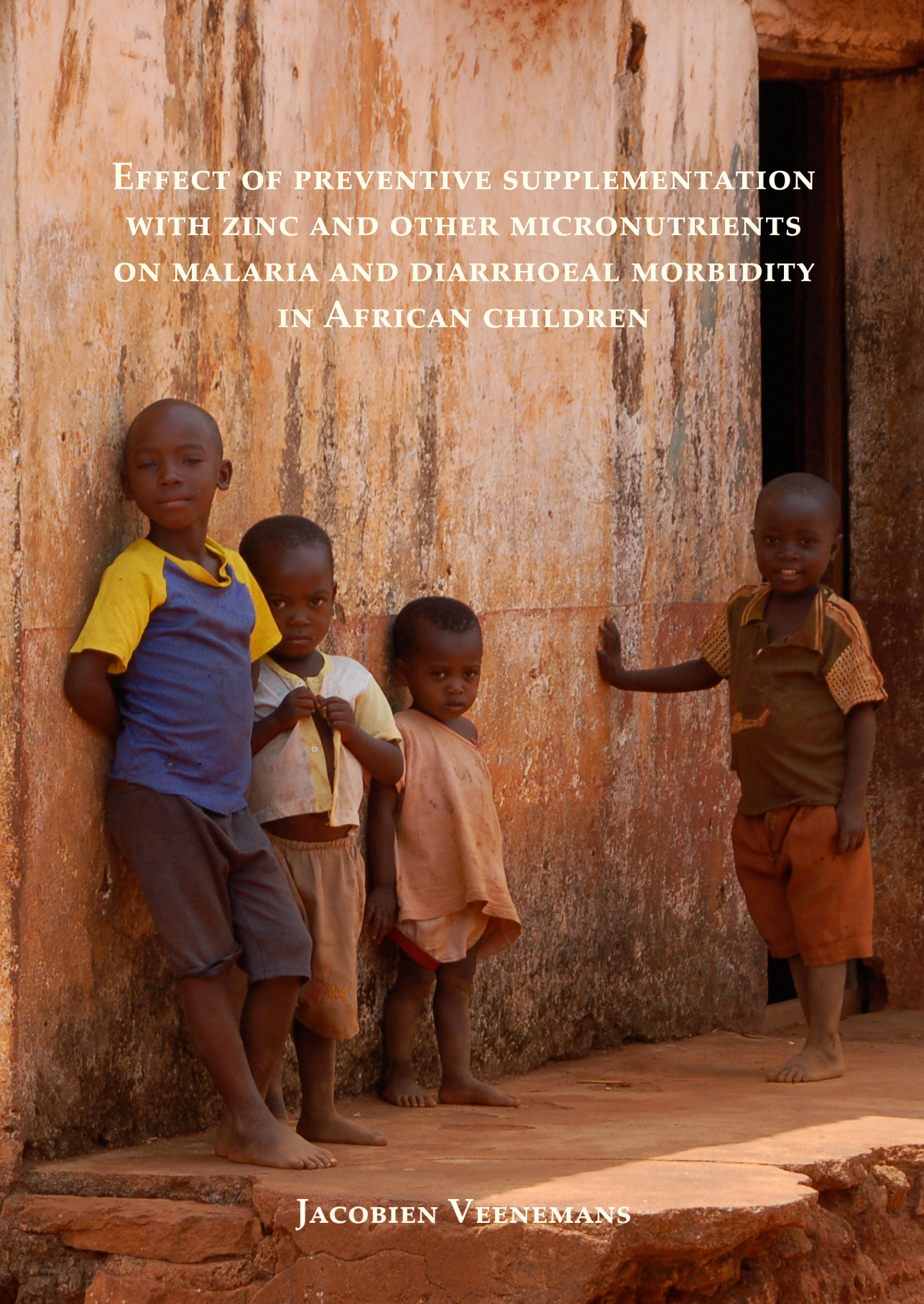
Effect of preventive supplementation with zinc and other micronutrients on malaria and diarrhoeal morbidity in African children



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Contents

Chapter 1

INTRODUCTION

Nutritional zinc deficiency

Nutritional deficiencies are estimated to be responsible for 21% of worldwide deaths in children aged below 5 years (2.1 million in 2004) and 21% of their total disease burden. This disease burden is mostly due to deficiencies in zinc and vitamin A, and is highest in Asia and sub-Saharan Africa (Black et al. 2008).

Zinc plays a critical role in maintaining epithelial barrier function (Keen and Gershwin 1990, Alam et al. 1994, Truong-Tran et al. 2001, Zalewski et al. 2005, Finamore et al. 2008), which is the body's first-line defence against intestinal and airway pathogens. In vitro studies using Caco-2 cells (human epithelial cells derived from a colon adenocarcinoma that, when appropriately cultured, become differentiated and polarised so that they morphologically and functionally resemble enterocytes lining the small intestine; Hidalgo et al. 1989) suggest that tight junctions between intestinal epithelial cells have impaired function in zinc deficiency (Finamore et al. 2008). Morphological studies and findings from a zinc supplementation trial in Bangladeshi children with diarrhoea suggest that zinc deficiency adversely affects intestinal permeability (Moran and Lewis 1985, Roy et al. 1992).

Zinc is central in the formation of 'zinc fingers', i.e. loops in proteins that typically bind with DNA and RNA and that are essential for gene expression and many other processes, including DNA replication and transcription. Thus zinc deficiency particularly affects short-lived enterocytes and cells of the immune system such as macrophages that must divide and differentiate rapidly in response to antigenic stimulation (Shankar and Prasad 1998, Klug 1999). Zinc deficiency is associated with decreased numbers and impaired functioning of neutrophils and natural killer cells, both important components of the innate immune system.

Zinc is also important for adaptive immune responses (Cuevas et al. 2002, Fraker et al. 1987, Prasad 2000, Rink and Kirchner 2000, Rink and Gabriel 2000, Salgueiro et al. 2000, Ibs and Rink 2003, Fraker and King 2004). Deficiency results in a reduction in thymic size and cellularity, mostly in the thymic cortex where thymocytes mature into T cells (Shankar and Prasad 1998), and in decreased activity of thymulin (Prasad et al. 1988, Keen and Gershwin 1990), a zinc-dependent thymic hormone in serum that stimulates proliferation, differentiation and function of T cells. This may account for the decrease in concentrations of cytokines such as IFN-γ and IL-2 that are produced by Th1 cells, whereas Th2 cell responses seem unaffected (Beck et al. 1997). Thus by regulating secretion patterns of these cytokine, zinc may be needed to

maintain balance in cell-mediated and humoral immunity pathways that are driven by Th1 and Th2 cell responses to infection, respectively (Prasad 2000, Kidd 2003). In addition, the decreased production of IL-2 may lead to decreased recruitment of naive T cells, a decreased percentage of T cytolytic cells, and decreased NK cell lytic activity (Prasad 2000).

The supply of zinc from exclusive breastfeeding is probably sufficient to meet the dietary needs of infants for at least the first 3 months after birth and possibly for as long as the first 6 months (IZiNCG 2004, Brown et al. 2009a). The risk of deficiency increases with the introduction of complementary foods, which generally have lower nutrient densities than breast milk. Because these foods are usually based on cereals, they also contain high concentrations of phytates and polyphenols that inhibit the intestinal absorption of dietary zinc. Consequently, two-thirds of sub-Saharan Africans are at risk of inadequately low zinc intake (Brown and Wuehler 2000).

Zinc status is usually measured using biochemical indicators such as serum or hair concentrations of zinc. These markers are useful at a population level but poorly predict zinc status or functional impairment in individuals (IZiNCG 2004). Thus randomised placebo-controlled trials remain the best available method to establish zinc deficiency in populations, and to determine the health gains that can be obtained by increasing the population's zinc intake. The outcomes in such trials usually concern morbidity or growth indices, because zinc deficiency is known to cause children becoming stunted and underweight (Brown et al. 2002, 2009b).

A recent meta-analysis showed that preventive supplementation with zinc can reduce the incidence of acute diarrhoea and lower respiratory tract infections by 20% and 14%, and overall child mortality by 6% (Brown et al. 2009b). The same meta-analysis, however, also found inconsistencies in trial results. Thus research priorities must shift from studies to measure efficacy to identifying factors that determine the magnitude of the effect of zinc supplementation. Most trials have so far been conducted in Asia and Latin America. The response to supplementation may be different in Africa, where different dietary and other environmental factors may influence the efficacy of zinc, and where children's health may be challenged by different pathogens (Galanis et al. 2006, Chimalizeni et al. 2010), malaria being the prime example. **Table 1** provides an overview of the trials have so far been carried out with zinc supplements in Africa.

