INTERACTIONS BETWEEN MALARIA AND IRON OR VITAMIN A STATUS IN RURAL ZAMBIAN CHILDREN

by

Maxwell A. Barffour, M.P.H.

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ABSTRACT

Background and objectives

Evidence regarding the bidirectional interactions between iron or vitamin A status and malaria remains inconclusive. We assessed the longitudinal associations between iron (defined by ferritin) or vitamin A (defined by retinol) and malaria, assessed 6 months later by microscopy. Additionally, the changes in ferritin, soluble transferrin receptor (sTfR) and retinol during malaria and/or inflammation (AGP >1 g/L) were estimated.

Design

We included 1024 children, 4-8 years old from Mkushi District, Zambia, participating in a 6-month trial, designed to evaluate the efficacy of a provitamin A intervention. We analyzed baseline (August, 2013) and endline (March, 2014) survey data, collected in the low and high malaria transmission seasons respectively. We estimated incidence rate ratios (IRR) comparing endline malaria risk across baseline iron or vitamin A status using, modified Poisson regression, controlling for inflammatory changes in the indicators of iron and vitamin A at baseline.

Results

Inflammation alone was associated with changes of up to 66% in ferritin, up to 12% in sTfR, and up to -13% in serum retinol relative to their respective reference groups. Malaria with inflammation was associated with changes of up to 280% in ferritin, up to 40% in sTfR and up to -36% in retinol. After controlling for baseline inflammation, the IRR, comparing the low (0.7-1.05 µmol/L) and adequate (>1.05 µmol/L) vitamin A groups to the deficient group (<0.7 µmol/L) were 0.54 (95% CI: 0.24-1.17) and 0.47
(95% CI: 0.21-1.07) respectively for incident malaria, and 0.51 (95% CI 0.23-1.13) and 0.36 (CI: 0.16-0.85) respectively for incident malaria with inflammation. In children <72 months (but not older), the IRR for malaria among the moderate (≤ 75 µg/L but not deficient) and high (>75 µg/L) ferritin groups, relative to the deficient group (<12/15 µg/L depending on age), were 2.49 (95% CI: 0.97-6.40) and 3.27 (95% CI: 1.21-8.81) respectively.

**Conclusion**

Our data suggests that vitamin A adequacy may protect against malaria, whereas iron adequacy beyond some threshold may increase malaria risk. Our results also suggest that the concurrent assessment of malaria, in addition to inflammation, may enhance the interpretation of retinol, ferritin and sTfR in endemic regions.

**DISSERTATION COMMITTEE**

**ADVISOR:**
Christian L. Coles, PhD
Assistant Professor, International Health

**READERS:**
Keith P. West, Jr. DrPH
Professor, International Health

William J. Moss, MD
Professor, Epidemiology

Douglas Norris, PhD
Professor, Molecular Microbiology and Immunology

Kerry J. Schulze, PhD (Alternate)
Associate Scientist, International Health
Michael Rosenblum, PhD (Alternate)
Assistant Professor, Biostatistics

Amanda C. Palmer, PhD
Assistant Scientist, International Health

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“But those who hope in the Lord will renew their strength. They will soar on wings like eagles. They will run and not grow weary, they will walk and not be faint” (Isaiah 40:31).

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<tr>
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<td>Artemisinin Combination Therapy</td>
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<td>ACT</td>
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<td>AGP</td>
<td>α-1-Acid Glycoprotein</td>
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<td>CD36</td>
<td>Cluster Determinant 36</td>
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<td>CRP</td>
<td>C-Reactive Protein</td>
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<td>DCytB</td>
<td>Duodenal Cytochrome B</td>
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<td>DMTI</td>
<td>Divalent Metal Iron Transporter</td>
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<td>DTP</td>
<td>Diphtheria Tetanus Pertussis</td>
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<td>EC</td>
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<td>Integrated Management of Childhood Illness</td>
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<td>Labile Iron Pool</td>
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<td>LOWESS</td>
<td>Locally Weighted Scatter Plot Smoothing</td>
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<tr>
<td>MUAC</td>
<td>Mid-Upper Arm Circumference</td>
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<td>Non Transferrin Bound Iron</td>
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<tr>
<td>P. falciparum</td>
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<td>PfEMP</td>
<td><em>Plasmodium</em> Falciparum Erythrocyte Membrane Protein</td>
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<td>Tumor Necrotic Factor</td>
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<tr>
<td>VA</td>
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<td>VAD</td>
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<td>VAS</td>
<td>Vitamin A Status</td>
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<td>VCAM</td>
<td>Vascular Adhesion Molecule</td>
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<td>VSA</td>
<td>Variant Surface Antigen</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Chapter 1: SPECIFIC AIMS AND CONCEPT

PROBLEM STATEMENT

Over 90% of the approximately 600,000 annual childhood malaria deaths occur in sub-Saharan Africa, where deficiencies of both iron and vitamin A are widespread.\textsuperscript{1-8} The evidence suggests that whenever these micronutrient deficiencies are superimposed on malaria infections, the risk for severe disease and death is increased, particularly in situations where interventions to control both malaria and these micronutrient deficiencies are limited.\textsuperscript{1,9} In Zambia, for instance, the prevalence of subclinical vitamin A deficiency (defined as serum retinol < 0.7 µmol/L), a known cause of malaria morbidity and mortality, has been previously estimated at 54% among children below the age of 5 years.\textsuperscript{10} In this same population, the prevalence of anemia, a historical proxy for iron deficiency (ID), is estimated at close to 60%.\textsuperscript{10} This overlap in the burden of malaria and deficiencies of vitamin A or iron, presents opportunities to intervene in an integrated, cost-effective manner. A first step toward such integrated strategies is an improvement in the understanding of the nature of the interactions between malaria and these micronutrients.

Several epidemiological studies over the last five decades, have attempted to elucidate the pathophysiologic pathways of these negative synergisms.\textsuperscript{11-13} Unfortunately, the findings have been inconsistent, and as a result, opinions vary on issues to guide iron and vitamin A programming in malaria-endemic regions. In particular, the evidence remains inconclusive regarding a) the extent, direction and causality association between vitamin A status and malaria outcomes; b) the extent, direction and causality of association between iron status and malaria outcomes and c) the reliability of current biochemical indicators of iron\textsuperscript{14-16} and vitamin A status\textsuperscript{17-20} in
the presence of malarial infections. To this end, this epidemiological study provides an opportunity to characterize the bidirectional interactions between malaria, defined serologically, and vitamin A defined by serum retinol and iron status defined by serum ferritin and soluble transferrin receptor (sTfR). The study was conducted among children 4-8 years in rural Mkushi District, amidst a malaria-endemic region in Zambia.

**Vitamin A**

Since the isolation of vitamin A about 8 decades ago,\(^2\) and the subsequent discovery of its anti-infective properties,\(^2\) there has been interest in its potential to protect against incident malaria infection and its severity.\(^2\) Although the mechanisms involved are not completely understood, it has been postulated that vitamin A, through its retinoic acid metabolites, enhances immune cell proliferation, up-regulates the secretion of anti-inflammatory cytokines, and down-regulates the secretion of pro-inflammatory cytokines during malaria, as part of the process of coordinating the resolution in immune response to malaria infection (figure 1.1).\(^2,26,30-32\) Regrettably, evidence from human studies evaluating the association between vitamin A status (or supplementation) and malaria outcomes have been insufficient to date to fully understand this nutrition-infection dynamics. So far, only two human trials have been designed to evaluate the effects of vitamin A supplementation on malaria outcomes, and their findings were inconsistent.\(^12,33\) In northern Ghana, Binka *et al* found no evidence of a protective effect of prophylactic vitamin A supplementation on malaria morbidity or mortality in children.\(^12,32\) In contrast, Shankar *et al*\(^33\) found that quarterly supplementation with high dose vitamin A among preschool children in Papua New Guinea reduced the incidence of clinical
malaria by 30%. In the present study, we sought to provide additional contextual data to this issue by investigating cross sectional and longitudinal associations between malaria and vitamin A status among children in a malaria-endemic region.

Iron

The health benefits of iron in malaria-endemic region remain debatable. Iron deficiency is the most important dietary cause of anemia and a leading risk factor for morbidity and mortality in malaria-endemic regions.\(^{34-36}\) Paradoxically, iron adequacy has also been associated with increased malaria risk (figure 1.2). In a large randomized controlled trial conducted in Pemba, Sazawal et al\(^{11}\) found that children receiving 12.5 mg of iron and folic acid with or without zinc were about 12\% (p=0.02) more likely to die or need treatment at a hospital, and about 15\% (p=0.03) more likely to be admitted to a hospital compared to the control group. Recent studies have also found that adequate iron nutriture, either acquired from birth or through routine dietary intake in the absence of iron supplementation, may exacerbate the risk for severe falciparum malaria in young children\(^{37}\). In a longitudinal birth cohort of Tanzanian children, Gwamaka et al found that iron deficiency (compared to adequacy) was associated with a 1.3 fold reduction in the odds of subsequent \(P. falciparum\) parasitemia and a 1.6 fold reduction in the odds of subsequent severe malaria.\(^{37}\) Considering that anemia, the leading cause of severe malaria disease and death among children, responds positively to iron interventions, these emerging findings warrant further investigation to better understand roles of iron in the etiology of malaria. Such level of evidence will inform efforts to integrate iron interventions into malaria control programs, as a means of reducing the burden of iron deficiency and anemia.
Defining iron and vitamin A status in the context of infections is daunting. The biochemical indicators of iron (including ferritin as a marker of iron stores and soluble transferrin receptor as a marker of tissue iron demand) or vitamin A status (retinol as an indicator of vitamin A stores) are substantially modified by inflammation-inducing infection, including malaria.\textsuperscript{36,38,39} During the acute phase response to infection, ferritin is up-regulated\textsuperscript{36} whereas serum retinol, owing to the downregulation of its carrier retinol binding protein, is reduced.\textsuperscript{38,39} The inflammation-induced changes in iron and vitamin A status indicators are considered transient and may not reflect body stores, potentially invalidating these biomarkers as indicators of true biologic status. In defining the interactions between iron or vitamin A and malaria, it is critical to understand and adjust, as necessary, potential confounding by inflammation. Failure to control for the influence of inflammation could bias estimates of the association between these micronutrients and malaria. The bi-directional relationships between iron, vitamin A and malaria necessitates a longitudinal study to permit assessment of potential causal associations. In addition, this study also sought to characterize malaria- and inflammation-associated changes in indicators of iron and vitamin A status.

This was a longitudinal study, nested into a randomized controlled trial designed to assess the effects of consuming carotenoid-biofortified maize flour on the vitamin A status of Zambian children. Specific aims are as follows:

**Specific Aims and Hypotheses**

**Aim 1:**
To quantify the differences in serum retinol concentrations comparing children with and without inflammation (AGP $\geq$ 1 g/L), and to quantify how the magnitude of the
difference is affected by malaria status. The estimated differences will subsequently be used to derive inflammation- and malaria- corrected serum retinol concentrations.

**Hypothesis 1:**

- The reductions in serum retinol during inflammation (defined as elevated AGP) will be more severe in the presence of concurrent *P. falciparum* parasitemia.

**Aim 2:**

To characterize the longitudinal association between vitamin A status defined by either the measured retinol concentrations, or the inflammation- and malaria- corrected concentrations (as determined in Aim 1) and incidence malaria, assessed 6 months later.

**Hypothesis 2:**

- The incidence of malaria will be higher in vitamin A-deficient children compared to vitamin A-adequate children.

**Aim 3:**

To quantify the differences in serum ferritin and sTfR concentrations comparing children with and without inflammation (AGP> 1 g/L), and to quantify how the magnitude of the difference is affected by malaria status. The estimated differences will subsequently be used to derive inflammation- and malaria- corrected ferritin or sTfR concentrations.

**Hypothesis 3:**

- The changes in ferritin and sTfR during inflammation (defined by AGP) will be more severe during concurrent *P. falciparum* parasitemia.

**Aim 4:**
To characterize the longitudinal association between iron status defined by either the measured ferritin concentrations, or the inflammation- and malaria- corrected concentrations (as determined in Aim 3) and incident malaria, assessed 6 months later.

**Hypothesis 4:**

- The incidence of malaria will be higher in iron-adequate children compared to iron-deficient children.
Vitamin A and anti-malaria immunity (Adapted from Stevenson et al., 2004): Vitamin A adequacy is associated with both the replication of immune cells and enhancement of their functions. Innate immune cells including macrophages and dendritic cells are involved in the first line of defense against malaria infections, through the production of cytokines and non-antibody dependent phagocytosis. Vitamin A may also enhance the adaptive immune response by increasing the numbers of both T- and B-lymphocytes, and by so doing, enhancing the process of antigen presentation, production of malaria-specific antibodies and ultimately, antibody-dependent parasite clearance. These processes may ultimately decrease both the intensity and duration of malaria episodes.
Host iron and blood-stage malaria pathology (adapted from Clark et al 201441): The blood stage parasite is responsible for the clinical outcomes of malaria infections. The role of iron during this stage may be explained by the Non-Transferrin Bound Iron (NTBI) hypothesis. Beyond some level of iron adequacy, concentration of NTBI increases. Non-bound iron, whether intra- or extra-cellular is more easily accessible to the parasite. It is believed that by gaining access to body iron, the parasite’s replication and survival is enhanced, leading to the cyclical invasion and hemolysis of red blood cells (RBC), and precipitating the known adverse outcomes of malaria.
Chapter 2: BACKGROUND LITERATURE

Vitamin A deficiency in children

It is estimated that globally, up to 202 million preschool children may be living in a vitamin A deficient state, defined as having serum (or plasma) retinol concentration below 0.7 µmol/L. The prevalence of vitamin A deficiency is highest in the WHO African region where the clinical and biochemical estimates are 2% and 44% respectively, although substantial differences in deficiency burden exists across the region. For example, according to WHO estimates, the prevalence of night blindness among preschool children in Zambia (6%) is about 3 times the regional average and the estimated percentage of preschool children with subclinical vitamin A deficiency (54%) is 10% higher than the regional average of 44%. Vitamin A deficiency is considered a public health problem in about 122 countries and responsible for about 22% of malaria-attributable mortality. Such high disease burden continues to persist despite increased coverage of vitamin A programs worldwide.

In young children, the major causes of vitamin A deficiency include low vitamin A content of breast milk, low intake of vitamin A from dietary sources, and high prevalence of infectious diseases. Poor maternal nutritional status during pre-gestation, gestation and early post-partum periods are associated with low vitamin A content of breast milk. Newborns typically have very low vitamin A stores at birth and thus rely on the vitamin A status of the mother to improve their vitamin A stores. Unfortunately, women in undernourished regions of the world may not consume sufficient quantities of foods containing bioavailable vitamin A and, as a result, infants born under these conditions are at an increased risk of VAD. Although the
vitamin A requirement of women increases only slightly during pregnancy, its need increases substantially during lactation (from 700 µg/d in the non-pregnant, non-lactating woman to 1300 µg/d during lactation). Unlike women in developed countries, intake of a daily vitamin A supplement is not a common practice in less developed countries leaving poorly nourished women and their infants vulnerable to vitamin A deficiency. The period of breastfeeding is a critical period for growth and development. Lapses in breastfeeding practices during this stage therefore have adverse and sometimes irreversible health consequences for the growing child. The risk of vitamin A deficiency during the post-weaning periods is particularly high in developing countries. In low income countries, meals served to children typically contain very little animal source foods such as liver, egg and dairy products known to be excellent sources of bioavailable preformed vitamin A (retinyl esters).

A third factor increasing risk of vitamin A deficiency in developing countries is the high prevalence of infectious diseases including malaria, measles, diarrhea and pneumonia. Infections in children are associated with anorexia, mal-absorption, impaired nutrient transportation and increased excretion. Compared to healthy children, retinol absorption may be reduced by about 30% during diarrhea or respiratory illnesses. Some evidence from animal studies suggests that malaria parasites are able to incorporate host vitamin A and that this may deprive the host of essential vitamin A. It has been estimated that during infections, up to 6.0 µmol of retinol (representing over 18% of liver stores in young children) may be lost daily via urine.
Iron deficiency in children

Although it is believed that iron deficiency is the most widespread micronutrient deficiency worldwide, its burden is unknown, largely because of limitations with the current biomarkers for defining population iron status. Estimates of the burden of iron deficiency at the population level have historically been projected from the assessed prevalence of anemia. The WHO estimates that about 40% of children below 5 years and about half of children between 5-14 years are anemic. It is also considered a ‘rule of thumb’ that the burden of iron deficiency is about 2.5 times the burden of anemia in developing countries. In malaria-endemic regions, it is estimated that about 50% of all anemias are attributable to iron deficiency, whereas in non-malaria-endemic regions, the proportion of all anemias attributable to iron deficiency is estimated to be 60%. Anemia in developing countries is multifactorial in causation including genetic predisposition and environmental factors (nutrition and infections) associated with deficient or inefficient erythropoiesis, and/or increased loss of red blood cells. Iron deficiency anemia is associated with increased perinatal mortality, and increased morbidity and considered a cause of impaired physical and cognitive development later in life.

Iron deficiency among women of child bearing age and children is attributable to inadequate dietary intake during the periods of highest growth, namely pregnancy, lactation and early life. Specifically, impoverished populations consume levels of highly bioavailable iron sources such as meat, fish or poultry, coupled with a plant-based diet containing less absorbable forms of iron. Animal sources contain the more absorbable heme iron, whereas plant sources of iron are predominantly non-heme and less absorbable. In addition, the typical high consumption of staple
grains increase the intake of inhibitors like phytates and polyphenols which reduces iron absorption. Physiologic demands for iron increase during infancy and childhood, and in low income regions, this demand is often unmatched by intake. In addition, most infants in poor resource areas are born with low iron stores including over two-thirds of Zambians. In full term, fully breastfed newborns, iron stores are usually adequate to last for about 6 months. However after 6 months, external iron supply usually through complementary feeding is necessary to maintain adequate iron status. Unfortunately in developing countries, complementary foods are usually low in essential nutrients including iron.

Infections, particularly malaria and helminthes, remain dominant causes of iron deficiency in most parts of sub-Saharan Africa. Malaria infections have been associated with reduced iron absorption from diet, and redistribution of absorbed iron, leading to anemia of infection. During malaria infections, the destruction of red blood cells decreases blood hemoglobin levels, resulting in anemia. It is hypothesized that the dominant mechanism through which malaria causes anemia is by inducing the lyses and/or sequestration of parasitized cells, and destruction of non-parasitized cells during the inflammatory response. The evidence suggests that the iron trapped in destroyed red blood cells is recycled by macrophages, and becomes available again when the infection and its associated inflammatory response are resolved. The mechanism of recycling iron into hemoglobin requires an efficient erythropoietic system which responds sufficiently to erythropoietin. Unfortunately, this is typically not the case in the context of malaria infection. Malaria infections impair erythropoietin production and reticulocyte response, ultimately resulting in decreased blood hemoglobin levels.
The problem of iron deficiency and anemia in malaria-endemic regions is compounded by the lack of consensus on the benefits of iron-based interventions in endemic regions. Views vary about the association between iron status or interventions and health in malaria-endemic regions. Emerging evidence overwhelmingly supports the notion that a state of iron adequacy may have adverse consequences for malaria outcomes, particularly in situations with inadequate malaria control. Evidence from non-malaria-endemic regions, however suggests that iron interventions are efficacious in improving health outcomes, including anemia, in vulnerable groups. This conundrum creates a troubling policy and programmatic stasis in malaria-endemic regions. The concerns about iron interventions are worsened by emerging evidence that normal physiologic iron levels—even in the absence of iron supplementation—may increase the risk for adverse malaria outcomes. In a recent study in a Tanzanian birth cohort, Gwamaka et al found that iron deficiency (relative to adequacy) was associated with a 1.3 fold reduction in the odds of subsequent *P. falciparum* parasitemia and a 1.6 fold reduction in the odds of subsequent severe malaria during a three year follow-up period. It is important, therefore, that more epidemiological studies be done to seek the balance between benefit and risk with respect to iron status and iron interventions, particularly among young children in malaria-endemic regions.

**Malaria among children**

Although the overall burden of malaria morbidity and mortality has declined in several endemic regions, including Zambia (figure 2.1), there are still over 200 million episodes of malaria each year and about 90% of these episodes occur in Africa. Malaria kills about 800,000 children annually and is responsible for about
16% of all deaths in children under five years in Africa. In Zambia, malaria is responsible for about 50,000 deaths annually, 50% of all hospital admissions and over 40% of all deaths in children less than 5 years each year. The prevalence of malaria parasitemia in Zambian children under 5 years of age may be close to 40% in rural areas. Malaria transmission in Zambia is seasonal and follows the pattern of rainfall (figure 2.1). Malaria transmission is stabilized or increased by a combination of several factors that directly or indirectly sustain the life cycle of the anopheline mosquito vector and its Plasmodium parasites. Environmental factors including irrigation and poor drainage, support the development of the larval stages of the mosquito, while tropical climate sustains the sexual reproductive phase, and hence transmission of the parasite. Temperature is a key factor that influences the stability of malaria transmission. At temperatures below 18 °C, the sexual reproductive phase in the mosquito takes more than three weeks to complete. At around 30 °C, it takes only approximately 9 days for replication. With an average life span of about 3 weeks for anopheline species, tropical weather conditions allow sufficient time for the mosquito to generate sporozoites and transmit to a human host before it dies.

Parasite resistance is the strongest justification for the current shift in anti-malaria therapy- from conventional drugs to artemisinin combination therapy (ACT). Although combination therapies are generally more effective, they are more expensive. Adoption of ACTs as first line malaria treatment requires that countries are adequately prepared to improve malaria diagnosis. Unfortunately in several parts of sub-Saharan Africa, the practice of diagnosing and treating malaria cases at home is still very popular. Unless enough awareness is created through research, health
policies and community advocacy, malaria will continue to threaten the health and lives of children in these endemic regions.

**Figure 2.1: Monthly malaria prevalence in Southern Zambia (Source: Sutcliffe et al, 2011)**

Presumptive malaria treatment, the practice of making malaria treatment decisions on the basis of fever, is a major iatrogenic contributor to the high malaria burden.\(^{109}\) Whereas fever is a sensitive indicator of clinical malaria, specificity of ‘fever’ for the diagnosis of malaria is poor owing to the other causes of febrile illness in developing countries.\(^{110,111}\) Algorithm-based diagnosis of malaria has been associated with over-diagnosis, over-treatment and parasite resistance to antimalarial drugs.\(^{109}\) Parasite resistance contributes substantially to the disease, death and financial cost of malaria in endemic regions. A recent study from South Africa found that the risk of malaria infections more than quadrupled and the case fatality doubled
during periods when chloroquine resistance reached 10% compared to periods when resistance was less than 10%.\textsuperscript{112} Similarly, the risk of malaria infections increased by 6 times, and case fatality rate doubled when resistance to sulfadoxine-pyrimethamine reached 10%.\textsuperscript{112}

Genetic diversity in the \textit{Plasmodium} species also helps to stabilize malaria transmission.\textsuperscript{113,114} Antigenic diversity enables the parasite to evade host immune mechanisms (particularly humoral responses).\textsuperscript{115} Antigenic diversity also makes the task of developing vaccines against the blood stage parasite more challenging.\textsuperscript{116} The risk of adverse malaria outcomes is highest in young children in whom immune response is suboptimal.\textsuperscript{60,117} Clinical immunity to malaria is slow to develop and may only occur after several years of repeated infections.\textsuperscript{118} Research into the role of potential immune enhancers such as vitamin A may be critical for reducing the burden of malaria in children. It is estimated that undernutrition is the underlying cause of over 50% of malaria attributable deaths in children and VAD may be responsible for about 20% of all malaria attributed deaths in children.\textsuperscript{9} More research is needed to elucidate the mechanisms through which VAD influences malaria morbidity and mortality in children.

\textbf{Interactions between malaria and vitamin A}

Since vitamin A was isolated 1937,\textsuperscript{21} there have been several accounts of the potential for vitamin A to improve resistance to infections and survival.\textsuperscript{119} Historical anecdotes of ancient Egyptians recovering from vision impairment by topical application of pig ‘liver juice’ on the eye,\textsuperscript{120} and in some cases, actual consumption of liver set the tone for later epidemiological studies on the role of vitamin A and its metabolites in infectious disease morbidity and mortality. The earliest experiments of
the role of vitamin A in malaria morbidity were conducted in ducks and showed that inoculation of ducks fed on a vitamin A deficient ration with *P. lophurae* resulted in significantly higher mortality than control ducks, although vitamin A deficiency did not increase parasitemia. The series of experiments and trials that followed this initial experiment suggest that vitamin A adequacy may provide protection against malaria although results have not been consistent, as under some circumstances, vitamin A supplementation may adversely affect malaria outcomes. Future policies regarding the benefits of vitamin A on malaria must be informed by a careful review of evidence and new studies.

Remarkably few trials in human have investigated the impact of vitamin A supplementation on malaria outcomes and the findings have been inconsistent. In a study by Binka et al, in which Ghanaian children 6-11 months were given age-appropriate, high-dose vitamin A supplements once every 4 months and followed for up to 24 months, there was no evidence of a protective effect of prophylactic vitamin A supplementation on malaria morbidity or mortality. Although the authors found a 23-30% reduction in probable malaria (defined as temperature > 37.5°C and parasites/L of at least 4 x 10⁹), this finding was not statistically significant. The study by Binka *et al* has been criticized for lacking adequate power to detect a reasonable effect size, and also for using non-specific indicators (fever and verbal autopsy) to define malaria outcomes. The study was not powered to detect a difference in malaria morbidity of less than 70%. A second study, conducted in Papua New Guinea (PNG) by Shankar *et al* found that vitamin A supplementation was associated with a 30% reduction in *P. falciparum* febrile malaria episodes. The study also found that vitamin A supplementation significantly delayed the onset of first malaria episodes.
Findings of a randomized double-blind, placebo controlled trial in Burkina Faso\textsuperscript{128} in which children 6-72 months were given 200 000 IU of vitamin A (plus 10 mg of elemental zinc taken 6 days in a week) versus placebo for 6 months, was consistent with the trial in PNG. Zeba et al observed a larger reduction in the prevalence of malaria in the treatment group (30\% higher) compared to the placebo group.\textsuperscript{128} More research is needed to elucidate the exact mechanism through which vitamin A may confer anti-malaria protection.

**Mechanisms involved in the malaria-vitamin A interaction**

Several mechanisms may explain the protective role of vitamin A on malaria outcomes. Vitamin A, when given to children in malaria-endemic regions may have adjuvant effects,\textsuperscript{24} may reduce the secretion of pro-inflammatory cytokines,\textsuperscript{25,26} may increase eryptosis (the suicidal death of red blood cells)\textsuperscript{129} and clearance of parasitized red blood cells,\textsuperscript{25,26} may enhance the potency of known antimalarial drugs\textsuperscript{27-29} and may improve erythropoiesis.\textsuperscript{130} Understanding of these mechanisms is critical in advancing research and subsequently, policies for improving beneficial health outcomes.

**Vitamin A and anti-malaria immunity**

Anti-malaria immunity, both clinical and anti-parasite, is only acquired after years of repeated exposure.\textsuperscript{131-134} Adequate vitamin A status has been associated with enhancement in both innate and adaptive immune response.\textsuperscript{31,60,135-137} It is believed that, vitamin A, by enhancing several of the pathways involved in antimalarial immunity, inhibits parasite growth, reduce disease severity, and the risk of death.\textsuperscript{31,60,135-137} Vitamin A has been associated with increased proliferation and
differentiation of immune cells, enhanced function of immune cells, improved antibody response, and regulation of parasite induced-cytokine imbalance.\textsuperscript{31,60,135-137} The up-regulation of immune pathways by vitamin A is achieved through the binding of its metabolites- retinoic acid- to nuclear receptors and the subsequent activation of these receptors, effectively up-regulating transcription and translation, cell proliferation and differentiation.\textsuperscript{138,139} In a recent study, Kain and Serghides showed that retinoic acid directly up-regulated CD36 expression, reduced tumor necrotic factor (TNF-\(\alpha\)) production and increase the phagocytosis of \textit{P. falciparum} parasitized erythrocytes.\textsuperscript{25,26} The authors found that treatment of human monocyte cell lines with 9-cis retinoic acid (or DMSO as control) resulted in a 46.5\% increase in the CD36 expression (95\%CI: 35.71-57.36), a 16 unit increase in phagocytic index (a locally developed definition of the extent of phagocytosis), and 60 to 82\% reduction in TNF-\(\alpha\) production depending on the parasite isolate used.\textsuperscript{25,26} There is also evidence that CD36 expression is up-regulated by a mechanism involving the hetero-dimerization of peroxisome proliferator activated receptor (PPAR) with retinoid X-receptor, whose only known natural ligand is 9-cis retinoic acid.\textsuperscript{97} The overall importance of the reduction in TNF-\(\alpha\) in the context of vitamin A adequacy is unknown. The common interpretation is that the reduction in TNF-\(\alpha\) is suggestive of reduced pro-inflammatory cytokine secretion, a phenomenon which may explain the observed protective effect of retinol against clinical malaria, as observed in the study by Shankar \textit{et al} in PNG.\textsuperscript{33} It is also plausible, that this phenomenon may be associated with increased parasitemia considering that the innate response is the first line immune defense against invading parasites.
There is divided opinion on the importance of CD36 up-regulation on the progression of malaria morbidity. Some earlier studies found that CD36-owing to its cyto-adherent properties- may increase the sequestration of parasitized erythrocytes into the vasculature and may increase the risk for severe malaria, particularly cerebral malaria.\textsuperscript{140-142} However, in the follow-up study by Serghides and Kain, CD36 up-regulation was primarily responsible for the observed increase in phagocytosis.\textsuperscript{97} In fact, Serghides and Kain observed that co-treatment of 9-cis retinoic acid- monocyte culture with CD36 antibodies inhibited phagocytosis by 75% in the retinoic acid group.\textsuperscript{26} Treatment of human monocytes with PPAR\textgreek{y}-RXR agonist (that included 1\textmu M 9-cis retinoic acid) yielded a 40-60\% increase in CD36 expression. In addition the up-regulation in CD36 was associated with a 30\% increased internalization of parasitized erythrocytes. The study also found that treatment of the monocytes with the PPAR\textgreek{y}-RXR agonist significantly reduced \textit{P. falciparum} induced TNF-\textalpha secretion. The observed increase in CD36 expression may have positive implication for malaria control in non-immune individual, particularly in young children, in whom antibody production is suboptimal. Without adequate antibody response, T-cell clearance of parasitized, non-opsonized erythrocytes may be key to reducing parasite growth and reversing disease progression. It is unclear the extent to which CD36-initiated phagocytosis is applicable to humans.

Cytokine imbalance and an exaggerated pro-inflammatory response are common during malaria infections.\textsuperscript{143-145} During malaria infections, there is dysregulation of cytokine secretion such that Th1-type cytokines (including interferon gamma, interleukin 1 (IL-1), IL-12 and tumor necrosis factor) are up-regulated, while secretion of Th2-type cytokines like IL-4, IL-10 are down regulated.\textsuperscript{143-145} The Th1
type reaction is the initial adaptive response against parasite infections, which ultimately leads to increased clearance by cytotoxic T-lymphocytes. Extended and amplified secretion of Th1 (as in the case of malaria) however results in unfavorable outcomes such as increased tissue damage, erythrocyte destruction, impaired erythropoiesis and fever. Vitamin A is hypothesized to reverse this imbalance in favor of Th2-type cytokine secretion although the cellular mechanisms involved are not clear. In the study by Serghides and Kain, treatment of the human monocytes/macrophages exposed to P. falciparum with 9-cis retinoic acid resulted in a significant decrease in TNF-α. TNF-α is an endogenous pyrogen, and once released, induces fever, a process mediated by IL-1.

The evidence from human studies regarding the potential protective effects have not been as strong and consistent as those seen in animal and in-vitro studies. In a recent randomized study by Cox et al, supplementation of pregnant and lactation women with vitamin A was associated with an increase pro- to anti-inflammatory cytokine ratio, suggesting that vitamin A supplementation may suppress Th2-type response. In another study, Tabone et al found that among a group of French adults with P. falciparum infections, IL-6 concentrations were inversely correlated with serum retinol and positively correlated with parasite density. IL-6 regulates an array of immune mechanisms including B-cell maturation, increase secretion of Th2-type cytokines and down-regulation of Th1-type cytokines. Hence a compromised IL-6 secretion as reported by Tabone et al may suggest a switch in favor of Th1 type cytokine. Current understanding regarding the mechanisms through which vitamin A increases pro-inflammatory cytokine secretion and the consequent effect on health remains incomplete. It is plausible that the negative correlation found between retinol
and IL-6 may have been due to the phenomenon of inflammation-induced hyporetinolemia.\textsuperscript{39,153}

Immune cell proliferation is associated with the binding of retinoic acid isoforms to nuclear receptors.\textsuperscript{31,60,135-137} The RXR receptor is activated by 9-cis retinoic acid whereas RAR binds to both 9-cis retinoic acid and all-trans retinoic acid.\textsuperscript{154} The two nuclear retinoic acid receptors, once activated by the binding of the appropriate ligands, heterodimerises either with each other, or with other nuclear receptors including Vitamin D\textsubscript{3} receptor, or the PPAR.\textsuperscript{154} The hetero dimers can then bind to response elements to up-regulate transcription and translation. The binding of retinoic acid to its nuclear receptors induces IL-2 production. IL-2 is key in conversion of T-lymphocyte and other immune cells from the G to the S phase of the cell cycle, where they begin to proliferate.\textsuperscript{155,156} Activation of RAR is also associated with enhance development of neutrophils.\textsuperscript{156} Impaired neutrophil development is associated with delayed clearance of foreign parasites.\textsuperscript{31,157} Vitamin A is also involved in the development and activation of macrophages and dendritic cells required for phagocytosis as well as antigen presentation to T-cells.\textsuperscript{137} Vitamin A is involved in the development of natural killer (NK cells)\textsuperscript{158} which are important for the early defense against Plasmodium parasite before the onset of T-cell mediated response.\textsuperscript{159}

\textbf{Adjuvant properties and anti-malaria antibody regulation by vitamin A}

Antibody response is an important component of the immune response to infections. Very few studies have looked at the effect of vitamin A on malaria-specific antibody responses. During VAD, antibody responses mediated through Th2-type cytokines is affected and this may lead to suboptimal levels of IgG, IgE, IgM and IgA.\textsuperscript{160,161} In an in-vitro system, B-cell treatment with retinoic acid increased the
secretion of IgG. The evidence from human studies in support of the direct role of vitamin A on anti-malaria antibody response is few and weak. In a recent study among Ghanaian primigravidae, Cox et al did not find a positive effect of VA supplementation (100,000 IU retinyl palmitate) on *P. falciparum* specific antibody response. Cox et al found that when blood samples collected at baseline, late pregnancy and 6 weeks post-partum were exposed to *P. falciparum* isolates, anti-body response to variant surface antigen (VSA) was lower in the vitamin A group. Overall, the evidence from animal models regarding the adjuvant properties of vitamin A remains inconclusive. In an experiment to determine the adjuvant properties of vitamin A, Dewotz et al, found no added benefit of vitamin A against soluble *P. berghei* antigen when administered to weanling rats exposed to a challenge infection. In weanling rats inoculated with 15 mg retinol (in addition to soluble *P. berghei* antigen) 24 hours before the injection with *P. berghei* infected erythrocytes, there was no significant improvement in the mortality, peak parasitemia, and survival times of the fatal infection. Although the combination treatment resulted in a 10% reduction in mortality, this difference was not statistically significant.

Some in-vitro studies investigating the potential of vitamin A as an adjuvant to vaccines (particularly DTP) seem to suggest that vitamin A supplementation may precipitate unintended adverse malaria outcomes. In a recent experiment, Jorgensen et al examined the effect of concurrent vitamin A (400 IU) and DTP vaccine (0.1 ml) supplementation on the intensity of infection and death among mice exposed to different stocks of *P. falciparum* ANKA isolates at concentrations in the range $10^3$ to $10^6$ infected RBCs. Seven day of pre-inoculation with vitamin A and DTP, followed by 5-6 days of infection with *P. falciparum* ANKA infected RBC resulted in
significant increase in parasitemia (defined as the number of infected RBCs in the murine blood samples) in the VA-DTP group. Geometric mean ratios comparing parasite density in the VA-DTP mice to the control mice ranged from 1.31 to 4.44, suggesting that VA supplementation may increase the risk for untargeted infection (malaria in this case) when administered concurrently with vaccines. A similar study by Hein-Kristensen et al,124 also supported the notion that VA when administered concurrently with DTP vaccine may have adverse untargeted effect on malaria outcomes. In female C5BL/6 mice strains, Kristensen et al found that concurrent administration of VA (400 IU) and DTP (0.1ml) approximately nine days before P. berghei exposure increased the rate of parasitemia. VAS/DTP appear to increase parasitemia especially among animals who received higher initial doses of infected RBC (10^5 and 10^6). The study reported a geometric mean ratio (comparing parasite densities in VAS/DTP group to controls) of 0.97 (95% CI: 0.62-1.50); 1.86 (95% CI: 1.03-3.37) and 1.69 (95% CI: 1.18-2.43) respectively for P. berghei concentration of 10^4, 10^5 and 10^6 infected RBCs. These findings raise questions about the current global practice in which children are given semi-annual doses of vitamin A, at a time when they are also receiving vaccines including DTP. In malaria-endemic regions, the concurrent supplementation of DTP and vitamin A may increase the risk of severe malaria outcomes by enhancing parasite multiplication. Research is needed to understand the extent to which the findings from animal models are applicable in humans.

