-left panel of **figure 3**). This seems supported by weak evidence of similar interaction in the same direction when examining the influence of iron deficiency anaemia on the association between IFN-γ and IL-5, and between IFN-γ and IL-13 (**table 3**). These associations may reveal that the regulatory T-cell responses (and possibly the T_h2-responses) in malaria are suppressed in iron deficiency anaemia. In addition, there is substantial evidence that the relationship between IFN-γ and IL-5 is influenced by the presence of malarial infection at the time of blood collection: the association between the responses in IFN-γ and IL-5 is steeper in children with malarial infection than in their peers without infection (**table 3**); this seems supported by weak evidence of similar interaction in the same direction between IFN-γ and IL-13 (**table 3**). These data provide evidence that in malaria, previous malarial infection suppresses the T_h2 (regulatory) responses to the disease. As regards to zinc and magnesium deficiencies, there is no evidence that the respective deficiencies influence the associations between IFN-γ and T_h2 cytokines (**table 3**). However, the findings that nutritional deficiencies and malaria status at time of blood collection are variably associated with T_h2 responses independent of IFN-γ alerts to the importance of nutritional component in boosting immune response to malaria. These associations, despite weak evidence, are indicative of a probable imbalance in cytokine concentration that may be influenced by nutrient deficiencies and malaria infection at the time of blood collection, the consequences of which may lead to exaggerated malaria pathology due to cytokine imbalance.

Not only zinc deficiency results into significant impact on cytokine responses to infections [7, 9, 14, 45-48] but also other nutrients, like magnesium and iron. The results show that zinc deficiency may have more impact on type I cytokine responses while magnesium has selective effects on type II responses. In addition, the results also seem to indicate that in iron deficiency anaemia, the prevalent cytokine response is more of type I than type II responses. A recent randomised controlled trial conducted in Burkina Faso has suggested that a combined vitamin A plus zinc supplementation reduced the risk of fever and clinical malaria episodes among children aged 6 to 72 months [49], and this combination may be included in control strategies to fight against malaria in African children. However, it does not exclude the contribution of other micronutrients that have not been reported in this paper which need to be further explored.

Our study have shown weak evidence [50] of effect of nutrients deficiencies on association between cytokine concentration and malaria status at time of blood collection. This could be

due to small sample size to detect differences, the use of confidence intervals in our analysis however, gives a strong reflection of what could be happening as it shows a range within which the true effect is likely to lie. A larger sample size could allow detection of even minor differences leading to a proposal to a more larger study particularly in the intervention study. The protective immune response to malaria is said to target a broad antigenic repertoire that go beyond parasitic developmental stages [51]. Our study used parasitized erythrocytes to induce cytokine response in PBMCs providing intimation that sterile immune protection focusing on whole parasite vaccines could be rewarding [52].

In conclusion, we have found that micronutrient deficiencies may variably be associated with impaired cytokine production. Zinc deficiency and iron deficiency anaemia have shown to be associated with remarkable increases in type I cytokine production, implying a shift in the balance of the immune towards pro-inflammatory and cellular type in these conditions. It may mean that zinc deficiency and iron deficiency anaemia directly induce increased production of pro-inflammatory cytokines or causes an imbalance in regulatory anti-inflammatory cytokines as reflected by increased pro-inflammatory cytokines. Since these pro-inflammatory cytokines have been associated with pathological consequences like cerebral malaria, it should be further assessed to what extent supplementation with zinc and iron is beneficial in children with deficiencies for these nutrients. Consideration of micronutrient supplementation may also be of value if incorporated in vaccine programs in endemic areas to boost immune responses to malaria.

Acknowledgement: Financial support was received from the Netherlands Organization for Scientific Research, NWO/WOTRO (grant numbers W 93-413, WAO 93-441 and WIZ93-465) and UN Children's Fund (UNICEF). We gratefully acknowledge the participating children, parents and the assistance given by community leaders, volunteers and colleagues in Tanzania and The Netherlands. Marga v.d. Vegte and Henry Witteveen for culturing PfRBC that were used in this study. Sincere gratitude is accorded to Marita Troye-Blomberg and Nele Wellinghausen for their complementary material support to the preparation of this manuscript. This paper was published with support of the Executive Director of KCMC, Moshi and the Director-General of the National Institute for Medical Research, Dar es Salaam, Tanzania. HV is supported by the European Community's Seventh Framework Programme under grant agreement no 211484.

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CHAPTER

5

Nutritional status and alteration in plasma levels of naturally acquired malaria-specific antibody response in Tanzanian children

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Submitted

Abstract

Immunoglobulin G (IgG) subclasses have been signaled to confer naturally acquired immunity to *Plasmodim falciparum* malaria. Cytophilic IgG1 and IgG3 by their potential for opsonization, phagocytosis and antibody-dependent cellular inhibition in association with monocytes have been suggestive for the critical role in malaria. The potential for production of antibodies is influenced by micronutrient status. We explored the effect of micronutrients, particularly zinc status on the profiles of IgG subclasses in 304 Tanzania children aged ≤ 5 yrs. An enzyme-linked immunosorbent assay was performed using whole asexual blood stage malaria antigens to determine plasma malaria-specific antibody titres. The findings reveal that zinc deficiency may influence the production of IgM, total IgG and several IgG subclasses in a malaria status-dependent manner. Of the four IgG subclasses, IgG3 and partly IgG2 displayed significant changes in the zinc deficient children with IgG3 predominating in subjects with malaria. Zinc, magnesium, iron deficiency anaemia and malaria status did not influence the association between IgG3 and IgG4. Under conditions of micronutrient deficiency and malaria status, an imbalance in IgG subclasses production may occur resulting into predominantly higher levels of IgG3 and IgG2 that may not confer sufficient protection. The profile of both cytophilic and non cytophilic IgG subclasses have shown to be variably influenced by zinc status, the effects that vary with age at least in under fives. These results provide an insight for inclusion of micronutrients, particularly precise amounts of zinc, in future malaria interventional programs in endemic areas.

Introduction

The humoral immune response is mediated by naturally acquired antibodies against *Plasmodium falciparum* blood-stage surface antigens and is vital in limiting parasite multiplication and the conferral of protection to clinical malaria. In malaria endemic areas the development of naturally acquired immunity to severe disease takes place in children at an age range of 1 – 5 years [1, 2]. This 'antiparasite' immunity is not absolute and is acquired through repeated exposure to *Plasmodium falciparum* [3]. Many parasite antigens are known to occur at different stages of the parasite in the human host with the body generating antibodies against the prevailing antigenic proteins. Protective immunity to falciparum malaria, however, has particularly been associated with cytophilic antibodies of immunoglobulin G (IgG) subclasses [1, 3]. Ferrante and Rzepczyk [3] pointed out the switch from Immunoglobulin M (IgM) on B-cells to different isotypes and different IgG subclasses (IgG1, IgG2, IgG3 and IgG4) upon encounter with malaria antigens. Antigens can differentially modulate immunoglobulin heavy-chain switching through induction of different cytokine secretory patterns by CD4+ T helper (Th) cells [3, 4]. The differential release of these cytokines (type I or type II) is influenced by nutritional status, particularly zinc [5, 6]. Studies by Tongren et al [7] reported the regulation of immunoglobulin class switching in murine malaria to be epitope-specific and that in human malaria, the IgG1/IgG3 class switching is independently regulated by the nature of antigen, cumulative exposure to the antigen and maturity of the immune system [8].

Several studies have highlighted the importance of naturally acquired antibody-mediated immunity through IgG subclasses to be crucial in limiting clinical malaria [3, 9-17] although these studies have targeted different parasite surface antigens. The levels of subclasses of IgG, in particular the proportion of cytophilic (IgG1 and IgG3) to non-cytophilic (IgG2 and IgG4) have been hypothesized to be more significant than the overall levels of antibodies in providing protection to the development of severe disease [1, 19, 20]. In addition, the fine specificity of antibodies towards specific antigenic epitopes on *P. falciparum* antigens is thought to be critical for the generation of an effective immune response [18, 19].

Poor nutrition in children may interfere with the development and function of the immune system. The immune regulatory mechanisms become impaired due to quantitatively and qualitatively altered immune cell populations [6, 20-23]. The whole immune response pathway from innate to adaptive antibody response and cellular responses is said to be affected [6, 24-35] and in malaria endemic areas the impact of the disease may be aggravated. Most children become vulnerable to infections at weaning when the passive immunity acquired

from prenatal maternal antibodies and breast milk wanes and nutritional support is poor. It is likely that poor nutrition interferes with the immune response to malaria in African settings where both situations prevail. Several reviews and studies [31-36, 38-41] have explored and the association between micronutrient particularly zinc deficiencies with infections but only few have specifically focused on the association with malaria [32, 36-38]. Even those studies that have extensively investigated the factors associated with a protective role of IgG subclasses, have been limited to individual effects of age, exposure, transmission intensity, ethnicity, geographical location of parasite and seasonality on the dynamics [11, 39] and fine specificity [14] of antibody-mediated protection to malaria. The fact that in some children the B-cell response undergoes isotype switching to a more cytophilic antibody response to malaria early in life and who remain protected from the disease attacks for quite longer time periods than other children [8, 40] even when exposure is limited by seasonality [3, 41], is indicative of the contribution of other factors like nutritional status in strengthening of an effective antibody response to malaria.

We hypothesized that zinc and other micronutrients deficiencies induce relative alterations in the plasma profile of naturally acquired *Plasmodium falciparum*-specific antibody responses that these antibodies may influence the induction of a protective immunity to manifestation of the clinical disease. To assess these associations, a direct enzyme-linked immunosorbent assay (ELISA) was performed to determine the plasma levels of isotype-specific antibodies responding to asexual *Plasmodium falciparum* stage antigens which are differentially involved in the parasite clearance and protection from clinical disease. Group differences were compared by determining the malaria infection status at the time when blood sample collection was done.

Subjects and Method

Study area and population: The cross-sectional study was conducted in the period of May-July 2006 in a lowland area around Segera village (S 05º 19.447', E 38º 33.249'), Handeni District, north-eastern Tanzania. Malaria is highly endemic in this area, with virtually all infections being due to *P. falciparum*. The residents in the study population comprise mostly poor farmer families growing maize and cassava for subsistence use. At the time of our study, only one health centre in Segera was available to serve all of the surrounding area. The study was approved by both Ethics Review Committees in The Netherlands and Tanzania. Informed consent was obtained from community leaders and local government officials, and from parents or guardians.

Sampling methods and eligibility criteria: A census list was made with all resident children aged 6-60 months in the study area. Using this list, 16 children were randomly selected from each of 19 communities, resulting in a total of 304 children. Children were eligible when they had no fever, and showed no signs of other severe disease or severe malnutrition (weight-for-height z-score below -3 SD). Further details are provided elsewhere [42].

Field procedures: All children were examined by clinical officers, who also measured axillary temperature by electronic thermometer. Information on the state of health such as malaria, fever, sickness and reports on fever in the past 14 days was diligently recorded. Venous blood (6 mL) was collected in containers suitable for mineral element analysis with sodium heparin as anticoagulant (Becton-Dickinson, Franklin Lakes, NJ). Immediately upon collection, the cap was sprayed with ethanol and allowed to dry; approximately 1.3mL blood was then drawn in a sterile manner from the collected blood for different measurements. Children were treated for common childhood infections and anaemia according to guidelines of Tanzanian Ministry of Health.

After arrival of blood samples at the laboratory in Moshi, they were immediately centrifuged (300´*g*) at ambient temperatures for 10 minutes. Plasma (1.2 mL) was stored in liquid nitrogen, and subsequently transported on dry ice to The Netherlands to measure additional biochemical indicators of micronutrient status and inflammation.

Parasite Enzyme-linked immunosorbent Assays (ELISA): Different ELISAs testing antibodies against various specific parasite antigens are described elsewhere [1, 10, 12, 13, 43-45]. We developed our own direct ELISA test protocol that was used to determine the plasma concentration of isotype-specific antibodies responding to *Plasmodium falciparum* asexual blood stages. Parasite extract (asexual blood stage antigens) were used to coat the plates, and these were covered and incubated overnight at 4^0C. The parasite extract was obtained as a kind gift from Professor Robert Sauerwein from the Medical Parasitology Laboratory, Radboud University Medical Centre Nijmegen, The Netherlands. The plates were blocked with 150µL of 1% w/v bovine serum albumin, (BSA) (grade V, Sigma P4417, St Louis MO, USA) in PBS. After blocking the plates were washed three times with an extensive volume of 0.05% Tween 20 (v/v) (Sigma-Aldrich, Missouri, USA) in PBS. Plasma samples were diluted 5 (IgG total, IgG1, IgM) or 20 (IgG2, IgG3, IgG4) times in 0.2% w/v BSA and 0.05% Tween 20 in PBS. Plasma samples were added to the wells at 50µL and malaria positive and negative samples were added as controls. The positive control consisted of a pooled sample of 25 highly immune Tanzanian individuals. Addition of plasma to the wells was

followed by incubation under cover for 3 hrs at room temperature. After incubation, the plates were washed 3x in 0.05% Tween 20 in an extensive volume. Thereafter, 50µL of horseradish peroxidise (HRP) conjugated sheep anti human antibody (The Binding Site, Birmingham, UK) was added. and incubated at room temperature in a shaker in the dark for 1.5 hrs. The following concentrations were used: 1µg/ml for IgG total (AP003) and IgG1 (AP006), 2µg/ml for IgM (AP012), IgG2 (AP007), IgG3 (AP008) and IgG4 (AP009). Plates were emptied and washed 6x in 0.05% Tween 20 and finally 100µL of substrate, (2.2'-azino-di[3-ethylbenzthi-azoline sulfonate (6)]), ABTS (Roche Diagnostics, Mannheim, Germany) was added and incubated in the dark for colour development. A plate reader (Anthos Photometer 2020, Anthos-labtec, Woerden, The Netherlands) was used to measure colour development at 405 nm.