Table 1. Overview of published trials to assess effects of preventive zinc supplementation in sub-Saharan African children

Country (reference)	**Population** (sample size)	**Design**	**Primary outcome**	**Secondary outcome**	**Main findings**
The Gambia (Bates et al. 1993)	Rural children, 0.6–2.3 years (n=111)	Zinc (70 mg as acetate and gluconate) versus placebo, twice weekly for 15 months (not randomized)	Linear growth	Mean number of clinic visits per child, also for malaria	No evidence of improved growth Seeming reduction in mean number of clinic visits for malaria
Zimbabwe (Friis et al. 1997a,b)	Rural schoolchildren, 11–17 years (n=313)	Zinc (30–50 mg as sulphate) versus placebo, 5 days/week for 7 months	Growth, *Schistosoma mansoni* infections		Intensity of *S. mansoni* reinfections reduced; lean body mass and weight marginally increased
Uganda (Kikafunda et al. 1998)	Nursery school children, age 33–89 months (n=153)	Zinc (10 mg sulphate) versus placebo, 5 days/week for 6 months	Growth		Mid-upper arm circumference increased but no or only limited evidence of marginal gains in height or weight
Ethiopia (Umeta et al. 2000)	Rural children, 6–12 months (n=153)	Zinc (10 mg as sulphate) versus placebo, 6 days/week for 6 months	Linear growth	Diarrhoea, respiratory tract infections	Growth rate dramatically increased in stunted children, and less so in non-stunted infants. Reduction of diarrhoea in stunted and non-stunted infants, whereby reduction was larger among stunted.
Burkina Faso (Müller et al. 2001, 2003)	Rural children, 6–31 months (n=709)	Zinc (12.5 mg sulphate versus placebo), daily for 6 months	Malaria incidence	Diarrhoea, respiratory tract infections, growth	No evidence of an effect on febrile episodes of malaria (IR: 0.98, 95% CI: 0.86–1.11) or on anthropometric indices. 13% reduction in reported episodes of diarrhoea (5–21%)

Country (reference)	**Population** (sample size)	**Design**	**Primary outcome**	**Secondary outcome**	**Main findings**
Ghana (Zlotkin et al. 2003)	Rural anemic children, 6–18 months (n=304)	Zinc (10 mg as gluconate) plus iron (80 mg as ferrous fumarate) versus iron alone, daily for 2 months [1]	Growth		No evidence of improved zinc status or catch-up growth
South-Africa (Bobat et al. 2005)	HIV-infected children, not receiving anti-retroviral therapy; 6–60 months (n=96)	Zinc (10 mg as sulphate) versus placebo, daily for 6 months	Plasma HIV-1 RNA	Diarrhoea and respiratory tract infections at cross-sectional surveys	No effect of zinc of plasma viral load or CD4 counts. Overall odds of diarrhoea (all surveys combined) lower in zinc group (p=0.001)
Ethiopia (Walker et al. 2007)	Infants aged 1–5 months with acute diarrhoea (n=163) (multi-country study also including children in India and Pakistan)	Zinc (10 mg as sulphate) versus placebo, daily for 2 weeks	Linear growth, rate of subsequent diarrhoea		No apparent benefit
Pemba, Tanzania (Sazawal et al. 2004, 2007, Olney et al. 2006, Kordas et al. 2009)	Children (without severe malnutrition), 1–35 months (n=42,546)	Zinc (5–10 mg as sulphate) versus placebo, daily until children reached the age of 48 months	Mortality	Time to walking unassisted, sleep duration, haemoglobin concentration, whole blood zinc protoporphyrin:haem (ZPP:H) ratio, plasma copper concentration	Zinc resulted in reduction in all-cause mortality by 7% (–19% to 6%). Effect seemed more pronounced among children aged > 12 m (18% [0–32%] reduction in all cause mortality). Zinc also resulted in longer sleep duration, increased haemoglobin concentration and decreased ZPP:H ratio

Country (reference)	**Population** (sample size)	**Design**	**Primary outcome**	**Secondary outcome**	**Main findings**
South Africa (Luabeya et al. 2007, Chhagan et al. 2009)	HIV-positive and HIV-negative children, 4–6 months (n=36 and 341)	Zinc (10 mg as gluconate) plus multi-nutrients plus vitamin A versus zinc plus vitamin A versus vitamin A alone, for 18 months. 21%–23% of children in all groups received therapeutic iron during the study (dose/ duration not specified)	2007: % of days with any reported diarrhoea and respiratory tract infections at weekly home visits 2009: incidence of diarrhoea detected at weekly visits	Growth, haemoglobin concentration	2007: No evidence that zinc, or zinc plus multi-nutrients reduce the prevalence of diarrhoea and respiratory tract infections. No difference in effect between subgroups. 2009: zinc and multi-nutrients reduce the incidence of diarrhoea among stunted, non-HIV infected children, but not among non-stunted children. 2010: Improved growth and increase in hemoglobin concentration due to multi-nutrients, as compared to zn+vit A, or vit A alone.

Allocation to treatment was by randomisation unless indicated otherwise.
[1] Nutrients were given as home fortificants with food

1. Zinc and malaria

More than 90% of global malaria deaths occur in African children, and up to 90% of African toddlers in many areas are symptom-free but infected with *Plasmodium* parasites (asymptomatic infection).

Unless restrained by immunity and anti-malarial drugs, populations of *Plasmodium* parasites expand logarithmically at around 10-fold every 48 h (Simpson et al. 2002), thus rapidly reaching potentially lethal densities. Both innate and adaptive immune responses are crucial for the control of *P. falciparum* infection and host survival (Stevenson and Riley 2004, Walther et al. 2006, Newman et al. 2008) but are also associated with the severity of the disease (**Figure 1**). Thus severe malaria is probably at least in part an immune-mediated disease (Stevenson and Riley 2004).

Zinc deficiency results in decreased percentage of precursors to cytotoxic T lymphocytes (Prasad 2000), thus possibly resulting in impaired control of liver stages of *Plasmodium* parasites. Because of its role in innate immunity and in maintaining balance of Th1 and Th2 responses to infection, however, one may expect zinc to have a particularly important role in the control of blood stages of *Plasmodium* spp. Zinc deficiency affects NK cells and neutrophils (see above), both of which play major roles in controlling *Plasmodium* infection (Nnalue and Friedman 1988, Good and Doolan 1990, Stevenson and Riley 2000). Through its effects on NK cells, zinc deficiency can interfere with the production of interferon (IFN)-γ (Figure 1), whilst a strong IFN-γ response is associated with reduced susceptibility to infection (Korbel et al. 2005). Neutrophils are important because they readily phagocytose *Plasmodium*-parasitised erythrocytes coated with opsonin (Perlmann and Troye-Blomberg 2000). Although the exact role of Th1 and Th2 immune responses in controlling *P. falciparum* infection is not entirely clear, the balance between Th1 and Th2 cytokines seems important (Perlmann and Troye-Blomberg 2000).

If, as suggested in the paragraph above, zinc is essential for the functioning of cells from the adaptive immune system, one may expect zinc deficiency to result in a reduced ability to control blood stage proliferation of *Plasmodium* spp. (**Figure 1**). If on the other hand, protection by zinc against infectious diseases were conferred exclusively through its effect on epithelial barriers, then little effect may be expected against uncomplicated malaria, because malaria parasites are injected by infectious *Anopheles* mosquitoes and do not pass through such barriers to enter the body.