**Vitamin A as adjunctive malaria therapy**

In recent years, there have been some attempts to explore the pharmacologic properties of retinol and its potential as an adjunctive malaria therapy.129,164,165 The
mechanisms through which retinol could potentially be used to directly halt parasite growth is unclear but it is hypothesized that retinol, owing to its ability to interact with lipid membranes, can be used to induce death of parasitized cells. In two studies, Hamza et al shed light on 1) the stage of *P. falciparum* infections which may be potentially targeted by pharmacologic retinol and 2) the metabolites of retinol which are most effective in a culture medium against *P. falciparum*. Hamza et al showed that retinol slowed the rate of radio-labeled hypoxanthine incorporation in the early ring forms and mature forms, as well as merozoite invasion of RBCs. In the second study, Hamza et al showed that in an in-vitro system, retinol was a more effective inhibitor of parasite growth than its other metabolites, namely all-trans retinol, 9-cis retinoic acid, retinyl palmitate and three retinoic acid receptor agonists. In the cultured systems, the concentrations of the retinoids needed to achieve 50% growth inhibition of *P. falciparum* (IC50) were 3.2 x 10^-5 nM for retinol, 7.0 nM for all-trans retinoic acid, 7.4 nM for 9-cis retinoic acid and about 1.2 to 2.9 nM for three RAR antagonists. The authors suggested that retinol (unlike its metabolites) can interrupt with, and hence disrupt parasitic membranes, and this may have led to increased lysis of parasitic cells. Although these finding are encouraging, replication of these finding in humans may be unlikely considering that the IC50 attained is 4 times higher than the normal physiologic retinol levels. Such high retinol concentrations (even if possible to attain in humans) have been associated with increased hemolysis of erythrocytes, and may in fact induce anemia. In addition, retinol in human is usually complexed to its transporter, retinol binding protein. It is not clear if retinol bound to RBP will achieve the same pharmacologic effect as free retinol, as seen in the animal models.
There is growing interest in the potential of retinol as an adjunctive antimalarial therapy, particularly in light of the widening trend in parasite resistance to antimalarial drugs. Kerschbaumer et al\textsuperscript{168} observed that the IC\textsubscript{50} and the full inhibition concentrations of the combination of mefloquine and artemisinin against \textit{P. falciparum} isolates from Thai patients were significantly lowered with the addition of retinol, in a dose dependent manner. The addition of retinol at concentration corresponding physiologic levels in healthy adults decreased the IC\textsubscript{50} of the mefloquine-artemisinin concentration by 3-4 times, and the IC\textsubscript{90} (the concentration drug needed to achieve 90\% inhibition) by about 2-3 times. In a similar study, Knauer \textit{et al} showed that retinol interacted synergistically with quinine, such that the addition of low, medium and high retinol concentrations corresponding to the 50\textsuperscript{th}, 65\textsuperscript{th} and 80\textsuperscript{th} percentile of the physiologic levels in healthy adults significantly reduce the concentration of quinine needed to inhibit growth of fresh \textit{P. falciparum} isolates.\textsuperscript{29} The EC\textsubscript{90} for the quinine-retinol combination against \textit{P. falciparum} isolates obtained from Thai patients (6 year and older) were 829 nM, 738 nM and 762 nM for the low, medium and higher retinol concentrations, which increased to 1950 nM when only quinine was used.\textsuperscript{29} Similarly, Reidl \textit{et al} found that incubation of fresh \textit{P. vivax} isolates in microplates pre-dosed with pyronaridine-retinol concentration resulted in a significant reduction in the concentration of pyronaridine alone needed to achieve partial or full inhibition of parasite growth\textsuperscript{169}. The addition of 1810 nM, 2206 nM and 2606 nM of retinol reduced the IC\textsubscript{50} for pyronaridine from 9.8 nM to 1.7, 0.51 and 0.17 nM respectively and the IC\textsubscript{99} from 162446 nM to 59379, 58891 and 16754 nM respectively. Overall, it does appear that the effect of retinol on the potency of
antimalarial drugs, with the exception of artemisinin compounds,\textsuperscript{126} have been consistently positive.

The evidence regarding the interactions between retinol and artemisinin-based compounds have not been consistent. An earlier study by Skinner-Adams \textit{et al}\textsuperscript{126} showed that retinol antagonized the inhibitory effect of artemisinin, artesunate, artemether and dihydroartemisin when applied to fresh \textit{P. falciparum} isolates. In contrast, Thriemer \textit{et al} found that the mean 50\% effective concentration (EC50) for artemisinin against fresh \textit{P. falciparum} isolates from Thai patients was reduced from 10.29 nM to 2.71 nM with the addition of 35 nmol retinol\textsuperscript{121} suggesting a synergistic interaction. More evidence is needed to explain these contradictory findings. The lack of consistency from the studies investigating that interaction between retinol and the artemisinin derivatives raises questions about the benefits of vitamin A supplementation in malaria-endemic regions where artemisinin combination therapy is the first line malaria treatment. Part of the mechanism of action of artemisinin derivatives involves the generation of free radicals that may be stressful to parasites. It is likely that retinol, through its antioxidant properties reduces the availability of these free radical, and hence the overall potency of artemisinin derivatives against \textit{P. falciparum}.

\textbf{Effects of malaria on vitamin A status}

The effects of malaria on vitamin A status can be categorized into two major components namely: a) the transient reductions, mainly resulting from the decreased mobilization of retinol from storage, during the acute phase reactions,\textsuperscript{153,170} and b) actual retinol lowering resulting from mechanisms such as infection-induced anorexia and increased excretion,\textsuperscript{30,58,171} A cross-sectional study among Indian children
presenting to the Ispat general hospital in India, found that retinol levels were about 0.4 µmol/L lower in children with malaria relative to healthy controls.\textsuperscript{172} In the same study, malaria was inversely associated with plasma levels of β-carotene, lutein, and α-carotene.\textsuperscript{172} In a group of Thai children, retinol levels were decreased during malaria infections in both rural and urban residents although the effect was more profound in rural children.\textsuperscript{39} Thurnham \textit{et al} observed that retinols levels were decreased by 56\% and 34\% respectively in rural and urban Thai children with malaria compared to malaria negative controls.\textsuperscript{39} In a group of Kenyan children, however, Nabakwe \textit{et al} found no difference in retinol concentrations comparing children with and without malaria parasitemia.\textsuperscript{173}

Malaria may lead to vitamin A depletion that may not resolve without a dietary intervention, even after full recovery from the disease. Depletion in vitamin A status could be due to one of several malaria-related mechanisms.\textsuperscript{30,171} Malaria is associated with illness-induced anorexia.\textsuperscript{30} In infants, illness is associated with inability or refusal to breastfed and in children it is associated with reduced dietary intake.\textsuperscript{174} Malaria is also associated with malabsorption and may be associated with impaired transportation of nutrients.\textsuperscript{175} These mechanisms may ultimately contribute to decreased availability of vitamin A at the tissues. Malaria is also associated with increased urinary excretion of retinol.\textsuperscript{61,171} It is hypothesized that the increased urinary loss of retinol during infections may be partly explained by the decreased renal reabsorption of retinol binding protein (RBP).\textsuperscript{58,60} Increased catabolism during infections also increases the requirement for vitamin A and hence the risk of deficiency.
Transient vitamin A loss during malaria infection is associated with the acute phase response to infections.\textsuperscript{60-62} In characterizing the interactions between malaria and vitamin A status, it is important that these transient losses are discriminated from the actual vitamin A losses that puts children at an increased risk for vitamin A related adverse health outcomes. To this effect, it is critical that vitamin A status and malaria morbidity are ascertained at different times in order to limit potential reverse causality.

**Acute phase response and vitamin A status**

Inflammation-induced hyporetinolemia in the acute phase response following infections or trauma is the host’s response to reducing tissue damage, initiate repair and to limit the growth of pathogens.\textsuperscript{176-179} The inflammatory response following infections involves the homeostatic regulation of a cascade of immunologic mechanisms.\textsuperscript{180} The liver is the primary source of both negative and positive acute phase proteins. Signals for the induction of inflammation are carried to the liver via cytokines produced by cells of the immune system, particularly macrophages and monocytes following the invasion by foreign pathogens.\textsuperscript{179} Levels of the acute phase proteins are regulated by hepatocytes in response to activation by cytokines particularly IL-1, IL-6 and TNF-\(\alpha\).\textsuperscript{180} Hepatic regulation typically involves an increased or decreased transcription of the mRNA for the various acute phase protein and a corresponding change in the rate of translation into proteins.\textsuperscript{181}

The class of proteins whose concentrations generally change by at least 25\% during the inflammatory response to infections are referred to as the acute phase proteins.\textsuperscript{179} Levels of protein such as fibrinogen, AGP and \(\alpha\)-1 antichymotrypsin (ACT) may increase by up to 5-fold while others such as C-Reactive Protein (CRP)
and Serum Amyloid A (SAA) may increase by up to a 1000-fold. The class of proteins whose levels increase during inflammatory reaction are referred to as positive acute phase reactants. Fast responding APP such as CRP and SAA rise quickly, as early as within the first 4 hours, and may peak within 72 hours following the initiation of inflammatory stimulus. Slow responders such as AGP, however, only begin to rise after 24 hours and peak after 7 days. Assessment of at least one fast responding and one slow responding protein is necessary to monitor the response in retinol across the various stages of an infection.

Levels of plasma proteins including albumin, retinol binding protein and transthyretin are lowered during the acute phase response and hence are referred to as negative acute phase proteins. Lowering of retinol levels following the initiation of inflammation is closely tied to changes in the circulating levels of retinol-binding protein and transthyretin. Retinol is transported in circulation bound to the RBP-transthyretin complex and a reduction in the levels of these transport proteins significantly affects the mobilization and distribution of retinol. It has also been observed in experimental rats that the levels mRNA for both RBP and transthyretin drop rapidly within the first 36 hours after induction of inflammation. In the absence of enough transport proteins, the rate of release of retinol by the liver is also reduced. Other postulated mechanisms for the observed decrease in levels of retinol and RBP during inflammation include the increased permeability of vascular tissues at the site of infection and hemodilution as a result of vasodilation or increase urinary excretion.

In practice, inflammation is defined using threshold concentrations for the various acute phase proteins. CRP is the most standardized and typically, levels above
5 or 10 mg/l are suggestive of active inflammation. Cut-offs of 5.0 mg/L, 0.65 and 1.4 g/L for SAA, ACT and AGP respectively are used to define inflammation. Although the use of these thresholds allows for easy interpretation and comparison across studies, there is evidence that even lower levels, as is often the case in asymptomatic infection, is associated with substantial reductions in the levels of retinol. The phenomenon of inflammation-induced hyporetinolemia has been demonstrated under several disease conditions including diarrhea, pneumonia, HIV and malaria. In a group of HIV infected patients from Kenya, Thurnham et al found that mean retinol concentration increased from about 1.16 µmol/L to about 1.33 µmol/L after correcting for inflammation. In a cross-sectional study of Bangladeshi children, the prevalence of VAD changed from 31% to 16% after correcting for raised levels of CRP and in a pooled sample of Indonesian infants enrolled in a zinc-iron-β-carotene supplementation trial, reductions in plasma retinol levels of 0.07 µmol/L and 0.12 µmol/L were associated with raised concentrations of CRP (>10mg/L) and AGP (>1.2mg/L) respectively. In a study involving 2519 apparently healthy Pakistani children, the prevalence of mild to moderate and severe VAD were elevated by 10% and 56% respectively during inflammation (as defined by raised concentrations of ACT or AGP). Several prior studies in both animals and humans have attempted to characterize the nature and impact of APR on the vitamin A status. Currently, there is no consensus on the optimal approach for utilizing acute phase protein in adjusting for the transient reductions in retinol levels.

There is evidence of a strong association between inflammation and retinol levels in the context of malaria. In a hospital-based study among children on admission for acute P. falciparum malaria, IL-6 levels correlated negatively and
significantly with levels of pre-albumin, RBP and retinol. In children 6-59 months from malaria-endemic region in northern Ghana, elevated AGP was associated with a 24% decrease in serum retinol concentrations. In a childhood population in Papua New Guinea, with 70% malaria prevalence, elevated AGP was associated with about 0.12 µmol/L reduction in serum retinol levels. In the same population, elevated CRP (>10 mg/L) was associated with a reduction in serum retinol of 0.26 µmol/L.

In a recent meta-analysis, Thurnham et al estimated that plasma retinol levels may be lowered by about 12-32% and that the prevalence of VAD may be inflated by up 10% in malaria-endemic regions if inflammation is not corrected.

Malaria presents a very unique situation in that with repeated exposure, infections tend to be sub-clinical. The potential to underestimate the effect of inflammation on vitamin A status in malaria-endemic regions is therefore high. In estimating the corrections factors for vitamin status in the presence of inflammation, Thurnham et al pooled studies from settings with different dynamics with respect to the burden and transmission pattern of prevailing infectious diseases. The evidence suggests that the magnitude of the strength of inflammation-induced hyporetinolemia may differ across diseases and hence across settings with different epidemiological profiles. Most of the studies that investigated the association between malaria and vitamin A status did not account for how the malaria-vitamin A associations might be influenced by concurrent elevations in one or more of the commonly used acute phase proteins. It is important that such assessments are done to improve the estimation of vitamin A deficiency in malaria-endemic regions. Evidence is also limited regarding how the phenomenon of inflammation-induced hyporetinolemia differs in children with sub-clinical infection versus those with febrile malaria illness.
Research is also needed to demonstrate how the association between inflammation and vitamin A status changes over a range of parasite densities.

In previous attempts to quantify the extent of inflammation-induced hyporetinolemia, the common practice has been to simply categorize a cross-sectional study population into those with and without raised acute proteins. Unfortunately, this approach can be very problematic. The goal of generating an adjustment factor is to quantify the transient lowering of retinol owing to infection-induced inflammation. Ideally, this goal warrants that retinol levels be measured at least at two time points. The cross-sectional model neglects the fact that a component of the difference may not be artificial, but may actually reflect reduction in hepatic stores. Adjustment factors generated by cross-sectional comparison of apparently healthy children with and without raised levels of APPs may overestimate the magnitude of the apparent transient changes. The potential overestimation of the effect of inflammation may also be due to the fact that vitamin A deficiency and infections tends to cluster within individuals and households such that sick children (compared to healthy children) are more likely to have vitamin A deficiency independent of the current inflammatory status. A better approach for estimating the adjustment factor is to monitor changes in levels of acute phase proteins and vitamin A indicators over time in the same group of individuals. The longitudinal approach ensures that the inflammatory and non-inflammatory groups are reasonably comparable with respect to the history of prior episodes of infections, and by so doing, may mitigate the potential to overestimate the adjustment factors to be used for correcting the inflammation-associated changes. In the current study, we proposed to assess the association between retinol and
inflammation (defined by AGP), using both cross-sectional and longitudinal models in a cohort of rural Zambian children.

**Epidemiological evidence of the interaction between iron and malaria**

The role of iron in the etiology of malaria in children remains a contentious issue. Evidence from controlled trials and observational studies, in both humans and animal models have been inconsistent. The overwhelming majority of studies seem to suggest that a state of iron adequacy may precipitate adverse malaria outcomes including increased parasitemia, clinical malaria and death. Although the exact mechanisms remain to be elucidated, evidence from both human and animal studies suggest that the observed role of iron in the pathology of malaria revolves around the systemic iron redistribution and utilization by both the parasite and human host infections.

Early studies of the association between iron and malaria were mostly conducted in clinical settings and typically reported no evidence of an association or evidence of increased risk of adverse malaria outcomes. In a study in Papua New Guinea in which children were randomized to receive 200 mg of ferrous sulphate for 16 weeks, there was no treatment effect on parasite rates, parasite density, spleen size or anti-malaria IgG response. However among this group of children, 92% were anemic at baseline and it is unclear if the outcomes would have changed if the anemia burden was lower at baseline as reported in other studies. Two other studies also reported evidence of no association between iron supplementation and malaria outcomes in children. Among a group of Tunisian children receiving treatment for severe malaria-related anemia, iron supplementation did not impact the incidence of malaria parasitemia or parasite density during a 12
week follow-up and, in Togo, a randomized, double-blind placebo trial found no effect of a daily dose of iron (2-3 mg) on malaria outcomes in children 6-36 months (n=229).199

In contrast, some early clinical investigations found evidence of increased risk for adverse outcomes following iron supplementation. A clinical study by Smith et al found that among Gambian children (n=216) aged 6 to 60 months with iron deficiency anemia, randomized to receive either oral iron or a placebo, the risk of malaria parasitemia increased among those who received the iron supplement (OR=2.35; 95% CI=1.30-4.25).201 Similarly a placebo-controlled trial of intramuscular iron dextran prophylaxis among 2-month old infants in Papua New Guinea found that iron supplementation was associated with an increased prevalence of malaria defined by parasite rates and spleen sizes at 6 and 12 months post intervention.95 A meta-analyses of these earlier studies found no overall increased risk associated with iron supplementation in a malaria-endemic setting.202,203 These studies were, however, mostly clinical, and participants typically received anti-malaria therapy for diagnosed cases.202,203

More recent evidence from observational studies point to a pattern of adverse outcomes following iron interventions. In a longitudinal birth cohort of Tanzanian children (n=785), Gwamaka et al found that ID was associated with a 1.3 fold reduction in the odds of subsequent P. falciparum parasitemia and a 1.6 fold reduction in the odds of subsequent severe malaria.37 In addition, ID, corrected for inflammation, was associated with a 66% reduction in the hazard of malaria related mortality.37 In another study conducted by Nyakeriga et al, ID was associated with a 30% reduction in the incidence of clinical malaria (defined as any parasitemia PLUS fever) during a
12 months follow-up among supplemented Kenyan children (n=240), 8 to 96 months old.²⁰⁴ The protective effect of iron deficiency has also been reported in animal models. Koka et al showed that in mice, iron deficiency was associated with a 20% increased malaria survival rate after 30 days of infection and that iron deficient erythrocytes are more rapidly cleared from the blood compared to iron replete erythrocytes.²⁰⁵

Much of the recent debate on the potential harmful effects of iron interventions among children in malaria-endemic regions has centered on the outcomes of a large, randomized iron supplementation trial, conducted in Pemba. In this study,¹¹ Sazawal et al found that children receiving 12.5 mg of iron and folic acid with or without zinc were about 12% (p=0.02) more likely to die or need treatment at a hospital, and about 15% (p=0.03) more likely to be admitted to a hospital compared to the control group. Interestingly, a similar trial in southern Nepal, a non-malaria-endemic region, found no adverse effect of iron or folic acid supplementation on severe morbidity or mortality.⁹⁴ Sub-analyses of the Pemba trial revealed that the risk of adverse malaria outcomes were lower in a subsample of the children who were iron deficient at baseline and whose malaria outcomes were properly managed. Specifically the incidence of severe malaria morbidity was reduced by 49% in iron-deficient anemic children who received the iron supplements,¹¹ This suggests that even in malaria-endemic regions, iron supplementation may be beneficial if delivered to those at risk of iron deficiency and if malaria outcomes can be properly managed. In fact, a more recent meta-analysis of oral iron supplementation seems to corroborate this notion. A meta-analyses by Ojukwu et al found that iron supplementation was
associated with improvement in hemoglobin, reduction in anemia, and no increase in the risk of clinical malaria (RR=1; 95% CI=0.88-1.13).\textsuperscript{206}

The lack of consistency from the evidence gathered so far regarding the interaction between iron and malaria underscores the need for further studies. There is an urgent need to elucidate the pathophysiological basis for the increase risk of adverse outcomes when iron is delivered in a malaria-endemic region.\textsuperscript{207} It remains to be known whether the risk imposed by iron is dependent on the exact formulation of iron used in intervention studies or the pathway through which iron is delivered. Recent evidence seem to suggest that unlike oral iron supplements, iron delivered through fortified foods is efficacious in improving indictors of iron status and safe with regards to increasing the risk of adverse malaria outcomes.\textsuperscript{208} A meta-analyses of studies predominantly conducted in non-malaria-endemic regions suggested that home-based fortification with micronutrients including iron is efficacious in improving micronutrient and growth status of children and does not increase the risk of adverse health outcomes, such as diarrheal and respiratory diseases.\textsuperscript{208} However, trials of iron and micronutrient fortified foods in malaria-endemic regions have been conducted among infants in whom the risk of both iron deficiency and malaria are relatively low compared to children entering the preschool years. Evidence from Zambia suggests that the risk for both malaria and iron deficiency is highest in the post-infancy period.\textsuperscript{209,210}

**Mechanisms of the role of iron in malaria pathology**

The intermediate steps leading to the iron-associated adverse outcomes include the increase available of non-transferrin bound iron,\textsuperscript{86,211-213} and increase
accessibility to both malaria and non-malaria parasites. The immediate steps include iron’s role in the generation of reactive oxygen species, iron redistribution and dyserythropoiesis through the hormonal activities of hepcidin, enhancement of co-infections by iron, delayed parasite clearance and sequestration, enhanced hemolysis and an exaggerated acute phase response through the secretion of high levels of pro-inflammatory cytokines. A thorough understanding of these pathways is a necessary step towards the development of safe iron interventions that may improve both iron status and malaria outcomes.

The redox potential of iron makes it an indispensable element in several biochemical reactions in both the human host and the Plasmodium parasite. Increased intra-erythrocyte iron concentrations increase the ‘labile iron pool (LIP)’, which is easily accessible by the Plasmodium parasite, enhancing its growth. The LIP hypothesis is consistent with findings from several studies in animal models and in-vitro experiments which reported that iron chelators inhibited the growth of malaria parasites. Extracellular NTBI, intracellular labile iron pool, and iron from the degradation of hemoglobin are associated with increased oxidative stress on the erythrocytes. Iron has been shown to catalyze the production of hydroxyl radicals which are capable of causing peroxidative damage to plasma membranes. In rats, treatment with hemin, a heme derivative, was associated with increased chemotaxis of neutrophils, secretion of interleukin-8, and production of reactive oxygen species. The generation of reactive oxygen species may result in death of both parasitized and non-parasitized RBC at a time when erythropoiesis is impaired.
The homeostatic control of iron at the point of absorption, transport, mobilization from stores, and recycling is critical in determining how the iron-dependent pathways interact to influence malaria outcomes. Under normal physiologic conditions, circulatory levels of iron are regulated at the point of absorption and mobilization from major storage sites such as the enterocytes, hepatocytes and macrophages. Absorption of dietary iron occurs in the duodenal enterocytes. The types of proteins involved in iron absorption are dependent on the form of dietary iron being ingested—heme or non-heme. Absorption of non-heme iron requires the divalent ion transporter (DMTI). Since most of the ingested iron is in the +3 oxidation state (Fe\(^{3+}\)), dietary iron must first be converted to Fe\(^{2+}\) using the duodenal cytochrome B (DcytB), a process that is enhanced by ascorbic acid, before it can be transported by DMT1. Although the absorption of heme iron is not completely understood, it is believed that the heme carrier protein (HCP 1) is involved in importing heme iron from the gut into the enterocytes. Absorbed iron, whether heme or non-heme is either stored as ferritin, or exported into plasma through the basolateral membrane bound protein, ferroportin. Hence iron may be released into the circulation bound to transferrin or may be lost when the enterocytes are sloughed off.

Hepcidin is the major regulatory protein involved in the maintenance of iron homeostasis. The hepatic synthesis of hepcidin, a 25-amino acid peptide hormone, is up-regulated by high extracellular iron concentrations and inflammation, and down-regulated by erythropoiesis. Upon synthesis, hepcidin binds to ferroportin in the storage cells, causing its internalization and degradation and hence prevents the release of iron into circulation. Hepcidin synthesis is
decreased during iron deficiency anemia and in other conditions that up-regulate the rate of red blood cell formation. Suppression of hepcidin is associated with an increase in levels of erythropoietin (EPO) which ultimately ensures that there is enough iron for erythropoietic activities.  

By virtue of its role in iron homeostasis, there is a lot of interest in whether hepcidin can be considered a putative target as a means to reducing the iron-related adverse malaria outcomes. The evidence suggests that hepcidin levels are up-regulated during malaria infections although the physiologic relevance is not completely understood. Increased synthesis of pro-inflammatory cytokines including IL-6 and TNF-α during P. falciparum infections have been associated with elevated serum hepcidin levels. This is thought to be a proactive mechanism to prevent the release of iron into circulation, and hence making it less accessible to the parasites. In a cross section of Indonesian school children (n= 1197) 5-15 years old, P. falciparum infection was positively associated with a high mean serum hepcidin concentration and among a cross-section of Ghanaian patients with uncomplicated P. falciparum infections, Howard et al found a positive dose response association between hepcidin and P. falciparum parasite density. The elevation in hepcidin during malaria infections may have consequences for iron absorption and hence the efficacy of iron interventions in malaria-endemic regions. Recently, Cercamondi et al found that P. falciparum infection was associated with about 40% reduction in dietary iron absorption. It is possible that iron loading in the gut, at a time when iron export from the enterocyte is compromised, may enhance microbial growth, and precipitate the adverse health outcomes associated with malaria.
The up-regulation of hepcidin and the resulting redistribution of iron during malaria infection impairs erythropoiesis and exacerbates the development of anemia, a major cause of malaria related death in children.\textsuperscript{218,229,257-259} Sequestration of iron in storage cells ensures that iron transport to the bone marrow, and hence differentiation of erythroid progenitor cells are inhibited. In addition, the up-regulation in hepcidin synthesis has been associated with a reduced sensitivity to erythropoietin. It has been suggested that the state of dyserythropoiesis may actually be protective against severe malaria outcomes although the evidence is inconclusive. It has been shown that production of reticulocytes, the preferred habitat for \textit{P. falciparum} parasites are compromised when hepcidin levels are high.\textsuperscript{230,260} It is therefore plausible that by sequestering iron and impairing the formation of reticulocytes, hepcidin may decrease parasite entry into these preferred, immature cells, and hence reduce the risk for severe malaria.\textsuperscript{260} Emerging evidence from animal models suggest that hepcidin may protect against secondary infections by inhibiting the development of sporozoites into merozoites in the liver.\textsuperscript{223,224} Recently, Portugal \textit{et al} showed that at some threshold of blood stage parasitemia, new liver-stage infections are inhibited, and that this may be related to the redistribution of iron under hepcidin control.\textsuperscript{223} Withholding iron from hepatocytes as a result of sequestration in enterocytes and macrophages may arrest parasite growth and inhibit development into blood stage.\textsuperscript{224} Consistent with the hypothesis that hepcidin may offer protection against severe malaria, Wang \textit{et al} recently showed that treatment of mice with anti-hepcidin antibodies increased the rate of parasitemia and death, and that the induction of hepcidin expression in the mice was associated with protection against severe \textit{P. berghei} malaria.\textsuperscript{261} It is not yet
known whether hepcidin, by facilitating iron sequestration, may offer the same protective effect in humans.

Increased availability of non-transferrin bound iron (NTBI) during malaria infection has been associated with an exaggerated pro-inflammatory cytokine response.\textsuperscript{211-213,256} NTBI may be elevated when the rate of iron influx into the plasma exceeds the rate of binding to the transport protein transferrin. As part of the innate immune response to infection, levels of the intracellular adhesion molecule (ICAM) and vascular adhesion molecule (VCAM) are elevated.\textsuperscript{262,263} These adhesion molecules, although useful for attracting immune cells to the site of infection, may also precipitate severe disease by facilitating the sequestration of parasitized erythrocytes in the microvasculature. During malaria infections, the surface of RBCs are altered by the increase expression of cyto-adherants including the \textit{P. falciparum} erythrocyte membrane protein (PfEMP1).\textsuperscript{264} The membrane protein are targets for ICAM and VCAM, whose expression are up-regulated by NTBI.\textsuperscript{265} \textsuperscript{212,256} NTBI have also been associated with bacteremia, a risk factor for severe malaria.\textsuperscript{212,256}

Some evidence regarding the adverse effect of iron repletion on malaria outcomes have come from studies investigating the association between iron deficiency and malaria. Iron deficiency has been associate with eryptosis and accelerated clearance of parasitized erythrocytes.\textsuperscript{266,267} In murine species fed on either an iron deficient diet or a normal diet, the rate of parasitemia and death after 30 days of infection with \textit{P. berghei} was 21\% and 20\% lower respectively in the iron deficient group compared to controls fed on an iron replete diet.\textsuperscript{205} In addition, the phosphatidylserine expression on the surface of infected RBCs was significantly higher in the mice fed the iron deficient diet.\textsuperscript{205}
erythrocytes is achieved through a mechanism involving the activation of Ca^{2+}-sensitive K^+ channels in the membranes of RCBs and subsequently increased expression of phosphatidylserine on the surface of RBCs.\textsuperscript{215-217} Activation of these channels cause the flow of KCl out of the cell, resulting in loss of osmotic potential, loss of water, shrinkage of cells and scrambling of cell membranes.\textsuperscript{215-217} In addition, the disruption in RBC membrane leads to exposure of phosphatidylserine, a target for macrophages, on the surface of the parasitized erythrocytes. Macrophages have phosphatidylserine receptors with which they are able to bind to, and engulf the parasitized RBC, degrading them in the process, and reducing parasitemia.

There is growing interest in role of iron in the development of co-morbidities during malaria infections.\textsuperscript{203,212,221,225,226} The several pathogens that infect humans, including bacteria, viruses and protozoans, rely heavily on host iron for survival, and have developed mechanisms to extract iron from the host’s storage sites.\textsuperscript{225,268} Bacteria in particular have siderophores and other compounds that enable them to retrieve iron from host feroproteins.\textsuperscript{269} Other microbes such as the Salmonella bacteria actually resides in macrophages, such that iron sequestration into macrophages may prove beneficial outcomes for these microbes. It is still debatable whether hepcidin, by regulating the redistribution of iron, enhances or slows the concurrent growth of pathogens. On one hand, it seems plausible that the increased iron availability through supplementation or hemolysis may enhance the replication of extracellular pathogens. On the other hand, sequestration of iron may enhance the growth of intracellular microbes that dwell in the iron storage compartments.
Associations between iron indicators and markers of inflammation

Several of the proteins involved in iron metabolism, and which are typically used for defining population iron status, are affected by the inflammatory response to infections including malaria. Malaria affects ferritin, total iron binding capacity, iron distribution and transferrin receptor levels. Historically, hemoglobin has been used to define population iron status, a practice that is linked to both the practicality of hemoglobin assessment (ease, duration and cost), as well as the several limitation of performing assays for the commonly used iron biomarkers, including ferritin and sTfR. enzyme-linked immunosorbent assay (ELISA) technologies for the assessment of ferritin, sTfR and transferrin are expensive, and require a functional laboratory. The use of hemoglobin, however, has several limitations. Hemoglobin is a non-specific indicator of body iron stores. Hemoglobin levels are affected by several factors including the rate of erythropoiesis (or the rate of destruction of RBCs), hemoglobinopathies, infections and other inflammatory conditions. It is impossible to discriminate between the several causes of anemia or iron deficiency with hemoglobin without the concurrent assessment of at least one additional biomarker of iron status. In fact, the WHO and CDC recommend the current use of hemoglobin and ferritin for the diagnosis of anemia and iron deficiency respectively. There is a need to discriminate between anemia due to iron deficiency and anemia due to other causes to properly inform the design of interventions to control anemia and iron deficiency.

Ferritin is the iron storage protein usually found in the reticulo-endothelial cells of the liver, spleen and bone marrow, and in small concentration in the plasma. The exact role of serum or plasma ferritin, or its source is not
completely known. Emerging evidence suggests that serum ferritin has the potential to act as an iron delivery system for cells with ferritin receptors. Serum ferritin levels are indicative of total body iron stores and hence a good measure of iron status. Unfortunately, ferritin is a positive acute phase protein and hence during infections its concentration is transiently increased. A recent meta-analysis showed that plasma ferritin concentration may be elevated by 1.6, 2.6 and 1.5 times during the incubation, early convalescence and late convalescence stages of infection as defined by Thurnham et al. The WHO recommends that concurrent assessment of one or more markers of inflammation be used to improve the interpretation of ferritin levels in regions where infections are endemic.

Transferrin receptor is expressed on the surface of all cells (with the exception of mature erythrocytes) and involved in the receipt of iron bound to transferrin. The truncated version of the membrane bound transferrin receptor, referred to as the soluble transferrin receptor, is present in the plasma and used for defining iron status. Concentration of the soluble transferrin receptor reflects the levels of the membrane bound receptor, whose rate of transcription, translation and expression are up-regulated when iron levels are low. Although the common perception is that sTfR is robust to inflammation, emerging evidence suggest that sTfR may actually be affected during the acute phase response to infections. In a recent study, Kasvosve et al showed that among Zimbabwean children less than 5 years (n=208), the concentration of sTfR was positively correlated with CRP, and IL-6, and that this association was independent of the rate of erythropoiesis, hypoxia, or iron status, the three major determinants of sTfR expression. In a related study, Verhoef et al showed that the mean serum transferrin concentration was significantly higher
(p=0.005) in children with asymptomatic malaria (11.4 mg/L) compared to children without malaria (7.8 mg/L). Similar results were found among a group of non-hospitalized Nigerian children in whom the mean sTfR concentration among children with *P. falciparum* infection (3.68 mg/L) was significantly higher compared to controls (2.99 mg/L, p = 0.0009).

A few studies have reported a negative dose response association between malaria and sTfR, raising questions about the uniformity of the association of sTfR with inflammation. Beesley et al found that in a cross-section of European adults hospitalized with malaria, sTfR levels were negatively and significantly correlated with both AGP and CRP but not ACT and a study by Williams et al concluded that sTfR levels were significantly reduced during clinical malaria in a sample of Vanuatu adults. A study by Kuvibidila et al however found that among a cross-sectional of children from the Democratic Republic of Congo (DRC), concentrations of sTfR were similar across groups of children with asymptomatic malaria, symptomatic malaria and no malaria. More research is needed to examine the robustness of transferrin receptor in the context of inflammation inducing conditions. There is also a need to develop standards to guide the interpretation of sTfR measures. Currently there is no global consensus on the threshold for defining iron deficiency using sTfR. Until sTfR is properly standardized, it will be impossible to make comparisons across studies using this indicator.

Recently, it has been suggested that using the sTfR-ferritin index, an index derived by calculating the ratio of serum transferrin receptor to serum ferritin (or the log-transformed ferritin) may be more reliable and superior than either ferritin or sTfR alone in distinguishing between iron deficiency anemia and other types of anemia.
Yang et al found that this index correlated highly and positively with body iron stores (Pearson correlation >0.95). In a study by Jain et al, a sTfR/log ferritin ratio >1.5 was found in all cases of iron deficiency anemia and in all cases of anemia of chronic inflammation with concurrent iron deficiency. This finding is consistent with outcomes of a study by Goyal et al, which concluded that in patients with rheumatoid arthritis sTfR/log ferritin ratio >1.5 can be used to distinguish between ID related anemia of chronic disease and non-ID related anemia of chronic disease. Recently, Phiri et al showed that the sTfR-F index has a sensitivity and specificity of 74 and 73% respectively when compared to bone marrow iron content, and that the accuracy was superior to that found with either of ferritin or sTfR alone. There is a need for more examination of this index, particularly among children in whom the burden of anemia and ID are greatest.

Several other indicators have been proposed for diagnosis of population iron status, as well as evaluation of the efficacy of interventions designed to improve iron or hemoglobin status. These include zinc protoporphyrin, serum or plasma iron, transferrin saturation, total iron binding capacity and mean cell volume. Zinc protoporphyrin is an indicator of iron deficient erythropoiesis, during which zinc instead of iron is inserted into the protoporphyrin molecule in the later stages of hemoglobin formation. Mean cell volume on the other hand indicates whether RBCs are of normal size or smaller as in the case of iron deficiency anemia. Mean cell volume is a non-specific indicator of iron status and zinc protoporphyrin does not adequately reflect body stores in the context of inflammation. More evidence is needed to validate and standardize the use of zinc protoporphyrin, serum or plasma iron, transferrin saturation, total iron binding capacity and mean cell volume.
In conclusion the evidence from prior studies have been inconsistent with respect to the: a) the effects of vitamin A adequacy on malaria outcomes, b) the effects of iron adequacy on malaria outcomes and c) the robustness of the current indicators of iron and vitamin A status in the context of inflammation. These are gaps in current understanding that must be filled by continuing research. In particular, there is a need to demonstrate whether there exists a threshold concentration for iron or vitamin A, beyond which harmful or beneficial malaria outcomes may be precipitated. To this effect, this study was designed to characterize the dose-response associations between iron or vitamin A status and malaria incidence in rural Zambian children. As a necessary step towards addressing this primary goal, this study was also designed to characterize the pattern of inflammation- and malaria-associated changes in the biomarkers of iron (ferritin and sTfR) and vitamin A (retinol) status.
Chapter 3: INTRODUCTION TO STUDY

Study objectives

This study has four main objectives: a) to characterize the pattern of inflammation- and malaria-associated changes in serum retinol concentrations; b) to characterize the pattern of inflammation- and malaria-associated changes in ferritin and soluble transferrin receptor concentrations; c) to evaluate the longitudinal associations between vitamin A status, defined by serum retinol and incidence of malaria, serologically defined; and d) to evaluate the longitudinal associations between iron status defined by ferritin and incidence of malaria. Data used in this study were collected as part of a cluster-randomized, controlled trial designed to test the efficacy of provitamin A carotenoid biofortified versus standard white maize flour consumption in improving the vitamin A status among children in rural Zambia. The study included children, 4-8 years old in Mkushi District, Central Province, Zambia. This age group was chosen partly because of the difficulty in getting a control group in the younger, high risk group who are the target of a national high-dose vitamin A program. In part, this group was also chosen because of the lack of evidence regarding vitamin A status.