Statistical analysis: The data were entered and analysed using SPSS for Windows (version 15.0. SPSS Inc., Chicago, IL, USA). Relative titres were calculated using the control sample which was added to each plate. For these calculations a standard curve was made. Antibody titre values were log-transformed to obtain normally distributed variables. A linear regression model was used to calculate the effect change in antibody levels in zinc replete and deficient individuals. Linear regression analysis was also used to assess whether different IgG subclasses associations were influenced by conditions of zinc, magnesium, iron deficiency anemia and malaria status. Cut off values were set for zinc (plasma zinc concentration <9.9 µmol/L), magnesium (plasma magnesium concentration < 750 µmol/L) and iron deficiency anaemia (iron deficiency, ferritin concentration <12 µg/L accompanied by anaemia, haemoglobin concentration <110 g/L). Average antibody titres were determined by comparison of means from which the standard error of the means were used to calculate confidence intervals and the absolute values were obtained by exponential transformation of previously log transformed values using Microsoft excel. The confidence intervals were used as these provide an estimate of the mean at a confidence range of 95%. The difference in antibody titres in relation to zinc status among groups was based on 'healthy' children (with neither of conditions) and effect change estimated for asymptomatic malaria (from malaria dipstick results); sick with malaria infection; sick with malaria infection and a history of fever in the past 14 days; and sick without malaria infection. The group differences were accounted for by the use of confidence intervals (95% CI and p-value ≤ 0.05). A generalized linear model (GLM) was performed to determine whether the effect of zinc deficiency on plasma IgG subclass was age-dependent. Lots were used as scaling weight variables to account for the diversity in malaria prevalence among lots. The analysis compared among age groups the differences in *P. falciparum* (schizont asexual blood stage parasites)-

specific antibodies plasma reactivity. Three age classes were established; 0.5 – 1.5 yrs, 1.5 – 3 yrs and 3 – 5 yrs.

Results

General effect change and plasma total IgM and IgG levels: We initially evaluated the effect change and relative antibody levels of IgM and total IgG class (**tables 1 and 2**). The relative antibody levels in the zinc replete group were moderately higher compared to the zinc deficient group. IgM levels were significantly increased relative to the reference group (healthy children) in children with asymptomatic malaria, 38% (95% CI; 16% to 65%) and those who were sick with malarial infection and reported fever in the past 14 days, 53% (95% CI 25% to 89%) in the zinc replete group. Significantly higher levels of IgM were detected in the zinc deficient group for asymptomatic children, sick with malaria and those who were sick with malaria and a report of fever in the past 14 days (**table 1**). The results in total IgG levels followed similar trends to the levels of IgM for the zinc replete as well as the zinc deficient group (**table 2**). In both cases the increase in IgM and total IgG levels were statistically not significant in sick children without malaria infection.

Table 1: The effect change in plasma total IGM levels in different health situations associated with malarial infections in zinc deficient subjects

Healthy status	IgM							
	Zinc replete				Zinc deficient			
	Mean level (n)	Effect change	95% CI	*P*-value	Mean level (n)	Effect change	95% CI	*P*-value
Healthy children	118.4 (47)				112.0 (46)			
Asymptomatic malarial infection	164.0 (38)	38%	16% to 65%	0.000	161.2 (41)	44%	23% to 69%	0.000
Sick with malarial infection	181.7 (09)	53%	25% to 89%	0.000	171.8 (10)	53%	17% to 100%	0.002
Sick with malarial infection and 14d history of fever	134.5 (22)	14%	-15% to 52%	0.389	175.6 (09)	57%	21% to 103%	0.001
Sick without malarial infection	109.7 (30)	-7%	-23% to 12%	0.419	106.1 (32)	-5%	-20% to 12%	0.529

Table 2: The effect change in plasma IgGT levels in different health situations associated with malarial infection in zinc-replete subjects

Health status	IgGT							
	Zinc replete				Zinc deficient			
	Mean level (n)	Effect change	95% CI	*P*-value	Mean level (n)	Effect change	95% CI	*P*-value
Healthy children	80.1 (47)				86.8 (46)			
Asymptomatic malarial infection	112.8 (38)	41%	13% to 75%	0.002	119.5 (41)	38%	14% to 66%	0.001
Sick with malarial infection	122.5 (09)	53%	18% to 98%	0.001	125.7 (10)	45%	6% to 98%	0.021
Sick with malarial infection and 14d history of fever	110.6 (22)	38%	-4% to 98%	0.081	123.8 (09)	43%	6% to 93%	0.021
Sick without malarial infection	68.3 (30)	-15%	-32% to 8%	0.177	81.5 (32)	-6%	-23% to 15%	0.534

Effect change in plasma levels of IgG subclasses: Evaluation of the general plasma changes in IgG subclasses in different malaria situations are shown in **figure 1**. There was a significant change in antibody titres for all IgG subclasses ($P < 0.05$) in zinc replete children with asymptomatic malaria and those who were sick and had malaria infection. In these subjects, the changes in IgG3 and IgG4 levels were almost similar with IgG4 being slightly higher than IgG3 in children that were sick with malaria infection and those who were sick with malaria infection and a report of fever in previous 14 days. With zinc deficiency (**figure 1**), only the changes in IgG3 levels were significant for asymptomatic malaria, sick with malaria infection and in sick children with malaria and who reported complaints of fever in the past 14 days ($P < 0.05$). On the other hand, IgG2 predominantly showed increased levels in sick children with malarial infection ($P < 0.05$). There was a marked difference with respect to zinc status in which only IgG3 and partly IgG2 predominated in zinc deficiency which may imply these IgG subclasses to remain as critical weapons to fight against malaria in deficiency situations. The insignificant change in IgG subclasses in sick children with no malaria infection strengthens the notion that the antibodies studied are specific for malaria antigens.

Comparison of levels of plasma IgG subclasses with respect to zinc status: The levels of *Plasmodium falciparum*-specific IgG subclasses in plasma were assessed to determine whether there were differences within individual IgG subclasses as a result of zinc status (**figure 2**). With 'healthy children' children set as reference group, the levels of IgG1 were significantly increased in asymptomatic malaria ($P = 0.007$; 95% CI, 13% to 120%) and in children who were sick with malaria infection ($P = 0.011$; 95% CI, 13% to 146%). There was no significant increase in IgG1 in the zinc deficient group. Increase in IgG2 levels followed a similar trend to IgG1 in zinc replete group but in the zinc deficient group, the IgG2 levels were significantly higher in the sick children with malaria infection ($P = 0.015$; 95% CI, 10% to 141%). Levels of IgG3 were significantly increased in both zinc replete and zinc deficient group ($P < 0.05$) relative to healthy individuals, except in sick children without malaria (**figure 2**). The levels of IgG3 in the zinc deficient group sick children with malaria ($P = 0.004$; 95% CI, 30% to 289%) and those sick with malaria and a reported fever in the previous 14 days (P = 0.002; 95% CI, 38% to 333%) were significant and relatively higher than their counterparts in the zinc sufficient group. With the exception of children sick without malaria, IgG4 levels were higher in zinc replete and the increase of IgG4 in the zinc deficient group in all cases was statistically insignificant (**figure 2**).

Table 3: Change in IgM, IgG total and IgG subclasses due to age in zinc deficient children compared to reference healthy group

Parameter	Age Group (Years)		
	0.5 – 1.5	1.5 – 3.0	3.0 – 5.0
IgM	$P < 0.001^{a-}$	$P = 0.019^{a-}$	$P < 0.001^{a-}$
	$P = 0.036^{c-}$	$P = 0.029^{b-}$	$P = 0.021^{b-}$
			$P = 0.002^{c-}$
IgG total	$P < 0.000^{a-}$	$P = 0.031^{b-}$	$P = 0.003^{a-}$
		$P = 0.05^{c-}$	$P = 0.027^{c-}$
IgG1	$P = 0.016^{a-}$	Δ-	Δ-
IgG2	$P = 0.045^{b+}$	$P = 0.049^{b+}$	$\Delta\text{-}/d^{+}$
		$P = 0.075^{d+}$	
IgG3	$P < 0.001^{a-}$	**$P = 0.062^{b-}$**	$P = 0.002^{a-}$
	$P = 0.048^{c-}$		$P = 0.022^{b-}$
			$P = 0.004^{c-}$
IgG4	$P < 0.001^{a-}$	Δ-	Δ-
	$P = 0.004^{c-}$		

a = asymptomatic malaria infection
b = sick with malaria infection
c = sick with malaria infection and history of fever in the past 14 days
d = sick without malaria infection
– = reduction
+ = increase
Δ = insignificant change ($P \geq 0.10$)
$\Delta\text{-}/d^{+}$ = variable insignificant change with a change in sick without malaria increasing
NB: Bolded values indicates borderline significant P-values

Association between IgG3 and IgG4 subclasses under different conditions of micronutrients and malaria status: Since the effect change and the levels of IgG subclasses showed a great potential for IgG3 and IgG4 to be in higher levels with respect to zinc status, we performed a linear regression analysis to determine whether these two immunoglobulin subclasses we associated under different conditions of micronutrient status (**figure 3**). The association between IgG3 and IgG4 were linear but the higher *P*-values reflected that the slopes of the regression lines in the four panels (**figure 3**) are not different. These results provide no evidence that the associations between IgG3 and IgG4 are influenced by deficiencies in zinc, magnesium, iron deficiency anaemia or malaria status.

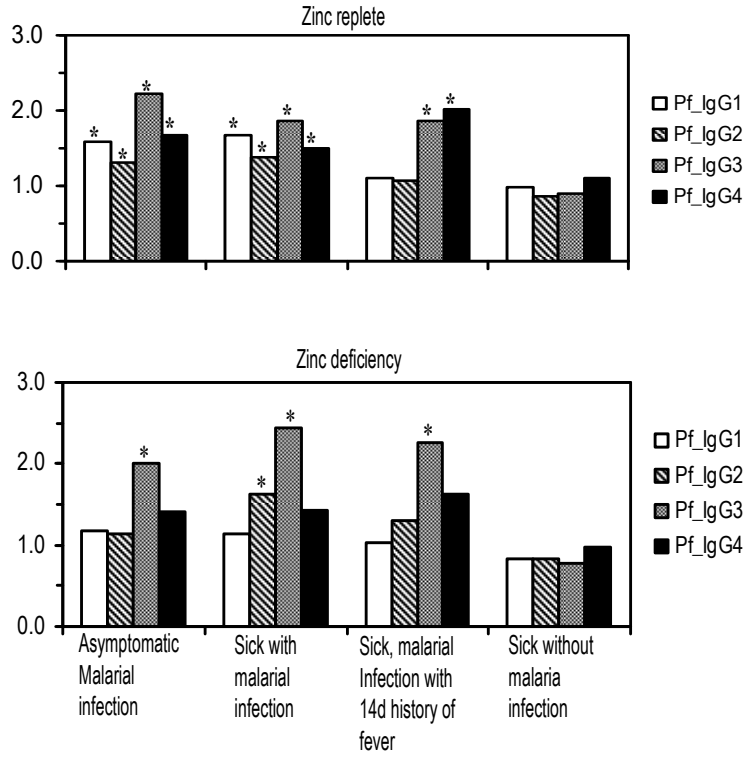

Figure 1: *Effect change in profile of relative plasma concentrations of malaria parasite-specific IgG subclasses in different malaria situations with and without associated clinical features in zinc replete and zinc deficient children. Value on the y-axis are log transformed values of antibodies titres as detected in plasma. Bars with asterisk (*) indicates significance at $P < 0.05$.*

Effects of zinc deficiency on IgM, total IgG and malaria-specific IgG subclasses with age group: Using a generalised linear model, we assessed whether the influence of zinc deficiency on the change of IgM, IgG total and IgG subclasses levels varied with age (**table 3**). Results showed zinc deficiency to significantly influence the change in antibody levels with variable conditions of malaria status and age groups. The reduction in levels of IgM and IgG3 had more impact in older children (3 – 5 yrs) as compared to other age groups. On the other hand, the impact of zinc deficiency on IgG1 and IgG4 was significant in the younger age (0.5 – 1.5yrs) with insignificant impact on the medium (1.5 – 3yrs) and the older age group. The interesting finding in this study is the increase in levels with zinc deficiency for IgG2 contrary to other IgG subclasses and that it was associated with sick children with malaria and those sick without malaria in at least all age groups. This could mean that IgG2 is more profoundly associated with sickness than protection against malaria. The effect of zinc deficiency on IgG2 in sick children without malaria and on IgG3 in sick children with malaria was borderline significant (**table 3**).

Discussion

In *Plasmodium falciparum* malaria protective immunity to clinical disease is mainly ascribed to immunoglobulin G subclasses. In human malaria, cytophilic IgG1 and IgG3 subclasses with high affinity to Fc receptors on monocytes, providing a crucial protection [1, 3, 18] gradually increasing with age, to target antigen and duration of exposure [8]. Their protective potential solely rests on their ability to fix complement, and facilitating opsonization and phagocytosis that limits parasite multiplication in an antibody-dependent manner. The kinetics of the antibody isotype formation are continually altered by reinfection [46] with the proportion of cytophilic IgG1 and IgG3 relative to non-cytophilic IgG2 and IgG4 considered more significant than the overall levels of antibodies in providing protection to severe disease [1, 19, 20]. We hypothesized that micronutrients, zinc deficiency in particular, could contribute to alterations in the levels of these antibodies to influence protection against malaria since in African children the two entities commonly co-exist. This study provides the first findings in associating micronutrient deficiency with specific IgG subclasses that confer protection to malaria in endemic areas.