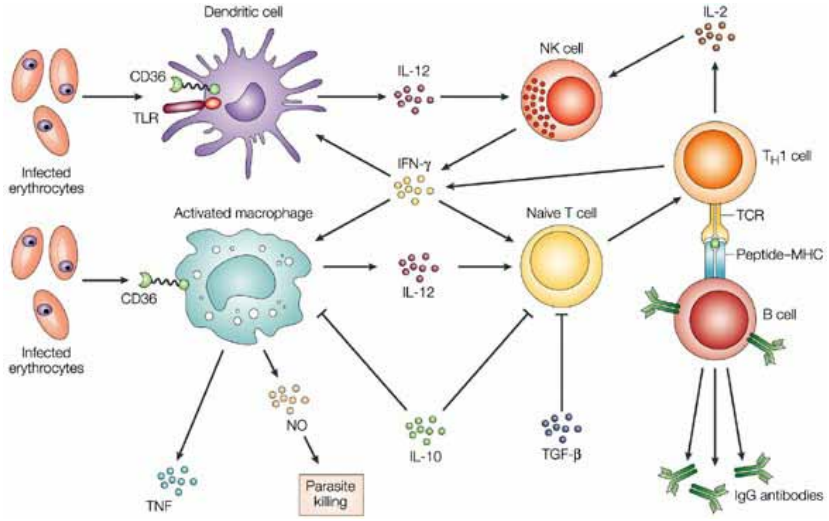

Figure 1. Putative innate and adaptive immune responses to *Plasmodium*-parasitized erythrocytes (Stevenson and Riley 2000)

One of the earliest events in the innate response to a new *Plasmodium* infection might be the activation of dendritic cells and possibly macrophages. Following recognition of parasitized erythrocytes through toll-like receptors (TLRs) or CD36 protein, dendritic cells eliminate these erythrocytes by phagocytosis and present *Plasmodium* antigens at their cell surface. Simultaneously, they upregulate CCR7, a chemotactic receptor protein that leads the dendritic cell to migrate through the blood stream to the spleen, the primary site of immune responses against blood-stage malaria parasites. Plasma concentrations of cytokines such as interleukin-12 (IL-12) derived from dendritic cells and macrophages increase within hours of the emergence of parasitized erythrocytes in the circulation. IL-12 results in activation of natural killer (NK) cells that are mainly found in peripheral blood, the spleen and bone marrow, and that can respond rapidly to *P. falciparum*-parasitized erythrocytes by producing interferon-γ (IFN-γ) and lysing parasitized erythrocytes. IFN-γ further stimulates the maturation of dendritic cells but also results in increased antigen presentation by macrophages, enhanced nitric oxide (NO) production and enhanced killing activity of macrophages of parasitized erythrocytes and free merozoites. The fast response by NK cells and macrophages following the release of *Plasmodium* parasites in the blood stream results in suppression of parasitaemia while allowing the body several days to develop an effective T cell response. In the spleen, by presenting antigens and co-stimulation with cell-surface receptors (CD80, CD86 and CD 40), dendritic cells activate antigen-naive T cells (Th0) and induce them to proliferate and differentiate under the influence of IL-12 into antigen-specific T helper 1 (Th1) cells. IFN-γ, initially produced by NK cells and later also by Th1 cells, assists in this process by promoting Th1 differentiation and suppression of Th2 cell activity. IL-2 produced by Th1 cells further activates NK cells and further amplifies the adaptive immune response. A Th1 response typically involves the proinflammatory cytokines INF-γ and tumour-necrosis factor-α (TNF-α), and is directed at maximising the killing efficacy of macrophages, the proliferation of cytotoxic T cells and the production of opsonizing antibodies. Cytotoxic T cells can affect liver stages but not affect blood stages of *Plasmodium* parasites, however, because erythrocytes, being anucleate cells, do not express major histocompatibility complex (MHC) class I molecules that are needed for recognition by cytotoxic T cell receptors. An

appropriate acquired immune response to malaria is characterised by the ability to control circulating proinflammatory cytokines at levels that facilitate parasite clearance but do not trigger pathology. Th1 responses are classically suppressed by subsequent Th2 responses, which also serve to stimulate B-cell proliferation, to induce B-cell antibody class switching, and to increase neutralizing antibody production. The mechanism by which this switch is achieved is unknown but key immunoregulatory cytokines involved seem to be IL-10 and transforming-growth factor-β (TGF-β) that are produced by macrophages and regulatory T cells, and either of which might contribute to the regulation of innate responses.

When we planned the studies described in this thesis, the effect of preventive zinc supplementation on malaria had been investigated in four trials, with discordant and inconclusive results. In The Gambia, zinc led to a statistically non-significant reduction by 33% in the mean number of clinic visits for malaria (Bates et al. 1993). This trial was designed to measure effects on child growth; illness episodes were recorded through routine clinic procedures and, accordingly, they were inappropriate to adequately measure effects on malaria rates. In Papua New Guinean children, zinc led to a reduction of the number of fevers attributable to malaria due to *P. falciparum* by 38% (Shankar et al. 2000). By contrast, in a recent trial in Burkina Faso, zinc provided no or only little protection against malaria (Müller et al. 2001), despite evidence that the children studied were zinc deficient.

The findings from these trials may have been discordant because the response to zinc supplementation is suppressed in children who are also deficient in other nutrients known to be essential for immunity, or in nutrients that impair zinc absorption or utilisation (Ronaghy et al. 1969, 1974). If so, then zinc and other micronutrients would act synergistically, and simultaneous supplementation with other nutrients might be required to overcome the lack of effect that may occur when zinc is given alone. To our knowledge, the effect of supplementation with multiple micronutrients combined on malaria morbidity has not yet been investigated.

The reports of several related trials became available after we started our studies. In Peru, there was no strong support that zinc reduced malaria risk (Richard et al. 2006). Among children aged 6–72 months in Burkina Faso, combined supplementation with zinc and vitamin A reduced malaria incidence by 30% (Zeba et al. 2008), but the design of this study does not allow attribution of the effect to either zinc alone or vitamin A alone. Lastly, a recent trial in Ghana reported that addition of zinc to the routine intervention package of malaria chemoprophylaxis, iron and folic acid for pregnant women in Ghana was associated with reduced densities of malaria parasites (Saaka et al. 2009). In children initially aged 1–35 months living in

a highly malaria-endemic area (Sazawal et al. 2007), supplementation with zinc resulted in a modest reduction of overall mortality (7%; 95% CI: –6% to 19%) but sub-group analysis suggested that it reduced overall mortality in children aged ≥ 12 months by 18% (0% to 32%), perhaps mostly due to a reduced fatality in children admitted to hospital of with severe malaria (Black et al. 2007).

In a multi-country study, zinc supplementation did not appear to provide benefit as an adjunct therapy in preschool children with uncomplicated malaria due to *P. falciparum* (Zinc Against Plasmodium Study Group 2002).

2. Safety of supplementation with other micronutrients

Despite the potential benefits of nutrient supplementation, its safety is controversial, particularly in malaria-endemic areas. Micronutrients, in addition to having a role in host immunity, may also serve as a nutritional source for pathogens, thus resulting in increased morbidity. A review of the associations between single micronutrients and malaria (Shankar et al. 2000), based on evidence from human and animal studies, shows how little is known about this subject; the evidence available should raise concern, however, about the safety of at least several of these micronutrients.

A randomised trial among children aged 1–35 months in Pemba, Tanzania showed that daily supplementation with iron (12.5 mg as ferrous sulphate) and folic acid increased rates of hospital admission and all-cause mortality (combined endpoint) by 12% (2% to 23%) (Sazawal et al. 2006). This report reinforced earlier concerns that iron interventions can increase the incidence of malaria and infectious disease, even in individuals without iron overload (Oppenheimer 2001, 2002).

The Pemba study is unique in scale and objectives, and important in view of some of the findings to be presented later in this thesis, so its design and findings are described here in some detail. The main trial (n=32,155) was conceived as a 2×2 factorial trial, with either iron, folic acid and zinc; zinc alone; iron and folic acid; or placebo. Because of excess adverse events, supplementation in the two arms receiving iron and folic acid (n=16,070) was stopped ahead of schedule, converting the trial into one with two arms, namely zinc versus placebo.

The study also included a smaller sub-study (n=3,171, with analyses based on 162 adverse events in 2,262 child-years of follow-up) with the same

interventions and surveillance for adverse events as the main trial; however, the sub-study differed from the main trial in that a blood sample was collected at baseline for haematological analysis and for assessment in whole blood of zinc protoporphyrin: haem ratio (used by the authors to indicate iron status) and *Plasmodium* parasite density (number of parasites per unit volume of blood); in addition, children were medically examined at 6 months and 12 months after randomisation, and the diagnostic work-up and therapeutic practices were more extensive. As per local guidelines at the time, the first-line anti-malarial therapy consisted of a combination of antifolate medicines (sulfadoxine-pyrimethamine). Paradoxically, in the sub-study it seemed that iron and folic acid *reduced* the rate of hospitalisation and death by 24% (–9% to 48%). The authors concluded that this discrepancy from the main study was caused by more intensive diagnosis and management of children with malaria and other infections in the sub-study. Lastly, as reported by the authors, subgroup analysis suggested that the supplementation effect depended on baseline iron status: in children with elevated zinc protoporphyrin:haem ratio, or with moderate anaemia at baseline, supplementation protected against adverse events (hazard ratios: 0.62, 0.41–0.93 and 0.59, 0.37–0.92, respectively), whereas in children with normal zinc protoporphyrin:haem ratio or without anaemia, there was no statistical evidence of effect: 1.63, 0.72–3.66 and 1.08, 0.58–1.98).