Data collected during the baseline (low malaria transmission season) and follow up (high malaria transmission season) were used in addressing the study questions (figure 3.1).
Following a mapping and census activity (June 2012), clusters of children were formed and randomized to receive either a β-carotene biofortified maize intervention, or a control intervention (non-biofortified maize). Baseline survey (August 20th through September 19th, 2012) was done in the low malaria transmission season to collect field data and blood, for subsequent laboratory analyses. Following baseline, children were fed twice daily during a 6-month follow-up. Monthly household surveys were done to collect child diet and morbidity data. Endline survey (March 4th through April 5th, 2013), conducted in the high malaria transmission season, involved a repeat of the baseline assessments. Field and laboratory data were pooled from the baseline and endline surveys to address the study questions.
Study design

Biofortified maize intervention trial

The parent study was a cluster-randomized, controlled trial designed to evaluate the efficacy of provitamin A carotenoid biofortified maize flour in improving the vitamin A status of children 4-8 years of age in Mkushi District, a rural, malaria-endemic community in Zambia. Nested into the trial was baseline and 6-month follow-up assessment of micronutrient (vitamin A, iron), inflammation (AGP) and malaria (blood smears and a rapid diagnostic test (RDT), which provided the basis for conducting the present study. Prior to the formation of clusters, a mapping and census activity was conducted to identify, map and enumerate all eligible children in the catchment area. Children were eligible to participate in the study if they were born between June 30, 2005 and July 1, 2008 and not enrolled in school at the time of the mapping and census activity. Children registered in the mapping and census were assigned clusters defined by geographic residence. The clusters were then used as units of randomization to assign children into the intervention arms. A total of 50 clusters were randomized into the biofortified maize (n=25) or a standard white maize (n=25) group. Feeding sites (1 per cluster), were chosen such that households were within a 1km distance. Beginning August 20, 2012, houses were visited to obtain consent, enroll children and conduct baseline assessments until September 19, 2012. Initiation of feeding by assigned cluster began in sequence following baseline assessments. Thus feeding started on August 22, 2012 and continued through March 19, 2013, at which time children in all clusters had fed each day approximately 6 months. Monthly follow-up visits were conducted to collect data on household and child food intake patterns, as well as child morbidity history. The endline survey was
conducted 6 months after baseline (April 5, 2013). Blood samples during these surveys were sent to Tropical Disease Research Centre (TDRC) for laboratory assessments of serum retinol, ferritin, soluble transferrin receptor, and AGP. Malaria morbidity was assessed at baseline and follow-up using microscopy and RDT. At baseline we enrolled a total of 1024 children -543 into the provitamin A group, and 481 into the control group- data from whom were used in this study.

**Study area and subjects**

The study catchment area included all village communities covered by five rural health centers- Nkumbi, Chibefwe, Nkolonga, Chalata, and Masansa in Mkushi District, Central Province, Zambia. Maize prepared as *Nshima* (a thick semi-solid porridge) is the staple food for people in Mkushi. The district has a tropical climatology with seasonal rainfall and disease patterns. The peak of malaria transmission coincides with the rainy season, typically the months of November through April. The entire district covers an area of 14808 km$^2$ and has a total population of about 165,000 with about 19% in the age group of 0-5 years. Malaria is highest among children below five years of age and contributes to the high prevalence of anemia and malnutrition. It is estimated that in Zambia, malaria is responsible for about 50 000 deaths annually, 50% of all hospital admissions and over 40% of all deaths in children less than 5 years each year.$^{100,101}$ Zambia has seasonal malaria transmission patterns, which is closely tied to the rainfall patterns. Malaria burden is especially high in rural areas where the estimated annual prevalence may be up to 40%.$^{102}$ In a recent national survey,$^{10}$ about 57% of children below 5 years were found to be anemic and about 54% were found to be vitamin A deficient. The prevalence of
Elevated CRP (>5mg/dl) and elevated AGP (>1g/L) among Zambian children was recently estimated at 40 and 76% respectively.\textsuperscript{288}

**Eligibility Criteria**

The study included children who were born between June 30, 2005 and July 1, 2008 and not enrolled in school as at April 2012.

**Sample size and power calculations**

**Study aim 1:**

We also estimated that to detect a difference in retinol levels of 0.1 $\mu$mol/L between children with and without malaria, and assuming a within-group standard deviation in serum retinol of 0.25 $\mu$mol,\textsuperscript{288} and a 20% prevalence of malaria, we would require a total sample size of 410 at 90% power and 5% type 1 error.

**Study aim 2:**

To be able to detect a relative risk of 1.4 ($\beta=0.2, \alpha=0.05$) comparing the incidence of malaria in vitamin A deficient to vitamin A adequate children, and assuming a 50% percent baseline VAD prevalence, and a 20% malaria prevalence in VA-adequate children (at follow-up), we estimated that we would require a total sample size of 892.

**Study aim 3:**

We estimated that to detect a difference in ferritin levels of 2.0 $\mu$g/L, assuming a within-group standard deviation in serum ferritin of 2.2 $\mu$g/L,\textsuperscript{289} and a 40% prevalence of elevated CRP(>5mg/L), we would require a total sample size of 55 at 90% power and 5% type 1 error.

**Study aim 4:**
We estimated the sample size requirements for determining the association between baseline iron status and the risk of malaria morbidity at follow-up \((\beta=0.1,\alpha=0.05)\). Assuming an ID prevalence of 50% and a 20% malaria prevalence in the iron-adequate group, we estimated that we would require 784 children to be able to detect a 10% difference in malaria incidence.

**Institutional review and approval**

Ethical clearance was obtained from the Johns Hopkins University (JHU), Baltimore USA, and the Tropical Disease Research Centre (TDRC), Ndola, Zambia. This included approval for preparatory work and implementation of both the main trial and nested study.

**Community sensitization**

Community sensitization activities, led by the project’s community liaison officer, were conducted throughout the course of the study. From the onset, the goals, needs and expected outcomes of the study were conveyed regularly to district health officers, civic leaders, and traditional leaders and to the community at large by local, Mkushi-based public radio broadcasts. In January 2012, meetings were held between representatives of the Johns Hopkins University (JHU), TDRC, the national food and nutrition commission (NFNC) of Zambia, and local members to discuss details of the project, concerns and ways forward. Following these leadership meetings, public announcements targeting the larger community were made via newspapers, flyers, postings on government buildings, community radio and herdsmen to explain the purpose, procedures and intended participants of the trial. Routinely, the community liaison officer received feedback from informants within the target villages, and organized meetings to address concerns whenever necessary. This involved visiting
households to talk to people on specific aspects of the trial as necessary. Sensitization activities were particularly critical in addressing deeply rooted ethnic and religious concerns about blood draw in the district.

**Mapping and census**

**Figure 3.2 Map of Mkushi District**

Mapping and census procedures conducted in June, 2012 by trained field interviewers provided the basis for identifying and enumerating households and potential study participants for subsequent recruitment into the study. Each field interviewer was given a tablet computer equipped with GPS to locate and geocode waypoints of interest into the electronic map system. In addition, field interviewers collected household level information including type of household, size of household...
and location of each household. For each individual identified in a specified household, the name, age, date of birth, gender and school enrollment status (for children in the 4-8 age group) were also documented. Geocoded data from the mapping and census activity formed the basis for cluster formation and feeding site selection. Field interviewers also sought permission to place barcode stickers bearing households IDs on household (doors). These barcode stickers were used to identify households during subsequent visits at baseline and monthly household visits thereafter.

Cluster formation

Following mapping and enumeration of eligible children, spatial analyses were done to identify and assign children into clusters for subsequent randomized allocation into the study arms. Fifty clusters (median=24.5; inter-quartile range=7) were formed. Clusters were aggregated into blocks of four and randomized in a 1:1 ratio into the provitamin A group (n=25) or the control group (n=25).

Feeding and clinical assessment site identification

Once clusters were formed, feeding sites were identified by food systems personnel working in conjunction with community supervisors and key community informants. Feeding sites were identified such that houses were within a 1 km radius from the site and near a water source for cooking. Typically this would be an enclosed or semi-enclosed hut made of mud and grass roof. Whenever it was impossible to identify an existing facility, shelters were constructed and used as feeding sites. In most clusters, feeding sites also served as clinical assessment sites. Whenever possible, health centers were conveniently chosen as clinical assessment sites for nearby clusters.
**Intervention**

The trial was designed to feed each child the equivalent of 200 g dry weight maize flour in the form of porridge and *nshima* each day. It was estimated that this amount would supply the RDA of vitamin A (400 µg RAE/d) for children in the 4-8 years age group, assuming a provitamin A density of 15 µg/g of the biofortified flour and a conversion factor (provitamin A to retinol) of 7:1. Standardized recipes were developed to guide the preparation of porridge, *nshima* and relish, taking into considering the cluster sizes. Relishes were carefully selected to ensure availability whiles limiting the additional provitamin A supply from sources other than the fortified maize. The relishes included beans, cabbage, groundnut, cowpea leaves, and *kapenta*. These were organized into a two-week rotating menu which specified the kind of accompaniments to be eaten with porridge and *nshima* for a given day. All children who consented to participate showed up at the sites daily (except for Sundays) to receive food. Photobooks which contained photos of the children taken at the time of consent were used to ensure that children were feeding at their assigned clusters. Cooks (two at each site) were trained to record on daily basis including the attendance at the feeding sites, the amount of food served, and leftovers if any. Typically children would report for breakfast, and play at the cooking site until lunch was served. In the event that children did not show up for two consecutive meals, cooks would visit the household to find out why children did not show up. When necessary, meals were packaged and sent to the house for the child to eat.

**Staff training**

All field staff were trained prior to the baseline assessments using a detailed training protocol. Six anthropometrists were trained on how to take weight, height, mid-upper
arm circumference and triceps skinfold thickness measurements. Four anthropometrists were ultimately hired to do anthropometry using a pre-defined criteria which included technical error of measure (TEM) and reliability coefficient. Several meetings were held between laboratory technicians from TDRC and the JHU team to harmonize the procedures for collecting and handling blood, and treatment or referral of sick children. All field interviewers, anthropometrists and laboratory technicians received extensive training on how to collect and save data on tablet computers. Training activities were repeated before the start of the endline survey.

**Data Collection**

**Baseline data collection summary**

The baseline survey was conducted from August 20th through September 19th 2012, comprising a sequence of activities that started at the household and ended at the clinical assessment sites. There were four clinical assessment teams, each composed of three field interviewers (FIs), one anthropometrist, one study nurse, and one laboratory technician. Early in the morning, FIs went to assigned households with rosters and maps indicating cluster IDs, community, location of household, household ID, child ID, household head name, child name, and community supervisor name and contact. In addition, field interviewers were given tablet computers loaded with: a) consent form, b) household interview form and c) child diet and morbidity form. Once at the household, the FIs checked the barcode sticker on the door (placed during the mapping and census activities) to confirm that the target child is still residing in house.

At the household, the field interviewer read the consent statement (Appendix I) in Bemba to the respondent (typically a parent of the child). At the end of the
statement, the interviewer asked the parents or other responsible adults if the household would agree for the child to be enrolled and participate in the study. In addition, the field interviewer asked permission for project staff to draw less than 2 teaspoons (7 ml) of blood from the child. Permission was also sought to take photos of the children, which were later used in the construction of photobooks used at the feeding sites to confirm their identity. Following consent, the field interviewers administered the household interview form, which sought to obtain information on membership, socioeconomic status, food security, and foods consumed. The field interviewer then administered the child diet and morbidity assessment form (Appendix G). This form contained four modules: a) recall foods eaten in the 24 hours prior to interview, b) a morbidity recall which asked the number of days children were observed to have symptoms of illnesses over the 2 weeks, c) a dark adaptometry module which asked questions to ascertain vision impairment in the children, and d) a health practice module which asked questions about the general health practices in the past 6 months. The morbidity recall was especially relevant to this study of nutrition and infections. The protocol involved asking parents to recall if their child had any of 13 pre-specified symptoms in the past 14 days, including fever, cough, difficulty breathing, headache and loose stools. If the child had a symptom, the respondent was asked the number of days in the past 7 days the child had the symptom. If present, the respondent was asked if the child had the symptom in the last 24 hours. These questions were repeated for all 13 symptoms. For children who consented to participate, the field interviewer accompanied them to the clinical assessment site (typically accompanied by another household member). Activities at the site included anthropometry, axillary temperature measurement, blood drawing and packaging (for
transportation to the field laboratory), onsite testing for malaria using RDT, hemoglobin measurement, malaria smear preparation and prophylaxis and treatment explained below.

**Anthropometry**

Anthropometric measurements were taken by trained anthropometrists, who also acted as site leaders for the clinical assessment teams. Each worker was given a roster which indicated all the assigned children for a particular day. They also had a tablet computer on which to electronically enter all data. Once a child showed up at the clinical assessment site, the anthropometrist would check the name on the roster to confirm their site assignment. Once confirmed, the form was opened, identifying barcode scanned, and height, weight, mid-upper arm circumference (MUAC) and triceps skinfold thickness measured. Height was measured with a Shorr board to the nearest 0.1 cm. Weight was measured on a SECA 874 digital scale to the nearest 0.1 kg. MUAC measurements were taken with insertion tapes to the nearest 0.1 cm and skinfold assessed with a Holtain caliper to the nearest 0.1 mm. Following anthropometry, children advanced to the blood draw and disease control substation.

**Axillary temperature**

Children had their axillary temperature taken with a digital thermometer by study nurses according to the manufacturer’s instruction. Children with high fever (axillary temperature >39.5°C) were referred to the nearest health center.

**Blood collection**

All metadata related to blood draw were entered into the electronic blood draw and disease control form (Appendix H). Trained laboratory technician (assisted by a study nurse) performed blood draw, field tests, data entry and disease control

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procedures, including referrals when necessary. At the blood draw station, the child’s identifiers were confirmed on daily rosters, and scanned into the electronic clinical assessment form. Approximately 7 ml of venous blood was collected from each child by syringe into 10 ml blood collection tubes (Covidien Monoject sterile tubes with no additives, Fisher Scientific Catalogue #22029308) with attached specimen ID that was linked to each child’s ID. Tubes were immediately put into cooler boxes containing ice packs, transported to the field laboratory in Mkushi for separation. Blood remaining in the syringe was used for measuring hemoglobin on a hemocue (Hemocue 201+, hemocue AB, Angelholm, Sweden), performing RDT and smear preparation for malaria assessment. All meta-data related to blood sampling and tests performed were documented on electronic clinical assessment forms. At the end of clinical assessments, each child was given a child report card containing key health data including weight, weight, temperature, and malaria status.

**Malaria rapid diagnostic testing (RDT)**

For each child, malaria status was ascertained using a malaria rapid diagnostic test (SD Bioline Malaria Ag P.f, Standard Diagnostics, Yongin, South Korea; 05FK50). The RDT is a rapid immune-chromatographic test for the detection of HRP-2 antigens in peripheral blood. A drop of blood from a syringe (described above) was added to the test packet of the RDT, and three drops of buffer added. The kit was then allowed to react for up to 15 minutes before reading. Results of the RDT test were entered into the tablet computer by the laboratory technician. Test-positive children were treated with Coartem® according to Zambia national guidelines.
Malaria smear preparations

Thick and thin venous blood films were prepared at baseline and follow-up, each about 2 µl and 10 µl of blood respectively. In preparing thin smears, the blood was dropped at one end of a clean slide using the syringe, and then spread to the other end using a spreader. To prepare a thick smear, the blood was dropped into the center of a clean slide and then spread into approximately 15 mm radius circle. The slides were allowed to dry for about 10 minutes and then packed into slide boxes. Smear preparation was confirmed on the electronic clinical assessment form. Slides were transported to the TDRC laboratory in Ndola for staining and reading.

Hemoglobin determination

Hemoglobin was assessed at baseline and at follow-up using venous blood from a syringe and a Hemocue® Photometer (Angelholm, Sweden). Hemocue readings were immediately entered into the clinical assessment form on the tablet. All children with severe anemia (Hb <7g/dl) were referred to the nearest health center for further evaluation and treatment.

Disease control procedures

At baseline, all children were given 400 mg of Albendazole by study nurses after blood collection. This is the standard for school aged children in Zambia. Children were also given Praziquantel tablets, as a control for endemic schistosomiasis, at the baseline in a weight dependent manner. At baseline and endline, all malaria RDT positive cases were treated with Coartem according to WHO guidelines. Coartem® is a combination of about 20 mg fast acting Artemether and 150 mg slow acting Lumefantrine and is currently the first line malaria treatment for children in Zambia. The treatment regimen for children (<16 years) consists of 6
weight-specific doses over a period of 3 days. Treatment began at the point of
diagnosis. Caretakers of the children were instructed to complete the weight-specific
regimen at home. Children noted to have severe malaria or danger signs were referred
to the health center for further management and care. These signs included severe
anemia (Hb <7g/dl), high fever, prostration, vomiting and obvious weakness. Reason
for referral were documented on a child referral card which was sent with the child to
the health center. Sick children were transported in project vehicles to the health
center.

**Monthly household visit and follow-up survey**

After baseline assessment and initiation of feeding, field interviewers visited
households on a monthly basis to administer household interviews about each child’s
diet in the past 24 hours and morbidity symptoms over the previous 2 weeks. A final
survey was conducted approximately 6 months after the initiation of feeding
following the same assessment protocols with the exception of helminthes and
schistosomiasis control procedures, which were implemented at baseline to limit
interference with the main interventions.

**Laboratory procedures**

**Serum preparation and transport**

On daily basis, a senior laboratory technician collected and transported to the
field laboratory in cooler boxes, blood samples from clinical assessment sites.
Samples were centrifuged at 3000 rpm for 10 minutes under dim light. Serum was
aliquoted into three pre-labeled cryovials bearing IDs corresponding to the clinic IDs
on the blood collection tubes. The cryovials were stored in liquid nitrogen tanks and
transported to TDRC laboratory for storage at -80 °C. Sample remained stored at -80 °C until analyzed.

**Microscopy**

Light microscopy was used to read malaria slides in the laboratory. Slides were stained with 3% Giemsa, washed, dried and read independently twice by two microscopists at the TDRC in Ndola. When readings were discordant, a third independent reading was done to resolve discrepancies. For each positive reading, the corresponding thin film was read to determine the *Plasmodium* species. Thick films were used to estimate parasite density by counting the number of parasites per 200 white blood cells (WBC). A slide was ruled negative if no parasites were detected after counting 200 oil immersion fields. The number of parasites per 1ul of blood was calculated by assuming 8000 WBC/µl of blood.

**Determination of serum retinol, AGP, ferritin and soluble transferrin receptor (sTfR)**

All laboratory assays were performed at the TDRC in Ndola. High pressure liquid chromatographic techniques were used for the determination of serum retinol concentrations. Enzyme linked immune-assays (ELISA) were used for the determination of serum AGP (Abcam®, Cambridge, USA), ferritin (Ramco Lab. Inc, Texas, USA) and sTfR (Ramco Lab. Inc, Texas, USA) according to manufacturer’s instructions. Detailed procedures for the determination of retinol, AGP, ferritin and sTfR are shown in the appendix (chapter 9).

**Data Management**

A data management officer, stationed at the central project office was responsible for ensuring that data were collected and managed in accordance with the
study protocol. Data collection procedures on the field were done electronically using tablet computers. The electronic forms were uploaded onto the tablet each night by the data management officer. Field workers signed up for their pre-assigned tablets each morning and checked-in their tablets at the end each day. Once tablets were returned to the central office, entries were pulled in “.xml” format using Open Data Kit (ODK) Briefcase into a folder, exported in ‘csv’ format, and then imported into a SQL server. Data were backed-up to an external hard-drive. Laboratory data were compiled at TDRC and transmitted electronically to investigators at the Johns Hopkins University using a Dropbox account. At JHU, data was reviewed, cleaned and saved into a separate computer with a Windows Server Operating System, for the management of access.

**Quality control**

Quality control procedures were designed to assure adherence to the protocol throughout data collection and entry. Personnel involved in data collection were trained extensively before the baseline and endline surveys. A detailed manual of operations was prepared and used in drill exercises designed to simulate the anticipated work flow during the actual data collection. Each staff was trained in the use of tablet computers. Several practice sections were organized for staff to practice data entry on the tablet. Prompts were inserted at key points in the electronic forms to ensure that field staff were following the protocol during the interviews, assessments and data entry. Among other things, the prompts served as reminders to the staff to make double entries where required, to make entries within a pre-specified range, to avoid skipping questions and to save all entries. Quality control supervision
guidelines were developed and used in the training and supervision of field and laboratory work.
Chapter 4 : PAPER 1

TITLE Malaria exacerbates inflammation-related reductions in serum retinol concentrations

ABSTRACT

In the context of inflammation, serum retinol concentrations are reduced. Whether the magnitude of the inflammation-associated reduction is exacerbated by concurrent malaria, a potent inducer of inflammation, is unknown. We determined the extent to which retinol concentration in apparently healthy children, defined as malaria negative and having no inflammation (AGP > 1 g/L), differed from the concentration among children with: a) inflammation without malaria and b) malaria without inflammation, and c) malaria with inflammation. Baseline (August - September, 2012) and endline (March - April, 2013) data collected in the low and high malaria transmission seasons respectively from rural Zambian children (4-8 years) assigned to the control arm of a provitamin A biofortified maize efficacy trial were analyzed (n=352). Percent reduction in retinol relative to the apparently healthy group was estimated based on cross-sectional data at the baseline and endline. Inflammation (AGP>1 g/L) alone, or with concurrent malaria (defined as positive microscopy), was associated with a change in retinol of -10% (CI: -15, -5) and -15% (CI: -21, -8) at baseline, and a change of -7% (CI: -16, 2) and -36% (CI: -44, -27) at endline, respectively. When the endline estimates were restricted to children who were apparently healthy at baseline (cohort approach, n=169), inflammation alone, or with concurrent malaria, was associated with a change of -2% (CI: -26, 38) and -28% (CI: -41, -13) respectively. Our results suggest substantial variation in the magnitude
of the inflammation-associated reduction in retinol across malaria status or season.
The concurrent assessment of malaria, in addition to inflammation, may enhance the interpretation of retinol values in endemic regions.

INTRODUCTION

In malaria-endemic regions, semi-annual high dose (200 000 IU) vitamin A supplementation of children remains a key public health strategy for improving population vitamin A status and related health outcomes. This practice is necessitated by the high burden of vitamin A deficiency (VAD), an important risk factor for early childhood morbidity and mortality. A key requirement for effective vitamin A programs is the estimation of the prevalence of deficiency at the population level. This is a critical step in evaluating the impact of vitamin A interventions, as well as ensuring that resources are appropriately targeted. The assessment of vitamin A status is, however, problematic particularly in regions with a high prevalence of infections. It has been shown that concentrations of serum retinol, the commonly used biomarker of vitamin A status, are reduced during the acute phase response to infections, including malaria. This phenomenon, referred to as inflammation-induced hyporetinolemia, is not completely understood and poses a threat to the validity of population estimates of vitamin A status. In a recent meta-analyses, Thurnham et al estimated that plasma retinol levels may be reduced by about 12-32% and that the prevalence of VAD may be inflated by up to 10% if inflammation-induced hyporetinolemia is uncorrected. There is currently no standardized procedure for correcting the prevalence of vitamin A status in the context of inflammation. In addition, evidence is lacking on whether the magnitude of the inflammation-associated changes in retinol is disease-specific. This study is an
attempt to characterize the response in serum retinol concentrations to malaria, a potent inducer of inflammation.\textsuperscript{298-300} The outcomes of this study will inform assessment of vitamin A status in a malaria-endemic region.

The World Health Organization recommends that to improve the interpretation of serum or plasma retinol concentrations, there is a need to define the presence of inflammation by concurrently assessing one or more acute phase proteins.\textsuperscript{301} Correcting for inflammation is complicated because, unfortunately, there is little consensus on which acute phase proteins to use, or how they should be used in correcting for the transient reductions in serum retinol concentrations.\textsuperscript{17,18,20,38,60,153,172,182,189,296,297,302-309} In addition, it is impractical to restrict the assessment of vitamin A status to healthy subjects. Such an attempt would require that in regions with a high burden of infections, a large number of individuals be sampled in order to obtain a sufficient number of apparently healthy individuals to estimate uncomplicated vitamin A deficiency. The idea of adjustment factors, proposed by Thurnham \textit{et al.}\textsuperscript{38,301} has gained traction among researchers in this area, mainly because it accounts for variations in the magnitude of the inflammation-induced hyporetinolemia across progressive stages of infection.\textsuperscript{38,301} This approach, while practical because it does not require the exclusion of subjects with inflammation, assumes that the effect of inflammation on retinol concentrations is independent of the type of infection. The evidence from populations with differences in the burden of prevailing infections suggests that this assumption, although convenient, may not be valid\textsuperscript{35,38,310,311}. In this study, we proposed to quantify the difference in serum retinol concentrations between apparently healthy children and
those with inflammation defined by AGP. In addition, we proposed to examine how the AGP associated changes differ by malaria status.

A major problem with the cross-sectional models for generating adjustment factors is the inherent tendency to neglect the effects of other determinants of vitamin A status, and with that the potential to overestimate the effect of the current inflammation. This kind of problem can be expected in places where the risk factors for infections and malnutrition tend to cluster within individuals and households, such that children with and without inflammation may not be comparable with respect to the history of vitamin A status. For example, children with poorer inherent vitamin A status may be more inclined toward illness and thereby more likely to have elevated AGP. As well, historical exposure to infections may contribute to lower retinol levels through mechanisms such as anorexia, increased urinary losses and impaired absorption. These losses can affect vitamin A hepatic stores. Unfortunately, deriving factors to “correct” retinol using cross-sectional data on retinol and inflammation alone attributes all the observed differences in serum retinol to the current inflammation. This problem can be mitigated by adopting a cohort approach, in which individuals without inflammation are followed until such a time when some become exposed to inflammation, at which point the serum retinol concentration among those with inflammation are compared to the concentration in those without inflammation. In our study population, this approach was applied to compare the difference in serum retinol across inflammatory groups at the follow-up, among children who were free of malaria and inflammation at the baseline. Considering the age-specific patterns in the progression of malaria, we also evaluated potential age interactions in the inflammation-associated changes in serum retinol.
The overall goal of this study, therefore, was to evaluate, using a cross-sectional or a cohort approach, the changes in serum retinol concentrations during inflammation, and whether the differences differ by malaria status and by age. Specific questions are as follows:

a. How does serum retinol concentration differ between groups of children defined by inflammation (AGP > 1g/L) and malaria status?
b. Does the difference in serum retinol concentrations across the inflammation groups differ between low and high malaria transmission seasons?
c. Is the magnitude of the difference determined with a cross sectional approach different from estimates based on a cohort approach?
d. Does the magnitude of the inflammation- and malaria-associated changes in serum retinol differ across age?

The current study used data collected from rural Zambian children participating in a 6-month cluster-randomized trial, designed to evaluate the efficacy of a provitamin A intervention on vitamin A status. Baseline and endline survey data, collected in the low and high malaria transmission seasons respectively, were used to address the study questions.

**METHODOLOGY**

Details of the study design, including the study population, blood draw and processing, data collection and data management are presented in chapter 3.
Data analyses

Definition of inflammation groups using cross-sectional approach

Children were assigned into four inflammatory groups defined by malaria and AGP as follows: reference (AGP ≤1 g/L and malaria negative); inflammation only (AGP >1 g/L and malaria negative); malaria only (AGP ≤1 g/L and malaria positive); malaria with inflammation group (AGP>1 g/L and malaria positive). Children were considered as having malaria if they had *P. falciparum* parasitemia of any density as defined by microscopy. To generate adjustment factors for retinol, we first calculated geometric means for the retinol distributions within the reference group and each of the three malaria-inflammation groups. We then calculated the ratio of the geometric mean retinol in each of the malaria-inflammation groups to that of the reference group. The percentage difference in retinol from the reference group was calculated by multiplying the geometric mean ratios by 100. Adjustment factors were established by taking the inverse of geometric mean ratios. All individual retinol values among members of the corresponding groups were then multiplied by the adjustment factor to derive an estimate of retinol assumed to be unaffected by inflammation or malaria exposure. Corrected concentrations were generated separately for both baseline (low malaria transmission intensity) and follow-up (high malaria transmission intensity).

Cohort approach for quantifying inflammation- and malaria- associated changes in serum retinol concentrations

In the cohort approach, analyses were restricted to individuals who had neither inflammation nor malaria at the baseline. Among this sub-group (n=169), adjustment
factors were defined on the basis of endline retinol, AGP and malaria data, using the same steps outlined in the cross-sectional approach above.

**Definition of vitamin A status**

Vitamin A deficiency was defined as serum retinol concentration <0.7 µmol/L.

**Definition of baseline socio-demographic, nutritional status, and morbidity history**

Socio-demographic variables included in the analyses include literacy of household head, assets, including ownership of motor vehicles and availability of electricity in the house, reported religious affiliation, and reported occupation of household head. We defined literacy as the ability to read or write in English. We defined stunting and underweight as height-for-age (HAZ) and weight-for-age (WAZ) z-scores respectively below -2 standard deviation of the WHO Growth Reference. Fever was defined as axillary temperature >37.5°C. Anemia was defined as hemoglobin <11.0 g/L for children <60 months, and <11.5 g/L for older children. We also included data on the reported prevalence of fever, cough and diarrhea in the 2 weeks preceding the interview.

**Statistical analyses**

We used exploratory analytic techniques including histograms, box plots, correlation matrices and normality tests to examine the distributions of relevant baseline and follow-up variables. We used the Shapiro-Wilk test to determine if the serum retinol concentrations at both baseline and follow-up were normally distributed. On the basis of the Shapiro-Wilk test, the concentration of retinol and
AGP were log-transformed before percent difference and adjustment factors were estimated. Using the log-transformed data, we examined the strength and direction of the linear and non-linear associations between these variables using correlation matrices and locally weighted scatter plot smoothing (LOWESS) techniques. For both baseline and follow-up data, we tested the difference in the serum retinol concentrations across the four groups using a one-way analyses of variance test. Differences between the uncorrected concentrations and the serum retinol concentration corrected for inflammation and malaria were tested using the paired t-test. Differences in prevalence of marginal vitamin A deficiency estimated using the corrected and uncorrected concentrations were tested using the McNemars chi-squared test. We used LOWESS techniques to visualize how inflammation and malaria associated changes in serum retinol concentration varied across age. We also tested for statistical interactions between age and inflammation or malaria. Consistent with the observed interaction, the age variable was dichotomized using cut-off of < 60 months versus older. We then ran linear regression models using the log-transformed serum retinol as the outcome variable, the malaria-inflammation indicator as the main exposure variable, the dichotomized age variable as a covariate, and the product of the age and inflammation indicator entered as an interaction term. All analyses were done separately for baseline and follow-up data. In all analyses, statistical significance was set at 0.05, except on the case of an interaction coefficient, in which case, significance was set at 0.1.
RESULTS

Biochemical, anthropometric and socio-demographic characteristics of study children

Our analyses were restricted to 352 children, 4-8 year from the control arm of the main trial, who had complete baseline and endline data for serum retinol, microscopy, and AGP (Figure 4.1). Our sample size of 352 allowed us to detect a difference in serum retinol concentration (between children with and without inflammation) of 0.1 µmol/L with 80% and 5% type 1 error rate. Table 4.1 shows the baseline characteristics of the sub-population included in this analysis. The mean age of children included in the analyses was 69 months, with 34% below 5 years, and 48% males. Prevalence of stunting and underweight were 25% and 11% respectively. Anemia prevalence was about 33% and the uncorrected prevalence of iron deficiency was 8%. At the baseline, 29%, 60%, and 5% of children reported having had fever, cough and diarrhea respectively in the previous two weeks. The subset included in this analyses were similar to the general population of study children with respect to relevant variables including age, retinol, inflammation and malaria, at both baseline and endline.

Vitamin A, inflammation and malaria status of children during low and high malaria transmission seasons

Table 4.2 shows the vitamin A, inflammation and malaria status of the subgroup included in the analyses, during the low and high malaria transmission seasons. The geometric mean retinol concentrations at both baseline and follow-up were 0.98 µmol/L. The uncorrected prevalence of VAD at the baseline and follow-up were 13% and 20% respectively. Confirmed malaria cases, defined as positive
microscopy, increased from 14% in the low transmission season to 21% in the high transmission season. Prevalence of inflammation was 47% and 74% during the low and high malaria transmission seasons respectively.

Figure 4.1 Consort diagram depicting the criteria used for selecting study participants for inclusion in paper 1

50 clusters (n=1280 children) randomized into two study arms

25 clusters (n=612 children) randomized to receive white maize

n=481 children enrolled at baseline

n=416 children with complete baseline data for retinol, AGP and malaria

n=352 children with complete baseline and endline data for retinol, AGP and malaria

25 clusters (n=668 children) randomized to receive provitamin A maize

n=131 not met or refused

n=65 children with incomplete baseline data for retinol, AGP and malaria

n=64 children with incomplete endline data for retinol, AGP and malaria
Table 4: Baseline socio-demographic, nutritional status and morbidity history of children included in the analyses

<table>
<thead>
<tr>
<th>Baseline Variable</th>
<th>N</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Literate household head</td>
<td>348</td>
<td>281 (80.8)</td>
</tr>
<tr>
<td><strong>Household assets</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electricity</td>
<td>348</td>
<td>20 (5.8)</td>
</tr>
<tr>
<td>Refrigerator</td>
<td>348</td>
<td>14 (4.0)</td>
</tr>
<tr>
<td>Motor vehicle</td>
<td>348</td>
<td>8 (2.6)</td>
</tr>
<tr>
<td><strong>Occupation of HH head (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farming/Farm labor</td>
<td>340</td>
<td>97 (28.5)</td>
</tr>
<tr>
<td>Self-employed</td>
<td>340</td>
<td>90 (26.5)</td>
</tr>
<tr>
<td>Salaried worker</td>
<td>340</td>
<td>56 (16.5)</td>
</tr>
<tr>
<td>Other</td>
<td>340</td>
<td>97 (28.5)</td>
</tr>
<tr>
<td><strong>Religious affiliation (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>348</td>
<td>8 (2.3)</td>
</tr>
<tr>
<td>Christian</td>
<td>348</td>
<td>294 (84.5)</td>
</tr>
<tr>
<td>Muslim</td>
<td>348</td>
<td>1 (0.3)</td>
</tr>
<tr>
<td>Traditional</td>
<td>348</td>
<td>45 (12.9)</td>
</tr>
<tr>
<td><strong>Child Characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Age in months (range)</td>
<td>352</td>
<td>69.1 (44.7-105.3)</td>
</tr>
<tr>
<td>Age&lt;60 months (%)</td>
<td>352</td>
<td>119 (33.8)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>352</td>
<td>168 (47.7)</td>
</tr>
<tr>
<td><strong>Nutritional Status (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stunting</td>
<td>350</td>
<td>88 (25.1)</td>
</tr>
<tr>
<td>Underweight</td>
<td>351</td>
<td>39 (11.0)</td>
</tr>
<tr>
<td>Anemia (%)</td>
<td>352</td>
<td>128 (36.4)</td>
</tr>
<tr>
<td><strong>Morbidity history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axillary temperature &gt; 37.5 °C (%)</td>
<td>352</td>
<td>4 (1.14)</td>
</tr>
<tr>
<td>Fever in past 2 weeks (%)</td>
<td>350</td>
<td>101 (28.9)</td>
</tr>
<tr>
<td>Cough in the past two weeks (%)</td>
<td>350</td>
<td>209 (59.7)</td>
</tr>
<tr>
<td>Diarrhea in the past 2 weeks (%)</td>
<td>350</td>
<td>18 (5.1)</td>
</tr>
<tr>
<td>Slept under mosquito net last night (%)</td>
<td>350</td>
<td>24 (6.9)</td>
</tr>
</tbody>
</table>
Table 4.2: Vitamin A, inflammation and malaria status of children during low and high malaria transmission seasons

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Low Malaria Transmission</th>
<th>High Malaria Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopy positive (%)</td>
<td>49 (13.9%)</td>
<td>84 (21.0%)</td>
</tr>
<tr>
<td>Retinol Conc. (95% CI)-µmol/L *</td>
<td>0.98 (0.95, 1.02)</td>
<td>0.98 (0.94, 1.02)</td>
</tr>
<tr>
<td>Retinol &lt;0.7 µmol/L</td>
<td>47 (13.4%)</td>
<td>69 (19.6%)</td>
</tr>
<tr>
<td>AGP Conc. (95% CI)-(g/L)*</td>
<td>0.95 (0.88, 1.03)</td>
<td>1.48 (1.40, 1.57)</td>
</tr>
<tr>
<td>Inflammation (AGP&gt;1 g/l)</td>
<td>164 (46.6%)</td>
<td>255 (72.4%)</td>
</tr>
</tbody>
</table>

*Geometric mean

Changes in serum retinol concentration during inflammation, with or without malaria, in the low and high malaria Transmission Seasons

Table 4.3 shows the geometric mean serum retinol concentration across the four inflammation-malaria groups, in both the low and high malaria transmission seasons. Retinol concentrations were highest in the reference group (no malaria and no inflammation) and lowest among children with elevated AGP and concurrent malaria in both the low and high malaria transmission seasons. The reference retinol concentration was 1.05 ± 1.40 µmol/L in the low malaria transmission season. This decreased by 13% among those with elevated AGP only (0.92 ± 1.38 µmol/L), by 9% among those with malaria only (0.96 ± 1.32 µmol/L) and by 15% among those with elevated AGP and concurrent malaria (0.90 ± 1.24 µmol/L). In the high malaria transmission season the reference serum retinol concentration was 1.11 ± 1.45 µmol/L. This was reduced by 7% among those with elevated AGP only (1.03 ± 1.45 µmol/L), by 7% among those with malaria only (1.03 ± 1.35 µmol/L) and by 36% among those with elevated AGP and concurrent malaria (0.65 ± 1.53 µmol/L) when the cross-sectional model was used. In the cohort approach (Table 4.4), the percentage among children with inflammation and concurrent malaria infection, relative to the
reference group was 27%. The retinol concentrations among those with inflammation only or those with malaria only was similar to that of the reference, when the cohort approach was used.