Effect change and plasma total IgM and IgG levels: This study used whole asexual blood stage malaria antigens to reflect the real *in vivo* milieu and providing broader antigenic targets for optimal induction of malaria-specific antibody profiles [13]. Primary responses to infection were assessed by measuring plasma levels of IgM and total IgG (Tables 1 and 2)

and the levels changed with malaria status and significant changes were associated with asymptomatic malaria, sick with malaria infection and sick with malaria infection together with a reported fever in the past 14 days especially in the zinc deficient group. The similar pattern of change in IgM and IgG levels support previous reports [3] of switching of IgM on B-cells to different isotypes and different IgG subclasses upon encounter with malaria antigens. This implies that IgM primarily determines the magnitude of protection by specific antibodies to malaria pathogen. In addition, Dodoo et al. [47] have reported recently IgM to confer protection and reduce incidence of malaria in African children. The levels of total IgG particularly reacting to glutamate rich protein (GLURP) of the parasite antigenic surface has been reported to be strongly associated with reduced malaria incidence in Africa [48]. The significant changes in zinc deficient group could be a reflection of persistent infection and concurrent new infection.

Effect change in plasma levels of IgG subclasses: This study found significant change in all IgG subclasses in zinc replete group especially in asymptomatic children and those who were sick with malaria infection (**figure 1**). The change in levels for sick children with malaria infection and a report of fever in the past 14 days was high only for IgG3 and IgG4. The predominantly significant change in IgG3 observed in this study concur with previous studies for the significant role of IgG3 in conferring protection to malaria [1, 8, 12, 14] and the trend that IgG1 and IgG2 are lower than IgG3 and IgG4, respectively in asymptomatic children is intriguing (**figure 1**). It may reflect the potential response that in asymptomatic malaria there is a shifting order of response dominance from IgG1 to IgG3 and IgG2 to IgG4 respectively to sustain protection, albeit studies need to be done to uncover this proposition. The dynamics of these isotype dominance may be due to dynamics of infection in the area with re-infection playing a role to keep the levels high [46].

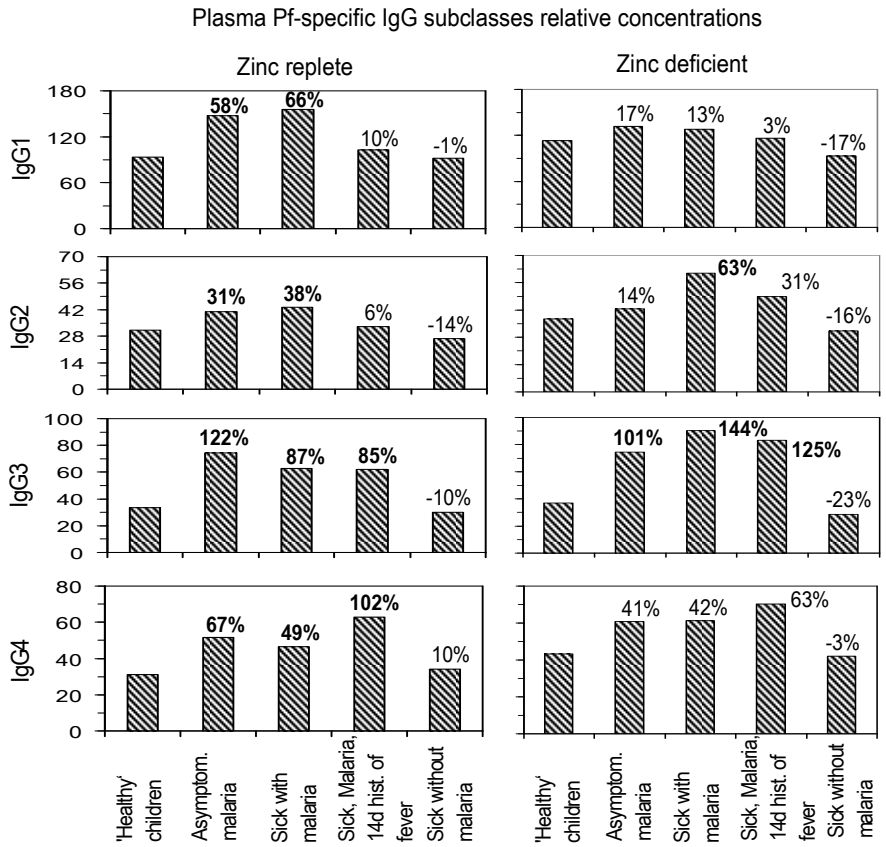

Figure 2: *Relative plasma levels and effect change under different malaria and zinc status. For each immunoglobulin G subclass the panels compare the levels in zinc replete and zinc deficient situations at different states of malaria infections. Percentages indicate paired group effect change differences in the relative plasma levels of antibodies. Bolded values: significant at* $P \leq 0.05$.

Higher changes in IgG4 levels in sick children with malaria and a report of fever in the previous 14 days may be explained by firstly, a long half-life of IgG4 (probably due to previous subclinical malaria infection) and secondly, the reports that IgG4 is more associated with disease than protection [49]. However, Nebie et al [48] have recently reported IgG3 and IgG4 to be associated with reduced risk of clinical malaria in African children. In zinc deficiency, however, the higher incremental change of IgG3 in all malaria situations further endorses the significant role IgG3 in protecting against malaria and that probably under de-

ficiencies, the role of this IgG subclass becomes more critical. The findings also contrasts previous propositions of downstream isotype switching from IgG3 to IgG1 later in the course of infection after displaying early comparable levels [3]. This is because in an area of intense malaria transmission, re-infection maintains the level of IgG3 isotype [17]. The significant change in levels of IgG2 in sick children with malaria may be associated with disease by the explanation that probably it replaces IgG4 in such situations of micronutrients deficiencies. Generally, there is a clear difference in alteration of IgG subclasses plasma levels in the zinc deficient group which may be explained by the probable influence of zinc status. IgG subclasses reacts to various sets of asexual stage parasite antigenic proteins that may account for the variable effects of zinc deficiency on these antibodies.

Comparison of levels of plasma IgG subclasses with respect to zinc status: Having assessed the effect change and levels of primary response by IgM and total IgG plasma levels and the effect change in IgG subclasses, we compared the levels of individual IgG subclasses with regard to zinc status (**figure 2**). Results showed higher levels of increase in the zinc replete group as compared to the zinc deficient group for at least each immunoglobulin subclass. This signifies the importance of these IgG subclass coexistence in conferring protection to malaria and that may be influenced by zinc status due to relatively lower increases in the deficient group. While the increase in IgG3 and IgG4 seemed to predominate in zinc replete group, IgG3 and partly IgG2 were predominant in the zinc deficient group. The trend of response for IgG1 and IgG2 and that for IgG3 and IgG4 were also similar in the zinc replete group but not in the zinc deficient group. Available reports [48] indicate that, in the course of conferring protection to malaria, the IgG subclasses target different surface parasite antigenic proteins with IgG3 and IgG4 responding against (glutamate rich protein (GLURP) and IgG1 against apical membrane protein 1 (AMA1). In addition, cytophilic IgG1 and IgG3 have been shown to be differentially regulated over time [50] with IgG3 remaining abundant [45]. This could further explain the association between IgG3 and IgG4 in the zinc replete group that was lost in the zinc deficient group. This may support previous findings that the proportion of these IgG subclasses is more important than their levels in conferring protection against the disease [1, 49, 51]. This proportion seemed to be disturbed under conditions of zinc deficiency. The increase in IgG3 levels can also be explained by its short half-life. IgG3 disappears fast (serum half life of 7 days) so that high amount can not be measured in healthy control, but there may be many IgG3 producing B-cells which rapidly start to produce IgG3 following infection.

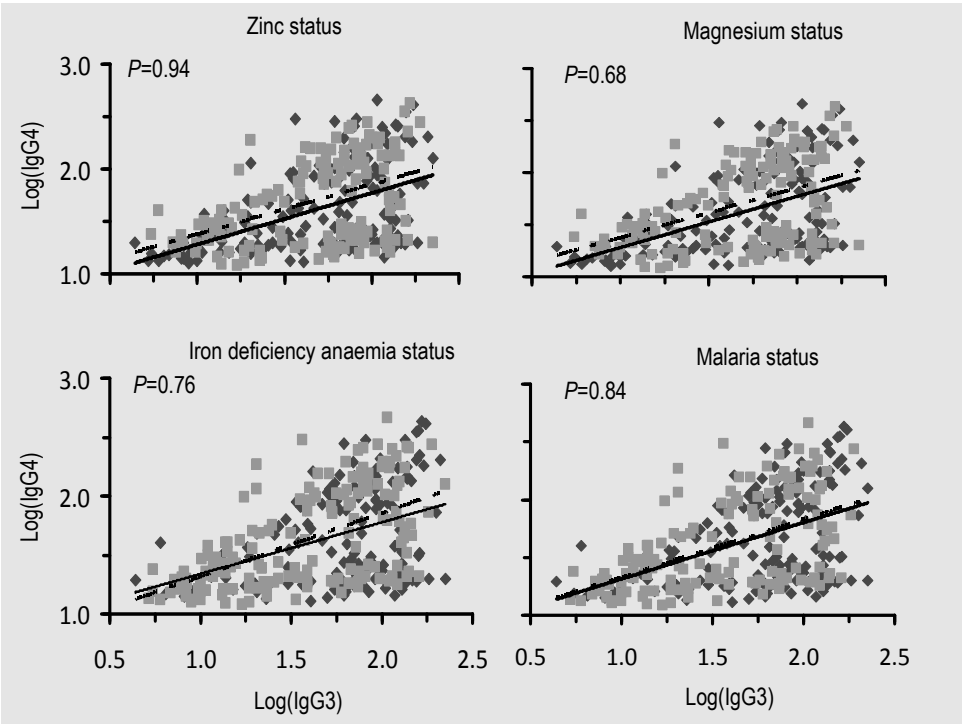

Figure 3: *Relationship between malaria specific plasma IgG3 and IgG4 under different situations of nutrition and malaria status. There were no significant differences between slopes in all four conditions being compared as indicated by P-values.*

Footnote: *scatter spots (black): zinc and magnesium replete, absence of iron deficiency anaemia and absence of malaria infection; scatter spots (grey): zinc, magnesium deficiency and iron deficiency anaemia and malaria infection. The black and dashed lines are their corresponding regression lines.*

In the zinc deficient group the predominance of IgG3 and that of IgG2 at least in sick children with malaria infection, reflect that probably under zinc deficiency these IgG subclasses are critical in providing protection against the disease. Garraud et al [52] reported the association between IgG2 and IgG3 in conferring protection to malaria and that certain individuals possess rare mutated allele encoding an FcR that can bind IgG2 along with IgG3 and IgG1. Other mechanisms have also been suggested [49, 53]. Another possibility is that, an isotype imbalance [49] occurred in zinc deficiency and thus the resulting IgG2 and IgG3

proportions may note be protective. These results are in contrast of previous findings by Groux and Gysin [54] that IgG1 and IgG3 were always predominant in serum. It may be that, in malaria infection as reported previously either of the cytophilic IgG subclass (IgG1 or IgG3) should be associated with a non cytophilic (IgG2 or IgG4) for perfect protection. Kinyanjui et al [46] reported the half-lives of these IgG subclasses to probably be shorter than what is known [3] with the half-life of IgG3 being shortest of all. The prevailing high levels of IgG3 in both zinc replete and zinc deficient group may be attribute to continued exposure to low but persistent malaria infections in an endemic area [1]. Rzepczyk et al [17] reported skewing of IgG response towards a short-lived IgG3 in response to *P. falciparum* infection and that plasma levels could be maintained through persistent infection or new infection.

Association between IgG3 and IgG4 subclasses under different conditions of micronutrients and malaria status: Assessment of whether the association between IgG3 and IgG4 are influenced by nutritional status indicated no impact of zinc deficiency, magnesium deficiency, iron deficiency anaemia or malaria status (**figure 3**). This implies that deficiency in micronutrients may influence production and consequently proportions of protective antibodies to malaria but not their association that may be programmed inherently by the body's immune system which has high fidelity.

Effects of zinc deficiency: Does age make the difference?: Several reports have evaluated the profile of antibody response to asexual blood stages of the malaria parasite. Some have in principle proposed the gradual change in the naturally acquired antibody responses with age [1, 3, 8, 40]. This study adds more information on zinc deficiency variably influencing the profile of antibody protection to malaria. Zinc deficiency seemed to significantly and negatively influence the profile of IgM and IgG in all age groups implying that the primary response is generally influenced by zinc deficiency regardless of children's age differences. Children of 0.5 – 1.5yrs have shown to be prone to lowered IgG1 and IgG4 production in zinc deficiency while the impact on IgG2 and IgG3 is significant in all age groups with IgG2 increasing with zinc deficiency in contrast to IgG3. As suggested by previous studies, IgG3 levels seem to be prevalent in all age groups implying its critical role in conferring protection across all young ages (within < 5yrs children). Deficiency in zinc may be more alarming in endemic areas due to its impact on the largely believed immunoprotective IgG3 subclass and possibly other IgG subclasses that have been shown in this study.

Our findings have shown preliminarily the variable effects that zinc may have on the profiles of IgM, IgG total and IgG subclasses, and that this effect seems to vary with age. IgG3 have been shown to be critically affected across all age groups as per this study classification. Inclusion of appropriately selected micronutrients could be a way forward towards boosting the production of protective IgG subclasses in endemic areas. Previously we found a profound impact of zinc and other micronutrients on the cytokine arm of the immune responses, both in the innate and anti-inflammatory cytokine profiles. This evidence brings us to firmly suggest for inclusion of micronutrients in future malaria vaccine programs pending thorough and extensive studies on these interactions are further done to strengthen the proposition. Special attention should be on the isotype switching under conditions of micronutrient deficiencies, malaria status and age.

Acknowledgement: Financial support was received from the Netherlands Organization for Scientific Research, NWO/WOTRO (grant numbers W 93-413, WAO 93-441, and PRIOR) and UN Children's Fund (UNICEF). We gratefully acknowledge the participating children, parents and the assistance given by community leaders, volunteers and colleagues in Tanzania and The Netherlands. Professor Robert Sauerwein is acknowledged for providing the parasite extract antigens for ELISA. This paper was published with support of the Executive Director of KCMC, Moshi and the Director-General of the National Institute for Medical Research, Dar es Salaam, Tanzania.