An expert group convened following the Pemba trial by the World Health Organization (WHO) recommended that iron supplementation in young children (6-24 months) should be restricted in areas where malaria transmission is intense and infectious disease highly prevalent (WHO 2006, 2007). Based on the results of the sub-study, the group recommended that iron supplementation should not be implemented without the screening of individuals for iron deficiency, because this mode of iron administration may cause severe adverse events in iron-sufficient children.

3. Strategies to control deficiencies of zinc and other micronutrients

Based on an analysis of possible interventions, an authoritative group, comprising some of the world's top economists, recently considered supplementation of undernourished children with vitamin A and zinc to be the best investment for advancing global health (www.copenhagenconsensus.com/Home.aspx). Much research is needed, however, to determine cost-effective methods of supplying zinc to deficient populations. Supplements can possibly be delivered to children using existing delivery platforms that provide reasonably frequent and reliable contacts with target groups and

high levels of coverage. Thus zinc supplementation may be integrated with twice-yearly vitamin A supplementation, growth monitoring activities, programmes to distribute anti-parasitic medicines, and delivered through private-sector distribution channels (Brown et al. 2009c).

A global consortium (www.harvestplus.org) led by some of the world's leading agricultural research institutes has embarked on a programme to breed and disseminate new staple crop varieties with high concentrations of micronutrients ('*biofortification*'). This initiative has generated the potential to create a safe, low-cost and self-sustaining approach to deliver zinc to poor farmer families (Hotz 2009). Development and dissemination of such 'biofortified' crops requires large initial research investments and sustained support by national and international policy makers; by contrast to other interventions, however, the long-term recurrent costs are low. Because of technical constraints, and contrary to other methods of delivery such as supplementation or fortification, the biofortification approach must focus on single nutrients or at most a few nutrients. This imposes a need to identify micronutrients that are critical to health, and reinforces the need to determine to what extent zinc is efficacious when given alone, or in combination with other micronutrients. Efforts to develop typically 'African' crops rich in zinc have so far been limited, and require increased support that is probably best obtained when showing compelling evidence of the health benefits of increasing zinc intake.

Fortification of staple cereal flours is a cost-effective, sustainable way to improve nutritional status in developing countries. Zinc fortification is technically feasible even if questions remain on the appropriate formulation of fortificant zinc and the target dosage that are required for efficacious fortification (Brown et al. 2010). International efforts to advocate flour fortification have nonetheless produced a small but rapidly increasing number of African countries planning to include zinc in national flour fortification programmes. The beneficiaries will be mainly urban and peri-urban populations who buy industrially processed flour; the prospects are more limited for small-scale fortification of flour for rural families relying in subsistence farming. In addition, it remains to be investigated to what extent fortification levels can be adjusted to meet the dietary needs of young children but without exceeding the tolerable upper intake levels for other population groups.

4. Aims and outline of the thesis

The long-term objective of this study was to develop efficacious and safe nutritional interventions to control common infectious diseases in African preschool children.

The specific aims were as follows:

1. To assess the effect of supplementation with zinc, alone or in combination with other nutrients, on the rates of malaria (primary objective);
2. To assess intervention effects on rates of other common diseases (diarrhoea, respiratory infections, non-malarial febrile illness);
3. To identify factors that determine the magnitude of the effect of the interventions;
4. To assess the effect of α^+-thalassaemia on rates of malaria and on the degree of anaemia associated with *Plasmodium* infection.

The field work was conducted in Tanzania and comprised two studies. First, we conducted a pilot survey (May–July 2006) with the primary aim to identify communities with a high prevalence of malaria that would be suitable for inclusion in a subsequent randomised controlled trial (February 2008–March 2009). Objectives 1–3 were pursued in the trial, the results of which are described in Chapters 2–5. This was primarily conceived as an explanatory trial (i.e. it aimed at measuring effects that can be achieved when supplements are administered under optimal, controlled conditions; Schwarz and Lellouch 2009) although some aspects were rather pragmatic (i.e. to guide practice by measuring effects under real-life conditions).

The trial also provided an opportunity to assess an issue not directly related to the primary objective: in Chapters 6 and 7, we assessed to what extent α^+-thalassaemia is associated with protection against malaria (objective 4). This haemoglobin disorder is highly prevalent in eastern Africa, and has recently been reported to protect against severe malaria. Another spin-off about the safety of iron fortification is provided in **Annex 1**.

The study site in Tanzania: Our studies were carried out in a lowland area (~250–350 m altitude) in Segera and Kwedizinga wards, Handeni District, northern Tanzania, close to the junction of the tarmac roads Tanga–Dar es Salaam and Moshi–Tanga (**Figure 2**). Virtually all villagers in the area live in self-constructed clay houses with thatch roofs, and there is no access to electricity or running water. The vast majority of people depend on subsistence farming, while some produce oranges as cash crops.

To our knowledge, no studies had been conducted previously in this area. We expected transmission patterns to be similar, however, to those in adjacent area towards the East, where previous entomological studies and parasitological surveys found transmission to be mostly by *Anopheles gambiae* s.s. and *An. funestus*, and to a small degree by *An. arabiensis* (Dr. Caroline Maxwell, personal communication, 2008) with people being bitten on average by *Plasmodium*-infected *Anopheles* mosquitoes 35–400 per person per year (Ellman et al. 1998).

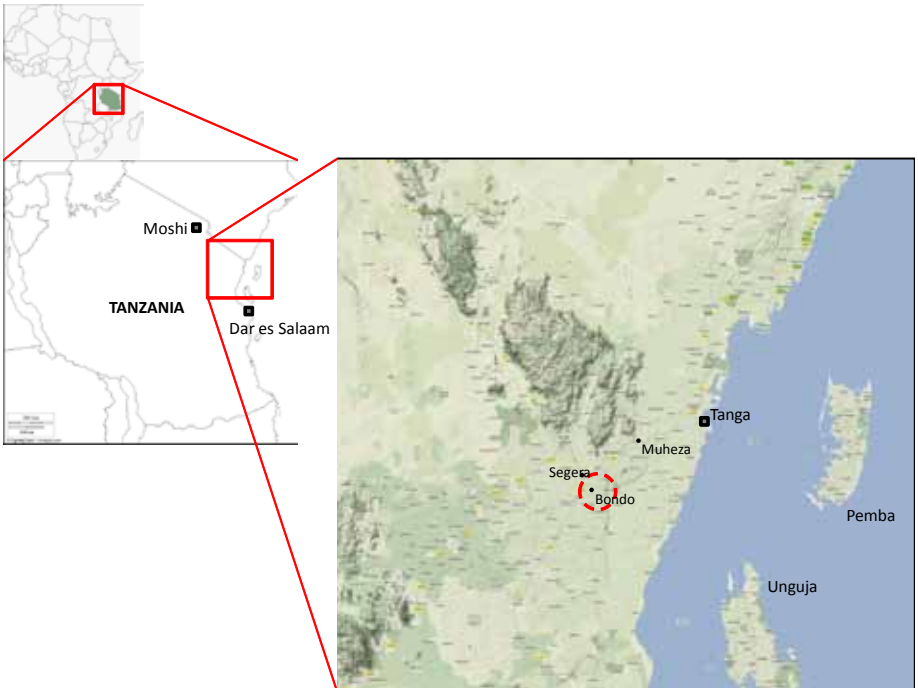

Figure 2. Location of the study site in north-eastern Tanzania
The study site is marked by a dashed red circle. Zanzibar, a semi-autonomous part of Tanzania, consists of two main islands: Pemba and Unguja.

The pilot survey was conducted in a predefined area that included several villages (Chan'gombe, Segera, Michungwani) along the tarmac roads, as well as an inland area to the east of Michungwani village (05°20.089 S, 38 °32.937 E). We used a lot quality sampling technique (Lanata and Black 1991, Lemeshow and Taber 1991) that is statistically efficient in distinguishing between 'lots' (communities) with high and low malaria prevalence. Thus, we conducted a census in the study area, and derived a list of all resident children aged 6–72 months (total: 2,472 children). Based on the existing

administrative boundaries, the overall population was divided into 19 lots.

By means of this list, 16 children were randomly selected from each of the 19 lots, resulting in a total of 304 sampled children. Communities were eligible for the trial when ≥ 9 of 16 children tested were infected with *P. falciparum* as assessed by an antigen test for *P. falciparum*-specific lactase dehydrogenase (pLDH). With communities of 100 children, the procedure provided 90% probability of including a community with a prevalence of infection of > 63%, and 90% probability of excluding a community with a prevalence of infection of < 37%. In smaller communities, this strategy would lead to even better performance to distinguish between communities with high and low prevalence, whereas this performance would be only marginally worse in larger communities (not shown).