**Table 4.3:** Cross-sectional estimates of the differences in serum retinol concentrations across malaria-inflammation groups during low and high malaria transmission seasons

<table>
<thead>
<tr>
<th>Malaria Season</th>
<th>N (%)</th>
<th>Mean (95% CI) / μmol/L</th>
<th>Ratio</th>
<th>Difference</th>
<th>AF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>169 (48.5)</td>
<td>1.05 (1.00, 1.11)</td>
<td>1.00</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Inflammation only</td>
<td>134 (38.4)</td>
<td>0.92 (0.87, 0.97)</td>
<td>0.94</td>
<td>(-19, -6)</td>
<td>(1.06, 1.23)</td>
</tr>
<tr>
<td>Malaria only</td>
<td>19 (5.5)</td>
<td>0.96 (0.83, 1.09)</td>
<td>0.91 (0.79, 0.87)</td>
<td>-9</td>
<td>1.10</td>
</tr>
<tr>
<td>Inflammation &amp; Malaria</td>
<td>30 (7.5)</td>
<td>0.90 (0.83, 0.87)</td>
<td>0.85 (0.78, 0.87)</td>
<td>-15</td>
<td>1.10</td>
</tr>
<tr>
<td><strong>High</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>89 (25.3)</td>
<td>1.11 (1.03-1.20)</td>
<td>1.00</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Inflammation only</td>
<td>189 (53.7)</td>
<td>1.03 (0.98-1.08)</td>
<td>0.93</td>
<td>-7</td>
<td>1.08</td>
</tr>
<tr>
<td>Malaria only</td>
<td>8 (2.3)</td>
<td>1.03 (0.80-1.33)</td>
<td>0.93</td>
<td>-16, 2</td>
<td>(0.98, 1.19)</td>
</tr>
<tr>
<td>Inflammation &amp; malaria</td>
<td>66 (18.8)</td>
<td>0.71 (0.64-0.79)</td>
<td>0.64</td>
<td>-36</td>
<td>0.56</td>
</tr>
</tbody>
</table>

*AF= Adjustment factors

**Age patterns in the inflammation- and malaria-associated changes in serum retinol concentration in the low and high malaria transmission seasons**

We observed a statistical interaction between malaria and age, such that the malaria associated reductions in serum retinol were more pronounced in younger children (< 60 months). This interaction was significant during the high malaria transmission seasons (p=0.023), but not in the low malaria transmission season. Table 4.5 and figure 4.2 show the age-stratified means of the serum retinol distribution.
across the inflammation-malaria groups, in the high malaria transmission season. In the high malaria transmission season, the reductions in serum retinol (relative to the reference group) among children with inflammation alone or inflammation with malaria were 18% and 51% respectively among children < 60 months, and 6% and 32% respectively among older children (≥ 60 months). We did not have sufficient sample size to generate reliable estimates of the age-specific reductions in retinol among children with malaria but not inflammation.

Table 4:4: Malaria- and inflammation-associated changes in serum retinol concentration in the high malaria transmission season, among children who had no malaria or inflammation at baseline (cohort approach)

<table>
<thead>
<tr>
<th>Malaria Season</th>
<th>N (%)</th>
<th>Mean (95% CI) / µmol/L</th>
<th>Ratio</th>
<th>% Difference</th>
<th>AF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>55 (32.5%)</td>
<td>1.07 (0.97, 1.18)</td>
<td>1.00</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Inflammation only</td>
<td>88 (52.1%)</td>
<td>1.04 (0.98, 1.12)</td>
<td>0.98</td>
<td>-2</td>
<td>1.02</td>
</tr>
<tr>
<td>Malaria only</td>
<td>5 (3.0%)</td>
<td>1.09 (0.71, 1.69)</td>
<td>1.02</td>
<td>2</td>
<td>0.98</td>
</tr>
<tr>
<td>Inflammation &amp; Malaria</td>
<td>21 (12.4%)</td>
<td>0.77 (0.64, 0.91)</td>
<td>0.72</td>
<td>-28</td>
<td>1.39</td>
</tr>
</tbody>
</table>

*AF= Adjustment factors

Changes in the estimated prevalence of VAD after correcting for malaria and inflammation

In the low malaria transmission season, the prevalence of marginal vitamin A deficiency, defined as retinol < 0.7 µmol/L (10% before correction) decreased by 4.8% (2.3, 7.4) after correcting for malaria and inflammation, when the cross-sectional adjustment factors were applied (Table 4.6). In the high malaria transmission season, the prevalence of marginal vitamin A deficiency (19.6% before correction) decreased by 8.0% (95% CI: 4.8, 11.1) after correcting for malaria and
inflammation using the cross-sectional approach. The corresponding reductions in the high malaria season following the application of adjustment factors generated from the cohort-based model was 5.4% (95% CI: 2.7, 8.0).

Table 4.5: Malaria- and inflammation-associated changes in serum retinol concentration in the high malaria transmission season, stratified by age

<table>
<thead>
<tr>
<th></th>
<th>N (%)</th>
<th>Mean- µmol/L (95% CI)</th>
<th>Ratio</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 60 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>13 (20.0)</td>
<td>1.26 (1.06, 1.51)</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Inflammation only</td>
<td>38 (58.5)</td>
<td>1.04 (0.91, 1.18)</td>
<td>0.82 (0.67, 1.01)</td>
<td>-18 (-33, 1)</td>
</tr>
<tr>
<td>Malaria only</td>
<td>0 (0.00)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inflammation &amp; Malaria</td>
<td>14 (21.5)</td>
<td>0.61 (0.51, 0.73)</td>
<td>0.49 (0.38, 0.62)</td>
<td>-51 (-62, -38)</td>
</tr>
<tr>
<td>≥ 60 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>76 (26.5)</td>
<td>1.09 (1.00, 1.19)</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Inflammation only</td>
<td>151 (52.6)</td>
<td>1.03 (0.97, 1.09)</td>
<td>0.94 (0.85, 1.05)</td>
<td>-6 (-15, 5)</td>
</tr>
<tr>
<td>Malaria only</td>
<td>8 (2.8)</td>
<td>1.03 (0.85, 1.26)</td>
<td>0.95 (0.77, 1.18)</td>
<td>-5 (-23, 18)</td>
</tr>
<tr>
<td>Inflammation &amp; Malaria</td>
<td>52 (18.1)</td>
<td>0.74 (0.66, 0.83)</td>
<td>0.68 (0.59, 0.79)</td>
<td>-32 (-41, -21)</td>
</tr>
</tbody>
</table>

DISCUSSION

This study was designed to characterize the pattern of inflammation-associated changes in serum retinol concentrations by malaria status, across malaria seasons and age of the child. Our results confirm the long-standing notion that plasma or serum retinol concentration is significantly reduced in the context of inflammation.\textsuperscript{35,153,172,189,193,312,313} More importantly, our data show that the lower distributions of serum retinol are more pronounced in children with concurrent malaria and clinically significant inflammation. We have shown that compared to malaria-negative children, serum retinol concentration are significantly lower in
malaria positive children, with or without elevated AGP. We have also shown that in a malaria-endemic region, the magnitude of the inflammation-associated change in serum retinol increases with increasing malaria transmission intensity, in an age dependent manner. Finally, we observed that cross-sectional adjustment factors were of a higher magnitude than adjustment factors estimated with a cohort approach.

Table 4:6: Comparability of corrected and uncorrected serum retinol concentrations in the low and high malaria transmission seasons

<table>
<thead>
<tr>
<th></th>
<th>Serum Retinol (µmol/L)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uncorrected (µmol/L)</td>
<td>Corrected (µmol/L)</td>
<td></td>
</tr>
<tr>
<td>Low malaria</td>
<td>n=352</td>
<td>n=352</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.98 (0.95, 1.02)</td>
<td>1.06 (1.04, 1.09)</td>
<td></td>
</tr>
<tr>
<td>High Malaria</td>
<td>Cross-sectional</td>
<td>0.98 (0.94, 1.02)</td>
<td>1.12 (1.08, 1.17)</td>
</tr>
<tr>
<td></td>
<td>Cohort approach</td>
<td>0.98 (0.94, 1.02)</td>
<td>1.05 (1.01, 1.10)</td>
</tr>
</tbody>
</table>

Our estimates of the inflammation-associated reductions in serum retinol are similar to those from two recent studies conducted in Ghana and Zambia, which found that among children (<5 years), elevated AGP was associated with 20-30% reductions in serum retinol concentrations. Our findings are also consistent with results from a study among a hyper-endemic population in Papua New Guinea (70% malaria prevalence), which found that serum retinol levels were reduced by 0.12 µmol/L in children with elevated AGP. These prior studies, however, did not evaluate whether the AGP-associated changes differed by malaria status. We observed that controlling for malaria substantially changes the magnitude of the AGP-associated reductions in serum retinol. We found a reduction of up to 36% in children with AGP and concurrent malaria, whereas a less pronounced reduction (of up to 13%) was seen in children with elevated AGP alone (without malaria), across the two malaria seasons.
Figure 4.2: Malaria and age-specific patterns in inflammation associated hyporetinolemia

The adjustment factor approach is increasingly being applied as a means of addressing inflammation-associated changes in micronutrient status, including serum retinol. A consequence of this growing trend in use is an attempt to generate “global adjustment factors” from a meta-analyses.\textsuperscript{38,301} In one such attempt, Thurnham \textit{et al}\textsuperscript{38} pooled data from 15 studies involving subjects from settings with significant differences in the burden and transmission patterns of prevailing infectious diseases, including malaria. Using the conventional biomarkers of inflammation, Thurnham \textit{et al} estimated adjustment factors of 13\%, 24\% and 11\% for children in three disease stages namely incubation stage (defined as CRP >5 mg/L and AGP ≤1 g/L), early convalescence stage (defined as CRP >5 mg/L and AGP >1 g/L) and the late convalescence stage (defined as CRP ≤5 mg/L and AGP >1 g/L). A concern with this
approach is the inherent assumption that the changes are independent of the type of stimulus or infections inducing the inflammation. We have shown that this assumption does not necessarily hold in a malaria-endemic region. By incorporating a direct indicator of malaria parasitemia into the definition of inflammation, we have shown that the magnitude of the inflammation-induced hyporetinolemia is disease-specific. Our results, therefore, suggest that the adoption of one set of adjustment factors in a malaria-endemic region may be problematic. Specifically, the use of such global adjustment factors has the potential to overestimate the bias among children with inflammation only (but not malaria) while underestimating the bias in children with inflammation and concurrent malaria.

Table 4:7: Comparability of corrected and uncorrected vitamin A deficiency prevalence estimates in the low and high malaria transmission seasons

<table>
<thead>
<tr>
<th></th>
<th>Uncorrected</th>
<th>Corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low malaria season</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=352)</td>
<td>86 (10.0%)</td>
<td>3.9 (2.4, 5.2)</td>
</tr>
<tr>
<td><strong>High malaria season</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross-sectional (n=352)</td>
<td>69 (19.6%)</td>
<td>8.0 (4.8, 11.1)</td>
</tr>
<tr>
<td>Cohort approach (n=169)</td>
<td>69 (19.6%)</td>
<td>5.4 (2.7, 8.0)</td>
</tr>
</tbody>
</table>

A second potential problem with the adoption of the Thurnham *et al* adjustment factors in a malaria-endemic region is the lack of accountability for the age-specific patterns. Our data suggests that the extent of malaria-induced hyporetinolemia may be age-dependent. We observed that the magnitude of the inflammation-associated changes was highest in the younger age group (<60 months), and this age pattern was especially conspicuous in the high malaria transmission season. Among children <5 years at the follow-up, we observed an average difference
of ~ 0.52 µmol/L in serum retinol concentration between those with and without malaria. In the older children (5+ years) however, we observed an average difference of only 0.24 µmol/L across malaria status. These age differences are consistent with the known age-specific differences in anti-malaria parasite immunity and disease severity.\textsuperscript{131,132} We know that anti-parasite immunity to malaria takes several years of repeated infections to develop, such that infections progress more quickly in younger children than in older populations.\textsuperscript{131-134} Consistent with this hypothesis, we observed that the parasite density among those infected progressively decreased with age. On average, the parasite density among malaria positive children <60 months was over 3 times the parasite density among positive cases who were older than 60 months. This age pattern make it less ideal to adopt the Thurnham \textit{et al} adjustment factors, which were estimated with a population comprising of both preschoolers and pregnant women.

The cohort approach used here, was an attempt to ensure that our inflammation and reference groups were reasonably comparable with respect to the history of prior exposures. Ideally, individuals should be assessed at least at two time points, namely, a time when they are exposed to inflammation, and a second relevant time point when the inflammation has resolved. This way, individuals serve as their own controls, effectively diminishing influence of historical exposures, which may also contribute to current vitamin A status. This is particularly important in low income settings, where infections tend to cluster within individuals and households. By definition, this cluster effect means that the probability of prior exposure to infections may be higher in individuals with current inflammation. It follows, therefore, that the retinol levels in individuals with current inflammation may be
further reduced by contributions from prior exposures through mechanisms such as increased excretion, reduced intake, and reduced absorption. Consequently, the effect of the current inflammation on retinol levels may be over-estimated with the cross-sectional analyses which assumes that the difference in retinol is wholly attributable to the current inflammation or infection. Our data also suggests that the common practice of estimating adjustment factors with a cross-sectional approach may overestimate the extent of the inflammation-induced hyporetinolemia. In our study population, correcting for inflammation and malaria using the cohort approach resulted in an average upward adjustment of 0.07 µmol/L, as opposed to 0.14 µmol/L when the cross-sectional approach was used. In the cohort approach, we excluded prevalent malaria and inflammation cases at the baseline, and reassessed the distribution of serum retinol at the follow-up, across the four malaria-inflammation groups. By so doing, we may have minimized potential disparities in the history of exposure between those with and without current inflammation. It is conceivable that because of the relatively longer lag time (6-month), children may have been exposed in the intervening period. Although we are unable to quantify the burden and impact of these potential intervening exposures, we expect any contributions from these sources towards the differences in retinol (across the malaria-inflammation groups) to be minimal. This is because in addition to treating all malaria cases at the baseline, all children received mosquito nets and were given the recommended doses of Albendazole and Praziquantel, for the control of intestinal helminthes and schistosomiasis. Collectively these measures may have reduced the exposure to these common infections in the intervening period.
Our data has shown that correcting for inflammation (with or without malaria) have substantial implications with respect to the level of public health importance attached to the burden of VAD. By definition, a population is considered to have vitamin A deficiency of public health importance if the prevalence of VAD is at least 15%. In the low malaria transmission seasons, the uncorrected prevalence of VAD was < 15%, and hence, correcting for inflammation had no effect on whether or not the burden of VAD be considered a public health issue. In the high transmission season, however, the uncorrected prevalence was ~20% (indicating an important public health problem), and correcting malaria and inflammation reduced the VAD prevalence to <15%, indicating a VAD burden of less public health importance.

In conclusion, our results suggest that in malaria-endemic regions, relative reductions in serum retinol levels are driven by both malaria and non-malaria related inflammation. Our observation that the relative reductions were especially high in those with elevated AGP and concurrent malaria likely reflects a consequence of increased malaria parasitemia, and an intensified pro-inflammatory response as a result. This is also the likely explanation for the observation that the relative reductions were higher in younger age group. Our results highlight the complexity in dealing with the inflammation-induced changes in serum retinol concentrations in a malaria-endemic region. We have shown that the estimated magnitude of the inflammation-associated hyporetinolemia depends on several factors including the model used (cohort approach vs. cross-sectional), the inclusion (or non-inclusion) of a marker of malaria infection, the malaria transmission intensity (low vs. high), and the age distribution of the population. There is a need for continuing research and discussions to standardize the procedures for dealing with the inflammation-induced
changes in serum retinol. Until such standardization becomes possible, our data suggests that adjustment factors, if at all used, should be context-specific, and should account for the malaria-attributable variance in serum retinol, in endemic regions.
Chapter 5: PAPER 2

TITLE Vitamin A status, corrected or uncorrected for inflammation, and the incidence of *P. falciparum* parasitemia in rural Zambian children

ABSTRACT

Evidence from animal and in-vitro studies suggests that vitamin A adequacy may confer protection against malaria incidence and severity. There is limited evidence from human studies regarding the associations between vitamin A status and malaria risk. We evaluated the association between vitamin A status and incidence of malaria (defined as *P. falciparum* parasitemia) or malaria with inflammation (defined as *P. falciparum* parasitemia with AGP > 1 g/L), as a proxy indicator for malaria severity. We included 381 children from the control arm of 6-month long provitamin A efficacy trial. Baseline and endline survey data, collected 6 months apart, were used to define vitamin A and malaria status respectively. Vitamin A status was based on either the measured serum retinol concentration (VAS) or the retinol concentration corrected for inflammation and malaria at the baseline (VAScorr), and was defined as deficient (retinol <0.7 µmol/L), low (retinol ≥ 0.7 but <1.05 µmol/L) or adequate (retinol ≥ 1.05 µg/L). After excluding baseline malaria cases, the incident rate ratio (IRR) for malaria, comparing the low and adequate groups to the deficient group, were 0.69 (95% CI: 0.37-1.27) and 0.50 (95% CI: 0.25-0.97) respectively when VAS was used, and 0.54 (95% CI: 0.24-1.17) and 0.47 (95% CI: 0.21-1.07) respectively when VAScorr was used. The IRR for malaria with inflammation in the low and adequate groups was 0.59 (95% CI: 0.31-1.13) and 0.41 (95% CI: 0.20-0.83),
respectively when VAS was used, and 0.51 (95% CI 0.23-1.13) and 0.36 (CI: 0.16-0.85) respectively when VAScorr was used. Our results are consistent with evidence that vitamin A adequacy may protect against malaria incidence and disease severity.

INTRODUCTION

Malaria and vitamin A deficiency (VAD) are independently associated with severe morbidity and mortality in children. The evidence suggests that when malaria and VAD overlap, as seen in Sub-Saharan Africa, the risk for disease and death increases.\(^7\)-\(^9\) Malaria accounts for about 800,000 deaths in children under five years each year,\(^8\) and about 500 million morbid episodes globally.\(^98\) A trial in Papua New Guinea, by Shankar \textit{et al},\(^33\) found that quarterly supplementation of children with high dose vitamin A reduced the incidence of clinical malaria by 30%. Although the mechanisms underlying this potential protective effect are not completely understood, evidence from animal and in-vitro studies suggests that vitamin A may confer anti-malaria protection through a number of immune-modulatory mechanisms including the up-regulation of anti-inflammatory cytokine secretion\(^144,146,150,152,161,315\) and down regulation of pro-inflammatory cytokines.\(^25,26,31,145,161\) There is a dearth of evidence from human studies demonstrating the potential association between vitamin A and malaria, and whether the protective effect may be achieved by mitigating the pro-inflammatory response. To this effect, this study was designed to evaluate the association between vitamin A status and the incidence of malaria or malaria with inflammation.

A major challenge with using observational study designs to characterize the interactions between vitamin A status and malaria is the potential misclassification bias resulting from the inflammation-induced changes in serum retinol concentrations.
It has been documented that during the acute phase response to infections such as malaria, serum retinol concentration is reduced. These reductions may not affect liver stores per se, and may lead to misdiagnosis of actual vitamin A status. To evaluate how this inflammation-induced bias may affect the characterization of the vitamin A-malaria interaction, this study was also designed to evaluate the association between vitamin A status, corrected for inflammation and malaria, and the incidence of malaria.

Therefore, the overall goal of this longitudinal study was to determine whether vitamin A status, corrected or uncorrected for inflammation, is associated with the incidence of malaria. This study was conducted among rural Zambian children (4-8 years), participating in a cluster-randomized controlled trial to assess the efficacy of provitamin A intervention. Specific aims are as follows:

1. Does vitamin A status, corrected or uncorrected for inflammation, modify the risk for malaria (defined as *P. falciparum* parasitemia), assessed 6 months later?
2. Does vitamin A status, corrected or uncorrected for inflammation, modify the risk for malaria with inflammation (defined as *P. falciparum* parasitemia with AGP >1 g/L), assessed 6 months later?

**METHODOLOGY**

Details of the study design, including the study population, blood draw and processing, data collection and data management are presented in chapter 3.
Data analyses

All data analyses were conducted with STATA 11 software (StataCorp, College Station, Texas)

Generation of inflammation-corrected retinol concentrations at the baseline

Children were assigned into four inflammatory groups defined by malaria and AGP as follows: reference (AGP ≤ 1 g/L and malaria negative); inflammation only (AGP > 1 g/L and malaria negative); malaria only (AGP ≤ 1 g/L and malaria positive); malaria with inflammation group (AGP > 1 g/L and malaria positive). Children were considered as having malaria if they had *P. falciparum* parasitemia of any density as defined by microscopy. To generate adjustment factors for retinol, we first calculated geometric means for the retinol distributions within the reference group and each of the three malaria-inflammation groups. We then calculated the ratio of the geometric mean retinol in each of the malaria-inflammation groups to that of the reference group. Adjustment factors were established by taking the inverse of geometric mean ratios. All individual retinol values among members of the corresponding groups were then multiplied by the adjustment factor to derive an estimate of retinol assumed to be unaffected by inflammation or malaria exposure.

Definition of vitamin A status

Baseline serum retinol concentration, corrected or uncorrected for inflammation, were used to define vitamin A status. Vitamin A status was defined as deficient (retinol < 0.7 µmol/L), low (retinol ≥ 0.7 but < 1.05 µmol/L) or adequate (retinol ≥ 1.05 µmol/L).15
**Definition of malaria outcomes**

Malaria was defined as the presence of *P. falciparum* parasitemia of any density. Malaria with inflammation was defined as the presence of *P. falciparum* parasitemia of any density and concurrent elevation in AGP (>1 g/L). Malaria parasite density was estimated from the count per 200 WBC assuming 8000 WBC/µl of blood. We calculated malaria incidence rates per 100 person-months using the number of cases at the follow-up and assuming a 6-month time at risk per child.

**Statistical Analyses**

Exploratory analytic techniques were used to examine the association between relevant demographic, anthropometric and biochemical variables, and vitamin A status or malaria. We used a modified Poisson regression approach as proposed by Lou *et al*[^16,317] to estimate the incidence rate ratios (IRR), comparing the incidence of both malaria and malaria with inflammation at the endline across baseline vitamin A status. In our regression models, we adjusted for variables that were associated with both baseline serum retinol and endline parasite density (P ≤ 0.1). These were age (baseline), hemoglobin (baseline and endline) and ferritin (endline). All covariates were entered as continuous variables. Skewed data including ferritin were log-transformed before parametric tests were performed. All models were run separately with the baseline measured vitamin A status, and the vitamin A status corrected for inflammation. In addition, all models were rerun by excluding children who had malaria (defined by both microscopy and RDT) at the baseline. Statistical significance was set at P=0.05.

[^16,317]: This is a citation example. It indicates that the information provided is based on the works cited by Lou et al. in 2016 and 2017.
RESULTS

Figure 5.1: Consort diagram depicting the criteria used for selecting study participants for inclusion in paper 2

- 50 clusters (n=1280 children) randomized into two study arms
- 25 cluster (n=612 children) randomized to receive Non-provitamin A intervention
- 25 cluster (n=668 children) randomized to receive provitamin A intervention
- N=481 children enrolled at baseline
- N=612 children randomized to receive Non-provitamin A intervention
- N=440 children with complete baseline retinol data
- N=668 children randomized to receive provitamin A intervention
- N=131 not met or refused
- N=352 children with complete baseline and endline data for retinol, AGP and malaria
- N=65 children with incomplete baseline data for retinol, AGP and malaria
- N=64 children with incomplete endline data for retinol, AGP and malaria
Table 5.1: Baseline biochemical, clinical and socio-demographic characteristics of study children

<table>
<thead>
<tr>
<th>Baseline Variable</th>
<th>N</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Literate household head</strong></td>
<td>377</td>
<td>305 (80.9)</td>
</tr>
<tr>
<td><strong>Household assets</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electricity</td>
<td>377</td>
<td>21 (5.6)</td>
</tr>
<tr>
<td>Refrigerator</td>
<td>377</td>
<td>15 (4.0)</td>
</tr>
<tr>
<td><strong>Occupation of HH head (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farming/Farm labor</td>
<td>367</td>
<td>106 (28.9)</td>
</tr>
<tr>
<td>Self-employed</td>
<td>367</td>
<td>97 (26.4)</td>
</tr>
<tr>
<td>Salaried worker</td>
<td>367</td>
<td>64 (17.4)</td>
</tr>
<tr>
<td>Other</td>
<td>367</td>
<td>100 (27.3)</td>
</tr>
<tr>
<td><strong>Religious affiliation (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>377</td>
<td>8 (2.1)</td>
</tr>
<tr>
<td>Christian</td>
<td>377</td>
<td>320 (84.9)</td>
</tr>
<tr>
<td>Muslim</td>
<td>377</td>
<td>1 (0.3)</td>
</tr>
<tr>
<td>Traditional</td>
<td>377</td>
<td>48 (12.7)</td>
</tr>
<tr>
<td><strong>Child Characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Age in months (range)</td>
<td>381</td>
<td>68.7 (44.9, 105.3)</td>
</tr>
<tr>
<td>Age &lt; 60 months (%)</td>
<td>381</td>
<td>130 (34.1)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>381</td>
<td>185 (48.7)</td>
</tr>
<tr>
<td><strong>Nutritional Status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinol &lt;0.7 µmol/L</td>
<td>381</td>
<td>54 (14.1%)</td>
</tr>
<tr>
<td>Mean height (SD)/cm</td>
<td>379</td>
<td>107.7 (9.4)</td>
</tr>
<tr>
<td>Stunting</td>
<td>379</td>
<td>103 (27.0)</td>
</tr>
<tr>
<td>Mean weight (SD)/kg</td>
<td>380</td>
<td>17.8 (3.4)</td>
</tr>
<tr>
<td>Underweight</td>
<td>380</td>
<td>43 (11.1)</td>
</tr>
<tr>
<td>Anemia (%)</td>
<td>381</td>
<td>135 (35.4)</td>
</tr>
<tr>
<td>ID (WHO-cutoff for ferritin)</td>
<td>376</td>
<td>27 (7.2%)</td>
</tr>
<tr>
<td><strong>Morbidity history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axillary temperature &gt; 37.5 °C (%)</td>
<td>381</td>
<td>4 (1.1)</td>
</tr>
<tr>
<td>Fever in past 2 weeks (%)</td>
<td>379</td>
<td>109 (28.7)</td>
</tr>
<tr>
<td>Cough in the past two weeks (%)</td>
<td>379</td>
<td>225 (59.4)</td>
</tr>
<tr>
<td>Diarrhea in the past 2 weeks (%)</td>
<td>379</td>
<td>20 (5.3)</td>
</tr>
<tr>
<td>Slept under mosquito net last night (%)</td>
<td>397</td>
<td>27 (7.2)</td>
</tr>
<tr>
<td>RDT positive (%)</td>
<td>380</td>
<td>75 (19.7%)</td>
</tr>
<tr>
<td>Microscopy positive (%)</td>
<td>361</td>
<td>49 (13.6%)</td>
</tr>
<tr>
<td>AGP &gt; 1 g/l</td>
<td>381</td>
<td>181 (47.5%)</td>
</tr>
</tbody>
</table>
Table 5.2: Vitamin A status and the incidence of *P. falciparum* parasitemia assessed 6 months later

<table>
<thead>
<tr>
<th>VA Status</th>
<th>Cases (%)</th>
<th>IR(^a)</th>
<th>Unadjusted IRR 95% CI</th>
<th>P-value</th>
<th>*Adjusted IRR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uncorrected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>14/54 (26%)</td>
<td>4.3</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>39/163 (24%)</td>
<td>4.0</td>
<td>0.92 (0.54, 1.56)</td>
<td>0.766</td>
<td>0.84 (0.48, 1.46)</td>
<td>0.427</td>
</tr>
<tr>
<td>Adequate</td>
<td>28/164 (17%)</td>
<td>2.8</td>
<td>0.66 (0.37, 1.16)</td>
<td>0.146</td>
<td>0.52 (0.30, 0.92)</td>
<td>0.024</td>
</tr>
<tr>
<td><strong>Corrected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>8/31 (26%)</td>
<td>4.3</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>30/142 (21%)</td>
<td>3.5</td>
<td>0.82 (0.42, 1.61)</td>
<td>0.563</td>
<td>0.63 (0.31, 1.30)</td>
<td>0.211</td>
</tr>
<tr>
<td>Adequate</td>
<td>37/188 (20%)</td>
<td>3.3</td>
<td>0.76 (0.39, 1.48)</td>
<td>0.424</td>
<td>0.57 (0.29, 1.14)</td>
<td>0.111</td>
</tr>
</tbody>
</table>

*Adjusted for ferritin (endline), age (baseline) and hemoglobin (baseline and endline); \(^a\)Incidence rate per 100 person-months

Baseline socio-demographic profile, nutritional status and morbidity of study children

Analyses were restricted to 381 children from the control arm of the study who had at least baseline retinol and endline malaria data (Figure 5.1). Table 5.1 shows the baseline characteristics of the children. The mean age of children included in the analyses was 69 months, with 34% below 5 years, and 49% males. Prevalence of stunting and underweight were 27% and 11% respectively. Anemia prevalence was about 33% and the prevalence of iron deficiency was 7%. At the baseline, 29%, 59%, and 5% of children reported having had fever, cough and diarrhea respectively in the previous two weeks. The prevalence of uncorrected vitamin A deficiency at the baseline was 14%. The proportion of children with serum retinol <1.05 µmol/L was 57%. The prevalence of iron deficiency was ~ 7% and the prevalence of anemia was 34%. About 47% of the children had elevated AGP (>1 g/l). The malaria prevalence...
as defined by microscopy or RDT was 14% and 20% respectively. Fever was diagnosed in only 1% of the children.

**Associations between vitamin A Status and incidence of *P. falciparum* parasitemia in rural Zambian children**

Table 5.2 shows the incidence rate ratios comparing the malaria incidence at 6 months across baseline vitamin A status. The unadjusted incidence rate ratio comparing the incidence of *P. falciparum* parasitemia in the low and adequate vitamin A groups, to that of the deficient group were 0.92 (95% CI: 0.54, 1.56) and 0.66 (95% CI: 0.37, 1.16) respectively. After adjusting for age, ferritin and hemoglobin, the IRR in the low and adequate groups (relative to the deficient group) were 0.82 (95% CI: 0.48, 1.46) and 0.52 (95% CI: 0.30, 0.92) respectively. When vitamin A status was defined with the inflammation-corrected retinol concentrations, the unadjusted IRR in the low and adequate vitamin A groups (relative to the deficient group) were 0.82 (95% CI: 0.42, 1.61) and 0.76 (95% CI: 0.39, 1.48) respectively. The adjusted IRR using the inflammation-corrected vitamin A status, were 0.63 (95% CI: 0.31, 1.30) and 0.57 (95% CI: 0.29, 1.14) for the low and adequate vitamin A groups respectively. When the analysis was restricted to children who tested negative for malaria at the baseline (Table 5.3), the adjusted IRR for the low and adequate vitamin A groups were 0.69 (95% CI: 0.36, 1.31) and 0.47 (95% CI: 0.23, 0.97) respectively when the definition of vitamin A status was based on the uncorrected serum retinol concentration. When vitamin A status was defined with the inflammation-corrected retinol concentration and analyses restricted to children who tested negative for malaria at the baseline, the adjusted IRR were 0.51 (95% CI: 0.21, 1.20) and 0.47 (95% CI: 0.21, 1.05) in the low and adequate vitamin A groups respectively.
Table 5:3: Vitamin A status and the incidence of \textit{P. falciparum} parasitemia among children who were free of malaria at the baseline

<table>
<thead>
<tr>
<th>VA Status</th>
<th>Cases (%)</th>
<th>IR$^a$</th>
<th>Unadjusted IRR</th>
<th>P-value</th>
<th>*Adjusted IRR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>11/48 (23)</td>
<td>3.8</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>26/13 (19)</td>
<td>3.2</td>
<td>0.83 (0.44, 1.55)</td>
<td>0.554</td>
<td>0.69 (0.36, 1.31)</td>
<td>0.259</td>
</tr>
<tr>
<td>Adequate</td>
<td>22/15 (14)</td>
<td>2.3</td>
<td>0.63 (0.33, 1.21)</td>
<td>0.164</td>
<td>0.47 (0.23, 0.97)</td>
<td>0.042</td>
</tr>
<tr>
<td>Corrected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>7/29 (24)</td>
<td>4.0</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>21/12 (17)</td>
<td>2.8</td>
<td>0.70 (0.33, 1.49)</td>
<td>0.358</td>
<td>0.51 (0.21, 1.20)</td>
<td>0.123</td>
</tr>
<tr>
<td>Adequate</td>
<td>25/16 (15)</td>
<td>2.7</td>
<td>0.63 (0.30, 1.32)</td>
<td>0.224</td>
<td>0.47 (0.21, 1.05)</td>
<td>0.066</td>
</tr>
</tbody>
</table>

$^a$Adjusted for ferritin (endline), age (baseline) and hemoglobin (baseline and endline); $^b$Incidence rate per 100 person-months

Associations between vitamin A status and incidence of \textit{P. falciparum} parasitemia with inflammation in rural Zambian children

Table 5.4 shows the incidence rate ratios comparing the incidence of malaria with concurrent inflammation across baseline vitamin A status. The unadjusted incidence rate ratio comparing the incidence of inflammation–associated \textit{P. falciparum} parasitemia in the low and adequate vitamin A groups to that of the deficient group were 0.81 (95% CI: 0.47, 1.39) and 0.54 (95% CI: 030, 0.99) respectively. After adjusting for age, ferritin and hemoglobin, the IRR in the low and adequate groups (relative to the deficient group) were 0.76 (95% CI: 0.42, 1.37) and 0.45 (95% CI: 0.25, 0.83) respectively. When vitamin A status was defined with the inflammation-corrected retinol concentrations, the unadjusted incidence rate ratio in the low and adequate vitamin A groups (relative to the deficient group) were 0.77 (95% CI: 0.39, 1.52) and 0.64 (95% CI: 0.32, 1.26) respectively. The adjusted IRR using the inflammation-corrected vitamin A status, for the low and adequate vitamin...
A groups were 0.60 (95% CI: 0.30, 1.21) and 0.50 (95% CI: 0.25, 0.99) respectively. When the analyses was restricted to children who tested negative for malaria at the baseline (Table 5.5), the adjusted IRR using the uncorrected vitamin A status, for the low and adequate vitamin A groups were 0.59 (95% CI: 0.30, 1.17) and 0.38 (95% CI: 0.20-0.83) respectively. When vitamin A status was defined with the inflammation-corrected retinol concentration and the analyses restricted to children who tested negative for malaria at the baseline, the adjusted IRR were 0.47 (95% CI 0.21, 1.07) and 0.38 (CI: 0.17, 0.87) in the low and adequate vitamin A groups respectively.

Table 5.4: Vitamin A status and the incidence of P. falciparum parasitemia with inflammation

<table>
<thead>
<tr>
<th>VA Status</th>
<th>Cases (%)</th>
<th>IR*</th>
<th>Unadjusted IRR (95% CI)</th>
<th>P-value</th>
<th>*Adjusted IRR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uncorrected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>14/53 (26)</td>
<td>4.3</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>35/163 (21)</td>
<td>3.5</td>
<td>0.81 (0.47, 1.39)</td>
<td>0.450</td>
<td>0.76 (0.42, 1.37)</td>
<td>0.360</td>
</tr>
<tr>
<td>Adequate</td>
<td>23/159 (14)</td>
<td>2.3</td>
<td>0.54 (0.30, 0.99)</td>
<td>0.045</td>
<td>0.45 (0.25, 0.83)</td>
<td>0.010</td>
</tr>
<tr>
<td><strong>Corrected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>8/31 (26)</td>
<td>4.3</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>28/141 (20)</td>
<td>3.3</td>
<td>0.77 (0.39, 1.52)</td>
<td>0.453</td>
<td>0.60 (0.30, 1.21)</td>
<td>0.155</td>
</tr>
<tr>
<td>Adequate</td>
<td>30/183 (16)</td>
<td>2.7</td>
<td>0.64 (0.32, 1.26)</td>
<td>0.192</td>
<td>0.50 (0.25, 0.99)</td>
<td>0.048</td>
</tr>
</tbody>
</table>

*Adjusted for ferritin (endline), age (baseline) and hemoglobin (baseline and endline); *Incidence rate per 100 person-months
Table 5:5: Vitamin A Status and the Incidence of Inflammation-Associated *P. falciparum* Parasitemia among Children who were Free of Malaria at the Baseline

<table>
<thead>
<tr>
<th>VA Status</th>
<th>Cases (%</th>
<th>IR*</th>
<th>Unadjusted IRR 95% CI</th>
<th>P-value</th>
<th>*Adjusted IRR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncorrected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>11/47 (23)</td>
<td>3.8</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>22/13 (16)</td>
<td>2.7</td>
<td>0.69 (0.36, 1.30)</td>
<td>0.252</td>
<td>0.59 (0.30, 1.17)</td>
<td>0.112</td>
</tr>
<tr>
<td>Adequate</td>
<td>17/14 (12)</td>
<td>2.0</td>
<td>0.49 (0.25, 0.98)</td>
<td>0.004</td>
<td>0.38 (0.17, 0.85)</td>
<td>0.018</td>
</tr>
<tr>
<td>Corrected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>7/29 (24)</td>
<td>4.0</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>19/12 (15)</td>
<td>2.5</td>
<td>0.64 (0.30, 1.38)</td>
<td>0.254</td>
<td>0.47 (0.21, 1.07)</td>
<td>0.098</td>
</tr>
<tr>
<td>Adequate</td>
<td>18/15 (11)</td>
<td>1.8</td>
<td>0.47 (0.22, 1.02)</td>
<td>0.057</td>
<td>0.38 (0.17, 0.87)</td>
<td>0.022</td>
</tr>
</tbody>
</table>

*Adjusted for ferritin (endline), age (baseline) and hemoglobin (baseline and endline); *Incidence rate per 100 person-months

DISCUSSION

This study was designed to evaluate whether baseline vitamin A status modified the risk for malaria incidence assessed 6 months later. Our evaluations were conducted among rural Zambian children (4-8 years) from the control arm of a trial in which clusters of children were randomized to receive a provitamin A biofortified maize meal intervention, or non fortified, traditional maize meal (controls). We found that vitamin A adequacy was associated with a lower malaria incidence of up to 53% (relative to the deficient group), with subtle differences in the effect size depending on whether or not adjustments were made to the serum retinol concentrations to account for inflammation. A more pronounced association of up to 62% reduction was observed when the outcome was defined as malaria with concurrent inflammation. We also found that the magnitude of the reductions were similar regardless of whether
the measured or inflammation-corrected retinol concentrations were used to define
vitamin A status.

Our findings, that adequate vitamin A status is protective against severe
malaria, is consistent with those of a study by Shankar et al, in which high dose
vitamin A supplementation was associated with a 30% reduction in the incidence of
clinical malaria among Papua New Guinean children. Our finding are also consisted
with an in-vitro experiment by Kain and Serghides, which showed that retinoic acid
directly up-regulated CD36 expression, reduced TNF-α production and increase the
phagocytosis of P. falciparum parasitized erythrocytes through a CD36-dependent
pathway.