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CHAPTER

6

CD36 deficiency protects against malarial anaemia in children by reducing *Plasmodium falciparum*-infected red blood cells adherence to vascular endothelium

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Trop Med Int Health, 2009; **14**(7):9810-9816

Abstract

CD36 is a receptor that occurs on the surface of activated immune cells, vascular endothelial cells and participates in phagocytosis and lipid metabolism. This receptor is known to be the major endothelial receptor molecule for field isolates of *Plasmodium falciparum.* A T1264G mutation in exon X of the gene leads to deficiency of CD36. This study aimed at determining associations between CD36 deficiency, *P. falciparum in vitro* adherence on purified CD36 and anaemia among children in an endemic area. Genotypes were determined by nested polymerase chain reaction of isolated DNA from filter blood spots followed by Restriction Fragment Length Polymorphism (RFLP). *Plasmodium falciparum* adherence assays were performed on immobilized purified CD36. The data indicate that CD36 is an important cytoadherence receptor that mediates adherence to most *P. falciparum* field isolates. Our findings also suggest that mutations causing CD36 deficiency may confer significant protection against malarial anaemia (MA) in children ($\chi^2 = 8.58$, $P < 0.01$). The protective role that CD36 deficiency may confer against MA in children seems to be mediated through reduced cytoadherence of infected red blood cells to vascular endothelium.

Introduction

A unique characteristic feature of infections with *Plasmodium falciparum* is the ability of infected red blood cells (IRBCs) to adhere to vascular endothelium by cytoadherence. The result of cytoadherence is accumulation of IRBCs in the deep microvasculature. This phenomenon is called 'sequestration' and is associated with disease outcomes [1-3]. In some instances, parasite populations with a predisposition to adhere to certain receptors are more commonly associated with certain disease outcomes, such as cerebral malaria and placental malaria [4-6], although the precise role of parasite–receptor interactions in determining disease severity remains to be understood. Sequestration plays a major role in the development of severe disease and occurs as a result of both cytoadherence of IRBCs to capillary endothelium [3, 7, 8] and the binding of IRBCs to uninfected erythrocytes (rosetting) [9, 10]. Adherence is mediated via knob-like structures at the surface of IRBCs [11] resulting from the deposition and aggregation of parasite proteins and their interaction with the host cell cytoskeleton underneath the RBC membrane [12, 13]. Studies have suggested that *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) expresses variant-specific epitopes and mediates adhesion to endothelium. CD36 is among the host ligands that have been found to mediate endothelial binding of IRBCs and has been identified in most field isolates [8, 14-21].

CD36 is one of the most characterized host receptors for *P. falciparum* IRBCs. CD36 is an 88-kDa glycoprotein expressed on endothelial cells, platelets, macrophages and dendritic cells, and participates in phagocytosis and lipid metabolism. However, it is not expressed on endothelial cells of brain capillaries. The gene encoding CD36 consists of 15 exons, and extends at least 32 kb on the q11.2 band of chromosome 7 in humans. As most *P. falciparum* field isolates bind CD36, CD36 is considered the major endothelial receptor for sequestration, although not all parasites bind this receptor [22, 23]. *CD36* exons X–XII occur in numerous polymorphic forms in the Gambia and in Kenya and several of these forms are associated with susceptibility to cerebral malaria. A subsequently described polymorphism of exon X confers protection against severe anaemia in heterozygotes in Kenya by mechanisms not clearly defined [24].

The mechanism by which CD36 confers protection against malarial anaemia (MA) in children is not clearly understood. This study was designed to determine role of cytoadherence in the protection of CD36 deficiency against MA in children below 5 years of age. In particular, we assessed the role played by CD36 deficiency in the development of MA in chil-

dren. The study specifically explored the frequency of CD36 deficiency among children in malaria endemic areas and associations between CD36 deficiency and status of MA in children and the role played by cytoadherence on the development of MA in children.

Materials and methods

Participants, recruitment and consent: Mothers were recruited among women presenting at Muheza Designated District Hospital for antenatal care in the third trimester or for delivery hospitalization. Mothers of prospective study children initially learned about the study through community meetings. Mothers who qualified for inclusion were requested to read and sign a consent form on behalf of their newborns. Verbal consent was given by those unable to read and write, followed by thumb stamp. Children whose mothers consented for participation, and whose mothers were willing to bring them fortnightly and when they are sick for sample collection were included in the study. Unwillingness to sign the informed consent form and to give samples when required were exclusion criteria for this study. In total, 155 children were recruited. Of these, 149 completed the follow up, and provided 204 samples used for data analysis. Six children were dropped at different time points due to various exclusion criteria.

Definition of categories: A malaria case was defined as a child testing positive for *P. falciparum* parasites with a fever (body temperature) of >38.5 °C and any of the typical malaria symptoms. MA was defined as a haemoglo+bin (Hb) concentration of ≤10 g/dl, in the presence of positive thin and/or thick blood smears at any parasitemia level. Non-anaemia (NA) was defined as an Hb concentration of >10 g/dl, in the presence of malaria parasites at any parasitemia level. Hb concentration was determined using a Cell-Dyn 1200® Haematology Analyser (Spectron Corporation, Burlington, WA, USA). In this study, an IRBC was considered to be a binder to CD36 if the number of IRBCs binding to CD36 was at least twice as much as the number of IRBCs binding to the control molecule, bovine serum albumin (BSA), observed under light microscope at 10 × magnification in 20 fields of the binding plate. This study was approved by both the Tanzanian and the Seattle Biomedical Research Institute (Seattle, WA, USA) Ethics Review Boards.

Sample collection: We genotyped 155 children for the CD36 gene at the start of the follow-up. The children were then followed for a period of 12 months from November 2003, for occurrence of malaria-associated anaemia. Only malaria positive blood samples were used in this study. Each collected sample was treated as a separate sample representing a different infection. Samples were collected as passive cases when a child presented with malaria.

Every time a sample was collected, Hb concentration was measured, and IRBCs cultured for binding assays. Approximately 1 ml of blood was collected by venipuncture into 10 ml vacutainers (Fisher Inc, USA) containing citrate phosphate dextrose (CPD) as anticoagulant, at a ratio of 1:10, CPD: blood. A blood drop from whole blood was placed on Whatman® filter paper strips (Whatman, USA) and stored at room temperature for genotyping.

Parasites quantification and in vitro culture: Small volumes (5 µl) of blood were used to prepare a thick and thin smear for detection of malaria parasites. Slides were stained with Giemsa stain at pH 7.2. The number of IRBCs with asexual parasites was counted against 2000 total red blood cells (both infected and non-infected) to obtain the percentage of IRBC. The infected blood was then diluted using a mixture of blood group O+ cells and AB sera from malaria non-immune volunteers to 3–5% IRBCs, and cultured in a complete media with RPMI 1640 (Gibco), 10% human sera, Pen-strep (Gibco) and Gentamycin (Gibco) for 24 h at 37 °C, 5% CO_2, humidified incubator, to allow them to develop into the mature form before subjected to binding assays to determine their CD36 binding phenotypes.

Determination of parasite binding phenotype: Parasites (IRBCs) were allowed to develop into the mature form by *in vitro* culture before being subjected to binding assays to determine their CD36 binding phenotypes. Purified CD36 (20 µl) and BSA at a concentration of 1 µg/ml (Sigma Chemicals Co. St Louis, USA) were placed onto a Petri dish and incubated overnight at 4 °C. Petri dishes were then blocked with 2% BSA for 30 min at 37 °C and washed three times with 1 × PBS (Dulbeco). The dishes were incubated with 5% Hematocrit of washed parasites for 30 min at 37 °C followed by a triple wash with PBS. Parasites on Petri dishes were fixed using 1% (w/v) Formaldehyde solution at room temperature for 1 min, and then stained with 1% Giemsa stain for 1 min. The number of parasites binding onto each molecule in relation to those binding to BSA was determined by observing under light microscope at 10 × magnification in 20 fields of the binding plate.

DNA extraction and PCR amplification of CD36: DNA extraction was performed using the Gentra DNA extraction protocol (Gentra Systems Inc, Minneapolis, USA) according to the manufacturer's instructions. DNA was used as 10% of the PCR reaction mix. Nested PCR was used throughout this experiment. The reactions were performed in 50 µl reaction tubes on a PTC-100 Programmable Thermal Controller (MJ Research Inc., USA). The primer sequences for the first PCR reaction were F: 5' ATG CTT GGC TAT TGA GT and R: 5' TAT CAC AAA TTA TGG TAT GGA CTG and those for the nested PCR were F: 5' CTA TGC TGT ATT TGA ATC CGA and R: 5' ATG GAC TGT GCT ACT GAG GTT ATT

CGT T. The nested primers were designed using DNAstar® software (Lasergene, Madison, USA). Distilled water was included in the control reactions instead of the isolated DNA. For both PCR reactions, the following PCR cycle was used: a initial denaturation step of 94 °C for 4 min followed by 45 cycles of 94 °C denaturation for 1 min, annealing at 55 °C for 30 s and elongation at 68 °C for 8 min. The first PCR reactions amplified fragments of 415 bp whereas the nested PCR reaction amplified fragments of 212 bp.

Genotyping by RFLP and agarose gel electrophoresis: The nested PCR product (20 µl) from each sample were placed into a 50-µl microtubes followed by 1 µl of restriction enzyme *Nde*I (BioLabs Inc, New England, USA). The mixture was incubated for 4 h at 37 °C and then was heated at 65 °C for 1 min to stop enzyme activity. The *CD36* gene has a wild-type *Nde*I restriction site, 5'-CA/TATG. This single nucleotide mutation eliminates the inherent restriction site. Thus, *Nde*I digestion of the wild-type CD36 allele gave two fragments, 148 bp and 64 bp, whereas the homozygous mutant was not cut and thus was a single fragment of 212 bp. The heterozygous allele gave a mixture of the three fragments from the wild-type and the mutant allele, i.e. 212, 148 and 64 bp fragments. Restriction digestion products, PCR products and molecular weight markers were subjected to agarose gel electrophoresis in a 3.5% (w/v) agarose gel (Sigma Chemicals Co) containing 5 µl of 10 mg/ml ethidium bromide (Sigma Chemicals Co). PCR products, restriction digests and molecular weight markers were loaded onto the wells as 1 µl of 6 × loading dye (0.2% bromophenol blue, 0.2% xylene cyanol, 60% glycerol and 60 mm EDTA) in 10 µl of sample, and run in 1 × TE buffer at constant voltage of 120 V for 25–30 min. The DNA marker FX174/*Hinf*I (BioLabs Inc) with fragment size range from 24 to 726 bp was used to determine the various band sizes for the samples.

Statistical analyses: For categorical (nominal) data, chi-square correlation tests were used to compare expected and observed frequencies for (genotypes, binding patterns and MA) parameters using SPSS version 14.0.1 (SPSS Inc., USA) computer software. Unpaired *t*-test comparisons of means were used to compare mean Hb readings among different genotypes.

Results

Frequencies of CD36 alleles and CD36 binders: Results for CD36 genotyping and IRBC binding profiles are presented in Fig 1 and Table. The gel presented in Fig 1 shows an agarose gel for the PCR-RFLP of genotyped CD36 gene for the CD36 T1264G mutation. Table 1 summarizes the frequencies of different CD36 genotypes among children. Results show that out of the 204 genotyped children, 176 (86.3%) children had the wild-type allele, 22

(10.8%) children were heterozygous for the studied mutation and 6 (2.9%) children were homozygous for the CD36 mutation studied.

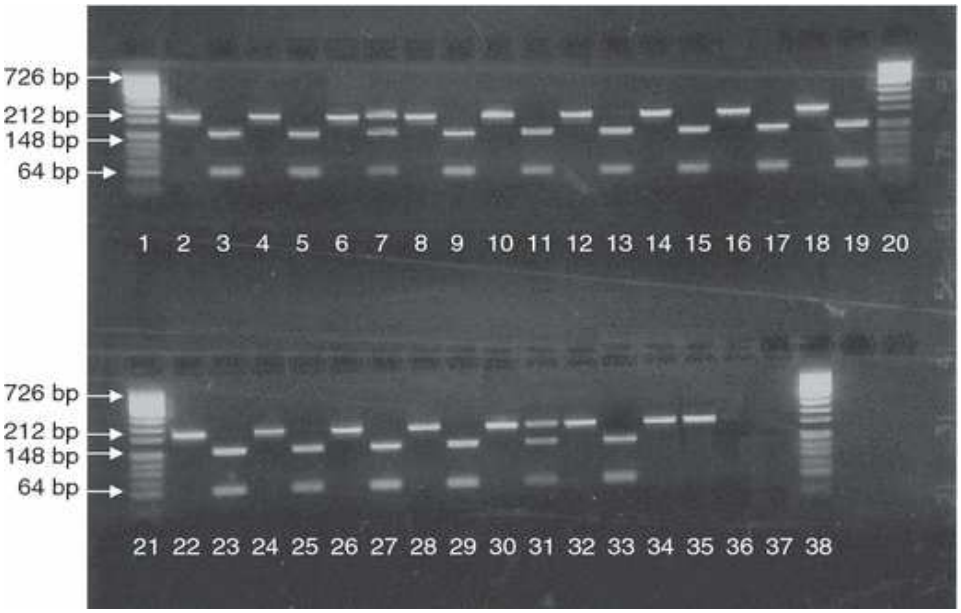

Figure 1: *Agarose gel showing restriction fragments for CD36 alleles. Lanes 1, 20, 21 and 38: molecular marker with the following band size (bp) (top–bottom): 726, 713, 553, 500, (427, 417, 413 together), 311, 249 200, 151, 140, 118, 100, 82, 66, (48, 42, 40 together), and 24 bp. Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 22, 24, 26, 28, 30, 32 and 34: undigested nested PCR product. Lanes 3, 5, 9, 11, 13, 15, 17 19, 23, 25, 27, 29: wild-type CD36 allele with two bands of 148 and 64 bp. Lanes 7 and 31: heterozygous for CD36 allele with three bands of 212, 148 and 64 bp. Lane 35: homozygous mutant with one band of 212 bp. Lanes 36 and 37: control samples (distilled water) digested and undigested, respectively.*