This process resulted in the selection of a contiguous area that we used for the intervention study, with an overall prevalence of *P. falciparum* infection of 71%. It was located along a dead-end, unpaved road to the east of Michungwani village, and contained four villages (Ngojoro, Bondo, Kwangwe and Kwadoya). There is no public transport to the main road, except on Saturday (market day), when there is limited service by local minibuses between the furthest village (Kwadoya) and the main road. The road becomes difficult to travel in the rainy season. Access to healthcare was limited before the trial, with the nearest health care facility in Segera village, which is located at a range of 8–25 km from the homesteads of the children studied. In collaboration with local villagers and authorities, we constructed and staffed a research clinic at a central location in the study area (Bondo village, 5°22'60'' S, 38°34'60'' E).

Several findings from the pilot survey are not described in subsequent chapters of this thesis but provide a context for our studies. We found the following prevalence estimates (methods described in subsequent Chapters): anaemia (haemoglobin concentration < 110 g/L): 50%; inflammation (whole blood C-reactive protein concentration > 8 mg/L): 34%; *P. falciparum* infection (positive test result for pLDH-based dipstick test): 46%; *Helicobacter pylori* antigenaemia (Amplified IDEIA Hp StAR enzyme immunoassay test, DakoCytomation, Ely, UK): 31%; *Giardia intestinalis* infection (by microscopical examination of a single stool sample per child): 30%; α^{+}-thalassaemia (by PCR test): 38%; being stunted, wasted and underweight (z-scores height-for-age, weight-for height and weight-for-age < –2 SD, respectively): 39%, 2% and 22%, respectively. Microscopic examination of a single stool sample per child revealed a single case of hookworm; no cases were reported of *Trichuris trichiura*, *Ascaris lumbricoides* or *Schistosoma* infestation. In a multivariate

regression model, we identified deficiencies of iron, vitamin B_{12}, and possibly vitamin A as independent risk factors for anaemia (**Table 2**). It was estimated that the prevalence of these disorders was 13%, 4% and 13%, respectively.

Table 2. Factors associated with haemoglobin concentration, pilot survey (n=304)

	Association with haemoglobin concentration, g/L			
	Crude	(95% CI)	Independent	(95% CI)
Nutritional factors [1]				
Iron deficiency	–7.8	(–13.6 to –2.0)	–12.7	(–18.5 to –6.8)
Vitamin A deficiency	–9.9	(–15.7 to –4.0)	–3.0	(–8.8 to 2.8)
Vitamin B_{12} deficiency	–15.8	(–25.9 to –5.8)	–12.4	(–21.5 to–3.4)
Infection-related factors				
Plasmodium spp.	–8.9	(–12.8 to –5.0)	–8.5	(–13.4 to –3.6)
Helicobacter pylori	–1.0	(–5.3 to 3.3)	–1.0	(–5.0 to 3.0)
Inflammation	–13.2	(–17.4 to –9.0)	–9.7	(–14.0 to –5.3)
α^+-thalassaemia				
Without *Plasmodium* infection				
Heterozygous	0.7	(–4.6 to 6.0)	–0.5	(–5.7 to 4.8)
Homozygous	–17.8	(–30.8 to –4.7)	–14.3	(–27.2 to –1.3)
With *Plasmodium* infection				
Heterozygous	0.3	(–6.5 to 7.1)	0.7	(–7.2 to 8.6)
Homozygous	–5.2	(–20.1 to 9.7)	–7.0	(–25.2 to 11.3)

Source: Veenemans et al. 2009

[1] Deficiencies of iron, vitamin A and vitamin B12 were defined by plasma concentrations of ferritin, retinol and cobalamin < 12 μg/L, < 0.70 mmol/L and < 150 pmol/L, respectively.

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Chapter 2

Issues in study design and analysis

Jacobien Veenemans, Hans Verhoef

The primary objective of the trial described in this thesis was to assess the effect of supplementation with zinc, alone or in combination with other nutrients, on the rates of malaria (see Chapter 1). The aim of this chapter is to describe several features of the design and analysis of the trial that are important for the interpretation of its findings. The results of the trial will be presented in subsequent chapters.

1. Factorial design

If adding vitamins and other minerals does not enhance the efficacy of zinc supplementation, then there is no use in combining zinc and other micronutrients. We employed a full factorial design, whereby each participating child was randomised to one of four groups receiving either placebo, zinc alone, vitamins and other minerals ('multi-nutrients alone'), or multi-nutrients including zinc. Such a design is unique in allowing the investigation of interaction between interventions. Thus, a key issue in our study was to what extent the rate ratio achieved by combined supplementation with multi-nutrients including zinc diverged from the product of the rate ratios achieved by supplementation with either zinc alone or multi-nutrients alone. A limitation of such a design, however, is that precision achieved is often insufficient to provide conclusive statistical evidence for such interaction if extant in the study population, unless the interaction effect is relatively large. Thus we conservatively determined the sample size of our trial on expected rate reductions by single interventions (zinc alone or multi-nutrients alone) relative to placebo, and a further rates reduction by combined intervention (multi-nutrients including zinc) relative to single interventions. We were guided in our exploration of interaction mostly by the magnitude of the estimated interaction effect and not only by the corresponding confidence interval or p-value (McAllister et al. 2003).

When assuming absence of interaction, i.e. the interventions act independently, data can be analysed 'at the margins' (McAllister et al. 2003), in our case, by comparing the pooled two groups receiving zinc (with or without multi-nutrients) versus the pooled two groups not receiving zinc (placebo or multi-nutrients); conversely, the effect of multinutrients can be analysed by comparing groups receiving multi-nutrients (with or without zinc) versus those without multi-nutrients (placebo or zinc). Under this assumption, the factorial design has the advantage that the number of participants required to evaluate the effects of two interventions is identical to the sample size required to assess single interventions. In this sense, a factorial design can 'achieve two trials for the price of one': every participant

contributes information to each of the randomized factors simultaneously (Lubsen and Pocock 1994).

Analysis of first episodes versus analysis of repeated events: Most trials have assessed intervention efficacy against malaria as group differences in time to first episode or incidence of first episodes. In highly endemic areas, however, children often experience multiple episodes of malaria over time, and the exclusion of second and subsequent episodes is controversial and may lead to biased estimates of intervention efficacy (Cheung et al. 2010).

Because of differences between individuals in exposure to infectious mosquito bites, immunity and therapy-seeking behaviour, some children experience malaria more frequently than others (**Figure 1**). In addition, when children are studied longitudinally, between-individual variation may occur in the frequency of malaria because of differences in the time of recruitment and follow-up in relation to the transmission season.

In the presence of such heterogeneity in susceptibility to malaria, 'high risk' individuals tend to become sick more rapidly than 'low risk' individuals, and once they have experienced an episode they are no longer considered in a time-to-first-event analysis. Thus, with the passing of follow-up time, only the 'low risk' individuals remain in the risk set and, accordingly, the event rate observed will decrease. In randomised trials with efficacious interventions, this decrease is generally more pronounced in groups with high overall event rates (placebo group), which have 'high-risk' individuals dropping out more efficiently. Thus in time-to-first-event analysis, group contrasts can become underestimated and efficacy may appear decrease with time.

Due to genetic, nutritional and immunological factors, some children may also benefit more from an intervention than others. Because of this heterogeneity in response to intervention, a protective effect introduces an additional selection process in time-to-first-event analysis: 'low-responders' tend to develop malaria and leave the risk set earlier than 'high-responders'. Because this process does not occur in the control group, it may result in an overestimate of intervention efficacy (Halloran et al. 1996, (Valim et al. 2008, White et al. 2010).

To better reflect the impact on the total burden of disease and to eliminate sources of bias that are described in the preceding paragraphs, we based our estimates of intervention efficacy on the total number of episodes and rather than time-to-first event analysis (**Annex 2**). We used a robust estimate of

the standard error to account for dependence among multiple events within persons, because confidence intervals will be underestimated when this is not done.

2. Case definition and detection

The validity of efficacy estimates greatly depends on a sensitive and specific detection of cases. In settings that are highly endemic for malaria, the performance of any diagnostic test is limited by the fact that, at any point in time, a considerable proportion of the paediatric population under study is asymptomatically infected. In such children, incident episodes of non-malarial febrile illness would be misclassified as being malaria. The resulting lack of specificity normally results in intervention efficacy being underestimated (O'Meara and Lang 2009).