The vitamin A-related immune mechanisms are believed to be initiated by the
activation of nuclear receptors following the binding of retinoic acid isomers, and the
subsequent up-regulation of transcription, translation, cell proliferation and
differentiation. In addition to evidence that vitamin A enhances
phagocytosis, there is also evidence that retinoic acid may enhance the clearance
of parasites by accelerating eryptosis, the suicidal death of parasitize erythrocytes.
Vitamin A is also associated with enhanced erythropoiesis, a process that may prevent
the development of severe malaria anemia, a leading cause of malaria related
mortality. A final mechanism through which vitamin A may protect against malaria is
by mitigating the pro-inflammatory response. Malaria is associated with an
exaggerated pro-inflammatory response, a process that may be reversed by
vitamin A. In the study by Serghides et al, treatment of human monocytes
exposed to P. falciparum with 9-cis retinoic acid resulted in a significant decrease in
TNF-α an endogenous pyrogen.
There were subtle differences between the definition of malaria outcomes used in this study and those used in other studies. In the study by Shankar et al, clinical malaria was defined as the presence of fever with parasite density of at least 8000 parasites per µl of blood. In a similar study by Binka et al, which found no evidence of effect of vitamin A supplementation on malaria, the outcomes used were malaria deaths and episodes of fever. Our sample size did not permit the assessment of severe and rare outcomes such as death or febrile malaria. Only 3% of our study population had fever at the follow-up. We also did not adopt the parasite density threshold used in the Shankar et al study. This threshold, although more specific, is arbitrary. There is no consensus on the level of malaria parasite density beyond which clinical symptoms begin to develop. Our use of malaria with inflammation as a separate outcome reflects the current understanding of the mechanisms through which vitamin A adequacy mitigates the progression of malaria infections to severe disease. The inclusion of elevated AGP in the definition of malaria provides a direct indicator of the potential for vitamin A to protect against inflammation during a malaria infection.

A limitation with our study design is its observational nature. Vitamin A and malaria interact bi-directionally, making the possibility of reverse-causality a real threat to the validity of inferences made in this study. Specifically, because baseline vitamin A and malaria were negatively correlated, and also because we observed a positive correlation between baseline and endline malaria, it follows that baseline vitamin A will be negatively correlated with endline malaria, even if the association was non-causal. A second source of bias in our study is the potential misclassification in vitamin A status as a result of the inflammation-induced reductions in serum retinol
concentrations. This misclassification in exposure has the potential to move the relative risk estimate closer to the null. We took steps to address these types of biases. First, the longitudinal approach adopted here, with the 6 months follow-up time, minimizes the threat of reverse causality. To further address the problem of reverse causality we reconstructed our regression models by excluding children who had malaria at the baseline. In fact, we observed a higher protective effect after excluding baseline malaria cases. When the measured retinol was used to define vitamin A status, the protective effect (seen in the adequate group) increased from 48% to 53% for malaria incidence, and from 55% to 62% for malaria with inflammation after excluding baseline malaria cases. We did not detect any measurable differences in the estimated IRR comparing models in which vitamin A status was defined with the measured serum retinol, as opposed to models in which vitamin A status was defined with the inflammation-corrected retinol concentrations.

In conclusion, we have shown that in a malaria-endemic region, vitamin A adequacy is associated with significant reductions in the incidence of malaria parasitemia, with or without concurrent inflammation in children. Our findings have important implication for vitamin A research and programs in malaria-endemic regions. The last decade has seen a decline in research specifically addressing the interactions between malaria and vitamin A, amidst a growing trend in the coverage of vitamin A interventions such as the semi-annual high dose supplementation of children. Our findings underscore the need for continuing research to demonstrate the overall health benefits of these vitamin A interventions, particularly as they relate to malaria outcomes.
Chapter 6 : PAPER 3

TITLE: Malaria exacerbates inflammation-associated changes in ferritin and soluble transferrin receptor concentrations

ABSTRACT

Ferritin and soluble transferrin receptor (sTfR), the commonly used biomarkers of iron status are affected by inflammation. Emerging evidence suggests that the inflammation-associated changes may be amplified in the context of malaria, although this effect modification by malaria is incompletely characterized. This study was designed to quantify the changes in ferritin and sTfR during inflammation, with and without malaria parasitemia. This study was also designed to estimate the changes in estimated prevalence of iron deficiency (ID), iron deficiency anemia (IDA) and the proportion of anemia associated with ID, after mathematically adjusting for the inflammation and/or malaria associated changes in ferritin and sTfR. We used baseline (August - September, 2012) and endline (March - April, 2013) survey data, collected in the low and high malaria transmission seasons respectively, from rural Zambian children (4-8 years), participating in a 6-month provitamin A efficacy trial (N=726). Children were classified into four inflammatory groups defined by malaria and AGP as follows: reference (AGP ≤1 g/L and malaria negative); inflammation only (AGP > 1g/L and malaria negative); malaria only (AGP ≤ 1g/L and malaria positive); malaria with inflammation group (AGP >1 g/L and malaria positive). Malaria was defined by microscopy. We defined ID as ferritin < 12/15 µg/L depending on age, or sTfR > 8.3 mg/L. Anemia was defined as hemoglobin<11.0g/L for children <60 months, and <11.5 g/L for older children. The ratio of the geometric
means of the ferritin distributions in the inflammation only, malaria only, and inflammation-malaria groups, relative to the reference category were 1.21 (CI: 1.03, 1.41), 1.17 (CI: 0.81, 1.69) and 1.55 (CI: 1.18, 2.00) respectively in the low malaria season, and 1.66 (CI: 1.39, 1.99), 1.90 (CI: 1.24, 2.89) and 3.80 (CI: 3.04, 4.74) respectively in the high malaria season. The ratio of the geometric means of the sTfR distributions in the inflammation only, malaria only, and inflammation-malaria groups, relative to the reference category were 1.07 (CI: 1.01, 1.14), 1.33 (CI: 1.17, 1.51) and 1.40 (CI: 1.26, 1.56) respectively in the low malaria season, and 1.12 (CI: 1.01, 1.24), 0.97 (CI: 0.77, 1.23) and 1.21 (CI: 1.06, 1.37) respectively in the high malaria season. The uncorrected prevalence of ID in the low and high malaria transmission seasons were 7.2% and 4.7% respectively when based on ferritin, and 26.9% and 53.3% respectively when based on sTfR. The inflammation and malaria-corrected prevalence of ID in the low and high malaria transmission seasons were 9.1% and 9.2% respectively when based on ferritin, and 20.1% and 47.9% respectively when based on sTfR. The estimates of the proportion of anemia with ID were low when based on ferritin (4-11%) and high when based on sTfR (28%-61%), regardless of whether or not adjustments were made to ferritin and sTfR. Our results suggest that in malaria-endemic regions, using either ferritin or sTfR may lead to contradictory conclusions about the burden of ID, and the contribution of ID to the burden of anemia. Our findings underscore the need to control for both inflammation and malaria when ferritin or sTfR are used to define ID or IDA in endemic regions.

INTRODUCTION

The WHO estimates that globally about 40% of children below 5 years and about half of children between 5-14 years are anemic. In malaria-endemic regions, it
is estimated that about 50% of all anemic cases are attributable to iron deficiency. Iron deficiency and anemia are an important risk factor for mortality, delayed or impaired cognitive and physical development, and low work capacity. The prevention of childhood iron deficiency and anemia is therefore a public health priority globally, but more so in malaria-endemic regions where the burden is greatest. A critical component of iron deficiency and anemia control programs is the definition of population iron deficiency or iron deficiency anemia (IDA). This is a necessary step towards defining the expected impact of iron interventions in a given populations. A recent technical consultation of the WHO and the US Centers for Disease Control and Prevention recommended the use of ferritin and soluble transferrin receptor (sTfR) for defining population iron status. Unfortunately ferritin and sTfR are affected by the acute phase response and therefore their utility in the context of infections is questionable.

Although it is recommended that one or more acute phase proteins be used to enhance the interpretation of ferritin and sTfR during infections, there is currently no consensus on the optimal procedure for controlling the inflammation-induced changes. The adjustment factors approach, involving the use of one or multiple acute phase proteins to quantify the extent of inflammation-induced changes, is being widely used. In the wake of new evidence suggesting that the inflammation-induced changes in ferritin and sTfR are modified by malaria, we adapted the corrections factor approach proposed by Thurnham et al, by including a direct marker of malaria into the definition of inflammation. Therefore, the overall goal of this study was to characterize how the inflammation-associated changes in ferritin or sTfR differ by malaria status and across malaria seasons. This study was
also designed to quantify the extent to which mathematical adjustments to ferritin and sTfR- to account for inflammation and malaria-changes the estimated prevalence of ID, IDA, and the proportion of anemia cases with ID.

The current study used data collected from rural Zambian children participating in a 6-month cluster-randomized trial, designed to evaluate the efficacy of a provitamin A intervention on vitamin A status. Baseline and endline survey data, collected during the low and high malaria transmission seasons respectively, were used to address the study questions. In a recent national survey, about 57% of children below 5 years from the district were found to be anemic and about 54% were found to be vitamin A deficient. Prevalence of malaria parasitemia in Zambian children less than 5 years is about 20% and may be close to 40% in rural areas.

**METHODOLOGY**

Details of the study design, including the study population, blood draw and processing, data collection and data management are presented in chapter 3.

**Data analyses**

**Sample size and power consideration:**

We estimated that to detect a difference in ferritin levels of 2.0 µg/L, assuming a within-group standard deviation in serum ferritin of 2.2 µg/L, and a 40% prevalence of inflammation (CRP >5 mg/L of AGP > 1 g/L), we would require a total sample size of 55 at 90% power and 5% type 1 error.

**Generation of inflammatory groups, adjustment factors and corrected concentrations:**

Children were categorized into four inflammatory groups defined by malaria and AGP as follows: reference (AGP ≤1 g/L and malaria negative); inflammation
only (AGP >1 g/L and malaria negative); malaria only (AGP ≤1 g/L and malaria positive); inflammation-malaria group (AGP >1 g/L and malaria positive). Children were considered as having malaria if they had *P. falciparum* parasitemia of any density as defined by microscopy. To generate adjustment factors for ferritin, the median ferritin concentrations in each of the three inflammatory groups were divided by the median ferritin concentration of the reference group. Group specific adjustment factors were defined as the inverse of these ratios. The same procedure was used for generating correcting factors for sTfR except that the mean, instead of the median concentrations, were used. Corrected concentrations were computed by multiplying the individual ferritin or sTfR concentrations by the group specific corrections factors. For both ferritin and sTfR, corrected concentrations were generated separately for both baseline (low malaria transmission intensity) and follow-up (high malaria transmission intensity).

**Definition of iron status and anemia**

We defined iron deficiency as serum ferritin levels (corrected or not) <12 µg/L in children < 5 years, and < 15 µg/L in children > 5 years. When sTfR was used to define iron status, deficiency was defined >8.3 mg/L. Anemia was defined as hemoglobin <11.0 g/L for children <60 months, and <11.5 g/L for older children. Iron deficiency anemia was defined as anemia with concurrent ID.

**Statistical analyses**

The distributions of baseline and follow-up variables were examined using exploratory techniques including histograms, box plots, correlation matrices and normality tests.
On the basis of the Shapiro-Wilk test of normality, we performed a natural-log transformation for ferritin and sTfR for both baseline and follow-up surveys. Using the log-transformed data, we examined the strength and direction of the linear and non-linear associations between these variables using correlations matrices and locally weighted scatter plot smoothing (LOWESS) techniques. We used the Wilcoxon rank-sum test to compare the ferritin distributions across the four groups. For sTfR, differences in concentrations across the four groups were tested using a one-way analyses of variance. Differences in prevalence of ID estimated using the corrected and uncorrected concentrations were tested using the McNemar’s chi squared test. All analyses were done separately for baseline and follow-up data. In all analyses, statistical significance was set at 0.05.

RESULTS

Baseline socio-demographic profile, nutritional status and morbidity of study children

The final study population included 726 children who had complete baseline (low malaria transmission season) and follow-up (high malaria transmission season) data for ferritin, sTfR, AGP, and malaria (Figure 6.1). This sample size allowed for the detection of a difference in serum ferritin of 0.35 µg/L at the baseline and 0.2 µg/L at the endline, between children with and without inflammation, with 90% power and 5% type 1 error. This sub-population was statistically similar to the general population of children included in the trial with respect to the distributions of ferritin, sTfR, AGP and malaria, at both baseline and endline. Table 6.1 shows the baseline distributions of relevant clinical, nutritional and socio-demographic variables among the subgroup included in the analyses. The mean age of children included in the analyses was 69 months, with 33% below 5 years, and 48% males. Prevalence of
Stunting and underweight were 27% and 13% respectively. Anemia prevalence was about 33%. At the baseline, 28%, 56%, and 6% of children reported having had fever, cough and diarrhea respectively in the previous two weeks.

**Figure 6.1: Consort diagram depicting the criteria used for selecting study participants for inclusion in paper 3**

50 clusters (n=1280 children) randomized into two study arms

N=1024 children (481 controls + 543 in pro-vitamin A group) enrolled at baseline

N=856 with complete baseline data for ferritin, AGP and malaria microscopy

N=726 children with complete baseline and endline data for Ferritin, sTfR, AGP and malaria microscopy

N=256 not met or refused

N=168 children with data missing/unavailable for one or more of ferritin, AGP or malaria microscopy at the baseline

N=130 children data missing/unavailable for one or more of ferritin, AGP or malaria microscopy at the endline
Table 6:1: Baseline clinical, nutritional and socio-demographic characteristics of study children

<table>
<thead>
<tr>
<th>Baseline Variable</th>
<th>N</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Literate household head</strong></td>
<td>714</td>
<td>587 (82.2)</td>
</tr>
<tr>
<td><strong>Household assets</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electricity</td>
<td>714</td>
<td>44 (6.2)</td>
</tr>
<tr>
<td>Refrigerator</td>
<td>714</td>
<td>19 (2.7)</td>
</tr>
<tr>
<td>Motor vehicle</td>
<td>714</td>
<td>14 (2.0)</td>
</tr>
<tr>
<td><strong>Occupation of HH head (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farming/Farm labor</td>
<td>715</td>
<td>188 (26.3)</td>
</tr>
<tr>
<td>Self-employed</td>
<td>715</td>
<td>198 (27.7)</td>
</tr>
<tr>
<td>Salaried worker</td>
<td>715</td>
<td>131 (18.3)</td>
</tr>
<tr>
<td>Other</td>
<td>715</td>
<td>198 (27.7)</td>
</tr>
<tr>
<td><strong>Religious affiliation (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>714</td>
<td>13 (1.8)</td>
</tr>
<tr>
<td>Christian</td>
<td>714</td>
<td>614 (86.0)</td>
</tr>
<tr>
<td>Muslim</td>
<td>714</td>
<td>1 (0.1)</td>
</tr>
<tr>
<td>Traditional</td>
<td>714</td>
<td>86 (12.0)</td>
</tr>
<tr>
<td><strong>Child Characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age in months (range)</td>
<td></td>
<td>69.1 (15.2)</td>
</tr>
<tr>
<td>Age&lt;60 months (%)</td>
<td>726</td>
<td>308 (33.4)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>726</td>
<td>350 (48.2)</td>
</tr>
<tr>
<td><strong>Nutritional Status (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean weight/kg (SD)</td>
<td>706</td>
<td>17.7 (3.3)</td>
</tr>
<tr>
<td>Mean height (SD) -cm</td>
<td>705</td>
<td>107.4 (9.4)</td>
</tr>
<tr>
<td>Stunting</td>
<td>705</td>
<td>192 (27.1)</td>
</tr>
<tr>
<td>Underweight</td>
<td>706</td>
<td>97 (13.7)</td>
</tr>
<tr>
<td>Mean hemoglobin (SD)-g/dl</td>
<td>726</td>
<td>11.7 (1.2)</td>
</tr>
<tr>
<td>Anemia (%)</td>
<td>726</td>
<td>239 (32.9)</td>
</tr>
<tr>
<td><strong>Morbidity history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axillary temperature &gt; 37.5 °C (%)</td>
<td>716</td>
<td>7 (0.96)</td>
</tr>
<tr>
<td>Fever in past 2 weeks (%)</td>
<td>716</td>
<td>197 (27.5)</td>
</tr>
<tr>
<td>Cough in the past two weeks (%)</td>
<td>716</td>
<td>403 (56.3)</td>
</tr>
<tr>
<td>Diarrhea in the past 2 weeks (%)</td>
<td>716</td>
<td>39 (5.5%)</td>
</tr>
<tr>
<td>Slept under mosquito net last night (%)</td>
<td>716</td>
<td>69 (9.6%)</td>
</tr>
</tbody>
</table>
Distributions of iron status indicators, malaria and inflammation in the low and high malaria transmission seasons

Table 6.2 shows the geometric means of the ferritin and sTfR distributions at the baseline and endline. The geometric mean ferritin and sTfR concentrations at baseline were 43.9 ± 2.7 µg/L and 7.0 ± 1.5 µg/ml respectively. The corresponding values at follow-up were 89.2 ± 3.4 µg/L for ferritin and 9.1 ± 1.8 µg/ml for sTfR. At baseline, uncorrected prevalence of ID in all children was 8% when based on ferritin, and 27% when based on sTfR. At the follow-up, the ferritin-defined ID prevalence decreased to 4% whereas the sTfR-defined ID prevalence increased to 53%. At the baseline, 46% of children had evidence of inflammation (AGP > 1g/L). The proportion of children with inflammation increased to 74% during the high transmission season. Suspected malaria cases, defined as positive RDT, increased from 19% in the low transmission season to 49% during the high transmission season whereas confirmed malaria cases, defined as positive microscopy increased from 14% in the low transmission season to 23% in the high transmission season.

Table 6.2: Distributions of iron status indicators, malaria and inflammation in the low and high malaria transmission seasons

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Low Malaria Transmission</th>
<th>High Malaria Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geometric Mean Ferritin ± SD (µg/l)</td>
<td>43.9 ± 2.7</td>
<td>89.2 ± 3.4</td>
</tr>
<tr>
<td>Iron deficiency (%)*</td>
<td>52 (7.2)</td>
<td>34 (4.7)</td>
</tr>
<tr>
<td>Geometric Mean sTfR ± SD (µg/ml)</td>
<td>7.0 ± 1.5</td>
<td>9.1 ± 1.8</td>
</tr>
<tr>
<td>sTfR &gt; 8.3 mg/L (%)</td>
<td>195 (26.9)</td>
<td>386 (53.2)</td>
</tr>
<tr>
<td>RDT positive (%)</td>
<td>175 (19.1)</td>
<td>419 (48.6)</td>
</tr>
<tr>
<td>Microscopy positive (%)</td>
<td>114 (13.6)</td>
<td>197 (22.9)</td>
</tr>
<tr>
<td>AGP &gt; 1g/l (%)</td>
<td>324 (44.6)</td>
<td>523 (73.6)</td>
</tr>
</tbody>
</table>

*Based on WHO cutoff for ferritin
Table 6.3: Malaria- and inflammation-associated changes in Ferritin in the low and high malaria transmission seasons

<table>
<thead>
<tr>
<th>Group</th>
<th>N (%)</th>
<th>Median (p25, p75)-µg/L</th>
<th>Ratio</th>
<th>% change</th>
<th>AF (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>359</td>
<td>39.2</td>
<td>1.00</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Inflammation only</td>
<td>268</td>
<td>47.3</td>
<td>1.21</td>
<td>21</td>
<td>0.83</td>
</tr>
<tr>
<td>Malaria only</td>
<td>43</td>
<td>45.9</td>
<td>1.17</td>
<td>17</td>
<td>0.86</td>
</tr>
<tr>
<td>Inflammation &amp; malaria</td>
<td>56</td>
<td>60.8</td>
<td>1.55</td>
<td>55</td>
<td>0.65</td>
</tr>
<tr>
<td><strong>High</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>175</td>
<td>50.8</td>
<td>1.00</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Inflammation only</td>
<td>381</td>
<td>84.4</td>
<td>1.66</td>
<td>66</td>
<td>0.60</td>
</tr>
<tr>
<td>Malaria only</td>
<td>17</td>
<td>96.1</td>
<td>1.90</td>
<td>90</td>
<td>0.53</td>
</tr>
<tr>
<td>Inflammation &amp; malaria</td>
<td>153</td>
<td>192.9</td>
<td>3.80</td>
<td>280</td>
<td>0.26</td>
</tr>
</tbody>
</table>

*AF= Adjustment Factor

Cross-sectional effects of inflammation and malaria on ferritin levels

Table 6.3 shows the geometric means of the ferritin distributions in each of the four malaria-inflammation groups, at the baseline and endline. Relative to the reference group, ferritin concentrations were increased in each of the three inflammatory groups and the increase was more pronounced during the high malaria transmission season. The geometric ferritin concentration in the reference group was 39.2 (CI: 35.6, 43.1) µg/L in the low malaria transmission season, and 50.8 (CI: 44.7, 57.8) µg/L in the high malaria transmission season. Relative to the reference group, the ferritin concentrations were increased by 21% (CI: 3, 41), 17% (CI: -19, 69) and 55% (CI: 18,100) in the inflammation only (47.3 µg/L), malaria only (45.9 µg/L), and
inflammation/malaria (60.8 µg/L) groups respectively during the low malaria transmission season. In the high malaria season, the increase in ferritin (relative to the reference) were 66% (CI: 39, 99), 90% (CI: 24, 189) and 280% (CI: 204, 374) in the inflammation only (84.4 µg/L), malaria only (96.1 µg/L), and inflammation/malaria (192.9 µg/L) groups.

**Figure 6.2: Box plots of the distributions of serum Ferritin across the malaria-inflammation groups in the low and high malaria transmission seasons**
Table 6.4: Malaria- and inflammation-associated changes in sTfR in the low and high malaria transmission seasons

<table>
<thead>
<tr>
<th>Group</th>
<th>N (%)</th>
<th>Mean (SD) mg/L</th>
<th>Ratio</th>
<th>% Change</th>
<th>AF (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>359</td>
<td>(49.5) 6.5</td>
<td>(6.3, 6.8)</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Inflammation only</td>
<td>268</td>
<td>(36.9) 7.0</td>
<td>(6.6, 7.4)</td>
<td>1.07</td>
<td>(1.14) (0.99, 0.88)</td>
</tr>
<tr>
<td>Malaria only</td>
<td>43</td>
<td>(5.9) 8.7</td>
<td>(7.6, 9.9)</td>
<td>1.33</td>
<td>33</td>
</tr>
<tr>
<td>Inflammation &amp; malaria</td>
<td>56</td>
<td>(7.7) 9.1</td>
<td>(8.2, 10.1)</td>
<td>1.40</td>
<td>(26, 56) (0.79, 0.64)</td>
</tr>
<tr>
<td><strong>High</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>175</td>
<td>(24.1) 8.3</td>
<td>(7.6, 9.0)</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Inflammation only</td>
<td>381</td>
<td>(36.9) 9.3</td>
<td>(8.8, 9.8)</td>
<td>1.12</td>
<td>(1.24) (0.99, 0.81)</td>
</tr>
<tr>
<td>Malaria only</td>
<td>17</td>
<td>(2.3) 8.1</td>
<td>(6.4, 10.2)</td>
<td>0.97</td>
<td>-3</td>
</tr>
<tr>
<td>Inflammation &amp; malaria</td>
<td>153</td>
<td>(21.1) 10.0</td>
<td>(9.1, 11.0)</td>
<td>1.21</td>
<td>(6, 37) (0.94, 0.73)</td>
</tr>
</tbody>
</table>

*AF= Adjustment Factor

Cross-sectional effects of inflammation and malaria on soluble transferrin receptor levels

The pattern of inflammation and malaria-associated changes in soluble transferrin receptor are presented in Table 6.4. In both the low and high malaria transmission seasons, inflammation was associated with elevations in sTfR and the increase was strongest among children with inflammation and concurrent malaria. The sTfR concentrations in the reference, inflammation only, malaria only and
inflammation/malaria groups were 6.5 mg/L (CI: 6.3 - 6.8), 7.0 mg/L (CI: 6.6 - 7.4), 8.7 mg/L (CI: 7.6 - 9.9) and 9.1 mg/L (CI: 8.2 - 10.1) respectively during the low malaria transmission season. This translated into an average percentage increase in sTfR of 7% (CI: 1 - 14), 33% (CI: 17 - 51) and 40% (CI: 26 - 56) in the inflammation only, malaria only and inflammation/malaria groups respectively. In the high malaria transmission season, the concentrations of sTfR in the reference, inflammation only, malaria only and inflammation/malaria groups were 8.3 mg/L (CI: 7.6-9.0), 9.3 µg/ml (CI: 8.8 - 9.8), 8.1 µg/ml (CI: 6.4 – 10.2) and 10.0 µg/ml (CI: 9.1 – 11.0) respectively. This translated into an average percentage increment of 12% (CI: 1-24). -3% (CI: -23
- 23) and 21% (CI: 6 - 37) in the inflammation only, malaria only and
inflammation/malaria groups

Table 6.5: Comparability of corrected and uncorrected geometric mean ferritin
or sTfR concentrations in the low and high malaria transmission seasons

<table>
<thead>
<tr>
<th></th>
<th>Geometric Mean sTfR (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uncorrected</td>
</tr>
<tr>
<td>Ferritin</td>
<td></td>
</tr>
<tr>
<td>Low malaria season</td>
<td>43.9 (40.8, 47.2)</td>
</tr>
<tr>
<td>High malaria season</td>
<td>89.2 (81.5, 97.6)</td>
</tr>
<tr>
<td>sTfR</td>
<td></td>
</tr>
<tr>
<td>Low malaria season</td>
<td>7.0 (6.8, 7.2)</td>
</tr>
<tr>
<td>High malaria season</td>
<td>9.1 (8.8, 9.5)</td>
</tr>
</tbody>
</table>

Changes in the estimated prevalence of iron deficiency after correcting for
malaria and inflammation in the low and high malaria transmission seasons

Correcting for both inflammation and malaria decreased the geometric means
ferritin concentration by 12.4% in the low malaria transmission season and by 37.4%
in the high malaria transmission season (table 6.5). The corresponding reductions in
sTfR after correction were 8.6% in the low malaria transmission season and 7.7% in
the high malaria transmission season. After correcting for malaria and inflammation,
the estimated prevalence of ID, as defined by ferritin, increased from 7.2% to 9.1% in
the low malaria season and from 4.7% to 9.2% in the high malaria season (Table 6.6).
After correction for malaria and inflammation, the estimated prevalence of ID, as
defined by sTfR, decreased from 26.9% to 20.1% in the low malaria season and from
53.2% to 47.9% in the high malaria season.
Table 6.6: Comparability of corrected and uncorrected iron deficiency estimates in the low and high malaria transmission seasons

<table>
<thead>
<tr>
<th></th>
<th>Prevalence (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uncorrected</td>
<td>Corrected</td>
<td></td>
</tr>
<tr>
<td>Ferritin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low malaria season</td>
<td>52 (7.2)</td>
<td>66 (9.1)</td>
<td></td>
</tr>
<tr>
<td>High malaria season</td>
<td>34 (4.7)</td>
<td>67 (9.2)</td>
<td></td>
</tr>
<tr>
<td>sTfR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low malaria season</td>
<td>195 (26.9)</td>
<td>146 (20.1)</td>
<td></td>
</tr>
<tr>
<td>High malaria season</td>
<td>386 (53.2)</td>
<td>348 (47.9)</td>
<td></td>
</tr>
</tbody>
</table>

Changes in the estimated prevalence of anemia, iron deficiency anemia, and the proportion of anemia cases with iron deficiency after correcting for Malaria and Inflammation

Table 6.7 shows the estimated prevalence of anemia, IDA, and the proportion of anemia cases with concurrent ID. Prevalence of anemia was 33% during the low malaria seasons, and 40% during the high malaria season. Adjusting the ferritin concentration for malaria and inflammation increased the IDA prevalence from 3.0 (95% CI: 1.8, 4.3) to 3.7 (95% CI: 2.3, 5.1) in the low malaria season, and from 1.7 (95% CI: 0.7, 2.6) to 3.2 (95% CI: 0.7, 4.5) in the high malaria season. Similarly, adjusting the ferritin concentration for malaria and inflammation increased the proportion of anemia cases with ID from 9.2 (95% CI: 5.5, 12.9) to 11.3 (95% CI: 7.3, 15.3) in the low malaria season, and from 4.1 (95% CI: 1.8, 6.4) to 7.8 (95% CI: 4.8, 10.9) in the high malaria season. The estimates for both IDA and the proportion of anemia cases with ID were significantly higher when sTfR (instead of ferritin) was used, with or without adjustment for inflammation and malaria. Adjusting the sTfR concentration for malaria and inflammation, decreased the IDA prevalence from 11.6
(95% CI: 9.2, 13.9) to 9.2 (95% CI: 7.1, 11.3) in the low malaria season, and from 24.8 (95% CI: 21.6, 27.9) to 23.0 (95% CI: 19.9, 26.1) in the high malaria season.

Similarly, making adjustments to sTfR increased the proportion of anemia cases with ID from 35.1 (95% CI: 29.0, 41.2) to 28.0 (95% CI: 22.3, 33.8) in the low malaria season, and from 61.4 (95% CI: 55.8, 67.0) to 57.0 (95% CI: 51.3, 62.7) in the high malaria season.

Table 6: Estimated prevalence of iron deficiency anemia corrected or uncorrected for inflammation-associated changes in ferritin and sTfR

<table>
<thead>
<tr>
<th></th>
<th>% Anemia (95% CI)</th>
<th>% Iron Deficiency Anemia (95% CI)</th>
<th>Proportion Anemia with Iron Deficiency (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uncorrected</td>
<td>Corrected</td>
<td>Uncorrected</td>
</tr>
<tr>
<td>Ferritin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>32.9 (29.5, 36.3)</td>
<td>3.0 (1.8, 4.3)</td>
<td>3.7 (2.3, 5.1)</td>
</tr>
<tr>
<td>High</td>
<td>40.4 (36.8, 43.9)</td>
<td>1.7 (0.7, 2.6)</td>
<td>3.2 (0.7, 4.5)</td>
</tr>
<tr>
<td>sTfR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>32.9 (29.5, 36.3)</td>
<td>11.6 (9.2, 13.9)</td>
<td>9.2 (7.1, 11.3)</td>
</tr>
<tr>
<td>High</td>
<td>40.4 (36.8, 43.9)</td>
<td>24.8 (21.6, 27.9)</td>
<td>23.0 (19.9, 26.1)</td>
</tr>
</tbody>
</table>

DISCUSSION

This study was an attempt to characterize the magnitude of the malaria and inflammation-associated changes in ferritin and sTfR and the to determine how the application of adjustment factors to these indicators changes the estimates of the prevalence of ID, IDA and the proportion of anemia cases with ID. Our data has shown that both ferritin and sTfR are significantly elevated during inflammation, but more so in the context of concurrent malaria parasitemia. This pattern was seen across both malaria seasons, with more dramatic changes in the high transmission seasons.
particularly for ferritin. Consistent with the malaria-associated patterns, we observed that the prevalence of ID, as defined by ferritin, was lower in the high malaria transmission season compared to the corresponding estimate in the low malaria season. The estimated prevalence of ID was significantly higher when based on sTfR, in both the low and high malaria seasons, even after controlling for malaria and/or inflammation. Consequently, our estimates of the proportions of anemia associated with ID differed widely depending on whether ferritin or sTfR was used to define iron status. The estimated proportion of anemia cases with ID, when based on ferritin, appears to suggest that the contribution of ID to the burden of anemia is low, whereas the sTfR-based estimate leads to different conclusions, that ID is a major contributor to the problem of anemia.

Our findings are consistent with evidence that both acute and chronic inflammation are associated with significant elevations in ferritin and sTfR, especially in the context of malaria-endemicity. Our findings, however, contradict the popular perception that sTfR concentrations are stable during inflammation. The mean sTfR concentration, whether corrected or uncorrected for inflammation, was higher in the high malaria transmission season, compared to low malaria transmission season. Consequently, the estimated prevalence of ID was also significantly higher in the high malaria transmission season, even after correcting for inflammation. Unlike ferritin, we also found that the magnitude of the malaria-associated changes in sTfR were actually higher during the low transmission season. Malaria (with elevated AGP) was associated with 40% and 20% increase in sTfR, in the low and high malaria seasons respectively, relative to the reference group. The relatively lower magnitude of the increase in sTfR during intense malaria
transmission may be explained by hypothesized inhibition in the synthesis and response to erythropoietin (EPO), and dyserythropoieses during malaria infections. The primary stimulus for the expression of transferrin receptor is the rate of erythropoiesis, such that inhibition of this process, as seen during intense malaria, may reduce the demand and hence expression of sTfR.

The exact relevance of the transient changes in ferritin and sTfR during the acute phase response remains debatable. The summary of the evidence seems to suggest that the rise in serum ferritin is part of the innate immune process to sequester and make iron less available to invading pathogens. In particular, ferritin levels are up-regulated by intracellular iron accumulation, a process induced by hepcidin during malaria infections. During *P. falciparum* infections, hepcidin regulates the redistribution of iron away from circulation, and into storage sites where they are incorporated into ferritin. In response, ferritin biosynthesis is up-regulated in hepatocytes and macrophages to accommodate the sequestered iron. Soluble transferrin receptor is a truncated version of the membrane bound receptor, whose expression is dependent on the rate of erythropoiesis and the iron demand for hemoglobin synthesis. The destruction of parasitized and non-parasitized erythrocytes during malaria infections increase the demand for iron in the bone marrow to replace the loss. For this reason, the specificity of sTfR as an indicator of iron status may be particularly compromised in the context of malaria infections.

Our findings have important implications for making decision regarding how to address the problem of ID or IDA. Unfortunately, the observed differences in the ferritin or sTfR-based estimates of IDA or the proportion of anemia related to ID lead
to conflicting conclusions about what is driving the problem of anemia in this population, and may elicit different reactions in terms of policy. The low prevalence of IDA when based on ferritin would suggest that much of the problem of anemia in this population is driven by causes other than iron deficiency, and that iron interventions will likely not have a significant impact on the problem of anemia. The reverse conclusion is implied when IDA estimate is based on sTfR instead of ferritin. Such polarizing findings regarding the exact burden of ID or IDA has the potential to stagnate the process of making decisions to address the problem. While it is impossible to make conclusions on which ID or IDA estimate -ferritin vs. sTfR- is most reliable in this context, it is plausible that the sTfR-based prevalence is likely overestimated considering that the expression of sTfR is directly up-regulated by several aspects of malaria pathology. Hence, within the limitations of the adjustment procedures applied here, the ferritin-based estimate of ID or IDA (instead of sTfR) is preferred in this malaria-endemic population.

In conclusion, our results, while demonstrating the associations between inflammation, malaria and the biomarkers or iron status, also highlight the challenges with the concept of adjustment factors. By definition, the concept of adjustment factors does not take into account the potential dose-response relationship that exist between disease severity, the inflammatory makers, and the biomarkers of nutritional status. We have shown that the extent of inflation in ferritin and sTfR may be disease-specific and dependent on disease severity. The decision on how best to address the inflammation-associated changes in these iron indicators should be informed by context and availability of resources to perform laboratory assessment. When resources allow for detailed laboratory analyses, our results indicate that a malaria
indicator be used in addition to the conventional inflammatory markers (CRP and AGP). This approach may help to explain more of the variance in ferritin and sTfR, compared to when the inflammatory markers are used alone. Our results also indicate that adjustment factors may not be generalized across settings with different infectious disease profiles. Our findings do not support the idea of generating correcting factors from meta-analyses of studies pooled from across settings with differences in infectious disease prevalence or transmission patterns. In the absence of a practical ‘gold-standard’ indicator of iron status, it is impossible to determine whether or not the application of adjustment factors improves our ability to discriminate between people with normal or abnormal iron status. Until such an indicator becomes available, adjustment factors should be context-specific, and should be interpreted cautiously.
Chapter 7 : PAPER 4

TITLE: Serum ferritin, corrected or uncorrected for inflammation, and the incidence of *P. falciparum* malaria among rural Zambian children

ABSTRACT:

Higher iron stores, defined by serum ferritin (SF), may increase malaria risk. Whether there exists a threshold ferritin concentration beyond which adverse malaria outcomes are precipitated remains unknown. We evaluated the dose-dependent association of SF, and SF corrected for inflammation (SFcorr), with incident malaria among 4-8 y old Zambian children participating in a 6 months provitamin A efficacy trial (August 2012 to March 2013). Baseline ferritin and malaria status (measured 6 months later) were used to define exposure and outcomes respectively. To generate SFcorr, children were classified into four groups on the basis of inflammation (AGP >1 g/L) and malaria. To calculate SFcorr, we multiplied each individual’s SF by the inverse of the group-specific ratio of SF in each group to that of the reference (AGP ≤1 g/L and malaria negative). Iron deficiency was defined as SF or SFcorr < 12/15 (depending on age). Consistent with results of our exploratory analyses, two categories of iron adequacy were created- moderate (<75 µg/L but not deficient) and high (≥75 µg/L). We defined malaria as positive microscopy. All malaria cases were treated at baseline. We observed an age-dependent, positive dose-response association between baseline ferritin and malaria incidence. Among children younger than six years, the incidence rate ratio (IRR) relative to the deficient group, in the moderate and high categories were 2.26 (95% CI: 0.77, 6.58) and 3.34 (95% CI: 1.14, 9.73) respectively when SF was used and 2.90 (95% CI: 0.95, 8.85) and 3.68 (95% CI: 1.19, 11.37) respectively when SFcorr was used. Similar, statistically significant IRR
estimates were obtained even when the analyses were restricted to children who had no malaria at the baseline in this age group. We found no evidence that higher baseline ferritin increased malaria risk in the older children (≥72 months). Our findings are consistent with evidence that higher iron stores may predispose young children to an increased malaria risk, even in the absence of exogenous iron administration.