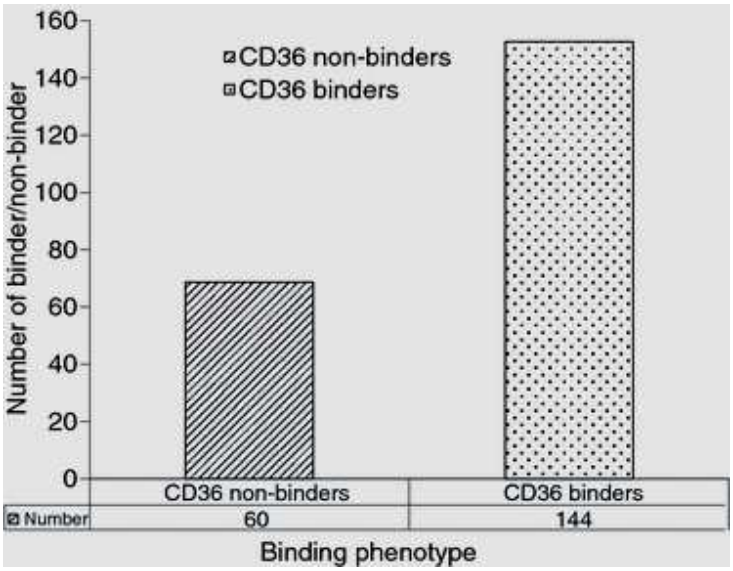

Figure 2: *Overall results for CD36- IRBC binding phenotypes. IRBC samples were either binders or non-binders if the number of CD36 binding IRBC were twice as much (or more) as the number of IRBC binding to BSA, tested on the same plate.*

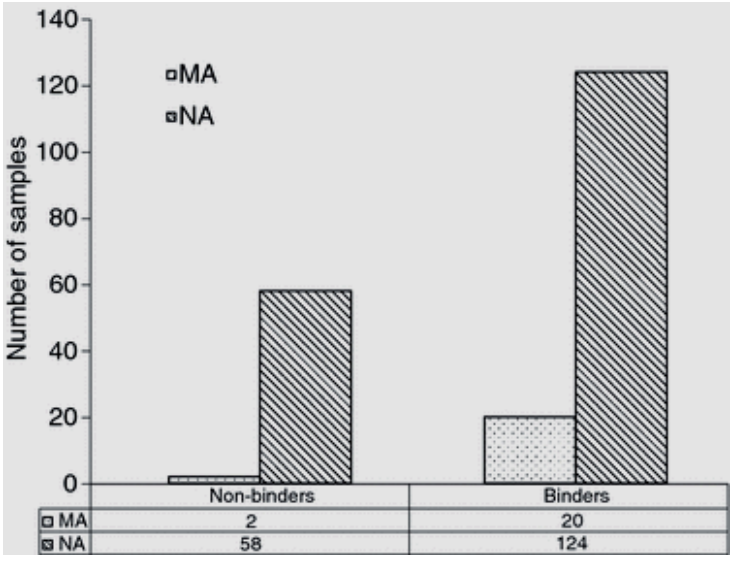

Figure 3: *Binding phenotypes of IRBCs isolated from different CD36 genotypes to CD36. The y-axis shows the number of anaemic cases from both binders and non-binder IRBCs to CD36. The binding phenotype was found to be statistically associated with occurrence of*

MA among children using the Fisher's exact test (P < 0.01). MA, malarial anaemia, NA, non-anaemia.

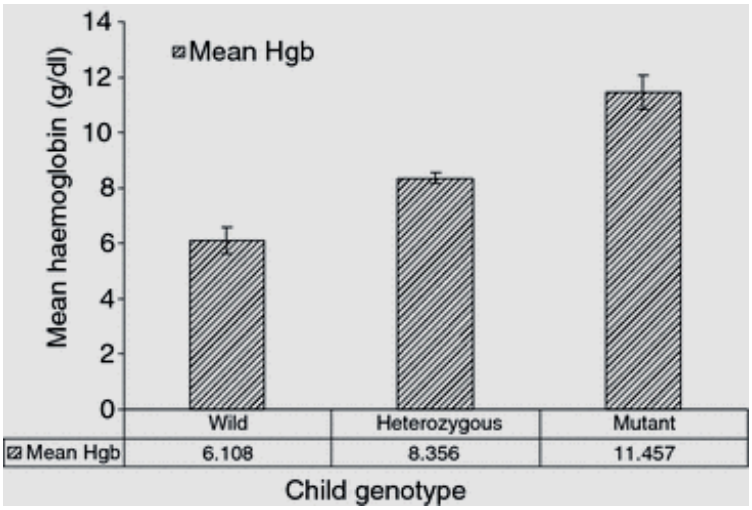

Figure 4: *Mean haemoglobin (Hb) levels among different genotypes of CD36. Bars represent the mean (mean ± SE) Hb level (g/dl) for each CD36 genotype. All means were statistically different from one another (P < 0.01).*

Table 1: Frequencies of CD36 genotypes and binders in each category

	Frequency	
	A	B
	Genotype	Genotype frequency (%)
Wild-type	176 (86.3)	124 (70.5)
Heterozygous	22 (10.8)	20 (10.8)
Homozygous	6 (2.9)	0 (0.0)

Data presented in column A: Numbers indicate the number of children that fall in each genotype (Wild-type, Heterozygous and Homozygous mutant). Numbers in parentheses show the percentage of respective genotypes out of 204 typed children. Column B: Data in column B show the number of IRBC samples binding to CD36. Numbers in parentheses indicate the respective percentage of binder IRBC samples in each genotype.

Discussion and conclusions

The absence of data that intimately defines, at the molecular level, the host–parasite interface during infection with malaria parasites leaves a major gap in our understanding of the critical phenomena that lead to severe malaria, particularly in children. The only available information provides statistical associations between polymorphisms that occur in genes encoding host proteins used by malaria parasites as ligands of cytoadherence and severe malaria. Consistent with previous observations in Kenya and The Gambia [22], results from this study show CD36 to be the major ligand for cytoadherence of field isolates of *P. falciparum.* In line with previous findings elsewhere [25-29], we have shown in our study that the prevalence of CD36 deficiency in Muheza, Tanzania is (2.9%).

A small proportion of IRBCs from CD36 deficient children bound to the purified CD36 protein. Binding to CD36 has been shown to be highest when IRBC used were from children with the wild-type CD36 allele. This association between CD36 genotypes and parasite adherence to CD36 in our study was found to be statistically significant. The association between IRBC binding phenotype and occurrence of MA provides a direct explanation that failure of adherence of IRBC to vascular endothelial cells may be responsible for reduced occurrence of MA. The findings in this study suggest that protection against MA in CD36 deficient children is most likely to be a result of changes (absence/reduction) in IRBC adherence to vascular endothelial CD36 receptors.

As CD36 deficient children do not express the CD36 receptor, it is likely that CD36 deficiency alters the ability of IRBCs to bind to CD36. A reduction/absence of *in vivo* IRBC adherence to CD36 may result into a reduction in the number of sequestered, agglutinating and rosetting IRBC and non-infected RBC, which would result to resistance to reduction of number of circulating erythrocytes due to parasite-induced hemolysis. This phenomenon is reflected as normal Hb level, and thus absence of anaemia. This is the first study that has linked polymorphisms in the CD36 gene, genotype-specific, IRBC CD36 binding profiles and MA.

Apart from acting as a receptor for *P. falciparum*-infected IRBC, CD36 serves as an important molecule in modulating host immunity. CD36 is expressed on endothelial cells, platelets, macrophages and dendritic cells and participates in phagocytosis and lipid metabolism [30-36] all of which are crucial processes of life. This may partly explain the mechanisms by which a polymorphism that protects against a severe malarial syndrome (MA) is kept at a relatively low and stable frequency in the study population and elsewhere. The results of

this study focus on the contribution of CD36 polymorphisms to the development of MA in children. The deficiency of CD36 on immune cells is most likely to interfere with immune processes, including phagocytosis of ageing RBCs, which may have significant implications in terms of Hb levels, aberrant lipid metabolism, predisposition to atherosclerosis insulin intolerance and many other fatal conditions [33, 37, 38].

A delicate equilibrium is therefore likely to exist between the protective role of CD36 deficiency and reduced CD36 adherence against MA and its predisposition to other equally fatal diseases. This observation calls for future studies to better explain how CD36 deficiency may influence host immunity, and ways in which such deficiency modulates the clinical outcomes of fatal syndromes.

Acknowledgements: The authors acknowledge the support of the MOMS Clinical Research Laboratories at Muheza Designated District Hospital, Muheza Tanzania for providing space for this work. The Seattle Biomedical Research Institute (USA) is acknowledged for providing financial and technical assistance. The authors also thank the mothers who by consent, decided to participate in our study in virtue of its outcome and future use.

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CHAPTER

7

General Discussion

General discussion

Interpretation of the results

Chloroquine has been a useful antimalarial drug for decades, but parasite resistance to this drug has led to policy changes in most malaria-endemic countries. Tanzania, for example, changed its policy from chloroquine to an antifolate drug combination, sulphadoxine-pyrimethamine (SP) for first-line treatment of uncomplicated malaria in August 2001. In addition, SP is used to control malaria in pregnancy: as part of antenatal care, pregnant women are provided with repeated curative doses of SP. Surveillance for antifolate-resistant parasites in the field was important to keep tracking the spread and intensity of drug resistance. Monitoring drug resistance through regular exploration of gene mutations pertaining to drug resistance bears critical importance. Results from surveillance of SP resistance parasite genetic markers have led to a policy change in Tanzania in late 2006 from SP as an interim first-line drug to artemether-lumefantrine (Coartem). Both effective drugs and naturally acquired immunity play important roles in controlling malaria. Such immunity is acquired in endemic areas through continued or repeated exposure to malaria infection.

In the immune defence against infection with *Plasmodium falciparum*, many innate factors were found to be involved. Genetic polymorphisms in the innate immune response of the host during falciparum malaria results in a partial protection against the development of severe disease. Thalassaemia trait, sickle cell anaemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency may partially protect against disease. The acquired resistance, on the other hand, is slow to develop and the underlying immune mechanisms involved are still only partially understood. It is believed that acquired immunity requires repeated exposure to malaria infection, possibly with different variants of the parasite. In areas of stable transmission, neonates are protected for the first 6 months of age by maternal antibodies, followed by a periods of increased susceptibility during which immunity is only slowly acquired. Depending on the level of transmission anti-parasite immunity appears around 10 years of age. Adults tend to get less severe disease and parasite densities are usually lower than in children..

A combined efficient innate and adaptive immune response is essential for limiting *Plasmodium falciparum* multiplication in its host's blood or at least to prevent the progression of infection to (severe) disease. The importance of protective immunity is evident from the fact that many people in endemic areas are infected yet symptom-free. This protective immunity can possibly be influenced by micronutrient deficiencies. Zinc plays a critical role in multi-

ple enzyme systems that are involved in gene expression, cell division and growth, and immunological functions. Zinc determination in plasma is difficult to link to the zinc status in the individual. Supplementation with zinc and observing the response is the most reliable method to diagnose zinc deficiency. Based on available evidence and the guidelines for a survey design, zinc deficiency and low zinc status were defined as plasma zinc concentrations <9.9 μmol/L and <10.7 μmol/L, respectively. Zinc deficiency has been found to result in an increased risk and severity of a variety of diarrhoeal diseases, acute respiratory infections and possibly malaria. We therefore hypothesized that supplementation with zinc and other micronutrients would boost the capacity of the immune system to brandish a protective response to falciparum malaria. To study this hypothesis, we adopted technical and conceptual advances to analyse the immune capacity *in vitro* without the need to wait for actual challenging the individuals with infectious organisms. This enabled us to assess various immunological parameters reflecting the capacity of monocytes to mount innate immune response, induction of T-cell subpopulations and alterations in cytokine-mediated immune regulation, and alterations in malaria-specific antibodies and their isotypes in the serum.

The studies described in thesis were conducted in Tanzanian preschool children, with the following aims:

1. To assess the prevalence of resistance-associated mutations on dhfr (dihydrofolate reductace) and dhps (dihydropteroate synthase) genes of the infectious pathogen, *P. falciparum*, and their relation to the risk of drug resistance to SP.
2. To assess the effect of nutrient deficiencies on cytokine responses of innate immunity to Plasmodium falciparum.
3. To assess the effect of nutrient deficiencies on adaptive immune responses to Plasmodium falciparum, measured as T-cell responses to in vitro stimulation with cultured Plasmodium falciparum-infected red blood cell preparations.
4. To assess associations between markers of micronutrient status and plasma levels of malaria-specific immunoglobulins.
5. To assess associations between the endothelial receptor molecule CD36 deficiency, Plasmodium falciperum adherence in vitro on purified CD36 molecules and anaemia status among children in a malaria endemic region.

Mutations in the *dhfr* and *dhps* genes alter the conformational structure of some crucial enzymes that are involved in the synthesis of parasite DNA. SP is a drug combination that was designed to produce an intracellular state of folic acid deficiency so that folate-dependant enzymes involved in DNA synthesis and cell division are inhibited, thereby killing the para-

site. Mutations in those genes can induce parasite resistance to SP. The field work for our study (January-August 2002) was conducted shortly before the formal introduction of SP as the first-line treatment for uncomplicated malaria (August 2002). Even so, we found that 11% of treatments resulted in failure within 14 days of follow-up (**Chapter 2**). These findings and subsequent reports [1-4] provide support for the decision by late 2006 of Tanzanian policy makers to change from SP to artemether-lumefantrine (Coartem) as the first-line antimalarial drug. The problem of drug resistance by the malaria parasite has been compounded by similar problems of resistance by the mosquito vector to insecticides used for bednet impregnation. Thus improving nutrition to boost immunity against malaria remains to be a promising control strategy in endemic areas.