To redress this problem, we tested all children at baseline with a highly sensitive malaria dipstick test, and gave all those with infection a 3-day course of anti-malarial drug combination (artemether-lumefantrine) that is highly efficacious in clearing asexual parasites (Annex 2). Thus we assumed that children subsequently presenting with fever or inflammation and with confirmed infection would have malaria due to a newly acquired infection. Our primary endpoint was defined accordingly as: *a)* reported febrile illness with axillary temperature ≥ 37.5 °C and a positive result for a *Plasmodium-*

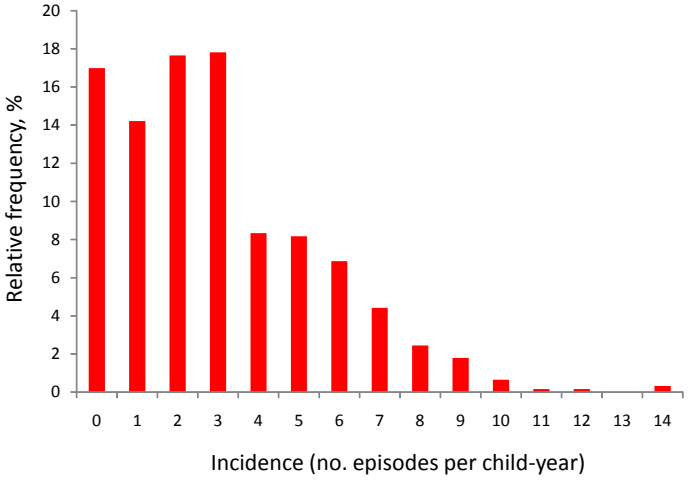

Figure 1. Number of malaria episodes per child, per year
Based on data collected during the trial described in this thesis (methods: see Chapter 3)

specific pLDH-based dipstick test; or *b)* reported (but unconfirmed) febrile illness with inflammation (whole blood C-reactive protein concentration ≥ 8 mg/L) and a positive result for a pLDH dipstick test.

Another, widely used approach to increase the specificity of the malaria case definition is to attribute a febrile episode to malaria only when the density of asexual parasites exceeds a pre-specified threshold value (Schellenberg et al. 1994, Smith et al. 2004). This method has been advocated by WHO for the use in malaria vaccine trials (Moorthy et al. 2007) and is based on the observation that high parasite densities are found less frequently among asymptomatic children in cross-sectional surveys, and the idea that the probability of fever is associated with parasite density. Based on a logistic regression model of this association, the probability can be calculated that a fever case is attributable to malaria given the parasite density observed at time of illness presentation. The pyrogenic threshold for uncomplicated malaria depends on age, transmission intensity and season (as proxy indicators for immunity) but is usually within the range of 0–6,000 asexual parasites/μL for infants, and 2,500–7,500 asexual parasites/μL for children aged 12–60 months (e.g. McGuinness et al. 1998, Bloland et al. 1999, Mwangi et al. 2005).

When designing our studies, we did not apply such threshold approach, for the following reasons:

a. Microscopy, the conventional standard for the detection and counting of malaria parasites in peripheral blood, is in many aspects deficient for this purpose. The method suffers from high inter- and intra-observer variability, particularly when the true parasite density is low (**Figure 2**) (O'Meara et al. 2007, Hänscheid 1999). A low precision can be inevitable even under ideal circumstances. For example, if the leukocyte count is 8,000/μL, and one counts an area of the slide in which a total of 200 leukocytes are found, as is typical in many studies, the volume of blood examined is only 0.0625 μL. At a true parasite density of 150/μL, one would expect this to result in only four parasitized cells being counted. However, repeated examination would result in 95% of times to 1–8 parasites being counted (calculations based on Poisson distribution), corresponding to a range of estimated densities of 40–320 parasites/μL. Particularly for infants, this range is disturbingly large in view of low thresholds reported in some studies (e.g. 100/μL and 150/μL in the dry and wet seasons in an area with an entomological inoculation rate of 8.5 infective mosquito bites per person per year, McGuinness et al. 1998). More studies seem warranted to assess the influence of this random error on the threshold value.

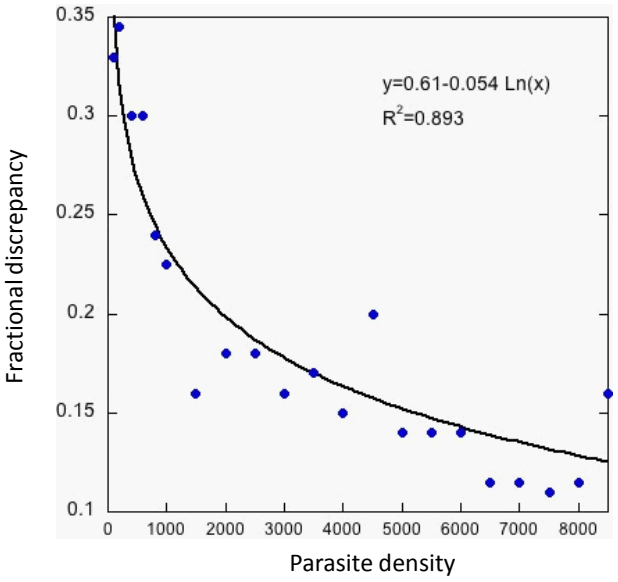

Figure 2. Discrepancy in density between two microscopists reading a single slide as a function of parasite density (O'Meara et al. 2007)

In addition, within individuals, great variations in parasite density have been observed within short periods of time: in a single untreated individual, the parasite density may vary by more than 100-fold within 6 h (Delley et al. 2000, Koram and Molyneux 2007). Asexual stages of *P. falciparum* have a 48-h life cycle, of which only the first half is visible to the microscopist. At 16–24 h after parasite invasion, erythrocytes start expressing adhesins on their surface, aggregate with uninfected erythrocytes (rosetting) and also begin to adhere to vascular endothelium, particularly in the venules (cytoadherence). Thus, depending on stage and synchronicity, a predominance in parasite biomass in the body may be either circulating and detectable, or sequestered in the deep vasculature and not detectable (White 1997). Pathophysiological processes in *P. falciparum* malaria are probably due to the mature forms of the parasite and subsequent release of pyrogens during erythrocyte lysis, and not to the younger circulating asexual stages. Thus patients tend to be more ill when the majority of their parasites are sequestered or just after schizont rupture (White 1997), which further complicates the loose relationship between parasitaemia and disease severity.

A practical problem, which also complicated the use of parasite density in our trial, is that it is not always logistically feasible to obtain leukocyte counts for blood samples collected from sick children. We would thus be restricted to using an assumed, fixed leukocyte count. Because malaria is known to suppress immunity and to produce leukopaenia (McKenzie et al. 2005), this could lead to substantially overestimated parasite counts. On the other hand, many children may have had concurrent bacterial infections, with an accompanying increase in leukocyte count, which could lead to substantial underestimates of parasite density. In addition, if the intervention would affect leukocyte counts during illness, as may theoretically occur with nutrient interventions, this would introduce additional bias.

b) Many assumptions must be made when establishing such threshold and its application does not guarantee an unbiased assessment of intervention efficacy (Smith 2007). First, because these thresholds are usually established based on a single survey, this approach does not account for seasonal and other external variations in the relation between density and fever risk, which may occur during the follow-up of trial participants.

Second, applying a threshold that uniformly applies to groups of children does not reflect heterogeneity in individual fever thresholds. Because malaria susceptibility will depend on immunity, age and several other factors, such a threshold will vary between individuals and – assuming that the threshold depends on previous malaria episodes – even within individuals. Thus misclassification of cases cannot be avoided. When affecting a similar proportion of cases in intervention groups being compared, such misclassification would increase the similarity between groups so that any true intervention effect would be underestimated (Hennekens and Buring 1987).

Third, the threshold approach assumes that the intervention under evaluation reduces malaria risk solely by reducing parasite densities and thus the proportion of infected children who exceed the fever threshold, but it fails when it also influences the fever threshold itself. In our trial, we expected this to be an important limitation because zinc and other micronutrients may achieve their protective effects at least in part by improving immunity, which is likely to modulate the relationship between parasite density and fever risk, and to influence the fever threshold. Thus the use of a single threshold would result in a differential specificity of the case definition between intervention arms, which causes

bias in the efficacy estimates that is difficult to control for, and of which magnitude and direction are difficult to predict. It can be argued that the use of different density cut-offs per intervention arm may partly solve this problem, but this is less easy to interpret, and such cut-offs cannot be specified in advance.