INTRODUCTION

Children in malaria-endemic regions are faced with a high burden of anemia and iron deficiency, and in need of iron interventions. Unfortunately, longstanding controversies regarding the associations between iron status or supplementation and malaria outcomes hinder efforts towards delivering iron-based interventions to children. The global debate regarding the role of iron in malaria pathology have predominantly focused on the effects of exogenous iron administration on malaria outcomes. This focus is a direct and justifiable reaction to the Pemba Trial, which concluded that in malaria-endemic regions, universal iron supplementation in young children may increase the risk for adverse health outcomes including severe malaria morbidity and mortality. An important finding of the Pemba Trial, which has received much less attention, is the revelation that the toxic effects seemed to have emanated, at least in part, from iron accumulated over time. In the Pemba Trial, the adverse effects were not apparent until after 6 months of daily supplementation and in addition, the effects were restricted to children who were iron adequate at baseline. This is consistent with emerging evidence suggesting that higher levels of storage iron, even in the absence of exogenous administration, may precipitate adverse malaria outcomes.
interventions that are safe for use in malaria-endemic regions, it is critical that we first understand the role of body iron status in malaria pathology. Such a level of understanding will inform decisions regarding the optimal dosing of iron interventions, as well as decisions concerning the integration of malaria and iron deficiency control programs.

A major challenge with evaluating the association between iron status and malaria is the bi-directionality of their interactions. Ferritin, the recommended marker for defining iron status,\textsuperscript{14} may be unreliable in the context of malaria-endemicity.\textsuperscript{62,153,182} Evidence suggests that ferritin levels are transiently increased during the acute phase response to infection, such that its use in defining iron status, may result in misclassification. Such misclassification, if uncorrected, can bias estimates of the association between iron status and malaria outcomes. Hence, this study was also designed to evaluate the iron-related risk for malaria, controlling for the inflammation-associated bias.

The overall goal of the study was to assess the association between ferritin, corrected or uncorrected for inflammation, and the risk for incident malaria defined by microscopy.

**METHODS**

Details of the study design, including the study population, blood draw and processing, data collection and data management are presented in chapter 3.

**Data analyses**

All data analyses were conducted with STATA 11 software (StataCorp, College Station, Texas)
Definition of iron status

Our primary exposure variable was baseline iron status defined by serum ferritin. Ferritin levels, either corrected or uncorrected for inflammation, were used to define iron status. To generate corrected ferritin values, children were categorized into four (4) inflammatory groups defined by malaria and AGP as follows: reference (AGP \( \leq 1 \) g/L and malaria negative); inflammation only (AGP >1 g/L and malaria negative); malaria only (AGP \( \leq 1 \) g/L and malaria positive); malaria with inflammation group (AGP >1 g/L and malaria positive). Details of the correction procedures are presented in Chapter 6. Children were considered as having malaria if they had *P. falciparum* parasitemia of any density as defined by microscopy. To generate adjustment factors for ferritin, we first calculated geometric means for the ferritin distributions within the reference group and each of the three malaria-inflammation groups. We then calculated the ratio of the geometric mean ferritin in each of the malaria-inflammation groups to that of the reference group. Adjustment factors were established by taking the inverse of geometric mean ratios. All individual ferritin values among members of the corresponding groups were then multiplied by the adjustment factor to derive an estimate of ferritin assumed to be unaffected by inflammation or malaria exposure.

We defined iron deficiency as serum ferritin levels (corrected or not) <12 \( \mu g/L \) in children <5 years, and <15 \( \mu g/L \) in children >5 years. In a three-group, dose-dependent analyses, children were defined as having deficient, moderate (\( \leq 75 \) \( \mu g/L \) but not deficient) and high (>75 \( \mu g/L \)) ferritin concentrations.

Definition of outcome

Our primary outcome of interest was confirmed malaria incidence defined by light microscopy at the follow-up. We calculated malaria incidence rates per 100 person-
months using the number of cases at the follow-up and assuming a 6-month time at risk per child.

**Sample size and power consideration**

We estimated the sample size requirements for determining the association between baseline iron status and the risk of malaria morbidity at follow-up (β=0.1, α=0.05). Assuming an ID prevalence of 50% and a 20% malaria prevalence in the iron-adequate group, we estimated that we would require 784 children to be able to detect a 10% difference in malaria incidence.

**Statistical Analyses**

We used exploratory analytic techniques to evaluate the association between relevant demographic, anthropometric and biochemical variables, and iron status or malaria. Variables explored included literacy (defined as the ability to read or write in English), stunting and underweight (defined as height-for-age and weight-for-age z-scores respectively below -2 standard deviation of the WHO Growth Reference), fever (defined as axillary temperature >37.5°C), anemia (defined as hemoglobin <11.0 g/L for children <60 months, and <11.5 g/L for older children). We also explored potential associations between reported prevalence of fever, cough and diarrhea in the 2-weeks preceding the interview and iron or malaria status. The variables were explored both as continuous variables or dichotomized where necessary. Differences in continuous variables by iron status were tested using T-test if the data were normally distributed. Differences in dichotomous variables by iron status were tested using Chi-squared tests. Skewed data including ferritin, sTfR, retinol, β-carotene and AGP were log-transformed before parametric tests were performed. To estimate the incidence rate ratios (IRR), we used a modified Poisson regression approach as
proposed by Lou et al.\textsuperscript{316,317} Separate models were constructed using the measured or inflammation-corrected iron status. As part of the exploratory analyses, we ran models to examine the presence of statistical interactions (P<0.1), as well as evidence of the presence of a threshold ferritin concentration beyond which the malaria risk is markedly increased. Consistent with findings from the exploratory analyses, we constructed age stratified models in which children were defined as deficient, moderate or high ferritin concentrations as defined above. A 72-month age cut-off was chosen because beyond this age, ferritin appeared to be either protective against malaria, or not significantly associated with malaria risk (figure 7.2). In addition we observed a statistically significant age interaction (P < 0.1). The three iron strata were based on finding from a logistic spline model, which showed that the slope of the ferritin-malaria function significantly changed beyond a ferritin concentration 75 µg/L. A variable was included in the final models if the exploratory analyses showed a statistically relevant association with either baseline ferritin or follow-up malaria (P <0.1), or if the evidence from prior studies suggest the existence of an association with either ferritin or malaria. All models were adjusted for vitamin A status, β-carotene, weight-for-age, reported intake of high-dose vitamin A capsule in the past 6 months, intervention group and age at the baseline, as well as sTfR and hemoglobin at the endline. Statistical significance was set at P=0.05. Significance of interaction terms was set at 0.1.
RESULTS

Baseline Socio-Demographic, Nutritional Status and Morbidity History of Children included in the Analyses.

The study enrolled 481 children into the white maize clusters and 543 children into the provitamin A maize clusters. Of these, 52 children from each of the white and provitamin A maize clusters either moved, or refused participation. Two children in the provitamin A group died and 2 were not met for follow-up assessment. Our analyses included 745 children from the provitamin A and white maize groups who had complete data for baseline ferritin, AGP, and malaria microscopy data, in addition to endline malaria microscopy data (Figure 7.1). Table 7.1 shows the baseline socio-demographic, nutritional status and morbidity history of the children included in the analyses. With this sample size, we estimated that we will be able to detect an incident rate ratio of 1.32 with 80% power assuming 5% type 1 error rate, and an incident rate ratio of 1.37 with 90% power and 5% type 1 error rate. About 82% of household heads were literate (ie. could read or write in English), and approximately 86 were Christians. The mean age of children included in the analyses was 69 months, with 33% below 5 years, and 51% males. Prevalence of stunting and underweight were 27% and 14% respectively. Anemia prevalence was about 33% and the uncorrected prevalence of iron deficiency was 8%. At the baseline, 28%, 56%, and 5% of children reported having had fever, cough and diarrhea respectively in the previous two weeks. Prevalence of malaria was 19% when based on RDT, and 14% when based on microscopy. Prevalence of elevated AGP was 45% among the children included in the analyses. We found no statistical difference with respect to iron,
malaria or inflammation status between the children included in the analyses, and those excluded for missing one or more required variables (Appendix A; Table 9.6)

Figure 7.1: Consort diagram depicting the criteria used for selecting study participants for inclusion in paper 4

50 clusters (n=1280 children) randomized into two study arms

N=1024 children (481 controls + 543 in pro-vitamin A group) enrolled at baseline

N=856 with complete baseline data for ferritin, AGP and malaria microscopy

N=745 children with endline malaria microscopy data

N=256 not met or refused

N=168 children with data missing/unavailable for one or more of ferritin, AGP or malaria microscopy at the baseline

N=89 children with data missing/unavailable for malaria microscopy at the endline
Table 7.1: Baseline socio-demographic, nutritional status and morbidity history of children included in the analyses

<table>
<thead>
<tr>
<th>Baseline Variable</th>
<th>N</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Literate household head</strong></td>
<td>733</td>
<td>602 (82.1)</td>
</tr>
<tr>
<td><strong>Household assets</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electricity</td>
<td>733</td>
<td>44 (6.0)</td>
</tr>
<tr>
<td>Refrigerator</td>
<td>733</td>
<td>19 (2.6)</td>
</tr>
<tr>
<td>Motor vehicle</td>
<td>733</td>
<td>14 (1.9)</td>
</tr>
<tr>
<td><strong>Occupation of HH head (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farming/Farm labor</td>
<td>734</td>
<td>191 (26.0)</td>
</tr>
<tr>
<td>Self-employed</td>
<td>734</td>
<td>203 (27.7)</td>
</tr>
<tr>
<td>Salaried worker</td>
<td>734</td>
<td>138 (18.8)</td>
</tr>
<tr>
<td>Other</td>
<td>734</td>
<td>202 (27.5)</td>
</tr>
<tr>
<td><strong>Religious affiliation (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>733</td>
<td>14 (1.9)</td>
</tr>
<tr>
<td>Christian</td>
<td>733</td>
<td>630 (86.0)</td>
</tr>
<tr>
<td>Muslim</td>
<td>733</td>
<td>1 (0.1)</td>
</tr>
<tr>
<td>Traditional</td>
<td>733</td>
<td>88 (12.0)</td>
</tr>
<tr>
<td><strong>Child Characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age in months (range)</td>
<td>745</td>
<td>69.4 (44.9-105.3)</td>
</tr>
<tr>
<td>Age &lt;60 months (%)</td>
<td>745</td>
<td>247 (33.2)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>745</td>
<td>383 (51.4)</td>
</tr>
<tr>
<td><strong>Nutritional Status (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stunting</td>
<td>724</td>
<td>197 (27.1)</td>
</tr>
<tr>
<td>Underweight</td>
<td>725</td>
<td>99 (13.7)</td>
</tr>
<tr>
<td>Anemia (%)</td>
<td>745</td>
<td>244 (32.8)</td>
</tr>
<tr>
<td><strong>Morbidity history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axillary temperature &gt;37.5 °C (%)</td>
<td>745</td>
<td>7 (0.96)</td>
</tr>
<tr>
<td>Fever in past 2 weeks (%)</td>
<td>735</td>
<td>205 (27.9)</td>
</tr>
<tr>
<td>Cough in the past two weeks (%)</td>
<td>735</td>
<td>413 (56.2)</td>
</tr>
<tr>
<td>Diarrhea in the past 2 weeks (%)</td>
<td>735</td>
<td>40 (5.4)</td>
</tr>
<tr>
<td>Slept under mosquito net last night (%)</td>
<td>735</td>
<td>72 (9.8)</td>
</tr>
<tr>
<td>AGP &gt;1 g/L (%)</td>
<td>745</td>
<td>336 (45.1)</td>
</tr>
<tr>
<td>RDT positive (%)</td>
<td>741</td>
<td>140 (18.9)</td>
</tr>
<tr>
<td>Positive microscopy</td>
<td>745</td>
<td>102 (13.7)</td>
</tr>
<tr>
<td>Axillary temperature &gt;37.5 °C</td>
<td>744</td>
<td>7 (0.9)</td>
</tr>
</tbody>
</table>
Iron status, corrected or uncorrected for inflammation, and the incidence of malaria

Table 7.2 shows the association between the measured iron status and the incidence of *P. falciparum* malaria. In all children, iron adequacy, as defined by the measured ferritin concentrations, was associated with an unadjusted incident rate ratio (IRR) for incident malaria of 1.27 (95% CI: 0.72, 2.26) and an adjusted IRR of 1.50 (95% CI: 0.84, 2.64), relative to the deficient group. Iron adequacy, as defined by the inflammation-corrected ferritin concentrations was associated with an unadjusted IRR of 1.20 (95% CI: 0.72, 1.99) and an adjusted IRR of 1.42 (95% CI: 0.88, 2.32), relative to the deficient group.

Table 7.2: Baseline iron status, corrected or uncorrected for inflammation and the incidence *P. falciparum* malaria at 6 months

<table>
<thead>
<tr>
<th>Iron Status</th>
<th>Cases (%</th>
<th>IR*</th>
<th>Unadjusted IRR (95% CI)</th>
<th>P-value</th>
<th>Adjusted IRR (95% CI)*</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uncorrected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>10/53 (18.9)</td>
<td>3.2</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Adequate</td>
<td>166/69 (24.0)</td>
<td>4.0</td>
<td>1.27 (0.69, 2.34)</td>
<td>0.440</td>
<td>1.50 (0.84, 2.64)</td>
<td>0.168</td>
</tr>
<tr>
<td><strong>Corrected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>13/65 (20.0)</td>
<td>3.3</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Adequate</td>
<td>163/68 (24.0)</td>
<td>4.0</td>
<td>1.20 (0.72, 1.99)</td>
<td>0.485</td>
<td>1.42 (0.88, 2.32)</td>
<td>0.152</td>
</tr>
</tbody>
</table>

*Adjusted for vitamin A status, β-carotene, weight-for-age, intake of high-dose vitamin A capsule, intervention allocation and age at the baseline, as well as sTfR and hemoglobin at the endline; incidence rate per 100 person-months
Age and dose-dependency of the association between baseline ferritin and malaria incidence

The exploratory analyses showed that the ferritin-malaria interaction was dependent on age. This age effect was most obvious when children were classified as either below the age of 72 months or older (Fig 7.2). We found a statistically significant interaction with age, such that the ferritin-effect was more pronounced in younger children (p for interaction <0.1). In addition, our exploratory analyses revealed that the increase in malaria risk across the range of baseline ferritin concentrations was non-uniform. In young children below 72 months, there was a steady but moderate
rise in malaria risk until ferritin concentration of approximately 75 μg/L, beyond which a marked increase in malaria risk was observed (figure 7.2).

Table 7.3: Dose-dependency of the malaria risk at 6 months across deficient, moderate and high baseline ferritin concentrations among children < 72 months

<table>
<thead>
<tr>
<th>Iron Status</th>
<th>Cases (%)</th>
<th>IR*</th>
<th>Unadjusted IRR (95% CI)</th>
<th>P-value</th>
<th>Adjusted IRR (95% CI)*</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uncorrected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>5/33 (15.2)</td>
<td>2.5</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Moderate</td>
<td>66/324 (20.4)</td>
<td>3.4</td>
<td>1.34 (0.52, 3.46)</td>
<td>0.539</td>
<td>2.26 (0.77, 6.58)</td>
<td>0.136</td>
</tr>
<tr>
<td>High</td>
<td>30/86 (34.9)</td>
<td>5.8</td>
<td>2.30 (0.89, 5.92)</td>
<td>0.084</td>
<td>3.34 (1.14, 9.73)</td>
<td>0.027</td>
</tr>
<tr>
<td><strong>Corrected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>5/39 (12.8)</td>
<td>2.1</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Moderate</td>
<td>72/332 (21.7)</td>
<td>3.6</td>
<td>1.69 (0.63, 4.52)</td>
<td>0.294</td>
<td>2.90 (0.95, 8.85)</td>
<td>0.061</td>
</tr>
<tr>
<td>High</td>
<td>24/72 (33.3)</td>
<td>5.6</td>
<td>2.60 (0.98, 6.88)</td>
<td>0.054</td>
<td>3.68 (1.19, 11.37)</td>
<td>0.024</td>
</tr>
</tbody>
</table>

*Adjusted for vitamin A status, β-carotene, weight-for-age, intake of high-dose vitamin A capsule, intervention allocation and age at the baseline, as well as sTfR and hemoglobin at the endline; 

Table 7.3 shows the IRR from the three group analyses among children <72 months. In these set of models, the malaria risk in the deficient group was compared to the risk in children with moderate (≤ 75 μg/L but not deficient) and high (> 75 μg/L) ferritin concentrations. The adjusted IRR in the moderate and high iron groups were 2.26 (95% CI: 0.77, 6.58) and 3.34 (95% CI: 1.14, 9.73) when iron status was defined by the measured ferritin concentration, and 2.90 (95% CI: 0.95, 8.85) and 3.68 (95% CI: 1.19, 11.37) respectively, when the inflammation-corrected ferritin concentrations were used. When the analysis was restricted to children who were negative for malaria (both microscopy and RDT) at the baseline in this age group (Table 7.4), the adjusted IRR in the moderate and high ferritin groups were 1.97 (95% CI: 0.66, 5.87)
and 3.03 (95% CI: 1.01, 9.06) respectively when the measured ferritin was used to define iron status. The corresponding IRR when the inflammation-corrected ferritin was used to define iron status were 2.36 (95% CI: 0.77, 7.19) for the moderate category and 3.26 (95% CI: 1.02, 10.48) for the high ferritin category (Table 7.4).

Table 7.4: Dose-dependency of the malaria risk at 6 months across deficient, moderate and high baseline ferritin concentrations among children < 72 months and free of malaria at baseline

<table>
<thead>
<tr>
<th>Iron Status</th>
<th>Cases (%)</th>
<th>IRa</th>
<th>Unadjusted IRR (95% CI)</th>
<th>P-value</th>
<th>Adjusted IRR (95% CI)*</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uncorrected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>5/33 (15.2)</td>
<td>2.5</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Moderate</td>
<td>49/294 (16.7)</td>
<td>2.8</td>
<td>1.10 (0.43, 2.83)</td>
<td>0.844</td>
<td>1.97 (0.66, 5.87)</td>
<td>0.221</td>
</tr>
<tr>
<td>High</td>
<td>19/68 (27.9)</td>
<td>4.7</td>
<td>1.84 (0.69, 4.95)</td>
<td>0.224</td>
<td>3.03 (1.01, 9.06)</td>
<td>0.047</td>
</tr>
<tr>
<td><strong>Corrected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>5/38 (13.2)</td>
<td>2.2</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Moderate</td>
<td>52/298 (17.5)</td>
<td>2.9</td>
<td>1.32 (0.50, 3.49)</td>
<td>0.568</td>
<td>2.36 (0.77, 7.19)</td>
<td>0.132</td>
</tr>
<tr>
<td>High</td>
<td>16/59 (27.2)</td>
<td>4.5</td>
<td>2.06 (0.73, 5.80)</td>
<td>0.171</td>
<td>3.26 (1.02, 10.48)</td>
<td>0.047</td>
</tr>
</tbody>
</table>

*aAdjusted for vitamin A status, β-carotene, weight-for-age, intake of high-dose vitamin A capsule, intervention allocation and age at the baseline, as well as sTfR and hemoglobin at the endline; Incidence rate per 100 person-months

We found no evidence that baseline ferritin modified malaria incidence in the older age group (Table 7.5). Among children ≥ 72 months, the adjusted IRR in the moderate and high iron groups were 0.74 (95% CI: 0.33, 1.65) and 1.32 (95% CI: 0.59, 2.95) when iron status was defined by the measured ferritin concentration, and 0.69 (95% CI: 0.36, 1.30) and 1.00 (95% CI: 0.50, 1.98) respectively, when the inflammation-corrected ferritin concentrations were used (Table 7.5).
Table 7.5: Dose-dependency of the malaria risk at 6 months across deficient, moderate and high baseline ferritin concentrations among children ≥ 72 months

<table>
<thead>
<tr>
<th>Iron Status</th>
<th>Cases (%)</th>
<th>IR^a</th>
<th>Unadjusted IRR (95% CI)</th>
<th>P-value</th>
<th>Adjusted IRR (95% CI)*</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncorrected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>5/20</td>
<td>4.2</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Moderate</td>
<td>41/199</td>
<td>3.4</td>
<td>0.82 (0.38, 1.82)</td>
<td>0.628</td>
<td>0.74 (0.33, 1.65)</td>
<td>0.463</td>
</tr>
<tr>
<td>High</td>
<td>29/83</td>
<td>5.8</td>
<td>1.40 (0.62, 3.15)</td>
<td>0.420</td>
<td>1.32 (0.59, 2.95)</td>
<td>0.502</td>
</tr>
<tr>
<td>Corrected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>8/26</td>
<td>5.1</td>
<td>1.00</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Moderate</td>
<td>48/211</td>
<td>3.8</td>
<td>0.74 (0.38, 1.44)</td>
<td>0.376</td>
<td>0.69 (0.36, 1.30)</td>
<td>0.246</td>
</tr>
<tr>
<td>High</td>
<td>19/65</td>
<td>4.9</td>
<td>0.95 (0.44, 2.04)</td>
<td>0.895</td>
<td>1.00 (0.50, 1.98)</td>
<td>0.989</td>
</tr>
</tbody>
</table>

^aAdjusted for vitamin A status, β-carotene, weight-for-age, intake of high-dose vitamin A capsule, intervention allocation and age at the baseline, as well as sTfR and hemoglobin at the endline.
^bIncidence rate per 100 person-months

DISCUSSION

This study was designed to evaluate the longitudinal associations between iron status and malaria incidence in a cohort of rural Zambian children. We found that in young children (<72 months), there exists a positive dose-response relationship between ferritin levels and future malaria risk. Baseline iron adequacy (relative to the deficient group) was associated with a significantly higher malaria incidence at the endline in this age group. We found no evidence that iron adequacy was associated with increased malaria incidence in older children. The relative risks estimated were similar when either the measured or inflammation-corrected iron status was used.

Our findings are consistent with evidence from five recent studies, including two randomized trial and three observational studies, all of which found that baseline iron status is a significant predictor of future malaria risk.11,37,93,339,340 The Pemba trial
found that the adverse effects of iron was only restricted to children who were iron adequate at baseline\textsuperscript{11} and in a recent trial designed to test the efficacy of an iron-based micronutrient powder in Ghanaian children, Zlotkins \textit{et al} reported that the risk for incident malaria was reduced by 33\% in children who were iron deficient at baseline relative to iron adequate children.\textsuperscript{93} We found three observational studies that looked at the longitudinal association between ferritin and incidence malaria.\textsuperscript{37,339,340} All three studies concluded that iron deficiency appeared to offer protection against malaria. In the first of these studies, Nyakeriga \textit{et al} found that among a cohort of Kenyan children 8-96 months, iron deficiency was associated with a 30\% reduction in the incidence of clinical malaria. In a Tanzanian birth cohort, Gwamaka \textit{et al} found that iron deficiency was associated with a 23\% and 38\% reduction in odds of incident malaria parasitemia and severe malaria during a 3 year follow up.\textsuperscript{37} In the third observational study, Jonker \textit{et al} estimated that iron deficiency was associated with a 45\% reduction in the risk of incident malaria in a cohort of Malawian preschool children.\textsuperscript{339} Our estimates translate into a reduction in incidence in the range of 7\% to 88\% in iron deficient group relative to the adequate group, and hence overlap the reported estimates from these previous studies.

Several plausible reasons may explain the observed relative increase in malaria risk in children who were iron adequate with respect to ferritin. Incident malaria cases reflect both hepatic stage survival of sporozoites as well as blood-stage development of merozoites.\textsuperscript{341-343} Ferritin’s role in malaria incidence may be linked to the availability of iron for the parasite’s metabolic needs.\textsuperscript{223,224} The adult human has about 2000-4000 mg of iron in the body, of which 65\% to 70\% are found in erythrocytes as heme.\textsuperscript{328,329,331,332} Approximately 60\% of the remaining iron (1000
mg) are stored in hepatic cells bound to ferritin. Several studies in mice have confirmed that both the sporozoite and merozoite stages are heavily reliant on iron, and that iron deprivation stymies their growth and development. A recent study by Portugal et al uncovered that a hepcidin-induced redistribution of iron away from hepatocytes and into macrophages significantly reduced the risk for secondary malaria infections in mice. The process through which the parasites capture and utilize iron is not completely understood, especially considering that most of the intra and extracellular iron is tightly bound to the ferro-proteins ferritin and transferrin respectively. New evidence points towards a cytosolic ‘labile iron pool (LIP), and the labile iron component of plasma non-transferrin bound iron (NTBI) as putative iron sources for the developing parasites. The labile iron pool has been defined as a transitory form of metabolically active, chelatable, non-bound iron. The LIP hypothesis is supported by several studies which have demonstrated the inhibitory effects of iron chelators on parasite growth and development. Considering that ferritin levels are up-regulated by higher concentration of both LIP and NTBI, it is plausible that iron repletion, as seen in our study children, is indeed a reflection of increased iron accessibility, and enhanced parasite development. There is also evidence that the apparent protection observed in iron deficiency may be attributable to ID-induced accelerated erythrocyte death and subsequent clearance from circulation. Through this hypothesis, parasite multiplication may be inhibited by the accelerated clearance of parasitized RBCs. It is unclear the extent to which the early death of RBCs affect the development of new sporozoites emerging from the liver into circulation. More research is needed to
understand the specific components of iron metabolism that sustains parasitic growth and survival in both the hepatic and blood-stage pathology.

Our study is limited by its observational nature, particularly considering the bi-directionality of the association between malaria and the indicators of iron status. In our study children, baseline malaria correlated positively with both baseline ferritin and follow-up malaria. It is therefore possible that the observed ferritin effect may be wholly or partly attributable to a residual baseline malaria effect. We took several steps to address these potential sources of bias. First, all positive malaria cases were treated at baseline with Coartem®, ensuring that the cases observed at the second survey were likely new cases, especially considering that parasite resistance has not been demonstrated with Coartem® treatment.\textsuperscript{354,355} Another limitation of our study is that the histidine-rich protein 2 (HRP-2), upon which the RDT is based\textsuperscript{356,357} may persist in circulation even after effective malaria treatment. It is however unlikely that the observed associations between ferritin and RDT is attributable to the phenomenon of persistence HRP-2 because most HRP2 are cleared with 2-4 weeks after effective treatment and unlikely to have persisted for the 6 months period.\textsuperscript{358-360} To further address the potential residual effect of baseline malaria, the models were reran by restricting analyses to children who were free of malaria at baseline. In this restricted analyses, we observed the same trend of increased malaria incidence in the iron adequate group. This confirmed that regardless of baseline malaria exposure, higher levels of ferritin predisposed children to an increased risk for malaria. We also corrected for inflammation to limit potential misclassification of iron status. In fact, our analyses of the effect of inflammation showed that correcting for inflammation increased the strength of the ferritin effect.
Our study has important implication for the future of iron-malaria research and programs. In the wake of the Pemba Trial, it is justifiably more difficult, on ethical grounds, to conduct trials that propose to give children iron supplements or even fortificants. This underscores the need to develop new research models to expand understanding of the interactions between iron and malaria. Our study has shown that there is a need for more research into the interaction between endogenous iron sources and malaria. We need to know if there exists a threshold of body iron beyond which iron becomes harmful. There is also a need to understand which stages of malaria pathology, hepatic, erythrocytic or both, are most affected by endogenous iron sources. In summary, we know from the Pemba trial that iron interventions are most beneficial when combined with active malaria control. We also know from the current study and several recent studies that withholding iron interventions from children in malaria-endemic regions does not necessarily eliminate the iron-induced malaria risk. It is logical that efforts are intensified to find feasible approaches for integrating iron intervention programs into existing malaria control programs.
Chapter 8: SUMMARY DISCUSSION OF FINDINGS, IMPLICATIONS, AND STRENGTHS AND LIMITATIONS OF THE STUDY

Advocacy for the integrations of malaria and iron or vitamin A deficiency control programs are justified not only by the overlap of malaria and these micronutrient deficiencies in the same population, but also by evidence suggesting that adequacy of these two essential micronutrients may improve malaria outcomes. Vitamin A, because of its involvement in immune response, and iron, because of its effect on iron deficiency, anemia and immunity, may both complement preventive and therapeutic strategies for reducing malaria morbidity and mortality. Unfortunately, the attempts toward integration are often thwarted by the lack of consistency in the evidence. Research into the role of vitamin A interventions on malaria outcomes have not always shown evidence of impact, and in the case of iron, there are concerns that a state of adequacy may actually increase the risk for adverse malaria outcomes. Consequently, there is a need for continuing research into how iron or vitamin A status may modify malaria outcomes.

The primary objective for this study was to evaluate the changes in malaria incidence as a function of prior iron or vitamin A status, as defined by either the measured concentrations of serum ferritin or retinol, or by the corresponding inflammation-corrected concentrations. Our secondary objective was to quantify how inflammation, with or without concurrent malaria, may modify the definition of iron or vitamin A status. This step was necessary to characterize how the inflammation induced changes in the biomarkers of iron (ferritin or sTfR) and vitamin (serum retinol) status affects the estimates of their effect on malaria outcomes.
We made four important observations. We found that: a) the inflammation-associated reductions in serum retinol are greater in the context of concurrent malaria infections (paper 1); b) the inflammation-associated elevations in both ferritin and sTfR were greater in the context of concurrent malaria infections (paper 3); c) adequate vitamin A status (as defined by serum retinol), corrected or uncorrected for inflammation, was associated with a significant reduction in the incidence of *P. falciparum* malaria compared to a deficient status; and d) adequate iron status (as defined by serum ferritin), corrected or uncorrected for inflammation, was associated with a significant increase in incidence of *P. falciparum* malaria compared to a deficient status.

**Iron status and malaria incidence in children**

The post-Pemba era\textsuperscript{11} has seen a surge in research studies investigating the associations between iron status in newborns or preschoolers, and the risk for incident malaria.\textsuperscript{37,204,205} The interest in iron status, as opposed to iron supplementation, may be partly attributable to the ethical concerns created by the Pemba trial, which found that universal iron supplementation to children in a malaria-endemic region increased the risk for adverse events including hospital admissions and death under some circumstances\textsuperscript{11}. The research focus on iron status- as opposed to iron supplementation- is also consistent with evidence that the potential harmful effects of iron likely emanates from iron accumulated over time (chronic effects).\textsuperscript{362} Sub-analyses of both the Pemba trial and a recent trial of iron-containing micronutrient power (MNP) by Zlotkins *et al.*,\textsuperscript{93} demonstrated that baseline iron status is perhaps the most important predictor of benefits or harm in the context of iron supplementation or fortification in young children. In the Pemba trial, the risk of adverse outcomes were
restricted to children who had adequate iron status at baseline (Zinc protoporphyrin ≥ 80 µmol/mol of haeme). Although an intention-to-treat analyses of the MNP trial (Zlotkins et al) found a lower incidence of malaria in the iron-containing MNP group, the reductions were not statistically significant after controlling for baseline iron status. In a longitudinal study of the effects of iron status at birth on the incidence of malaria among Tanzanian newborns, Gwamaka et al found a 23% reduction in the risk for incident malaria parasitemia and a 38% reduction in the risk for severe malaria, among the iron-deficient newborns relative to the iron-deficient group relative to the adequate group, during a 3 year follow-up period. These results have been corroborated by additional evidence from both human37,339 and animal studies, suggesting that iron deficiency appears to protect against malaria. In our study populations, we found that the risk for malaria in the iron adequate group (relative to the deficient group) increased about 3 times, with slight differences in the magnitude of the relative risk estimate, depending on the definition of iron status used, and whether or not children with baseline malaria were excluded from the analyses.

Our findings regarding the interactions between iron status and malaria, along with the several other studies that have previously documented the same pattern of association, have important implications for research and programs. In response to the publication of the findings of the Pemba trial, the WHO revised its guideline, essentially prohibiting universal iron supplementation of young children in a malaria-endemic regions. Prior to the publication of the findings of the Pemba trial, the WHO recommendation with respect to the prevention of iron deficiency and anemia was that in settings where the prevalence of anemia was ≥ 40%, children born with
normal birth weight be supplemented with daily iron (12.5 mg) and folic acid (50 µg) from 6 to 24 months. In addition, it was recommended that for children who were born with low birth weight, the daily iron and folic acid supplements be initiated at 2 months of age until 24 months. In the immediate aftermath of the Pemba trial, this recommendation was changed to a targeted approach, in which a requirement to first screen out children without iron deficiency was imposed. The revised recommendation was that universal supplementation of children should not be implemented without the screen of children for iron deficiency. This recommendation was heavily criticized for its lack of practicality, and hence its potential to halt progress of iron deficiency control programs. A consequence of this policy initiative is that in most countries in sub-Saharan Africa, including Zambia, where the burden of iron deficiency and anemia are greatest, there is a conspicuous absence of programs or policies to address the problem of iron deficiency and its consequent anemia. After years of debate, and especially in the wake of new evidence demonstrating the efficacy of relatively safer modes of delivering iron, the WHO recently revised its guideline again. The new WHO recommendation is that in malaria-endemic settings where the prevalence of anemia among preschool (24-59 months) or school aged children (5-12 years) is >20%, intermittent use of iron supplements, as a public health intervention, be used to reduce the burden of anemia among children. The current policy also recommends that the intermittent use of iron supplements be combined with strategies to prevent, diagnose and treat malaria. Our findings, that iron adequacy is associated with increased malaria incidence, supports this new recommendation to integrate iron deficiency and malaria control programs.
Early evidence from trials involving micronutrient powder (MNP) generated optimism towards the discovery of effective iron-based interventions which would be safe to use in both malaria-endemic and non-endemic regions. While most studies suggest that MNP do not increase the risk for adverse malaria outcomes, we have not yet seen any trial the size of the Pemba study, sufficiently powered to test for safety. Consequently, any conclusion about the safety of MNPs may be premature. In addition, evidence from three recent trials conducted in Tanzania, Pakistan and Ghana, suggest there is a reason to be concerned about safety, even with MNPs. In Tanzania, the early phase of an iron-containing MNP trial reported increased incidence of malaria among the MNP group and in Pakistan, children receiving iron-containing MNPs were more likely to have diarrhea, severe diarrhea and respiratory infections. A sub-analyses of a recent trial in Ghana found that the risk for hospital admissions was about 23% higher in the iron-containing MNP group. So far, the one thing that experts seem to agree on is the fact that iron interventions, whether as an oral supplement or a point-of-use fortificant, are safe when combined with measures to manage infections. If we are to control iron deficiency, amidst the unresolved concerns about safety, it is imperative that we intensively pursue the idea of integrating with malaria control programs. To this effect, there should be more research to explore integrated strategies which may ultimately increase both the coverage and the demand for iron intervention at the community.

**Vitamin A status and malaria outcomes in children**

Experimental investigations into the associations between vitamin A and health did not commence until the late 19th century, although there had been several prior anecdotal accounts of the potential of vitamin A to improve health, night
blindness in particular, either by consumption or topical application of vitamin A-rich foods.\textsuperscript{373} Studies by Lunin in the late 19\textsuperscript{th} century,\textsuperscript{372} and by McCollum in the early 20\textsuperscript{th} century\textsuperscript{371} generated momentum for further investigations into the potential for vitamin A (then known as fat soluble factor A) to improve resistance to infections, promote growth and improve survival. Since its discovery in the 1930s, a large body of research has demonstrated that vitamin has the potential to reduce morbidity and save life. Today, vitamin A interventions, including high-potency vitamin A capsules and fortified food, are widely used in several countries, including malaria-endemic regions. Unfortunately, there is very little evidence of impact, apart from the improvement in coverage, of these vitamin A programs. In malaria-endemic regions, the impact of vitamin A programs may be demonstrated by monitoring how changes in vitamin A status influence malaria outcomes. Characterization of the interactions between vitamin A status and malaria has the potential to sustain the interest and momentum in vitamin A programming in endemic regions.

The earliest experimental study into the role of vitamin A in malaria morbidity was conducted in 1946, nearly a decade after vitamin A was discovered.\textsuperscript{121} This pioneering study by Rigdon showed that inoculation of ducks fed on a vitamin A deficient ration, with \textit{P. lophurae} resulted in significantly higher mortality compared to control ducks fed on a normal diet.\textsuperscript{121} Several of the subsequent experiments in animal models demonstrated that vitamin A adequacy has the potential to improve malaria outcomes by boosting the proliferation and functions of immune cells,\textsuperscript{25,26,138,139} and may complement the potency of anti-malaria drugs.\textsuperscript{129,164,165} In the two human trials specifically designed to evaluate the efficacy of vitamin A supplementation in improving malaria outcomes, vitamin A supplementation was
associated with statistically significant reductions in clinical malaria incidence of 
~30% among Papua New Guinean children\textsuperscript{33}, but not in Ghanaian children.\textsuperscript{127} In this
population of rural Zambian children, we found that adequate vitamin A status was
associated with a reduced risk for incident malaria assessed 6 months later. Compared
to vitamin A deficient children at the baseline, children with low (retinol \( \geq 0.7 \)) but
<1.05 µmol/L) or adequate (retinol \( \geq 1.05 \) µg/L) vitamin A status had a relatively
reduced malaria incidence, and the difference was statistically significant in the
adequate vitamin A group. In an analyses in which baseline serum retinol
concentrations were first corrected for inflammation-associated hyporetinolemia, the
incident rate ratio (IRR) for malaria, comparing the low and adequate groups to the
deficient group, were 0.54 (95% CI: 0.24-1.17) and 0.47 (95% CI: 0.21-1.07)
respectively. After excluding baseline malaria cases and defining vitamin A status on
the basis of the measured, uncorrected serum retinol concentrations, the IRR for
malaria, in the low and adequate groups relative to the deficient group were 0.69
(95% CI: 0.37-1.27) and 0.50 (95% CI: 0.25-0.97) respectively.