The findings presented in **Chapter 3** show variable effects of micronutrient deficiencies on innate cytokine responses. Previous studies have shown the immunoregulatory potential of zinc [5-12]. Our results concur with previous reports that zinc deficiency is associated with increased production of malaria-extract induced and monocyte-derived inflammatory cytokines (TNF-α and IL-1β) that can be restored through zinc supplementation [8, 11, 12]. Our results contradict reports, however, that deficiency in zinc resulted in a reduced production of TNF-α. It can be derived from these contradictory findings that zinc deficiency might be causing an imbalance in cytokine production [13]. This is critical in malaria because increased inflammatory cytokine levels are widely associated with pathological consequences of the disease. The association between magnesium and innate cytokine regulation, particularly in malaria, had not been studied before. In our study, we found a relative increase in IL-10 concentration in magnesium deficiency as compared to zinc deficiency.

In **Chapter 4,** effects of micronutrients deficiencies are assessed on T-cell responses that, based on their associated cytokine profile, are indicated by type I and type II. The results concur with previous reports that zinc deficiency is typically associated with the production of signature type I cytokines, like IFN-γ, that results in a cell-mediated immune response [8, 15]. By contrast, magnesium deficiency appeared more associated with signature type II cytokines, notably IL-13. IL-13 production coincides with the generation of a humoral immune response [15], and is particularly associated with isotype switching from IgG to IgE [16-18]. This may be critical because IgE contributes to protection against malaria, but also to disease severity [17, 19]. Collectively, our findings indicate that deficiencies in zinc and magnesium may lead to an imbalance between type I and type II responses, which may be associated with poor protection or even pathology. Iron deficiency anaemia was found to be associated with variable changes in concentrations of both type I and type II cytokines, par-

ticularly in children with malaria infection.

The associations between zinc deficiency and plasma antibody responses to malaria are reported in **Chapter 5**. Specifically, the study determined the plasma concentrations of IgM, total IgG, and malaria-specific IgG subclasses to provide the profile of a specific antibody response to malaria antigens. The malaria-specific IgG3 concentration was predominantly high in both zinc deficiency and zinc replete groups although the levels were relatively higher in zinc deficient than in zinc replete individuals. The malaria status, both in asymptomatic and symptomatic children, was always associated with increased IgG3 concentrations, and the change was always associated with an age dominating in 'older' (3–5 yrs) as compared to younger age (< 3 yrs of age). The dominance in IgG3 responses is in agreement with previous reports that this IgG subclass is the main antibody class conferring protection against malaria parasites [20-25]. We report that the association of at least one cytophilic with one non-cytophilic IgG subclass may be crucial in conferring protection at least in zinc deficiency and this association is also influenced by age. This is because at younger age the IgG1 and IgG4 classes were dominant and IgG2 and IgG3 were more prevalent in older children providing the probability of the latter to be protective [26, 27] in relatively older children. These suggestions needs further studies but largely reflect an imbalance in antibody responses due to zinc deficiency following a similar effect as previously observed in T-cell cytokine responses. This is not surprising as the isotype switching potential of the malaria-specific B-cells is largely dependent on exposure to helper T-cell derived cytokines. Our study specifically underscores the notion that the variation in profiles of IgG subclasses are the combined result of zinc deficiency, malaria status and an age < 5 yrs. These findings support the idea that zinc (and other micronutrients) may influence the malaria-specific immune response at all steps ranging from the induction of the response to the generation of effector mechanisms by cells and antibodies [8, 28].

Our findings from chapters 3-5 can be presented in a model showing possible impacts of nutrient deficiencies on a range of immune responses to malaria parasites **(figure 1)**.

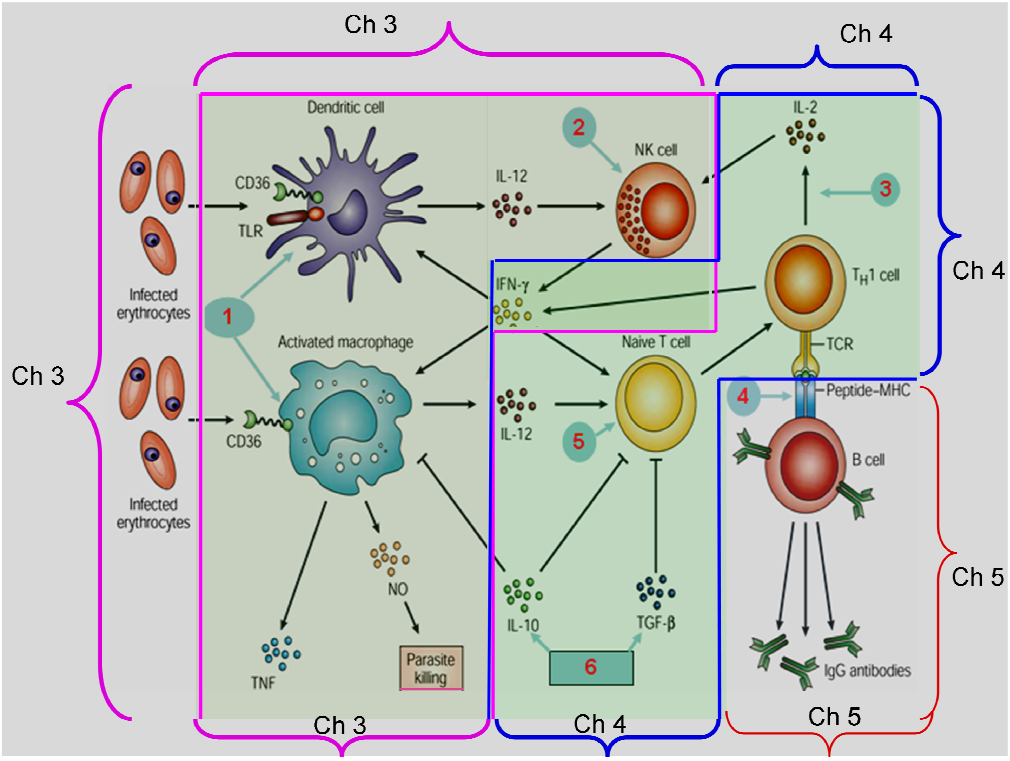

Figure 1: *Different points in the network of the proposed immune response to malaria parasites where possible effects of zinc deficiency are noticed; 1: impaired activation, phagocytosis; 2: impaired killing activity; 3: decreased production of IL-2 and IL-2 receptors; 4: impaired MHC class II expression; 5: impaired T-cell differentiation; 6: impaired production of anti-inflammatory cytokines by Tr cells (modified from: Stevenson & Riley [14]).*

Previous reports have proposed a stepwise fashion in *P. falciparum*-parasitized erythrocytes cytoadherence within the microvasculature, reflecting a synergistic activity between receptors. According to Ho and White [29], parasite ligands expressed on the surface of infected erythrocytes interact with endothelial receptor CD36, and in synergy with intercellular adhesion molecules-1 (ICAM-1), P-selectin and vascular adhesion molecule-1. Consequently, cytoadherence may activate intracellular signalling pathways of mediators, particularly cytokines, which can influence the outcome of infection. CD36 participates in phagocytosis and lipid metabolism and is known to be the major endothelial receptor molecule for field isolates of *Plasmodium falciparum.* A T1264G mutation in exon X of the gene leads to deficiency of CD36. **Chapter 6** explored the association between this CD36 deficiency, *P. falci-*

parum in vitro adherence with purified CD36, and anaemia in children. The findings indicate the protective role of CD36 deficiency against the development of malarial anaemia in children, which may be mediated through reduced cytoadherence of infected red blood cells to vascular endothelium. These findings necessitate a more focused study on the nature of the protective mechanisms that could be exploited and combined with malaria interventions to limit severe forms of malaria.

From the results of this thesis, we have generated a model that represents the effects of zinc and magnesium deficiencies on the generation of the immune response in the course of malaria infection **(figure 2)**. In this model, we propose that following an infection with malaria, of the presence of a zinc deficiency can induce Th1 cell activation with subsequent production of mainly type I signature cytokines (TNF-α, IL-12 and IFN-γ) which activate and induce B-cells, in a series of events to produce antibody switching from IgM to IgG1 and IgG3 subclasses. IgG3 is responsible for malaria parasite clearance (**Chapter 5**). On the other hand, we hypothesise that malarial infection under conditions of magnesium deficiency will lead more towards a Th2 response phenotype through the production of IL-5, IL-10 and IL-13. IL-13 is well known for its pathological role through induction of IgG4 and IgE isotype switching and CD23 expression by human B cells [17-19]. In malaria, Th2 responses will induce B-cells to produce IgM switching to IgG2, IgG4 and possibly IgE. As discussed in **Chapter 5**, IgG2 may be involved in protection against malaria in association with IgG3, but IgG4 and IgE may be associated with the development of disease. It is important to note that while zinc deficiency showed insignificant changes in innate cytokine responses (**Chapter 3**) in the absence of infection but a high cytokine response in malaria infection, magnesium deficiency seemed to associate with increased IL-10 levels independent of a malaria infection.

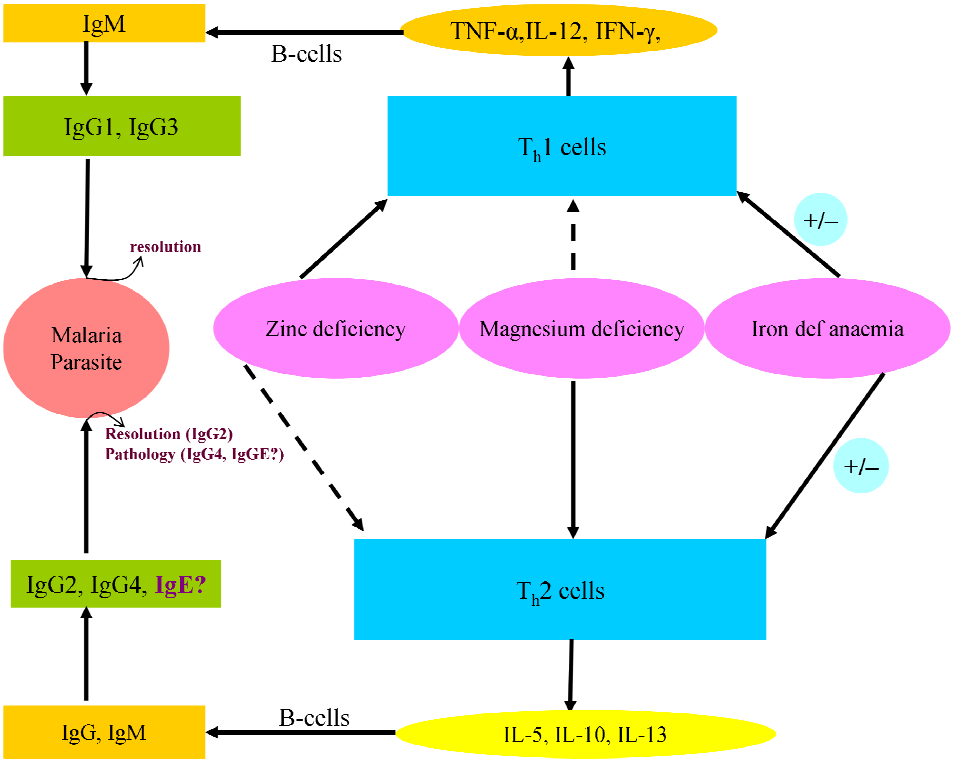

Figure 2: *Proposed model for the effects of zinc and magnesium deficiencies on immune responses in the course of infection by the malaria parasites. Iron deficiency anaemia variably influenced production of cytokines especially depending on malaria infection status (+/-). Dashed line: negative effects; solid line: positive effects.*

Intervention strategies

Micronutrient deficiencies have been widely regarded as contributing factors to morbidity from infections. Several studies have indicated a reduction in disease morbidities and mortalities as the result of micronutrient supplementation. Nutrient deficiencies lower the resistance to infection. Conversely, fever from infection speeds depletion of calories and thus nutrients by influencing satiety centres that control appetite in the brain. As a result, loss of appetite and other symptoms (e.g. weakness, nausea and mouth lesions) limit the ability to eat. Programmes that prevent or control infection (e.g. immunization, improvement in hygiene and sanitation, safe water supply, and access to medicine and medical care) can all indirectly improve nutrition status. This thesis evaluated the impact that some micronutrients may have on the immune response to malaria focusing on both innate and adaptive re-

sponses. The intervention strategies should focus on precise selection of specific nutrient components given in combination at proper dosages and with suitable supplementation vehicles. Preschool children are the primary beneficiaries from such supplementation, but one of the main operational problems is that infants and toddlers have problems with swallowing tablets or capsules that are conventionally used to supplement minerals and vitamins. The International Research on Infant Supplementation (IRIS) initiative has been evaluating newly created supplementation vehicles in the form of chewable tablet-cookies and nutrient-dense spreads suitable for use in tropical environments. The efficacy, safety and acceptability is being evaluated in populations of children where this study was carried and the initial results are promising. Another possibility is the breeding and dissemination of new staple crop varieties with high concentrations of micronutrients. This has the potential to create an entirely new, safe, low-cost and self-sustaining approach to deliver micronutrients to poor farmer families that are difficult to reach through alternative approaches. Biofortification focusing on single nutrients or at most a few nutrients will be rewarding especially those which have shown potential impacts on the immune response to malaria as reported in this thesis. Malaria vaccine programmes should consider incorporating at least few micronutrients that have shown the potential to boost the immune response to malaria, like zinc, and to strengthen protection against the manifestation of clinical disease.