3. Subgroup analysis

Subgroup analyses are conducted to assess to what extent individuals with certain characteristics respond differently to the intervention than those without. In quantitative interaction, the intervention is either superior or inferior for all subgroups but its effect is more marked in one subgroup. In qualitative interaction, the treatment effect is in opposite directions in different subgroups. Although indications for interaction may be obtained by stratified analysis, it should be formally investigated by assessing interaction between the subgroup variable and the intervention in a multiple regression model (Yusuf 1991, Parker et al. 2000).

Subgroup analyses feature prominently in this thesis. Even though their results can be informative, the extent to which they should influence the interpretation and conclusions in a trial report is open to dispute (Yusuf et al. 1991, Assmann et al. 2000, Fletcher 2007, Wang et al. 2007, Sun et al. 2010). On one hand, the sample size is often insufficient to provide adequate statistical support for interaction. On the other hand, the abundance of baseline factors that are usually measured creates the risk of assessing intervention effects in multiple subgroups in the hope that something will 'come up' that is 'statistically significant'. Selective reporting of such factors leads to publication bias.

Criteria to evaluate the credibility of subgroup analyses are provided by several authors (e.g. Oxman and Guyatt 1992, Wang et al. 2007, Sun et al. 2010). The evaluation of subgroup effects should not be framed in terms of absolute rejection or acceptance but one should rather assess the likelihood that a subgroup effect is real. To enhance their plausibility, anticipated subgroup effects (and their direction) should be predefined based on expected mechanisms and preferably in the context of similar findings from other studies. Statistical examination of the interaction effect should confirm that the observed differences are indeed unlikely to have occurred by chance. Consistency of subgroup effects across closely related outcomes enhances its credibility. Ideally, it should be shown that differences in effect between subgroups are independent of factors that may be associated with the criteria

that define the subgroups, although this assessment is usually compromised by limitations in the number of events that occurred. Exaggerated subgroup claims that are not sufficiently supported by the evidence or even coincidental findings may mislead decisions about intervention policies and should be avoided (Pocock et al. 2002).

On the basis of previous evidence and presumed mechanisms, we anticipated several interactions and, accordingly, predefined a limited number of subgroup analyses in a pre-established analysis plan for the primary outcome variable. In accordance with established guidelines (ICH 1998), we reviewed and updated this plan before breaking the blind (Annex 2).

4. Ethical aspects

Justification to include placebo: The use of a placebo is essential to reach conclusions about efficacy. We felt that the use of zinc placebo was justified because there are no national policies for micronutrient supplementation in Tanzania, and there was substantial uncertainty about the benefits of zinc and multi-nutrients in our study population (Verhoef 2007). We also anticipated that the implementation of the trial would provide benefits to all participating children, including those who received placebo. Because of the research dispensary that we constructed for this trial, all children in the area had 24h/day access to primary care and received medical free of charge for common childhood illnesses. Those receiving placebo were probably better off than non-participating children because they were seen daily by community health workers and, when they were sick, we administered diagnostic tests that were not available to non-participating children.

Based on evidence for age interaction and significant mortality reduction in children older than 12 months found in the Pemba trial (Sazawal et al. 2007), Black and colleagues (2007) questioned the ethical acceptability of using placebo for zinc in children older than 12 months. To some extent, this claim seems supported because the authors reported to have pre-specified such subgroup analysis in their study protocol. It is inappropriate, however, to assess effect modification by statistical significance within subgroups, because it uses a within-subgroup comparison to draw inferences about between-subgroup differences (Parker et al. 2000), whilst the p-value reported (0.07) (Sazawal et al. 2007) does not provide conclusive evidence for such effect modification by age, particularly in the context of a trial with multiple subgroup analyses (Lagakos 2006, Sun et al. 2010).

Although the uncertainty principle is widely used to ethically justify a randomised trial, 'substantial uncertainty' is a subjective judgment, and it is difficult to see who should make this judgment, how it should be practically interpreted and how consensus can be reached (Senn 2003). There may be sceptics who disagree with the 'substantial uncertainty' about the benefits of zinc, and argue that it is not ethical to include a placebo, on the grounds that poverty prevents the study population from having access to zinc and prevents the implementation of policies to increase zinc intake. In this view, the use of placebo would be unacceptable, and our trial would lack a rationale for assessing the efficacy of zinc. However, an alternative defence is possible: because zinc is not generally available to the study population, our trial represents the only chance for eligible children of getting zinc. Thus a prohibition of the trial on ethical grounds would be against the interest of the eligible child (or his/her guardian).

Justification to include iron and folic acid in the supplement: Critics may also argue that universal supplementation with iron and folic acid is unethical in view of the findings of the Pemba study that was reviewed in the preceding sections of this chapter. In the main trial of the Pemba study, however, children were not offered malaria prevention, diagnosis or treatment services other than those available through routine care by the primary health system in Zanzibar. By contrast, we treated all *Plasmodium*-infected children at baseline; excluded those with haemoglobin concentration < 70 g/L; provided iron and folic acid in a supplement that also contained other micronutrients, at least one of which (vitamin A) has been reported to protect against malaria (Shankar et al. 1999); improved access to care through construction of the research dispensary and provision of free transport for children referred to hospital; conducted a medical examination at a survey during the follow-up period; provided medical examinations, diagnostics and treatment for common childhood diseases free of charge; and we were warned of severe disease in study children through daily reports from community workers. These measures were probably more extensive than those used in the Pemba sub-study, where iron and folic acid protected against severe adverse events. A recent systematic review, which included data from the Pemba study, also concluded that iron does not increase malaria incidence or death when regular malaria surveillance and treatment services are provided (Ojukwu et al. 2009).

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Chapter 3

Preventive supplementation with zinc and other micronutrients to control malaria and other febrile illnesses in Tanzanian children: a randomised trial

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Submitted for publication

Summary

Background: Protection by zinc supplementation against malaria may depend on concomitant supplementation with other nutrients. We measured the effect of supplementation with zinc and other nutrients on the rates of malaria and other febrile illnesses.
Methods: In a highly malaria-endemic area in Tanzania, we selected children aged 6-60 months with height-for-age z-score ≤ –1.5 SD. *Plasmodium*-infected children were treated with artemether-lumefantrine at baseline. Children (n=612) were randomly assigned to daily oral supplementation with zinc alone (10 mg), multi-nutrients without zinc, multi-nutrients with zinc, or placebo. The primary outcome, episodes of malaria, was assessed in children reported sick at a primary care research clinic. In the primary, intention-to-treat analysis, we adjusted for pre-specified baseline factors that were prognostic for outcome (age class, *Plasmodium* infection status, distance between homestead and clinic, height-for-age z-score and mosquito net use).
Findings: The primary analysis included 1,572 episodes of malaria during 526 child-years of observation. Supplementation with zinc, multi-nutrients without zinc and multi-nutrients with zinc resulted in adjusted hazard ratios of 0.99 (95% CI: 0.82-1.18), 1.04 (0.87-1.23) and 1.14 (0.96-1.35), respectively. Corresponding values for non-malarial febrile illnesses were 0.79 (0.63-0.99), 0.83 (0.65-1.06) and 0.85 (0.68-1.07). In the first 100 days of intervention, and in the analysis of first events, supplementation with multi-nutrients, with or without zinc, increased the hazard of malaria by one-third.
Interpretation: In this zinc-deficient population, we found no evidence that zinc supplementation protected against malaria. However, it reduced the rate of non-malarial fevers. Supplementation with multi-nutrients including iron may be unsafe in malaria-endemic areas, even when recipients have excellent access to health care. (ClinicalTrials.gov number, NCT00623857).

Introduction

Preventive zinc supplementation can reduce the burden of diarrhoea and respiratory tract infections (IZiNCG 2009). If effective against malaria, it would be a tremendous advance in public health, particularly in Africa, where 90% of malarial deaths occur. Anti-malarial efficacy has been investigated in four trials, with discordant results. In Gambian toddlers, twice-weekly zinc seemed to reduce the mean number of clinic visits for malaria by 32% (Bates et al. 1993). In Papua New Guinea, daily zinc reduced rates of malaria due to *Plasmodium falciparum* by 38% (Shankar et al. 2000). By contrast, in Burkina Faso and Peru (Müller et al. 2001, Richard et al. 2006), there was no evidence of protection, despite zinc deficiency being highly prevalent at baseline and reversed by supplementation.

Malaria was detected among children self-reporting at clinics in Papua New Guinea and The Gambia, whereas cases were detected through regular home visits in Burkina Faso and Peru, possibly indicating that zinc supplementation is more efficacious in preventing severe malaria than mild episodes. Alternatively, the response to zinc may be suppressed when children are also deficient in other nutrients (Ronaghy et al. 1969, 1974). Simultaneous supplementation with other nutrients may be required to overcome a lack of effect of zinc supplements when given alone.