A decade ago, it was estimated that about 20% of the burden of both clinical
malaria and malaria mortality was attributable to vitamin A deficiency.\textsuperscript{9} This
population attributable-risk was based on the results of a single study\textsuperscript{33} which found
that high-potency vitamin A supplementation was associated ~30% reduction in the
incidence of clinical malaria among children from PNG. Although a more recent
study has confirmed a protective effect of vitamin A supplementation of similar
magnitude, the children in this study also received daily zinc in addition to the high-
potency vitamin A supplement.\textsuperscript{128} These findings are consistent with results from a
recent meta-analyses which found that high potency vitamin A supplementation may
reduce all-cause mortality by 25% among children 6-59 months, although the study did not demonstrate the effects of vitamin A supplementation on malaria-specific outcomes.\textsuperscript{374} The recent decline in the burden of malaria, mostly because of increase coverage of malaria control interventions.\textsuperscript{375} has coincided with a concurrent increased in coverage of high-potency vitamin A intervention in endemic areas.\textsuperscript{376} Whether or not there is a causal link remains unknown. This coincidence, nonetheless, provides yet another reason to design new studies to estimate the potential impact of wide-spread vitamin A interventions on malaria outcomes. Such new evidence, along with the several evidence from animal models, may help to remove the cloud of doubt regarding the overall benefits of continuing vitamin A interventions in malaria-endemic regions.

**Inflammation, with or without malaria, and the biomarkers of vitamin A and iron status**

Subclinical infections and their consequent inflammation present a major challenge to the diagnosis of micronutrient deficiencies including those of iron and vitamin A in individuals and populations.\textsuperscript{36,62} In the context of inflammation, the concentration of serum retinol is substantially decreased\textsuperscript{62,185} and that of ferritin is increased\textsuperscript{36} as part of the innate immune mechanisms to either conserve these micronutrients or withhold them from invading parasites\textsuperscript{179}. The evidence regarding the response in sTfR to inflammation is inconclusive, with some studies showing elevations in sTfR\textsuperscript{271,277} while others found a decrease,\textsuperscript{281} or no change\textsuperscript{282} with accompanying inflammation. This interference may lead to over- or under-diagnosis of vitamin A and iron status, depending on the direction of inflammation-induced
changes. There is therefore tremendous research interest in finding ways to control for this potential bias in the context of defining population micronutrient status.

The idea of using acute phase proteins to improve the interpretation of inflammation-susceptible nutritional biomarkers has previously been proposed,\textsuperscript{179,185} The fact that there are currently no standard procedures for correcting or even characterizing the inflammation induced changes in nutritional biomarkers reflects both the complexity of the pattern of inflammation associated changes as well as limitations with current level of knowledge. The evidence suggests that the pattern of changes, both in direction and magnitude, depends on the type of inflammatory marker used in defining inflammation and the duration of the infection or stimulus inducing the inflammation\textsuperscript{36,38,39,270}. In addition, we have shown in our study subjects that the magnitude of the inflammation-induced changes may be age- and disease-specific. We found that the reductions in retinol associated with elevated AGP are more pronounced in the context of concurrent malaria parasitemia. We also found that the elevation in ferritin and sTfR during inflammation is more pronounced in the context of concurrent malaria infections. Finally we, observed that inflammation induced changes were higher in younger children (<60 months) compared to older children.

The observed age- and disease-specific patterns in the inflammation-associated changes in retinol, ferritin and sTfR certainly provides yet another reason to be concerned about the current practice of generating adjustment factors solely on the basis of one or multiple acute phase proteins.\textsuperscript{36,38} The four-group adjustment factor approach, in which subjects are categorized into four disease stages on the basis of one fast-reacting acute and one slow-reacting acute phase protein, is the most
popular of all the current approaches for characterizing the inflammation induced changes in the biomarker of nutritional status.\textsuperscript{38,301} This approach assumes that magnitude of the inflammatory response, as defined by a specific acute phase protein, is independent of the type of stimulus triggering the inflammation. This assumption forms the basis of a recent meta-analyses by Thurnham \textit{et al} in estimating ‘global adjustment factors’ for adjusting the inflammation induced changes in ferritin and retinol.\textsuperscript{36,38} Our data suggests that the application of this overly simplified assumption may be inappropriate, particularly in regions where malaria is endemic. In a malaria-endemic region, adjusting the retinol or ferritin concentrations in children with inflammation by the same factor may result in over-adjustment among the children with non-malaria related inflammation, and under-adjustment among those with malaria-related inflammation. Therefore, if adjustment factors are to be used at all, our findings suggest that the procedure for generating the adjustment factors should at least account for the malaria-specific differences.

At the recent global micronutrient forum in Ethiopia (June, 2014), the issue of controlling the inflammation-associated changes in micronutrients, particularly the indicators of iron status, was extensively discussed. One strategy that emerged was the idea of using regression models to estimate adjustment factors, an initiative currently being spearheaded by the CDC’s International Micronutrient Malnutrition Prevention and Control Program (IMMPaCt). The advantage of this idea is that it allows for customized or, at least, context specific adjustment factors. The challenge is that the regression modeling of corrections factors is perhaps most difficult to standardize. To be able to standardize and implement this idea, one must make a decision regarding a finite set of variable which affect the inflammatory response, and
which should be included in the regression model. Assuming that any such finite list of inflammatory modifiers can be agreed upon, one must also assume that these variables will be available for inclusion in the regression models. The assessment of one or more inflammatory biomarker is challenging in itself. The imposition of a requirement to include more variables enroute to define inflammation, will make it almost impossible to implement the regression model approach in poor resource regions, where the problem of subclinical infection or inflammation is greatest.

In conclusion, our results show that the magnitude of the inflammation-induced changes in the biomarkers of iron and vitamin A status are dependent on several factors including the malaria status, the malaria transmission season, the approach used in estimating adjustment factors (i.e. cross-sectional vs. longitudinal), and the age distribution of study subject. The currently available options for addressing this potential bias include: a) the exclusion of subjects with inflammation, b) the use of adjustment factors, c) making adjustments to the conventional cut-off for defining micronutrient deficiencies and d) the use of regression models to quantify the inflammation-associated changes, controlling for relevant variable. None of these methods is currently regarded as a gold standard, although the adjustment factors approach has become increasingly popular. For programmatic purposes, there is a need for continuing global discourse to standardize the approaches used for quantifying the effect of inflammation. Until the procedure become standardized, or until more robust nutritional biomarkers become available, it is recommended that the reporting of estimates of the prevalence of micronutrient deficiencies or their association with health indicators, either during research or micronutrient programing, be done such that evidence is reported for: a) all children; b) children without
inflammation as defined by one or more acute phase proteins, and c) all children after correcting for inflammation using one or more acute phase proteins. Where appropriate, estimates should also be provided in all children by making informed-adjustments to the conventional threshold for defining micronutrient deficiencies.

**Strength and weaknesses of study**

This study has several strengths, but also some limitations, among which is its observational nature. Although the parent study was randomized, this nested study was observational in design, and as a result, we are unable to infer causality from our results. This approach, as opposed to an experimental design, was informed by both the ethical implications and the context in which the study was conducted. To be able to characterize the effects of inflammation on the biomarkers of nutritional status with an experimental approach, one would have to expose a random sample of subjects with a challenge infection, or an inflammatory agent. This action will be ethically inappropriate in a human population.

Although efficacy trials of vitamin A or iron interventions are regarded as the best design for addressing the two primary questions posed in this study, doing so in our study populations will be particularly challenging for two reasons. First, our study population included children below the age of five years, who are the targets for the semi-annual, high-potency vitamin A delivery program currently underway in several countries, including Zambia. Unless this age group is excluded, any efficacy trial of vitamin A interventions is bound to be affected by this global program. To enhance the public health importance of studies investigating the interactions between vitamin A and malaria, it is imperative that studies are designed to include children below 5 years, in whom the burden of micronutrient deficiency and malaria is greatest. In such
a context, an observation approach, as used here, presents an option to avoid the indirect route of having to extrapolate results from an older population.

The evaluation of the association between iron status (instead of iron intervention) and malaria outcomes is consistent with the current research needs regarding the interaction between iron and malaria. The summary of the previous research findings suggest that iron status at baseline is the most consistent predictor of the risk or benefits of iron intervention among children in malaria-endemic. Among the several key unresolved questions, there is a need to understand if there exists a threshold of body iron level, beyond which iron becomes easily assessable to parasites, effectively enhancing the progression to adverse outcomes. In defining iron status, we used the conventional, age-specific cutoff of 12 µg/L (for children <60 months) and 15 µg/L (for older children). In addition to the observation that malaria incidence was higher in the iron adequate children, we also found that among children <60 months, the risk for malaria only began to increase beyond a baseline ferritin concentration of 75 µg/L. There is a need to further investigate whether this is the iron threshold beyond which adverse malaria outcomes can be expected and if so, there is the need to explore the dose of iron interventions needed to keep children adequate, without exceeding this threshold.

Another limitation with our study is that malaria incidence was defined on the basis of a one-time follow-up survey, conducted 6 months following the assessment of baseline iron or vitamin A status. The decision to do less frequent parasitological surveillance was informed by the context in which the study was implemented. This study was conducted in the context of trial which was designed to evaluate the efficacy of a provitamin A intervention in improving vitamin A status. Zambian
national guideline requires that all RDT positive cases be treated upon diagnosis. Therefore, doing more frequent parasitological assessments may have resulted in more frequent malaria treatment, which in itself, could be regarded as an intervention for improving vitamin A status. Malaria treatment may improve vitamin A status by reducing anorexia and increased urinary excretion, and this may have interfered with the main study outcomes.

There are advantages and disadvantages to the 6-month lag time between the assessment of iron or vitamin A status, and the determination of malaria incidence. Against the assumption that the treatment of prevalence malaria cases at baseline were effective, the 6 month lag time will almost certainly ensure that the cases detected at the 6-month survey were truly incident cases. By minimizing the correlation between baseline and endline malaria, the 6-month interval may have also decreased the probability of reverse causality, a real threat considering the bi-directionality of the interactions between malaria and the biomarkers of iron or vitamin A status. A longer lag time is also important when malaria is defined by an HRP-2 based RDT. It is well documented that HRP-2 from prior *P. falciparum* infection may persist in circulation for several weeks following clearance of active parasitemia.\(^{356,359}\) Allowing sufficient time between test administrations, will therefore ensure that new diagnosis are indeed new infections. Still, it is possible that within the 6 months period in which no parasitological surveillance was done, some new cases may have been missed. It is possible that these missed cases, had they be diagnosed, may have changed the magnitude of the incident rate ratio.

It is also possible that within the 6 month intervening period, the nutritional status of children may have changed. Children may have transitioned from iron
deficiency to adequacy and vice-versa, or from vitamin A deficiency to adequacy and vice-versa. Considering that the children were not given any iron supplements, and also considering that the evaluations involving vitamin A were restricted to children in the control arm of the study, we expect this transition to be minimal. In fact, we observed that among children who were malaria negative at both baseline and endline, about 90% of those classified as having adequate vitamin A status and 97% of those classified as iron adequate at the baseline remained in adequate status at the endline. It is unclear whether the minimal switch in nutritional status- iron or vitamin A- may have impacted our results. The exact impact of these potential transitions on our risk estimates would depend on the time point within the intervening period when these transitions occurred, and more importantly, the duration for which children remained in a particular status. Unfortunately, our data does not allow us to construct transitional models to evaluate how these changes may have affected our estimate of the risk for malaria.

In conclusion, the limitations of this study should not detract from the public health importance of our findings. To the extent possible, the limitations in design were addressed during the analytic stage. In particularly, we addressed reverse causality by redefining vitamin A or iron status using their respective biomarkers after correcting for inflammation. We also repeated all models by excluding children who had malaria at the baseline. Overall, we found that vitamin A adequacy appears protective against malaria, whiles iron adequacy appears to increase the risk for malaria. Moving forward, research should be designed to address the following questions: a) what is the threshold iron status beyond which the risk for malaria is increased and what extent of risk constitutes a public health problem? b) What is the
optimal strategy for delivering iron such that children remain in adequate but not excess iron status? c) Do wide-spread vitamin A programming contribute to reducing malaria morbidity and mortality in endemic regions and by how much? d) What is the optimal strategy for integrating micronutrient programs, particularly those for delivering iron, into existing or new malaria control programs? In addition, efforts should be intensified in the search for new biomarkers of iron and vitamin A status, which would be robust to inflammation. This is critical in light of evidence from this study that the idea of generating adjustment factors, will almost certainly yield incomparable results from study to study. Together, addressing these set of questions will inform the implementation and monitoring of vitamin A and iron programming in malaria-endemic regions.
Chapter 9 APPENDIX

Appendix A: Supplemental data

Table 9:1: Estimated power to detect difference in malaria incidence across vitamin A status based on observed baseline vitamin A status and endline malaria incidence (n=381)

<table>
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<tr>
<th>Case in adequate group (%)</th>
<th>Number with adequate vitamin A</th>
<th>Ratio of adequate to deficient children</th>
<th>Type 1 error rate</th>
<th>Unadjusted incidence rate ratio</th>
<th>Power to detect (%)</th>
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<td>0.16</td>
<td>0.05</td>
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<td>0.05</td>
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Table 9:2: Estimated power to detect difference in malaria incidence across iron status based on observed baseline iron status and endline malaria incidence (n=745)

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<tr>
<th>Case in adequate group (%)</th>
<th>Number with adequate iron</th>
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<td>692</td>
<td>0.08</td>
<td>0.05</td>
<td>1.5</td>
<td>43</td>
</tr>
<tr>
<td>0.24</td>
<td>692</td>
<td>0.08</td>
<td>0.05</td>
<td>1.8</td>
<td>83</td>
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<tr>
<td>0.24</td>
<td>692</td>
<td>0.08</td>
<td>0.05</td>
<td>2.5</td>
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Table 9:3: Baseline comparison between children included in paper 1 (chapter 4) and those not included

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</tr>
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<tbody>
<tr>
<td>Mean age in months (SD)</td>
<td>n=352</td>
<td>n=627</td>
</tr>
<tr>
<td>Positive microscopy (%)</td>
<td>n=352</td>
<td>n=519</td>
</tr>
<tr>
<td>RDT positive (%)</td>
<td>n=352</td>
<td>n=608</td>
</tr>
<tr>
<td>Anemia (%)</td>
<td>n=352</td>
<td>n=626</td>
</tr>
<tr>
<td>Inflammation (%)</td>
<td>n=352</td>
<td>n=574</td>
</tr>
<tr>
<td>Vitamin A deficiency (%)</td>
<td>n=352</td>
<td>n=568</td>
</tr>
<tr>
<td>sTfR &lt; 8.3 mg/L (%)</td>
<td>n=348</td>
<td>n=572</td>
</tr>
<tr>
<td>Iron Deficiency (%)</td>
<td>n=347</td>
<td>n=576</td>
</tr>
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</table>
### Table 9:4: Baseline comparison between children included in paper 2 (chapter 5) and those not included

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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>value</td>
<td>n</td>
<td>value</td>
</tr>
<tr>
<td>Mean age in months (SD)</td>
<td>381</td>
<td>68.8 (15.1)</td>
<td>597</td>
<td>68.9 (15.4)</td>
</tr>
<tr>
<td>Positive microscopy (%)</td>
<td>361</td>
<td>13.6</td>
<td>510</td>
<td>12.9</td>
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<tr>
<td>RDT positive (%)</td>
<td>380</td>
<td>19.7</td>
<td>579</td>
<td>17.8</td>
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<tr>
<td>Anemia (%)</td>
<td>381</td>
<td>35.4</td>
<td>597</td>
<td>29.0</td>
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<tr>
<td>Inflammation (%)</td>
<td>381</td>
<td>47.5</td>
<td>545</td>
<td>45.0</td>
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<tr>
<td>Vitamin A deficiency (%)</td>
<td>381</td>
<td>14.2</td>
<td>539</td>
<td>7.8</td>
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<tr>
<td>sTfR &lt; 8.3 mg/L (%)</td>
<td>377</td>
<td>27.1</td>
<td>543</td>
<td>28.2</td>
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<tr>
<td>Iron Deficiency (%)</td>
<td>376</td>
<td>7.2</td>
<td>547</td>
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### Table 9:5: Baseline comparison between children included in paper 3 (chapter 6) and those not included

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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>value</td>
<td>n</td>
<td>value</td>
</tr>
<tr>
<td>Mean age in months (SD)</td>
<td>726</td>
<td>69.4 (15.4)</td>
<td>252</td>
<td>67.1 (15.0)</td>
</tr>
<tr>
<td>Positive microscopy (%)</td>
<td>726</td>
<td>13.6</td>
<td>252</td>
<td>11.0</td>
</tr>
<tr>
<td>RDT positive (%)</td>
<td>722</td>
<td>18.7</td>
<td>237</td>
<td>18.1</td>
</tr>
<tr>
<td>Anemia (%)</td>
<td>726</td>
<td>32.9</td>
<td>252</td>
<td>27.4</td>
</tr>
<tr>
<td>Inflammation (%)</td>
<td>726</td>
<td>44.6</td>
<td>200</td>
<td>51.0</td>
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<tr>
<td>Vitamin A deficiency (%)</td>
<td>725</td>
<td>11.0</td>
<td>195</td>
<td>8.2</td>
</tr>
<tr>
<td>sTfR &lt; 8.3 mg/L (%)</td>
<td>726</td>
<td>26.7</td>
<td>194</td>
<td>30.9</td>
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<tr>
<td>Iron Deficiency (%)</td>
<td>726</td>
<td>7.2</td>
<td>197</td>
<td>9.6</td>
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### Table 9:6: Baseline comparison between children included in paper 4 (chapter 7) and those not included

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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>value</td>
<td>n</td>
<td>value</td>
</tr>
<tr>
<td>Mean age in months (SD)</td>
<td>745</td>
<td>69.4 (15.3)</td>
<td>233</td>
<td>67.1 (15.2)</td>
</tr>
<tr>
<td>Positive microscopy (%)</td>
<td>745</td>
<td>13.7</td>
<td>218</td>
<td>10.3</td>
</tr>
<tr>
<td>RDT positive (%)</td>
<td>741</td>
<td>18.9</td>
<td>218</td>
<td>17.4</td>
</tr>
<tr>
<td>Anemia (%)</td>
<td>745</td>
<td>32.8</td>
<td>233</td>
<td>27.5</td>
</tr>
<tr>
<td>Inflammation (%)</td>
<td>745</td>
<td>45.1</td>
<td>181</td>
<td>49.7</td>
</tr>
<tr>
<td>Vitamin A deficiency (%)</td>
<td>743</td>
<td>11.2</td>
<td>177</td>
<td>7.3</td>
</tr>
<tr>
<td>sTfR &lt; 8.3 mg/L (%)</td>
<td>744</td>
<td>27.0</td>
<td>176</td>
<td>30.7</td>
</tr>
<tr>
<td>Iron Deficiency (%)</td>
<td>745</td>
<td>7.1</td>
<td>178</td>
<td>10.1</td>
</tr>
</tbody>
</table>
Figure 9.1: Baseline age distribution of study population
Figure 9.2: Baseline serum retinol distribution depicting the cutoff used for defining vitamin A deficiency
Figure 9.3: Baseline age-stratified serum ferritin distribution depicting the cutoff used for defining iron deficiency
Figure 9.4: Baseline sTfR distribution showing the cutoff for defining iron deficiency
Figure 9.5: Scatter plot of ferritin against malaria parasite density among children who tested positive by microscopy during the low and high malaria transmission seasons.
Figure 9.6: Scatter plot of ferritin against AGP concentration during the low and high malaria transmission seasons
Figure 9.7: Scatter plot of sTfR against malaria parasite density among children who tested positive by microscopy during the low and high malaria transmission seasons.
Figure 9.8: Scatter plot of sTfR against AGP concentration during the low and high malaria transmission seasons
Figure 9.9: Scatter plot of retinol against malaria parasite density among children who tested positive by microscopy during the low and high malaria transmission seasons
Figure 9.10: Scatter plot of retinol against AGP concentrations among children during the low and high malaria transmission seasons
Figure 9.11: LOWESS plots of baseline ferritin against endline malaria incidence stratified by age
Figure 9.12: LOWESS plot of baseline retinol against endline malaria
Appendix B: Laboratory determination of serum retinol concentrations by reverse-phase high-performance liquid chromatography

**Principle**
- Serum is denatured with ethanol containing an internal standard. The sample is extracted with hexane and the retinol and carotenoids are determined using Reverse-phase High-Performance Liquid Chromatography.

**Precautions taken to limit retinol losses in the laboratory**
- Samples processed under dim lightening conditions
- Extracted samples dried under a hood using current of nitrogen

**Quality Control procedures**

**Internal standards**
- Four internal standards for each of retinol (retinyl acetate) and β-carotene (β-apo-8’-carotenal) were included in each batch of samples inserted into the HPLC system. These separate runs were used to estimate the maximum peaks for retinol and β-carotene and later used to estimate the efficiency of the assay.
- Internal standard were also added to the samples before extraction. The peak areas determined from these internal standards are combined with the peaks determined from the internal standards run separately to estimate the efficiency of the assay.
- The efficiency was multiplied by the individual peak areas to generate the adjusted peak areas, which were then converted into concentration using the standard curve.

**Internal Control sera**
- A pooled serum sample was prepared and run with each batch of samples and internal standards. The pooled serum was run after every 10th sample to check for consistency of the HPLC system.
Table 9:7: The HPLC System

<table>
<thead>
<tr>
<th>Component</th>
<th>Retinol</th>
<th>B-Carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equipment type</td>
<td>LC-2010C HT, Cat #:228-45103-38, Ser #: c21254808339 LP</td>
<td>LC-2010C HT, Cat #:228-45103-38, Ser #: c21254808339 LP</td>
</tr>
<tr>
<td>Injection volumes</td>
<td>60ul</td>
<td>60ul</td>
</tr>
<tr>
<td>Columns</td>
<td>Supercosil LC-18, 25cm x4.6mm, 5um; cat#58298</td>
<td>Supercosil LC-18, 25cm x4.6mm, 5um; cat#58298</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Acetonitrile (80%) THF(14%), Methanol(6% containing 0.2% Ammonium acetate), 0.1% Triethylamine</td>
<td>Acetonitrile (80%) THF(14%), Methanol(6% containing 0.2% Ammonium acetate), 0.1% Triethylamine</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.5ml/min</td>
<td>1.5ml/min</td>
</tr>
<tr>
<td>Wavelength</td>
<td>325</td>
<td>450</td>
</tr>
<tr>
<td>Internal standard</td>
<td>Retinyl Acetate</td>
<td>β-apo-8’-carotenal</td>
</tr>
<tr>
<td>Retention Times</td>
<td>~3min</td>
<td>~25-30min</td>
</tr>
</tbody>
</table>

Table 9:8: Materials and sources for HPLC

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinyl Acetate</td>
<td>Sigma –Aldrich Chemie GmbH.Switzerland-Cat# R7882-1G</td>
</tr>
<tr>
<td>Retinol</td>
<td>Sigma –Aldrich Chemie GmbH.Switzerland #17772-1G</td>
</tr>
<tr>
<td>β-apo-8’-carotenal</td>
<td>Came as capsules without manufacturer information. Capsules given by Marjorie.</td>
</tr>
<tr>
<td>Beta carotene</td>
<td>Came as capsules without manufacturer information. Capsules given by Marjorie.</td>
</tr>
<tr>
<td>Methanol</td>
<td>Burdick &amp;Jackson, Michigan, USA. cat#AH230-4</td>
</tr>
<tr>
<td>THF</td>
<td>Burdick &amp;Jackson, Michigan, USA. cat##AH340-4</td>
</tr>
<tr>
<td>Hexane</td>
<td>Fisher Scientific, USA. Cas # 110-54-3</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>HIMEDIA, India Cas # 75-05-8</td>
</tr>
</tbody>
</table>

Purification of Retinol
1. About 5 mg of All-trans retinol was dissolved in 500 µl of ethanol.
2. About 2 µl of this solution was applied to the HPLC system to check the size of the peak.
3. The pure retinol was then collected in a clean tube.
4. The amount of impure retinol solution applied was increased until a desired amount of pure retinol was collected.
5. An equal amount of water was added to the purified retinol, mixed and the retinol extracted with about 1 ml portion of n-hexane. The hexane layer was transferred into a new tube and evaporated to dryness in a current of nitrogen.

6. The residue is dissolve in about 500 µl of ethanol the absorbance adjusted to about 0.208 at 325 nm on the UV-VIS spectrophotometer.

7. The concentration of this standard was determined using the extinction coefficient and absorbance as follows:

   \[ \text{Conc} = \frac{A}{1850} \times 10^6 \mu g/dl = 112.4 \mu g/dl \]

8. This standard solution was later used in the generation of the standard curve as follows:

**Generation of retinol standard curve**

- Different volumes of the standard retinol solution were injected into the HPLC system and the maximum area at 325 nm determined from the chromatogram for each volume injected.
- The peak area, determined from the chromatogram- is proportional to the volume injected.
- Seven different chromatograms were generated by injecting standard volumes of 5, 10, 25, 35, 55, 75, and 95 µl respectively into the HPLC system.
- The concentration of retinol per volume injected was determined by multiplying the original concentration of the standard by the volume injected.
- The standard curve was generated by plotting the concentrations against the peak areas for each volume injected.

**Preparation of internal standard (Retinyl acetate)**

The internal standard was prepared using retinyl acetate following the same procedures used for the preparation of retinol standards. The retinyl acetate solution was purified, extracted with hexane, dissolved in about 500 µl of ethanol and the final concentration adjusted to about 0.208 at 325 nm. The concentration of the purified internal standard solution was estimated by dividing the absorbance by the extinction coefficient at 325 nm. The internal standard preparations were kept and used for up to 2 days by storing in the freezer (at -20°C) and reused when necessary.

**HPLC daily work flow**

A typical day in the HPLC lab begun with sample extraction. Samples were extracted and prepared for HPLC by a team of three laboratory technicians. About 30-48 samples were extracted each day. The number of samples extracted in a day constituted a batch. Once all samples, standards and controls had been extracted and prepared, the batch was inserted into the HPLC system and run overnight. The batch continued to run until late morning the following day, when the last sample in the batch was analyzed. By late-morning the following day, newly extracted samples were ready to be pushed into the HPLC system again. Once each sample in the batch had been run, the peaks were integrated by a laboratory technician. Peak integration involved the reading of individual peak areas. For peaks whose base were not resting on the x-axis, a manual adjustment to the peak was done to prevent overestimation of the peak area.
Appendix C: Competitive Enzyme-Linked Immuno-Sorbent Assay (ELISA) for determination of α-1 Acid Glycoprotein (AGP)

**Principle**
Competitive ELISA technique was used to determine the concentration of AGP in the serum samples. ELISA kits for serum AGP (Abcam®, Cambridge, USA) had 96 wells pre-coated with AGP-specific antibody and blocked. Standards and samples are added to the wells and subsequently, biotinylated AGP is added and then washed with wash buffer. Avidin-Biotin-Peroxidase Complex is added and unbound conjugates are washed away with wash buffer. TMB is then used to visualize horseradish peroxidase (HRP) enzymatic reaction. TMB is catalyzed by HRP to produce a blue color product that changes into yellow after adding acidic stop solution. The density of yellow coloration is inversely proportional to the amount of Alpha 1 acid Glycoprotein captured in plate.

**The AGP kit (Abcam, Cambridge, MA, USA)**
- 96-well micro-plate (12 x 8 well strips)
- Alpha 1 acid Glycoprotein Standard (1 vial)
- 10X Diluent N Concentrate (30 ml)
- One vial biotinylated human AGP (lyophilized)
- 80 µL of 100X Streptavidin-Peroxidase Conjugate (SP Conjugate)
- Chromogen Substrate (8 ml)
- Stop Solution (12 ml)
- 20X Wash Buffer Concentrate (30 ml)
- Sealing Tapes 3

**Preparation of samples:**
All Samples were equilibrated to room temperature prior to use.
- A 1:10 diluent was prepared from the 10X diluent using deionized water.
- The 20X wash buffer concentrate was diluted 1:20.
- We then added 4 mL 1X diluent N to the lyophilized biotinylated AGP vial to generate 1X biotinylated AGP. This was allowed to sit for 10 minutes with gentle agitation prior to use.
- A desired volume of 100X streptavidin-peroxidase conjugate (SP Conjugate) is diluted 1:100 with 1X diluent.
Table 9:9: Dilution of standards for competitive ELISA assay

<table>
<thead>
<tr>
<th>Volume to dilute (µL)</th>
<th>Volume of diluent (µL)</th>
<th>Total Volume (µL)</th>
<th>Starting Concentration (µg/ml)</th>
<th>Final concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>120</td>
<td>240</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
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<td>120</td>
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<td>1</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>120</td>
<td>240</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>120</td>
<td>120</td>
<td>240</td>
<td>0.25</td>
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<tr>
<td>8</td>
<td>120</td>
<td>120</td>
<td>240</td>
<td>0.063</td>
</tr>
</tbody>
</table>

Assay Procedure
- All materials, samples and prepared reagents were equilibrated to room temperature (18-25°C) prior to use.
- Add 25 µL of AGP standard or sample per well.
- Immediately add 25 µL of 1X biotinylated AGP to each well (on top of standard or sample) and mix gently. Cover wells with a sealing tape and incubate for two hours at room temperature. Start the timer after the last sample addition.
- Wash five times with 200 µL of 1X wash buffer. Invert the plate each time and decant the contents by tapping it 4-5 times on absorbent paper towel to completely remove the liquid.
- Add 50 µL of 1X SP conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash microplate as described above.
- Add 50 µl of chromogen substrate to each well and incubate for 8 minutes.
- Add 50 µl stop solution to each well.
- Read the absorbance on a microplate reader at a wavelength of 450 nm immediately.

Conversion of absorbance into concentrations
The Magellan software was installed and programmed to automatically convert absorbance’s into concentrations. The software was programmed to first generate blank reduction by subtracting the absorbance of the blank from each individual sample’s absorbance. The blank reductions are then converted into equivalent concentrations against the standard curve. The Software first generates a four-order logistic curve using the concentrations of the standards. The standard curve was then used to convert the individual absorbance into corresponding concentrations.

Appendix D: Double antibody ELISA for determination of Soluble Transferrin Receptor (sTfR)

Principle
The double antibody sandwich ELISA was used for the determination of sTfR levels in serum samples. The sTfR present in the serum is first bound to anti-sTfR
antibodies adsorbed to the surface of the polystyrene micro-titer wells. The bound sTfR then reacts with an enzyme labeled anti-sTfR antibody, the anti-sTfR horseradish peroxidase conjugate. The Anti- sTfR antibody in this complex reacts with the free site on the sTfR. The conjugated enzyme then reacts with a chromogenic substrate (3’3’5’5’-tetramethyl benzidine (TMB)). The enzyme-substrate reaction yields a colored product, the absorbance of which is read at 450nm. The amount of colored product formed is proportional to the amount of sTfR in the serum sample.

The sTfR KIT (Ramco Laboratories Inc., Texas, USA, cat# TFC-94)
- Diluent (phosphate buffered saline, detergent and an inert coloring agent).
- Wash solution.
- Enzyme-antibody conjugate.
- Chromogen-substrate (TMB) solutions A and B.
- Human sTfR calibrator solution with concentrations of 0, 10, 20, 40, 100,200, 400 ng/ml.
- Anti-Human sTfR ELISA microplate
- Acid stop solution (0.3 M sulfuric acid)
- Controls (a normal control and a high control)

Dilution of samples
A 1/100 dilution of all serum samples and control is prepared following adding 10ul of sample to 1m of sample diluent. The Standards are pre-diluted and should not be diluted.

Preparation of 1X wash solution
- The 1X solution is prepared by transferring the entire content of the 5X wash solution into a 250ml graduated cylinder and bringing the volume to 250ml with distilled water.

sTfR Assay
- Remove the appropriate number of micro-well strips and place in a frame
- A blank solution was prepared with 200 µl of substrate solution and 100 µl of the potassium ferricyanide.
- 50 µl of all standards and blanks were then pipetted in duplicates into the adjacent pre-designated well.
- 50ul of each sample and control were then transferred into pre-designated wells.
- 150ul of HRP-conjugate are pipetted into all the micro wells.
- The plate were sealed with the self-adhesive plastic film, placed in a horizontal rotating table and mixed for 10 minutes at 190 rpm.
- The wells were then incubated for 2 hours at room temperature.
- While incubating, the substrate solution was prepared by adding and mixing equal volumes of substrate solutions A and B. The amount of substrate solution prepared should equaled the number of wells multiplied by 0.2 ml. The substrate solution was prepared immediately before use.
- Following incubation, the contents of the wells were then aspirated
- The wells are then washed three times with 1X wash solution.
- 200ul of substrate were transferred into each well and shaken for 1 minutes.
The plate were incubated at room temperature for a further 30 minutes.

After the 30 minutes, 50 µl of acid stop solution were added into each well and the color allowed to develop.

The absorbance of the solutions were then read at 450nm using the TECAN micro-plate reader.

**Conversion of absorbance into concentrations**

- Concentrations were estimated using the same procedures are described for AGP above.

**Appendix E: Determination of serum ferritin concentration using ELISA**

**Principle**

Human ferritin is first bound to a solid phase anti-human ferritin adsorbed to the bottom of the micro-plate wells. The complex then reacts with an anti-human ferritin conjugated to an enzyme alkaline phosphatase. In the final reaction, the phosphatase reacts with a substrate, consisting of phenylphosphate disodium and 40-amino-antipyrine. The addition of potassium ferricyanide stops the enzyme reaction, and develops a colored solution, whose absorbance is read at 500nm.

**The Ferritin ELISA kit (Ramco Laboratories Inc., Texas, USA, cat# S-22)**

- Dilution buffer
- Conjugated anti-human ferritin
- Potassium ferricyanide stop solution
- Chromogen-substrate solution
- Six pre-diluted ferritin calibrator solution at concentration 6, 20, 60, 200, 600 and 2000 ng/ml.
- Anti-Human Ferritin ELISA microplate

**Ferritin Assay**

- The micro-well is placed in a holder and shaken dry.
- A blank solution is papered with 200 µl of substrate solution and 100 µl of the potassium ferricyanide.
- Pipette 10 µl of all standards and blanks are in duplicates into the adjacent pre-designated well.
- 10ul of each sample is transferred into pre-designated wells.
- Pipette 200ul of conjugated Antihuman ferritin into all the wells.
- The wells are then incubated for 2 hours on a vibrator at room temperature.
- Following incubation, the contents of the wells are aspirated.
- The wells are then washed three times with 1X wash solution.
- 200 µl of substrate is transferred into each well.
- The plate is then incubated at room temperature for a further 30 minutes.
- After the 30 minutes, 100 µl of stop solution are added into each well and the color allowed to develop.
- The absorbance of the solutions are then read at 500nm using the TECAN micro-plate reader.
Conversion of absorbance into concentrations
- Concentrations were estimated using the same procedures as described for AGP above.

Appendix F: Malaria microscopy
Malaria smears, both thin and thick, were transported from the field to the lab in slide boxes, each box containing a maximum of 100 slides, 50 thick smears and 50 thin smears. In the laboratory, all thick slides are first stained, washed and dried before being read under a microscope. For all positive thick smears, the corresponding thin smear are also stained, washed, dried and examined under a microscope to determine the species.

Staining
Staining of both thick and thin smears were done with a Giemsa solution. The Giemsa solution was prepared by dissolving dry Giemsa powder in a 50:50 (v: v) mixture of glycerol and methanol. The stain was prepared by dissolving 7.6 g of Giemsa powder in 500 ml glycerol and 500 ml of methanol. The slides were stained by pouring enough of the Giemsa solution to cover the entire smear. About 15-20 minutes was allowed for the staining process. The glycerol facilitates the fixing of the smear to the slide. The stained slide was washed with water and dried.

Slide examination
- A drop of emersion oil was applied to the smear before the slide was examined under a 100X objective lens.
- For each field examined, one counter is used to enumerate the parasites and another used to enumerate the number of white blood cells (WBC) in a field. Separate fields are examined until 200 WBCs are enumerated.

Estimation of parasite density:
- The number of parasite per 200 WBC is determined from the smear examination. From this number, the parasite density is estimated as the number of parasite per μl of blood is estimated assuming 8000 WBC per μl of blood. A smear is adjudged negative if no parasite is seen after counting 200 WBC.

Quality Control
- All thick slides were read twice by two independent readers. Where necessary, a third reading was done by a third reader to resolve discrepancies in the paired readings.

Examination of thin smears
- Thin smears are examined for individual with positive thick smears. Thin smear are examined to identify the Plasmodium species present in the smear.
Appendix G: Child diet and morbidity form

Device id
Date stamp
Start time
Enter FI ID: __ __ __
Scan household barcode
Scan child barcode
1. Enter child’s name:
2. Enter respondent’s name: _______________________
3. Identify respondent’s relationship to child subject
   01 mother
   02 father
   03 legal guardian
   04 other ➔ specify: ______________

Section A: 24-hour recall
4. First, I’m going to ask you about all of the foods and drinks that [child’s name] ate yesterday, from when [s/he] woke up yesterday morning through last night. It is important that you report everything [s/he] had to eat or drink yesterday. Let’s discuss the first foods or drinks that [child’s name] had when [s/he] woke up yesterday morning.

   Early morning
5. What were the first things [child’s name] had to eat when [s/he] woke up yesterday? Prompt if necessary: what time did [she/he] get up in the morning? Did [s/he] eat or drink anything then?

   Enter food loop to select and describe food item

<table>
<thead>
<tr>
<th>food loop</th>
<th>to be repeated for each food consumed within each time period</th>
</tr>
</thead>
<tbody>
<tr>
<td>l1 – l5.</td>
<td>staged selection and description of food item</td>
</tr>
<tr>
<td>l6. select cooking method:</td>
<td></td>
</tr>
<tr>
<td>01</td>
<td>raw / fresh</td>
</tr>
<tr>
<td>02</td>
<td>boiled</td>
</tr>
<tr>
<td>03</td>
<td>fried</td>
</tr>
<tr>
<td>04</td>
<td>roasted</td>
</tr>
<tr>
<td>05</td>
<td>steamed</td>
</tr>
<tr>
<td>06</td>
<td>scrambled</td>
</tr>
<tr>
<td>88</td>
<td>other cooking method ➔ specify cooking method</td>
</tr>
<tr>
<td>99</td>
<td>don’t know</td>
</tr>
<tr>
<td>i1 – i8.</td>
<td>ingredient selection question(s) corresponding to food item selected in l1-l5</td>
</tr>
<tr>
<td>p1 or p2.</td>
<td>portion size question corresponding to food item selected in l1-l5</td>
</tr>
</tbody>
</table>

s1. Where did this food come from?
   01 this household / kitchen
   02 neighbor or relative
   03 purchased and eaten outside the home
   04 feeding program
   88 other
Mid-morning
6. Now I’d like to ask you about what [child’s name] ate from mid-morning yesterday. That is, from about 0800 to 1200 hours, what did [child’s name] have to eat or drink? Prompts if necessary: you’ve just told me about what [s/he] had for breakfast, now I’d like to know about what other snacks or drinks [s/he] had during the morning before lunch time. Where was [child’s name] yesterday during the morning? At home? At a relative’s house? At the market? Did [s/he] have anything to eat or drink there? What was s/he doing during that time? Did [s/he] have any foods or drinks while doing that activity? Did [s/he] have any small foods to eat?