Potential challenges for micronutrient interventions

Nearly half of the world's population suffers from deficiencies in vitamins and minerals. Development of effective strategies for increasing the intake of micronutrients by people, most of them being the poor, bears a critical importance. The concern is how to produce strategies that are cost effective, acceptable and the most likely to succeed. Distribution of dietary supplements can be very efficacious in the short term. This approach may be difficult to sustain, however, because of poor adherence to supplementation, particularly if supplements cause unpleasant side effects, cost, and difficulties in the distribution of supplements. The best strategy would thus be fortification of staple foods with vitamins and minerals. This has been used and has proven to be successful in many developed countries; it can be used also in developing countries but can only be used with centrally processed foods and probably has limited impact in rural self-sustaining populations. Another strategy could be the use of nutritionally enhanced staple food crops through conventional breeding or genetic engineering ('biofortification'). These strategies could be very rewarding but may require relatively high initial capital investments. The main potential challenge is the acceptance of biofortified foods. Genetically modified foods have been facing wide spread problems of ethical and public acceptance. There have also been concerns that

genetically modified foods can cause allergic reactions or other health hazards. Community education and sound scientific advice to policy authorities is needed to clear misconceptions and to promote the use of these promising approaches.

Potential challenges from parasite and vectors genetic factors

Several host and parasite genetic factors will determine the fate of malaria infection and its consequences. Drug resistance of the parasite has been critical in malaria control. In addition, the extensive diversity in parasite surface proteins, which are the targets for the induction of the protective immune responses, and this combination proved to be catastrophic for malaria control. Malarial parasites are capable of evading the induction of a protective immunity by displaying an unprecedented level of antigenic variation, and in addition have developed several specific genetic mutations that confer resistance to the employed antimalarial drugs. Integrated disease control is furthermore hindered by the resistance of *Anopheles gambiae* – the most important malaria vector in Africa – to commonly used insecticides, the inability of transgenic mosquitoes to compete under field condition and the ban of previously effective insecticide (DDT). In this context, the strengthened immune response to malaria through micronutrient supplementation could still be a preferable potential strategy particularly in poor communities. Our findings show potential associations between nutrient deficiencies and cytokine responses although they lack sufficient evidence to warrant development of public health interventions as a strategical measure to improve body's immune defence as yet. However, the study provides insights for further research that can bring this goal within reach. More large scale dietary intervention studies at the community level can substantiate and elaborate on the findings described in this thesis.

Potential challenges from host genetic factors

An emerging challenge in chapter 3 is the IL-12B gene promoter polymorphisms that have been detected in Tanzania, and that have been reported to be associated with low IL-12 production and increased malaria mortalities in children [33]. Suboptimal levels of IL-12 in early phases of infection may result in delayed or reduced triggers of subsequent cellular responses to infection. These polymorphisms may underestimate the real IL-12 cytokine response that could be expected under normal conditions. With the results of chapter 5, the recently reported ethnicity difference in malaria-specific antibody subclasses [27] poses a challenge on the variability in the response to malaria that can be expected in the population. This is in addition to the differences due to micronutrient deficiencies. Some genetic polymorphisms like those occurring in TNF-α and TLR-genes are challenging the potency of the immunological reactions. For example, it has been recently reported that single nucleotide

polymorphism haplotypes of TNF-α genes will result into significantly higher plasma levels of TNF-α [34]. Strong TNF-α responses have furthermore been associated with intestinal blockade of iron absorption leading to its systemic deficiency. On the other hand, the reported TLR-4-mediated response to malaria *in vivo* and the polymorphisms on the TLR-4 genes and their association with severe disease [35] are potential challenges to intervention programs aimed at correcting such nutrient deficiencies. Research efforts should be directed to address these challenges together with possible contribution of Th17 cells that produce a potential inflammatory cytokine IL-17 that may also contribute to malaria severity in endemic countries.

Conclusion

Despite the variable effects of micronutrients on several parameters of the immune response to malaria, the findings from all chapters in this thesis, add to our current knowledge of how useful micronutrient supplementation can become part of malaria control programs. In the face of the prevailing malaria parasite resistance potential to conventional antimalarial drugs, and given the potential development of protective immunity in endemic areas, the use of micronutrients supplementation may become an option for prevention, especially in young children.

Recommendations for future research

The potential effects of zinc and magnesium in boosting effective malaria-specific immune response as shown in Chapters 3 – 5 may help poor communities to prevent malaria. However, large scale dietary intervention trials are needed to confirm the medical importance of the findings that zinc deficiency and possibly magnesium, influence the balance in the production of crucial cytokines that play a role in limiting the dangers of malaria. Focusing on supplementation trials may be rewarding in providing sufficient evidence for policy changes towards complementary strategies for malaria control programs, but more emphasis should be placed on elucidating the underlying mechanisms by which micronutrients interact with or promote the development of effective innate and adaptive responses. In addition, African countries should support the development of research facilities so that most of advanced immunological researches can be near the field, where malaria occurs.

Food fortification of staple foods with vitamins and minerals, particularly those found to be deficient, should ideally, be implemented in developing countries. Lay people should be educated about the advantages of such programs, not only because they may improve the nutritional quality of foods but also because they may provide protection from malaria. Ac-

ceptance of nutritionally enhanced staple food crops through genetic engineering has been debated among scientists and lay people. Sufficient evidence and clarification, will be rewarding if focused on populations in need. Fortification of locally acceptable foods, and dietary modification to consume greater amounts of animal products and less phytates may contribute to better zinc and iron nutritional status. Focused researches on improving or designing the best supplementation vehicles should be sought to suit children, because these are at highest risk of malaria and other infectious diseases. With expected goals clearly explained to the community in need of the service, whatever useful strategy will hopefully be acceptable.

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Summary

This thesis aimed at investigating the role of genetic and nutritional factors that affect the immune response to malaria in Tanzanian children. The introductory chapter (**Chapter 1)** reviews the importance of nutritional deficiencies, particularly of zinc, and presents the hypothesis that such deficiencies lead to impaired immunity and contribute to the burden of malaria. The chapter also describes current efforts to prevent malaria through intermittent preventive treatment, both in infants (IPTi) and pregnant women (IPTp). Sulfadoxine-pyrimethamine is still used for first-line treatment of uncomplicated malaria, or, in many countries, to prevent malaria and anaemia in pregnancy. In malaria endemic areas, development of resistance to previously valuable antimalarial drugs has been continuously reported for decades. Thus our initial longitudinal study aimed at measuring the prevalence of resistance-associated mutations on dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) genes (*dhfr* and *dhps*) that confer parasite resistance to sulphadoxine-pyrimethamine (SP) that was used as an interim antimalarial drug after chloroquine resistance. Although SP resistance-associated point mutations were highly prevalent, we observed an adequate parasite response to SP (**Chapter 2**). We speculated that the impact of the *dhfr* and *dhps* mutations on SP resistance may be dependent at least in part on the protective immunity that has developed in response to frequent exposure to infection and may be weighed down by host immunity in endemic areas and thus impacts in the continued use of the drug for treatment of malaria. The impact of other drugs with similar mechanisms of action used as antibiotics in selecting mutations responsible for SP resistance needs therefore to be studied for their modulating activity of the immune response. These findings underscore the relevance to further study the crucial involvement of the immune system in the development of protection against malaria but also affecting the efficacy of treatment modalities of malaria in various African conditions.

In the subsequent cross-sectional studies, we assessed the effect of deficiencies of zinc and magnesium as well as iron deficiency anaemia on malaria-specific cytokine responses indicative of innate immunity to *Plasmodium falciparum* **(Chapter 3).** In this study, we used *Plasmodium falciparum*-parasitised red blood cells (pRBCs) as antigens for *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs). Cytokines were measured in the supernatant of cultured PBMCs after 24 hours of stimulation. Zinc deficiency was associated with a marked increase in monocyte-derived TNF-α concentration in children with malarial infection but not in their uninfected peers. In children with malarial infection, iron deficiency anaemia was associated with elevated concentrations of TNF-α, whereas magnesium deficiency in children without malaria seemed to be associated with increased concentrations of IL-10. Our findings reflected plasticity in cytokine profiles of monocytes reacting to

malaria infection under conditions of different nutrient deficiencies. Following the observation of the variable impact of micronutrients on innate cytokines, we evaluated the profile of both type I and type II cytokines and whether they were influenced by nutritional and malaria status **(Chapter 4)**. The cytokine measurements were performed at day 7 of stimulation anticipating that this timing was optimal for measuring effects on these cytokines mainly derived from activated T-cells. The results indicated a variable influence of nutrient deficiencies on increased cytokine response with zinc deficiency and iron deficiency anemia having greater impact on type I and magnesium deficiency on type II cytokines. The subsequent study evaluated the plasma levels of naturally acquired antimalarial antibodies of various IgG subclasses plus the total IgG and IgM levels and whether they were associated with zinc deficiency based on preceding chapters **(Chapter 5)**. The results indicated a high variability in antibody levels with zinc deficiency, varying with age of the affected child. IgG3 appeared to be predominant across all age subgroups within < 5 yrs aged children providing clues that IgG3 might confer immune protection to malaria under conditions of zinc deficiency. **Chapter 6** explored the association between CD36 deficiency, *P. falciparum in vitro* adherence on purified CD36 and anemia in children. CD36 is a receptor that occurs on the surface of activated immune cells and vascular endothelial cells and participates in phagocytosis and lipid metabolism. We hypothesized that it could play a fundamental role in cytoadherence of erythrocytes that are parasitized by *Plasmodium*. Our results showed that CD36 deficiency was associated with protection against the development of malarial anemia in children and that it may be mediated through reduced cytoadherence of infected red blood cells to vascular endothelium.

These studies demonstrate that despite antimalarial drug resistance, there is a potential for optimizing the immunological protective capacity in the population to confer parasite clearance that can be variably influenced by micronutrient status. Improving nutritional status in this population could be rewarding not only to increase protection to malaria but possibly also to other infections.

Samenvatting

In dit proefschrift worden de bijdragen van genetische en nutritionele factoren onderzocht op hun vermogen de immuunrespons tegen *Plasmodium falciperum* malaria in Tanzaniaanse kinderen te beïnvloeden. Het inleidende hoofdstuk (**Hoofdstuk 1**) word teen overzicht gegeven van het belang van dieet gebonden nutritionele deficiënties, in het bijzonder van zink, en wordt de hypothese ontwikkeld dat dergelijke deficiënties leiden tot verstoorde immuniteit die bijdraagt aan de ziektelast van malaria infecties. In dit hoofdstuk worden eveneens de huidige vormen van therapeutische interventies beschreven via zogenaamde intermitterende preventieve behandeling bij zowel jonge kinderen (IPTi) als zwangere vrouwen (IPTp). Sulfadoxine-pyrimethamine wordt nog steeds in vele landen gebruikt als eerstelijns behandeling van ongecompliceerde malaria of om malaria en daaraan geassocieerde bloedarmoede (anemie) tijdens de zwangerschap te voorkomen. In endemische malariagebieden wordt al gedurende vele jaren het probleem gerapporteerd van opkomende resistentie ontwikkeling tegen voorheen goed werkende antimalaria medicatie. In onze eerste longitudinale studie werd de prevalentie bepaald van resistentie geassocieerde mutaties in de genen die coderen voor dihydrofolate reductase (DHFR) en dihydropteroate synthase (DHPS) (*dhfr* and *dhps*) en die de parasieten resistentie bepalen tegen sulphadoxine-pyrimethamine (SP) dat gebruikt werd als interim antimalaria medicatie na de ontwikkeling van resistentie tegen chloroquine. Ofschoon aan SP resistentie geassocieerde puntmutaties veelvuldig voorkwamen vonden we dat de parasiet een adequate respons ontwikkelde tegen SP (**Hoofdstuk 2**). Wij speculeren dat het belang van de *dhfr* en *dhps* mutaties bij de ontwikkeling van SP resistentie (gedeeltelijk) berust op de beschermende immuniteit die in respons op veelvuldige blootstelling aan infectie optreedt op de jonge kinderleeftijd. Tegelijkertijd wordt deze beschermende immuniteit in endemische gebieden onderdrukt en maakt daarmee het continue gebruik van deze behandeling van malaria met SP noodzakelijk. Het belang van andere medicatie met vergelijkbare werkingsmechanismen als antibioticum om mutaties te selecteren die verantwoordelijk zijn voor SP resistentie dient daarom onderzocht te worden op het vermogen de beschermende immuunrespons te moduleren. Deze bevindingen onderstrepen het belang om de cruciale rol van het immuunsysteem nader te onderzoeken voor het opwekken van bescherming tegen malaria maar ook de beïnvloeding van de effectiviteit van de behandeling van malaria onder condities in Afrika.