Enter food loop to select and describe food item

Mid-day
7. Around mid-day, between 1200 and 1400 hours, what did [child’s name] have to eat or drink? Prompts if necessary: you’ve just told me about what [s/he] had during the morning, now I’d like to know about what [s/he] had for lunch, as a snack or to drink around 1200 – 1400 hours. Where was [child’s name] mid-day yesterday? At home? At a relative’s house? At the market? Did [s/he] have anything to eat or drink there? What were all the foods that [s/he] had with lunch? Did [s/he] have anything to drink with or right after lunch?

Enter food loop to select and describe food item

Afternoon
8. What did [child’s name] have to eat or drink during the afternoon yesterday? Prompt if necessary: you’ve just told me about what [s/he] had around lunch-time, now I’d like to know about what foods or drinks [s/he] had during the afternoon, before dinner time, that is, between about 1400 and 1700. Where was [child’s name] yesterday afternoon? Did [s/he] have anything to eat or drink there? What was s/he doing during that time? Did [s/he] have any foods or drinks while doing that activity?

Enter food loop to select and describe food item

Evening
9. Next, I’d like to ask you what [child’s name] ate yesterday evening. From about 1700 to 2000 hrs. what did [child’s name] have to eat or drink? Prompts if necessary: I’d like to know about what [s/he] had for dinner, as a snack or to drink around 1700 - 2000 hours. Where was [child’s name] yesterday evening? What was s/he doing during the evening yesterday? Did [s/he] have any foods or drinks while doing that activity? What were all the foods [s/he] had with dinner? Did [s/he] have anything to drink with dinner?

Enter food loop to select and describe food item

Night-time
10. What did [child’s name] have to eat or drink last night, from late evening through the whole night? Prompts if necessary: you’ve just told me about what [s/he] had yesterday evening, now I’d like to know about what [s/he] had to eat after dinner-time and during the night, after 2000 hours. Did [child’s name] have anything to eat or drink before going to bed last night? Did [s/he] get up during the night to eat or drink anything?

Enter **food loop** to select and describe food item

**Review of picture chart**
11. Now I’d like to review the picture chart you filled out yesterday to identify any other foods or drinks [child’s name] had yesterday. Are any foods ticked on the picture chart? If so, add group for each food which was ticked. Enter loop of questions 11a – 11d for each food ticked off on the picture chart

11a. what is the next item on the picture chart is marked as having been consumed? Enter the number of the next ticked item: __ __

{If 11a = 1} 11b. What times of day did [fill from q1] have this food?
Select all that apply
- Early morning (waking – 0800)
- Mid-morning (0800 – 1200)
- Mid-day (1200 – 1400)
- Afternoon (1400 – 1700)
- Evening (1700 – 2000)
- Night-time (2000 thru night)

{If 11a = 1} 11c. [Is this / are these] the item(s) we already discussed? Prompt to remind respondent of when this type of food was described.

0 no
1 yes
9 don’t know

{If 11d = 0} 11d. Let’s record this food item now.

Enter **food loop** to select and describe food item

**Check for any other foods**
12. What other foods or drinks did [fill from q1] have yesterday?
Review the day once more with the respondent, probing for any other foods or drinks the child may have had. Prompt if necessary: we have discussed foods and drinks that [fill from q1] had yesterday. Did [fill from q1] have anything to eat or drink yesterday that we haven’t already talked about? Do you want to add any other foods or drinks? Did the child go anywhere else yesterday where [s/he] had something to eat or drink? Did [s/he] receive any snacks or drinks from friends, neighbors or relatives? Did [s/he] eat any small foods from your kitchen or that [s/he] gathered [him/herself]?

Enter **food loop** to select and describe food item

**Editing foods already recorded**
13. Are there any foods that needed to be edited?

0 no → q15
1 yes

14. Describe edit that needs to be made to food record.
14a. description of food:
14b. time of day consumed:
Select all that apply
Early morning (waking – 0800)
Mid-morning (0800 – 1200)
Mid-day (1200 – 1400)
Afternoon (1400 – 1700)
Evening (1700 – 2000)
Night-time (2000 thru night)

14c. change to be made:

15. Was [child’s name] ill yesterday?
00  no  ➔ q17
01  yes
09  don’t know

16a. did illness affect [his/her] appetite?
00  no  ➔ q17
01  yes
09  don’t know

16b. was [his/her] appetite:
01  less than usual?
02  greater than usual?

17. Was yesterday a feast day?
00  no
01  yes
09  don’t know

18. was it a market day?
00  no
01  yes
09  don’t know

19. was it a fasting day?
00  no
01  yes
09  don’t know

20. Was [child’s name] food intake unusual yesterday for any other reason?
00  no
01  yes  ➔ specify: ______________________________________________________
09  don’t know

21. Did [child’s name] take any of the following yesterday:
…iron supplements?
…vitamin syrup? ➔ Specify brand:
…other supplements? ➔ Specify brand:

Section B: Morbidity recall
Now I’d like to ask you about any illness that your child may have experienced in the last two weeks.

22. In the last 14 days, has [child’s name] had a fever?
00  no  ➔ q23
22a. how many days in the past week, that is, since last [fill day of week], has [child’s name] had a fever?
   ___ 00 none
   01-07 no. of times
   08 8 or more times
   09 don’t know

22b. did [child’s name] have a fever in the past 24 hours?
   00 no
   01 yes
   99 don’t know

23. In the last 14 days, has [child’s name] had a headache?
   00 no \(\rightarrow\) q24
   01 yes
   09 don’t know \(\rightarrow\) q24

23a. how many days in the past week, that is, since last [fill day of week], has [child’s name] had a headache?
   ___ 00 none
   01-07 no. of times
   08 8 or more times
   09 don’t know

23b. did [child’s name] have a headache in the past 24 hours?
   00 no
   01 yes
   99 don’t know

24. In the last 14 days, has [child’s name] had chills?
   00 no \(\rightarrow\) q25
   01 yes
   09 don’t know \(\rightarrow\) q25

24a. how many days in the past week, that is, since last [fill day of week], has [child’s name] had chills?
   ___ 00 none
   01-07 no. of times
   08 8 or more times
   09 don’t know

24b. did [child’s name] have chills in the past 24 hours?
   00 no
   01 yes
   99 don’t know

25. In the last 14 days, has [child’s name] had convulsions?
   00 no \(\rightarrow\) q26
   01 yes
   09 don’t know \(\rightarrow\) q26

25a. how many days in the past week, that is, since last [fill day of week], has [child’s name] had convulsions?
   ___ 00 none
   01-07 no. of times
25b. did [child’s name] have convulsions in the past 24 hours?
00 no
01 yes
99 don’t know

26. In the last 14 days, has [child’s name] had a cough?
00 no  27
01 yes
99 don’t know  27

26a. how many days in the past week, that is, since last [fill day of week], has [child’s name] had a cough?
—
00 none
01-07 no. of times
08 8 or more times
09 don’t know

26b. did [child’s name] have a cough in the past 24 hours?
00 no
01 yes
99 don’t know

27. In the last 14 days, has [child’s name] had difficulty breathing?
00 no  28
01 yes
99 don’t know

27a. how many days in the past week, that is, since last [fill day of week], has [child’s name] had difficulty breathing?
—
00 none
01-07 no. of times
08 8 or more times
09 don’t know

27b. did [child’s name] have difficulty breathing in the past 24 hours?
00 no
01 yes
99 don’t know

28. In the last 14 days, has [child’s name] had rapid breathing?
00 no  29
01 yes
99 don’t know

28a. how many days in the past week, that is, since last [fill day of week], has [child’s name] had rapid breathing?
—
00 none
01-07 no. of times
08 8 or more times
09 don’t know

28b. did [child’s name] have rapid breathing in the past 24 hours?
00 no
01 yes
99 don’t know
29. In the last 14 days, has [child’s name] had a poor appetite?
   00  no \(\rightarrow\) **q30**
   01  yes
   09  don’t know \(\rightarrow\) **q30**
29a. how many days in the past week, that is, since last [fill day of week], has [child’s name] had a poor appetite?
   _  00  none
   01-07  no. of times
   08  8 or more times
   09  don’t know
29b. did [child’s name] have a poor appetite in the past 24 hours?
   00  no
   01  yes
   99  don’t know
30. In the last 14 days, has [child’s name] vomited?
   00  no \(\rightarrow\) **q31**
   01  yes
   09  don’t know \(\rightarrow\) **q31**
30a. how many days in the past week, that is, since last [fill day of week], has [child’s name] vomited?
   _  00  none
   01-07  no. of times
   08  8 or more times
   09  don’t know
30b. did [child’s name] vomit in the past 24 hours?
   00  no
   01  yes
   99  don’t know
31. In the last 14 days, has [child’s name] had four or more loose or watery stools in one day?
   00  no \(\rightarrow\) **q32**
   01  yes
   09  don’t know \(\rightarrow\) **q32**
31a. how many days in the past week, that is, since last [fill day of week], has [child’s name] had four or more loose or watery stools in one day?
   _  00  none
   01-07  no. of times
   08  8 or more times
   09  don’t know
31b. has [child’s name] had four or more loose or watery stools in the past 24 hours?
   00  no
   01  yes
   99  don’t know
32. In the last 14 days, has [child’s name] had blood or white mucus in [his/her] stools?
   00  no \(\rightarrow\) **q33**
   01  yes
   09  don’t know \(\rightarrow\) **q33**
32a. how many days in the past week, that is, since last [fill day of week], has [child’s name] had blood or white mucus in [his/her] stools?
   — 00 none
   — 01-07 no. of times
   — 08 8 or more times
   — 09 don’t know

32b. has [child’s name] had blood or white mucus in [his/her] stools in the past 24 hours?
   00 no
   01 yes
   99 don’t know

33. In the last 14 days, has [child’s name] had blood in the urine?
   00 no \(\rightarrow\) q34
   01 yes
   09 don’t know \(\rightarrow\) q34

33a. how many days in the past week, that is, since last [fill day of week], has [child’s name] had blood in the urine?
   — 00 none
   — 01-07 no. of times
   — 08 8 or more times
   — 09 don’t know

33b. has [child’s name] had blood in the urine in the past 24 hours?
   00 no
   01 yes
   99 don’t know

34. In the last 14 days, has [child’s name] had pus or liquid dripping from the ears?
   00 no \(\rightarrow\) q35
   01 yes
   09 don’t know \(\rightarrow\) q35

34a. how many days in the past week, that is, since last [fill day of week], has [child’s name] had pus or liquid dripping from the ears?
   — 00 none
   — 01-07 no. of times
   — 08 8 or more times
   — 09 don’t know

34b. has [child’s name] had pus or liquid dripping from the ears in the past 24 hours?
   00 no
   01 yes
   99 don’t know

35. In the last 30 days, did you seek advance or treatment for any of these symptoms or illnesses?
   00 no \(\rightarrow\) q36
   01 yes
   09 don’t know \(\rightarrow\) q36

35a. where did you seek advice or treatment? Code all that apply. Prompt:
   anywhere else?
   — 01 government hospital
   — 02 government health center / post
03 mobile clinic
04 community health worker / neighborhood health committee member
05 private hospital / clinic
06 pharmacy / shop
07 traditional practitioner
08 other, specify: __________
09 don’t know

36. Has your child taken any medications in the last 30 days?
00 no → q37
01 yes
09 don’t know → q37

36a. what medications did your child take? Code all that apply.
01 Amodiaquine
02 Amoxicillin/Ampicillin/Amoxil
03 Antiretroviral medication
04 Aspirin
05 Artesunate
06 Brufen/ Ibuprofen
07 Chloramphenicol
08 Chloroquine
09 Coartem
10 Coflyn
11 Cotrimoxazole/ septrim / bactrim
12 Dihydroartemisinin (dhart)
13 Flagyl / metronidazole
14 iron/ iron-folate
15 Mebendazole/ albendazole / vermox
16 Paracetamol/panadol
17 Quinine
18 SP/Fansidar
19 Tetracycline
20 vitamins /folic acid
21 Herbal medicine
22 other, specify: __________
99 don’t know

37. Has your child taken any *malaria* medications in the last 30 days?
00 no → q38
01 yes
09 don’t know → q38

37a. what malaria medications did your child take? Code all that apply.
01 Amodiaquine
02 Artesunate
03 Chloroquine
04 Coartem
05 Dihydroartemisinin (dhart)
06 Quinine
07 Sulfadoxine / Pyrimethamine / SP / Fansidar
08 herbal medicine
38. Has your child taken any medications for bilharzias in the last 30 days?
00  no
01  yes
09  don’t know

**Section C: Dark adaptometry**

39. Does your child have *kafifi*?
00  no
01  yes
09  don’t know

40. Does your child have *bumpofu bwabushiku*?
00  no
01  yes
09  don’t know

41. Does your child have *ubwafya bwakukana mona ubushiku*?
00  no
01  yes
09  don’t know

42. Does your child see adequately in the daytime?
00  no
01  yes
09  don’t know

43. Does your child have any difficulty during the evening or night:
   43a. … finding the food on his/her plate at dinner?
         00  no
         01  yes
         09  don’t know
   43a. … going outside to the bathroom?
         00  no
         01  yes
         09  don’t know
   43a. … playing with other children?
         00  no
         01  yes
         09  don’t know
   43a. … doing other activities, needing help from others?
         00  no
         01  yes
         09  don’t know
   43a. … moving around?
         00  no
         01  yes
         09  don’t know

44. Does your child have any difficulty playing normally during the daytime?
00  no
01  yes
09  don’t know
Section D: Health Practices

45. At any time in the past 6 months, has [child’s name] participated in a child health week?
   00  no  \rightarrow q46
   01  yes
   09  don’t know

45a. during that child health week, did [child’s name] receive a vitamin a capsule?
   00  no
   01  yes
   09  don’t know

45b. did [s/he] receive a deworming tablet?
   00  no
   01  yes
   09  don’t know

46. At any other time in the past 6 months, has [child’s name] received a vitamin a capsule?
   00  no
   01  yes
   09  don’t know

47. At any time in the past 6 months, has anyone sprayed the interior walls of your dwelling against mosquitoes?
   00  no  \rightarrow q48
   01  yes
   09  don’t know  \rightarrow q48

47a. how many months ago was the house sprayed?
   __  00-06  no. of months
   09  don’t know

48. Did [child’s name] sleep under a mosquito net last night?
   00  no
   01  yes
   09  don’t know

Thank you very much for answering these questions. For the health check-up, our team has set up their equipment at a central location. Can I accompany you and [child’s name] to that location?

49. Form status
   01  child diet & morbidity form complete
   02  child diet & morbidity form suspended

Appendix H: Blood draw and disease control form

Device id
Date stamp
Start time
Enter lab technician id: __ __
Enter community id: __ __
Enter cluster id: __ __
Scan child’s zed card:
Confirm consent status with FI
01 full consent
02 consent to participate; refused biospecimen collection
03 consent to participate & biospecimen collection; refused biospecimen storage
04 refused all participation

Section B: Biospecimen collection
Please enter child’s name directly as it appears on hhid/zid card
First name ______________________
Last name ______________________

5. Enter child’s axillary temperature
___ ___  value in °C
6.6  refused → q6
9.9  don’t know → q6
5a. re-enter child’s temperature
___ ___  value in °C
6.6  refused
9.9  don’t know
If q5≠q5a [warning: values do not match] → q5

6. How many hours has it been since your (or your child’s) last meal or snack? A snack may include any liquid other than water.
___ ___
00 <1 hour
01-12 no. of hours
13 more than 12 hours
99 don’t know

7. Blood collected
00 no → q8
01 yes → q9
02 finger stick only → q8
06 refused → q8

7ai. enter vid: 2- ___ ___ ___ ___
7aai. re-enter vid: 2- ___ ___ ___ ___
If vids do not match→ [warning: both vid entries should match] → 7ai

7b. record timestamp

8. Hemoglobin level
___ ___  value in g/dl
6.6  refused → q9
9.9  incomplete / don’t know → q9
8a. [next screen] reenter hb value
___ ___  value in g/dl
If q8≠q8a [warning: values do not match] → q8

9. Confirm thick slide preparation
01 complete
06 refused
09 not done

10. Confirm thin slide preparation
01 complete
06 refused
09 not done
11. Malaria RDT result
01 positive
02 negative
03 not valid
06 refused
09 not done
12. Danger signs present
00 no
01 yes
13. [if q11=1] confirm treatment with coartem
00 no
01 yes
06 refused
14: was saliva collected?
00 no
01 yes
99 ineligible
06 refused
15. Form status
01 blood draw form complete
02 blood draw form suspended
Appendix I: Consent statement:
Intervention groups – informed consent document
(Completed by field interviewer)

Study title: efficacy of β-carotene biofortified maize in improving vitamin a status and reducing the prevalence of vitamin a deficiency among children in rural Zambia

Principal investigator: Keith P. West, Jr.
IRB no.: 00004150
Version date: v2, 1 august 2012

Introduction
Muli shani. My name is [field interviewer name]. I work for the [project name]. We are doing a study on a new type of “orange” maize developed in Zambia using traditional methods. This project is being done by Johns Hopkins University, the Tropical Diseases Research Centre, and the national food and nutrition commission.

Purpose of research project
In this study, we want to see whether feeding young children “orange” maize, compared to “white” maize, will improve their health and nutrition. The results of this study will help us learn whether the government should promote this new crop in Zambia.

Why are you being asked to participate?
Your household is being asked to take part in the study because your child is between four and eight years of age and s/he is also not yet going to school.

Procedures
If you agree to let your child participate, s/he will be asked to come to a location close to your home to receive meals of porridge or nshima made with either orange or white maize. Meals will be served every day, except Sundays, over the next six months. We will ask your child to wear a special bracelet for the next six months. This will help a local worker make sure that s/he received every meal. If your child is unable to come to the feeding site, a project worker may bring meals to your home. The type of maize that your child receives will be decided by chance, just like flipping a coin.

Today, we would like to ask some general questions about your household. This will take about 60 minutes. We will ask about household members’ education and occupation, items that you own, and food that you grow in your fields or purchase in the market. We will also ask about your child’s diet and his/her health.

After this interview, we will ask you to bring your child to [location] for a check-up. This will take an additional 45 minutes. First, we will measure your child’s height, weight, arm size, and skin thickness on his/her arm. Then, a nurse will take about 2 teaspoons of blood from your child’s arm to check for substances that can tell us more about your child’s nutrition. We will test your child’s blood today for malaria and anemia. If positive for malaria, the nurse will give your child medicine for treatment. If your child is anemic, s/he will be referred to the health clinic. [For dark
adaptometry subsample: finally, we will then test your child’s eyes to see how well s/he can see in the dark using a special pair of eyeglasses.

After today, a project worker will visit your home once every month for the next six months. S/he will ask about what your child ate in the previous 24 hours and about foods available in your home. We will also ask general questions about your child’s health. At one of these visits, we may ask your child to spit into a small tube. We will use the material in this tube to examine how your child’s body uses the food that s/he eats. These visits will take about 45 minutes. We will make sure that we do these at a time that is convenient for you.

At the end of the study, our teams will come again to your home to repeat the interview and check-up that I just described.

**Risks/discomforts**
Your child may feel a slight pinch when measuring skin thickness on his/her arm. Taking blood can hurt for an instant, but otherwise poses little risk when done correctly. All measurements that we will take on your child are safe. There are no known risks or discomforts other than those I have just described. The questions we will ask are not sensitive, but you are free to not answer a question if you wish.

**Benefits**
There may be some benefits to your child as well. S/he will be fed six days a week for six months. This may help to improve his/her nutrition. We will provide a mosquito net and deworming medicine. If your child tests positive for malaria today or at the end of the study, s/he will receive treatment at no cost to you. If your child becomes severely ill, we will refer him/her to a local doctor. If we advise you to see a doctor, the project will pay your travel cost. Any treatment will be at your cost. The results of this study will help the government decide if “orange” maize can improve children’s health and nutrition. Your child’s participation may therefore help other children.

**Payment**
There is no payment for participation.

**Protecting data confidentiality**
All personal information you provide will be kept confidential by project staff. Your identity or that of your child will not be revealed when the information is used.

**Protecting subject privacy during data collection**
We will perform all interviews and measurements in a private area. No one will be allowed to listen in or be told about the information you provide. Only members of your family or neighbors you wish to be there will be allowed at the interviews.

**Biological specimens**
The blood, saliva, and data collected from your child are important to science. You will not own these after you give them to the study. The blood and saliva may be stored in our laboratories for up to ten years to test for substances that tell us about your child’s nutrition. During this period of time, we will keep the samples secure.
from anyone outside of the project. If we decide to test your child’s blood or saliva for a purpose not explained here today, we will return to ask your permission.

**What happens if you leave the study early?**
We are asking you to join our study. Your decision to participate is up to you. If you or your child wants to stop an interview, refuse meals, or refuse any measurements, you are free to do so at any time. This will not affect your child’s right to receive meals. If you permit us to collect blood and saliva for long term storage, you may change your mind in the future. You can ask at any time for these samples to be removed from storage and destroyed.

**Who do I call if I have questions or problems?**
If you have any questions or concerns, I can answer them now or our coordinator can answer these during the check-up. You may also contact Dr. Amanda Palmer or Mr. Ward Siamusantu, senior project officers, at our office in Mkushi (tel: 0975-570-685) or the ethical review committee at the tropical diseases research centre in Ndola (tel: +260-212-610-961).

Will you allow your child to participate in this study?

Do we have your permission to collect about two teaspoons of blood and saliva?

Do you agree to allow the project to store the blood and saliva for up to ten years?
Thank you

**Appendix J: Funding**
This work was funded by HarvestPlus Challenge Grant #8251, with support from the UK Department for International Development. The views expressed do not necessarily reflect those of HarvestPlus. MAB received partial support from the DSM Scholars Program through the Sight & Life Global Nutrition Research Institute at Johns Hopkins University and from Foreign Affairs, Trade and Development Canada Grant #112305.
Chapter 10: BIBLIOGRAPHY

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nutrition in children under two years of age. Cochrane database of systematic reviews (Online). 2011(9):CD008959.


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years of age. *Cochrane database of systematic reviews (Online).* 2011(12):Cd009085.


Curriculum Vitae

MAXWELL A. BARFFOUR, M.P.H.
(Born September 4, 1982, Kumasi, Ghana)

PART 1

Home
3550 Watermelon Rd
Northport, AL, 35473
Email: mbarffour@gmail.com

Office
Johns Hopkins University
Bloomberg School of Public Health
North Wolfe St, Room 2501
Baltimore MD, 21205
Tel (410) 585 5979
Email: mbarffou@jhsph.edu

EDUCATION:

2008-2014
Doctor of Philosophy (PhD), International Health, Johns
Hopkins University, Bloomberg School of Public Health,
Baltimore, MD, USA.

Thesis: Interactions between malaria and iron or vitamin A
status in rural Zambian children.

2006-2008
Master of Public Health (MPH), Epidemiology Concentration,
Missouri State University, Springfield, MO, USA.

Practicum: Determinants of iron deficiency among children
enrolled in the Special Supplementary Program for Women,
Infants and Children (WIC) in Taney County, MO.

2001-2004
Bachelor of Science (BSc.), Nutritional Biochemistry,
Department of Biochemistry, University of Ghana.

Dissertation: Determination of the zinc and iodine nutritional
status among Ghanaian Preschoolers.

PROFESSIONAL EXPERIENCE:

09/13- 12/13
Field Research Supervisor- Breast milk vitamin A study. A
randomized controlled trial jointly implemented by the
University of California, Davis, (UC Davis) and the Johns
Hopkins University School of Public Health to determine the
efficacy of two vitamin A interventions in improving breast
milk vitamin A status. Responsibilities: Training, coordination
and supervision of field assessment teams, supervision of data
collection and data management.

06/13- Present  **Research Assistant**- Johns Hopkins University Bloomberg
School of Public Health; Systematic review of evidence
regarding the benefits, safety and coverage of prenatal
corticosteroids in low-income countries. **Responsibilities:**
systematic review and manuscript preparation.

08/11-Present  **Field Research Assistant**- Johns Hopkins University,
Bloomberg school of Public Health; A randomized control trial
to evaluate the efficacy of a pro-vitamin A intervention in
reducing the burden of malaria and vitamin A deficiency
among children in rural Zambia. **Responsibilities:** Assisted
with proposal writing, recruitment and training of staff,
development of forms, supervision of field and laboratory
work, data collection, data cleaning, data analyses and
manuscript writing.

08/11 - Present  **Field Research Supervisor**- Johns Hopkins University,
Bloomberg School of Public Health; Portable Field Dark
Adaptometer Study; **Responsibilities:** Recruitment and training
of dark adaptometrists, testing and management of equipment,
writing of technical reports to donor (Canadian International
Development Agency-CIDA), supervision of field data
collection, data management, data analyses and manuscript
preparation.

11/10-09/12  **Research Scientist**- Johns Hopkins University/World Health
Organization/ World Bank /Bloomberg
Foundation/International Injury Research Unit: RS10 Project to
estimate burden and risk factors for road traffic injuries and
deaths in 10 low and middle income countries;
**Responsibilities:** Systematic literature review, data analyses,
write-up of manuscripts and technical reports to the Bloomberg
Foundation.

05/11-08/11  **Research assistant**- John Hopkins University Malaria
Research Institute (JHMI) and the International Centre of
Excellence for Malaria Research (ICEMR), Zambia; Malaria
Control in Southern Africa Project; **Responsibilities:**
Visibilities studies and study site recommendation, community-
based malaria diagnosis and treatment.
<table>
<thead>
<tr>
<th>Dates</th>
<th>Position</th>
<th>Organization</th>
<th>Responsibilities</th>
</tr>
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<tbody>
<tr>
<td>05/11-08/11</td>
<td>Statistician</td>
<td>John Hopkins University School of Medicine; Virtual- Reality Simulator Project</td>
<td><strong>Responsibilities</strong>: Analyses of data to assess the impact of training resident medical students on the use of a virtual-reality temporal bone simulator on their motor and technical skills. Results have been published in the Laryngoscope.</td>
</tr>
<tr>
<td>01/10-05/10</td>
<td>Data Analyst</td>
<td>WHO’s Child Health Epidemiology Reference Group (CHERG)/Johns Hopkins University Bloomberg School of Public Health</td>
<td><strong>Responsibilities</strong>: Analyses of data to determine the importance of preterm delivery and birth weight on neonatal and infant mortality.</td>
</tr>
<tr>
<td>09/09-01/10</td>
<td>Research Assistant</td>
<td>Institute for International Projects/WHO’s Child Health Epidemiology Reference Group (CHERG)/Johns Hopkins University</td>
<td><strong>Responsibilities</strong>: Study to investigate the importance of breastfeeding to child morbidity and mortality.</td>
</tr>
<tr>
<td>05/09-09/09</td>
<td>Research Assistant</td>
<td>Institute for International Programs; Johns Hopkins University</td>
<td><strong>Responsibilities</strong>: Systematic literature review.</td>
</tr>
<tr>
<td>02/09-09/09</td>
<td>Laboratory Research Assistant</td>
<td>Johns Hopkins University Bloomberg School of Public Health; Centre for Human Nutrition</td>
<td><strong>Responsibilities</strong>: Laboratory determination of soluble transferrin receptor levels in rural Bangladesh Women.</td>
</tr>
<tr>
<td>08/08-12/08</td>
<td>Laboratory Research Assistant</td>
<td>Lewis B. and Dorothy Cullman Cancer Chemo-protection Center; Johns Hopkins University School of Medicine</td>
<td><strong>Responsibilities</strong>: running of retinol assays as part of an experiment to investigate the role of estrogen in cellular retinol uptake.</td>
</tr>
<tr>
<td>04/08-08/08</td>
<td>Public Health Intern</td>
<td>City of Branson Health Department, Branson, MO</td>
<td><strong>Responsibilities</strong>: Impact analyses of Special Supplementation Program for Women, Infants and Children (WIC).</td>
</tr>
<tr>
<td>05/07-07/07</td>
<td>Program Coordinator</td>
<td>Public Health Emergency Response Training jointly organized by the Centre for Disease Control(CDC) and the State of Missouri</td>
<td><strong>Responsibilities</strong>:</td>
</tr>
</tbody>
</table>
Recruitment of students participants, participation in training, and analyses of data.

12/06-01/07 Graduate Teaching Assistant- School of Social Work, Missouri State University; Responsibilities: Management of program website, data analyses and assisting students with data analyses.

05/07-08/08 Public Health Assistant- Ozarks Public Health Institute, Springfield, MO; Responsibilities: Data analyses, website development and management, public health advocacy and write-up of reports and manuscripts.

01/07-08/08 Assistant Program Coordinator- Greater Ozarks Counseling Centre; Responsibilities: Data analyses, website development and management.

01/07-08/08 Vice President of Public Health Response- Missouri State University’s Public Health Professionals Association; Responsibilities: Liaison between student body and working public health professionals in the Green County and State of Missouri Public Health Association.

05/05-08/06 Research and Teaching Assistant- University of Ghana School of Public Health, Department of Population, Family and Reproductive Health; Responsibilities: Involved in all aspects of research, including proposal writing, literature reviews, data analyses and conference organization.

05/04-05/05 Project Coordinator- Rural Development and Education Project (RUDEP- Ghana); Responsibilities: Visibility studies and project supervision in 13 low income communities along the Ghana-Burkina Faso Border.

08/03-03/04 Quality Assurance Intern- Ghana Agro Foods Company Limited; Responsibilities: Quality Assurance supervision.

05/00-05/01 Senior Laboratory Technician- Science Resource Centre, Nkwatia-kwahu, Ghana; Responsibilities: Lead laboratory instructor and coordinator.

03/01-05/01 District-Level Examination Supervisor- West African Examination Council Certificate Examination for Senior High Schools (WAEC); Responsibilities: Supervision of physics, experimental biology and practical chemistry examinations.
AWARDS:

2014  DSM Doctoral Research Fellowship- Johns Hopkins University Bloomberg School of Public Health

2014  Elsa Orent Keiles Fellowship in Nutritional Biochemistry- Johns Hopkins University Bloomberg School of Public Health

2013  Federik Bang Immunology Research Award- Johns Hopkins University Bloomberg School of Public Health

2012  DSM Micronutrient Scholarship, Johns Hopkins University Bloomberg School of Public Health

2011  Centre for Global Health Research Grant- Johns Hopkins University Bloomberg School of Public Health

2011  Delta- Omega Research Scholarship, Johns Hopkins University Bloomberg School of Public Health

2011  Johns Hopkins University- Canadian International Development Agency Student Investigator Support

2011  Johns Hopkins University- HarvestPlus Student Investigator Support

2010  Johns Hopkins University- Department of International Health Tuition Scholarship

2006  Missouri State Outreach Graduate Scholarship, Missouri State University

PROFESSIONAL ACTIVITIES:

MEMBERSHIP:

- American College of Epidemiology
- American Association of Nutrition
- Biomedical Scholars Association, Johns Hopkins Medical Institutions
- Global Health Council Member
- International Injury Research Unit
- African Public Health Network
- Ghana Nutrition Association

EDITORIAL ACTIVITIES:


LABORATORY EXPERIENCE:

2013 Laboratory Determination of serum retinol levels in children using High Pressure Liquid Chromatography; Tropical Disease Research Centre, Ndola, Zambia.

2013 Determination of the serum C - reactive protein (CRP) levels in children using Enzyme-linked Immuno-assays (ELISA).

2013 Determination of serum Alpha-1- Glycoprotein (AGP) level in children using Enzyme-linked Immuno-assays (ELISA).

2013 Determination of serum ferritin levels in children and pregnant women using Enzyme-linked Immuno-assays (ELISA).

2013 Determination of serum soluble Transferrin Receptor levels (sTfR) in children and pregnant women using Enzyme-linked Immuno-assays (ELISA).

2013 Microscopic examination of thick and thin malaria smear and immunoassays for determination of malaria antigens.

2012 Determination of Aflatoxin levels in β-carotene bio-fortified maize using the Aflacheck rapid diagnostic kit.

PROPOSAL/GRANT WRITING EXPERIENCE

Grant Proposal 1:

Title: Iron-Malaria interaction: examining the robustness of iron status indicators to inflammation and evaluating the risk of adverse malaria outcomes as a function of prior iron status, a prospective study among rural Zambian children.

PI: Keith P. West, Maxwell A. Barffour

Submitted to: The Johns Hopkins University Bloomberg School of Public Health

Responsibility: Student Investigator; drafted the entire proposal

Outcome: Proposal selected as recipient of the 2013 Federik Bang Award
Grant Proposal 2

Title: *Quantification of the extent of malaria-induced hyporetinolemia and validation of the ‘icheck™’, a portable fluorometric tool for assessment of vitamin A status in rural Zambian children.*

PIs: Keith P. West, Christian Coles, Maxwell A. Barffour

Submitted to: Johns Hopkins University Bloomberg School of Public Health

Responsibility: Development of entire proposal

Outcome: Awarded the Delta Omega Scholarship

Grant Proposal 3

Title: *Cluster randomized controlled trial to evaluate the efficacy of β-Carotene Bio-fortified maize in improving the vitamin A status of rural Zambian children: Proposal submitted to Harvest-Plus Responsibilities- Drafted the malaria related component of the Proposal.*

PI: Professor Keith P. West

Submitted to: Harvest Plus

Responsibility: Drafted proposal for a nested sub-study to evaluate the association between vitamin A, malaria and anemia in children.

Outcome: Grant was awarded to implement proposed study

Grant Proposal 4

Title: *Effect of home-based fortification with multiple micronutrients including iron on anemia, growth and malaria morbidity among children in a malaria endemic region-a randomized trial in Nchelenge, Zambia.*

PI: Keith West, William Moss, Kerry Schulze, Alain Labrique, Christian Coles, Maxwell A. Barffour

Submitted to: Site & Life, Geneva, Switzerland
Responsibility: Drafted the initiation expression of interest (EOI) and full proposal

Outcome Research team invited to submit a full proposal after submission of expression of interest. Proposal selected as one of top-two applications.

COMPUTER LITERACY/STATISTICS:
- STATA, SPSS, S-PLUS, EPI-INFO, ‘R-Statistical Software’, LiST Software
- Microsoft Office applications (Word, Excel, PowerPoint, Access, Publisher etc.)

PUBLICATIONS:


Barffour A. M., Bell L., 2006, Medical Nutritional therapy for Type 2 Diabetes; Abstract published in the “Annual Research Symposium”, Missouri State University. Pp. 54.


PART 2

RELEVANT PRESENTATIONS:

- **Oral Presenter**: Experimental Biology Conference, April 2014, San Diego California. Presentation on the topic “*Ferritin, when corrected for inflammation, is associated with increased malaria incidence in rural Zambian children*”.

- **Poster Presenter**: Micronutrient Forum, June, 2014, Addis Ababa, Ethiopia. Presentation on the topic “*Malaria exacerbates inflammation-induced inflammation in ferritin and soluble transferrin receptor*”.

- **Oral Presenter**: malaria-micronutrient interaction- evaluating the role of iron and vitamin in malaria pathology among rural Zambia children; presentation to DSM executive & Advisory Board as a scholarship recipient. June 2012.

- **Poster Presenter**: Medical Nutritional Therapy for Type 2 diabetes, Annual Research Forum, Missouri State University, Springfield, MO. April, 2007.

CONFERENCES/SEMINARS/MEETINGS:

<table>
<thead>
<tr>
<th>Year</th>
<th>Event Description</th>
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<tbody>
<tr>
<td>2014</td>
<td>Experimental Biology Conference, San Diego, CA.</td>
</tr>
<tr>
<td>2012</td>
<td>International Conference on Traffic Injury Prevention organized at the Johns Hopkins University Bloomberg School of Public Health.</td>
</tr>
<tr>
<td>2010</td>
<td>Training in the use of LiST (lives Saved Tool), a new Maternal and Child Health software for estimation of the number of deaths that could be prevented by scaling up available interventions in low income countries.</td>
</tr>
<tr>
<td>2008</td>
<td>Week long Childhood obesity teleconferencing organized by the State of Missouri Department of Health and Human Services, and the City of Branson Health Department.</td>
</tr>
<tr>
<td>2008</td>
<td>Public Health Emergency Response Training, jointly organized by the Centre for Disease Control(CDC), and the Missouri State Health Department, Columbia, MO.</td>
</tr>
<tr>
<td>2006</td>
<td>Annual Meeting of the Missouri Public Health Association, Springfield, MO.</td>
</tr>
<tr>
<td>2005</td>
<td>Training in Epidemiology and Public Health Intervention Network (TEPHINET), University of Ghana School of Public Health.</td>
</tr>
<tr>
<td>2005</td>
<td>Emergency experts’ consultation on the avian flu outbreak,</td>
</tr>
</tbody>
</table>
RESEARCH INTERESTS:

- Research into the benefits of prenatal corticosteroid for preventing preterm related mortality.
- Interventions for reducing the burden of morbidity and mortality in pregnancy, infancy and early childhood in low income countries.
- Interactions between iron, anemia and malaria in children.
- Inflammation and the biomarkers of nutritional status.
- Development of novel models for diagnosis of vitamin A deficiency in the context of concurrent infections.
- Efficacy of bio-fortified foods in improving health and nutritional status in children, pregnant and lactating women.
- Safety and benefits of iron-based intervention in malaria endemic regions.
- Portable Field Dark Adaptometry for diagnosis of functional vitamin A deficiency.
- Disease burden attributable to comorbidities among children in low income countries.