In de hierop volgende cross-sectionele studies hebben wij het effect onderzocht van zink- en magnesiumdeficiëntie in combinatie met ijzer deficiëntie anemie op de malaria-specifiek geïnduceerde cytokinenresponsen die indicatief zijn voor de aangeboren immuniteit tegen *Plasmodium falciperum* infectie (**Hoofdstuk 3**). In deze studie hebben we *Plasmodium falciparum*-geparasiteerde rode bloed cellen (pRBCs) als antigeen gebruikt voor de *in vitro*

stimulatie van perifere bloed mononucleaire cellen (PBMCs) van jonge kinderen. De cytokines werden gemeten in het kweeksupernatant van PBMCs na 24 uur stimulatie. Zink deficientie bleek geassocieerd aan een sterke stijging in het van monocyten afkomstige cytokine TNF-α bij kinderen met een malaria infectie maar niet bij kinderen zonder deze infectie. Bij kinderen met een malaria infectie bleek ijzer deficiëntie anemie geassocieerd aan een verhoogde concentratie TNF-a, terwijl magnesium deficiëntie in kinderen zonder malaria infectie geassocieerd bleek te zijn aan verhoogde productie van het anti-inflammatoire cytokine IL-10. Onze bevindingen reflecteren de plasticiteit in de cytokinenprofielen van monocyten die verschillend reageren op malaria infectie afhankelijk van de nutriënt deficiëntie van het desbetreffende kind. Na het vaststellen van de variabele invloed van micronutriënten op de productie van cytokines die de innate immuniteit reflecteren, hebben wij het profiel van type I (IFN-γ) en type II (IL-5, IL-13) cytokines en de invloed daarop van malaria infectie en micronutriënt status onderzocht **(Hoofdstuk 4)**. De cytokinenmetingen werden uitgevoerd na 7 dagen stimulatie waarbij de effecten op de uitgroei en differentiatie van geactiveerde T-cellen waarneembaar zijn. De resultaten geven aan dat er een variabele invloed is van micronutriënt deficiëntie op de cytokinenproductie waarbij zinkdeficiëntie en ijzer deficiëntie anemie leiden tot een verhoogde productie van type I cytokines, terwijl magnesiumdeficiëntie de productie van type II cytokines verhoogt. Vervolgens hebben wij de plasmaspiegels van verschillende typen malaria-specifieke antistoffen bepaald die via natuurlijk voorkomende infecties waren gevormd. Speciaal is er gekeken naar malariaspecifieke antistoffen van de IgG subklassen en naar totale IgG en IgM antistoffen en of deze geassocieerd waren aan zink deficiëntie gebaseerd op voorgaande onderzoeken **(Hoofdstuk 5)**. De resultaten gaven aan dat zinkdeficiëntie leidt tot een grote mate van variabiliteit in antistofspiegels bij de individuele kinderen en dat deze spiegels afhankelijk blijken te zijn van de leeftijd van het kind. Malariaspecifieke antistoffen van de IgG3 klasse waren dominant in alle leeftijdsgroepen van kinderen jonger dan 5 jaar waarbij aanwijzingen werden verkregen dat IgG3 antistoffen immuunbescherming tegen malaria kunnen geven onder zinkdeficiëntie condities. In **Hoofdstuk 6** hebben wij de associatie onderzocht tussen CD36 deficiëntie, *P. falciparum in vitro* adherentie aan gezuiverd CD36 en het optreden van ijzer deficiëntieanemie bij jonge kinderen. CD36 is een receptor die voorkomt op het oppervlak van geactiveerde immuuncellen (zoals dendritische cellen en macrofagen), erytrocyten en vasculaire endotheelcellen en die participeert in fagocytose en lipide metabolisme. Wij onderzochten de hypothese dat CD36 een fundamentele rol zou kunnen spelen in cytoadherentie van erytrocyten die geïnfecteerd zijn met *Plasmodium*. Onze resultaten toonden aan dat CD36 deficientie geassocieerd was aan bescherming tegen de ontwikkeling van malaria geïnduceerde

anemie en dat dit mogelijk een gevolg was van gereduceerde cytoadherentie van geïnfecteerde rode bloedcellen aan het vasculaire endotheel.

Deze studie laat zien dat er in de populatie van Afrikaanse kinderen een grote potentie aanwezig is om via optimalisatie van de immunologische bescherming tegen infectie met *Plasmodium falciperum* malaria parasieten te verkrijgen ondanks het voorkomen van resistentie van de parasiet tegen de gebruikte geneesmiddelen en dat deze potentie verschillend beïnvloed wordt door de micronutriënt status van het individuele kind. Het verbeteren van de voedingsstatus van deze populatie kan daarmee de aanwezige beschermende immuniteit tegen malaria infectie versterken en wellicht ook tegen andere infecties.

Acknowledgement

This PhD thesis was done as part of research work by The Micronutrient and Child Health Project, a collaborative project that was undertaken by The Wageningen University in The Netherlands (at Cell Biology and Immunology Group) and Kilimanjaro Christian Medical Centre (KCMC) in Moshi, Tanzania. I feel to have honoured working with people of different calibre, cultural background, experience with motives for encouragement, support and give help whenever was needed.

I firstly wish to express my sincere gratitude to my Promotors, Prof. dr. ir. Huub F.J. Savelkoul (Cell Biology and Immunology Group) for his endless support and encouragement. Him, together with Prof. John F. Shao (KCMC) was the major firm stand towards success of this study.

I secondly appreciate the guidance and continued assistance from my co-promotor Dr. Hans Verhoef as a first-line field supervisor and excellent advisor in statistics and translation of field and laboratory language into 'book' language. I thank him very much! As a field supervisor, he worked tirelessly with his counterpart, Prof Raimos M. Olomi (KCMC) to keep the project going despite ups and downs that occurred along with this very long journey.

I thirdly, thank my employer Muhimbili University of Health and Allied Science for granting permission to undertake these studies and for encouragement. Special appreciation is accorded to Dr. Thomas B. Nyambo, my Head of Department for useful guidance and advice during the whole course of my studies. The support from all other colleagues at Muhimbili is highly valued.

I feel guilty not saying a word to Prescilla Jeurink, Yvonne Vissers, Marjolein Meijerink and Gerco den Hartog (mkuu kabisa sana) who were directly influential in my work. Special regards to Marjolein and Prescilla for their contributions from labwork to the development of some manuscripts constituting this thesis, as they were always available for questions when needed. I thank Maria Forlenza, together with all other colleague PhD and MSc students from CBI for their encouragement which started at the first Fish Immunology workshop (10^{th} – 14^{th} April 2005) all the way till writing this thesis.

Lidy Kamenade: Can you please accept these words of thank on behalf of other senior staff at CBI. I do not want to repeat many words, your team has been so wonderful and made me feel at home. Trudi, Nico and Henk: because of ink and papers, may you please accept to represent other technical staff for the support I received? I highly appreciate your kind and

always available assistance. I won't forget for the kind assistance you offered to me including your time and thoughts.

The Secretariat Desk: Hilda Valk, without you I would not knock the door to Huub, neither I would not go to Immigratie en Naturalisatie Dienst in 's-Hertogenbosch for a residence permit and preparation of this thesis. Your tireless link between Netherlands and Tanzania and within Netherlands has made this work get done. Thank you very much.

Arnold Ndaro, Liselotte and Ndealilia on behalf of other technical staff at KCMC-Biotechnology Laboratory in Moshi, receive my sincere gratitude for making my life easier at that lab. I felt at home. I also appreciate the company and support from all PRIOR/APRIORI PhD students, Charles Mwanziva, Seif Shekalaghe, Gibson Kibiki and Reginald Kavishe, in Moshi, fellow Tanzanian students at Wageningen University and The Tanzanian community in The Netherlands are thanked for their company.

I wish to extend my sincere thanks to the parents and guardians who consented on behalf of the children of Handeni district to participate in the study. Support from Dr Mhina, the district medical officer, the ward and village executives and the general community of Handeni district added value to the success of this thesis.

Similar gratitude is accorded to my paranymphs Marjolein and Gerco, for devoting their time to support me and being instrumental in the whole process of making this thesis done.

I am also grateful to the preliminary support from my parents, my mother Esterina Chogimembe Sekisinda and my late father Vitus Lyambangi Mbugi for sending me to school. I feel honoured to have such parents. I also thank my mother in-law Teresia Ndenyaro and may late father in-law Mathias Mbuya for bringing my lovely wife in this world.

With grateful sincere, I thank my wife Romana and my daughters; Faraja and Pelagrina for their patience during my whole on and off period of studies. The courage and support from my wife has resulted in all these successes. Sincere gratitude to my relatives, brothers and sisters and my friend Dr Damas Philip for their prayers, back up and support in preparation of this Thesis.

Without God, all the beautiful words above are as good as nothing!! I thank God for his creation, bringing this life in place and for keeping me healthy.

Training and Supervision Plan, Graduate School WIAS

The Basic Package (3 ECTS)
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The Dutch Society of Immunology Conference on Self-Non-self Recognition (2005)
The Gordon Research Conference on Malaria: frontiers of Science (2005)
The 4th MIM Pan-African Malaria Conference 2005 (2005)
The Allergy Consortium Wageningen: Mini-symposium (2005)
The African Malaria Network Trust (AMANET) Workshop on Malaria Vaccinology in Developing Countries (2005)
Joint Malaria programme Annual Workshop (2005)
WIAS Seminar: Immune response to viruses: a comparable approach (2006)
Seminar on Macrophage polarization and immune response (2006)
Workshop MACH project: preliminary results pilot study, planning randomised trial (2006)
Workshop MACH project: oral presentation of results randomised trial (2008)
Effects of supplementation of zinc and other micronutrients on the health, development and well-being of African children, poster (2005)
Meeting of Collaborators in the Micronutrients and Child Health, oral presentation (2005)
The effects of zinc and other micronutrients supplementation on cellular and serological indicators of immunity against malaria, oral (2005)
Drug resistance to Sulphadoxine-pyrimethamine (SP) in *Plasmodium falciparum* malaria, poster (2005)
Nutritional zinc deficiency and cellular immune response to malaria, electronic poster (2007)

In-Depth Studies (25 ECTS)
Training course on functional genomics of insect vectors of human diseases (2005)
Theoretical and Practical Training Course on DNA-Extraction and PCR for α-thalassaemia (2005)
Fish Vaccination course, Wageningen University (2006)
Design and analysis of randomised trials, two weeks (2006)
Fish Immunology Workshop (2005)
Workshop: Good Clinical Practice (GCP) and Good Clinical Laboratory Practice (GCLP) Training Course (2007)
The Basic Vaccinology Course: Developing vaccinology expertise for Africa (2007)
The Fourth EDCTP Annual Forum: Building Bridges for Better Health (2007)
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Bi-weekly Journal club with PRIOR PhD students under supervision by Dr Hans Verhoef (2005-2009)
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WGS Course: Information Literacy, including Introduction Endnote (2008)
WGS Course: Project- and Time Management (2008)

Research Skills Training (2 ECTS)
Preparing own PhD research proposal (2005)
Subtotal Research Skills Training

Didactic Skills Training (4 ECTS)
Supervising two MSc students (2007-2009)

List of Publications

1. **Mbugi EV,** Mutayoba BM, Malisa AL, Balthazary ST, Nyambo TB, Mshinda H. Drug resistance to Sulphadoxine-pyrimethamine (SP) in *Plasmodium falciparum* malaria in Mlimba, Tanzania. *Malaria Journal*; October 2006; 5:94.
2. **Mbugi EV,** Meijerink M, Veenemans J, McCall M, Olomi RM, Shao JF, van der Wielen J, Verhoef H, Savelkoul HFJ. Alterations in early cytokine-mediated immune responses to *Plasmodium falciparum* infection in Tanzanian children with mineral element deficiencies: a cross-sectional survey *(submitted).*
3. **Mbugi EV,** Meijerink M, Veenemans J, McCall M, Olomi RM, Shao JF, van der Wielen J, Verhoef H, Savelkoul HFJ. Effect of nutrient deficiencies on *in vitro* T_h1 and T_h2 cytokine response of peripheral blood mononuclear cells to *Plasmodium falciparum* infection *(submitted).*
4. **Mbugi EV**, den Hartog G, Veenemans J, Olomi RM, Shao JF, van der Wielen J, Verhoef H, Savelkoul HFJ. Nutritional status and alteration in plasma levels of naturally acquired malaria-specific antibody response in Tanzanian children *(submitted).*
5. **Mbugi EV,** Mutayoba BM, Balthazary ST, Malisa AL, Nyambo TB, Mshinda H. Multiplicity of infections and level of recrudescence in *Plasmodium falciparum* malaria in Mlimba, Tanzania. *African Journal of Biotechnology*; September 2006; **5**(18):1655-1662.
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Curriculum Vitae

Erasto Vitus Mbugi was born in Kibaha, Tanzania on 05th January 1972. He grew and undertook his primary school education in Tanangozi, Iringa south-western highlands of Tanzania. In 1987, he joined secondary school education at Tosamaganga Secondary School in Iringa and completed studies in November 1990. The author joined High School secondary education in July I991 at Kibaha High School in Pwani region, Tanzania. In 1994, he joined Sokoine University of Agriculture where he graduated 1999 with a Bachelor of Veterinary Medicine.

The author, worked as a veterinary consultant at DARVETCARE in Dar es Salaam for four months and then was employed by the Ministry of Home Affairs with a post at Moshi Police Training School as a Veterinary Officer, where he worked for a year. Erasto joined Muhimbili University of Health and Allied Sciences in 2001 as a Tutorial Assistant, his current employer to date as an Assistant Lecturer. Erasto holds a Master of Veterinary Medicine of Sokoine University of agriculture since November, 2004 that was funded by The African Malaria Trust Network (AMANET) researching on malaria. He has been involved in malaria research since 2003, Ifakara Health Research and Development Centre being the basis for his outstanding performance in research. He has previously investigated molecular markers for malaria parasite multiplicity of infections and SP drug resistance from which one of his publication constitute chapters of this Thesis. In January 2005, he joined Wageningen University as a sandwich PhD student at Cell Biology and Immunology Group under Micronutrients and Child Health Project, financed by WOTRO/NWO grant. The project is in corroboration with Kilimanjaro Christian Medical Centre in Tanzania as a host institution.

During his study period at Wageningen University, he has attended numerous local courses, international workshops and conferences relevant to his subject research with substantial oral and poster presentations.

The cover of this Thesis was kindly designed by Gerco den Hartog and is an integration of the field study site, the laboratory in Moshi including near mount Kilimanjaro, where initial sample processing was carried and representative children of malaria endemic area.

Financial sponsors: This study was conducted under the project 'Micronutrients and Child Health' project. Financial support was received from the Netherlands Organization for Scientific Research, NWO/WOTRO (grant numbers W 93-413, WAO 93-441 and WIZ93-465) and UN Children's Fund (UNICEF). HV is supported by a grant from the European Community's Seventh Framework Programme [Fp7/2007-2013] under grant agreement no 211484. We furthermore acknowledge support by the Clinical Chemistry Laboratory of Meander Medical Centre, Amersfoort; Medisan, Heerenveen; and Pilots without Borders; all in The Netherlands.

Cover design: Gerco den Hartog

Layout: Erasto Vitus Mbugi

This Thesis was printed by Wöhrmann Print Services