Our study aimed to assess the effect of supplementation with zinc, alone or in combination with other nutrients, on the rates of malaria and other febrile illnesses among Tanzanian children.

Methods

Study population: We conducted the study between February 2008 and March 2009, in a rural area in Handeni District, Tanzania (Veenemans et al. 2008) where malaria transmission is intense (Ellman et al. 1998). As per national guidelines, we treated uncomplicated malaria with artemether-lumefantrine (Novartis Pharma, Basel, Switzerland). This drug combination is highly efficacious (Ogbonna et al. 2008, White 2008), and was available for free at government health-facilities but not in local shops. Participating children received free medical care for common illnesses. Because of the strategic location of the research clinic, and based on interviews with local informants, we believe that very few sick participants were brought to other health facilities, or were treated at home.

The study (ClinicalTrials.gov: NCT00623857) received ethical clearance in The Netherlands and Tanzania; parents/guardians gave written consent. There were no important changes to methods after trial commencement.

Study design: In this explanatory efficacy trial with a 2×2 factorial parallel design, children randomly received daily supplements with: a) both zinc and multi-nutrients (**Annex 3**); b) zinc; c) multi-nutrients without zinc; or d) placebo.

Recruitment: In four villages, we listed all resident children aged 6-60 months and screened them in daily batches until attaining the target number (n=600) in August 2008 (**Figure 1**). We computed anthropometric indices as the average of two recordings, taken on consecutive days. Following a physical examination, we collected venous blood in EDTA-tubes suitable for trace element analyses (Becton-Dickinson, Franklin Lakes, NJ). We centrifuged one aliquot immediately after collection and stored plasma in liquid nitrogen; we examined a second aliquot by haematology analyzer (Sysmex KX21, Kobe, Japan).

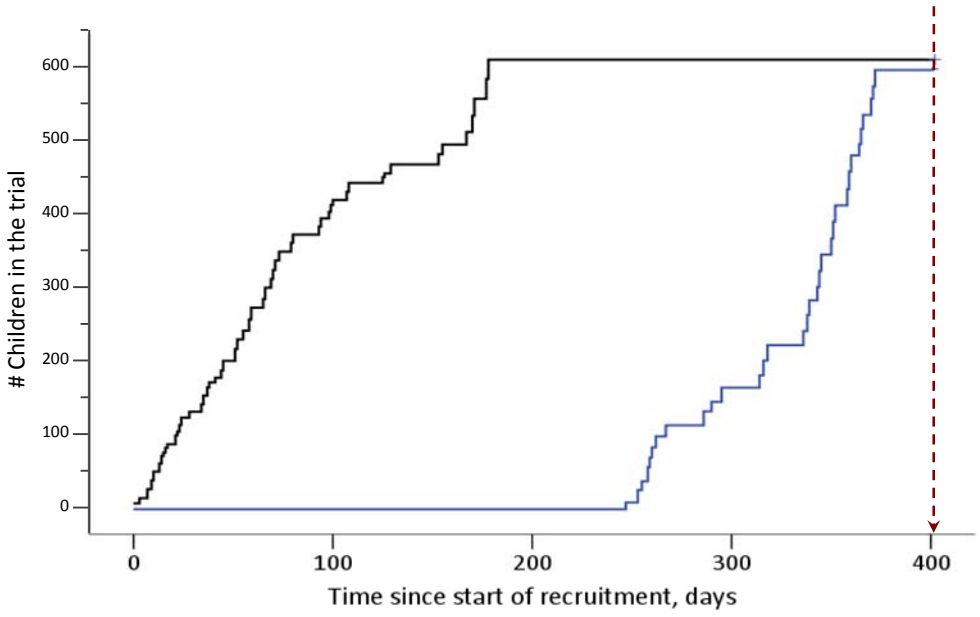

Figure 1. Participant flow over time. The black line indicates the cumulative number of children in the trial; the blue line indicates the cumulative children who had been included in the second survey; the dashed line indicates the date that the trial was stopped.

We used malaria dipstick tests (CareStart, G0121, Access Bio, Monmouth Jct, NJ, USA) to detect lactate dehydrogenase produced by live *P. falciparum* or other human *Plasmodium* species. This test has a sensitivity of 96% for samples with > 50 *P. falciparum* parasites/mL (Piper et al. 1999). Blood films were prepared for all children. Children with *Plasmodium* infection were treated with artemether-lumefantrine.

We excluded children with height-for-age z-scores > –1.5 SD (who are at lower risk of zinc deficiency) (Brown et al. 2002), weight-for-age z-score < –3 SD, haemoglobin concentration < 70 g/L, those unlikely to comply with interventions, whose parents/guardians refused consent, or with signs of severe or chronic disease.

Randomisation and blinding: A colleague not otherwise involved in the trial used tables with random numbers to generate randomly permuted blocks with random size 4 or 8 within each of 6 strata defined by *Plasmodium* infection (yes/no infected) and age class (6-17 months, 18-35 months and 36-60 months). Interventions were indicated by colour code on paper slips in opaque, consecutively numbered envelopes that were prepared in advance, in excess of the expected number required. A simple colour code was employed to minimize the possibility of children receiving the wrong supplements.

Supplements, as powder in colour-coded capsules, were contained in blister packs, and administered orally after suspending capsule contents in clean water or breastmilk. All types of powder had similar appearance, smell and taste. At the end of each screening day, when eligibility had been fully established, children were individually allocated in order of their screening number to intervention groups by drawing successive envelopes from a box corresponding to the infection- and age-specific stratum for that child. The number of the envelope was then recorded on a list before the envelope was opened. The randomisation code was not revealed to the researchers until data collection and the database had been completed.

Follow-up and case detection: Community volunteers administered supplements 7 days per week close to the homes of participating children and reported daily to field staff, who followed up the same day in case of non-compliance. Field staff made regular, unannounced spot checks to ensure adherence to procedures. Supplementation and follow-up continued for all children until 12 March 2009, when the trial was stopped.

At recruitment, we asked parents to bring study children to the clinic if their

child would develop fever or became unwell. A clinical officer was on 24h-duty and collected medical information on standardised forms. According to standard procedures, axillary temperature was measured and dipstick tests administered for any child with current or recent guardian-reported fever; for those with positive dipstick test results, we prepared blood films and measured whole-blood C-reactive protein concentrations and haemoglobin concentrations using point-of-care tests (QuikRead, Orion Diagnostica, Espoo, Finland and HemoCue, Ängelholm, Sweden, respectively).

A second survey, at 251 days (median; 95% reference range: 191-296 days) after enrolment, followed similar procedures.

Laboratory analyses: Plasma concentrations of C-reactive protein and ferritin were measured (Meander Medical Centre, Amersfoort, The Netherlands) on a Beckman Coulter Unicel DxC880i system according to the manufacturer's instructions. Plasma zinc concentrations were determined by inductively-coupled plasma-mass spectrometry (Varian 820-MS; CV: 9% at 26.8 μM; 13% at 21.25 μM and 13% at 15 μM; n=32, V=10 μL).

Statistical analysis: Data were analysed following a pre-specified plan, by intention-to-treat, using SPSS (v15·0 for Windows, SPSS, Chicago, IL, USA), CIA (v2.1.2) (Altman et al. 2000) and STATA (v11; College Station, Tx, USA). Compliance was measured as the proportion of children who consumed > 95% of scheduled supplements. Nutritional status was defined by the presence of iron deficiency (plasma ferritin concentration < 12 μg/L; UNICEF/UNU/WHO 2001), zinc deficiency (plasma zinc concentration < 9.9 μmol/mL; IZiNCG 2004) or being stunted (height-for-age z-score < –2 SD). Because inflammation can influence these plasma concentrations independently of iron and zinc status, we also conducted analyses that were restricted to individuals without inflammation.

The primary outcome, an episode of malaria, was pre-defined as a positive result for the malaria dipstick test in children with guardian-reported fever and any of the following: a) confirmed fever (axillary temperature ≥ 37.5°C), or b) unconfirmed fever with inflammation (whole blood C-reactive protein concentrations ≥ 8 mg/L), separated by at least 14 days from a previous malaria episode. We considered episodes with pre-defined parasitaemia thresholds as secondary outcomes.

Because we considered *a priori* a reduction in overall disease burden of primary public-health importance, and because analysis of first episodes may give biased estimates of intervention effects (O'Meara and Lang 2009,

Cheung et al. 2010), our primary analysis included all events. We used Cox models with robust methods to account for correlation between episodes within children. Following the analysis plan, we adjusted for prognostic factors at baseline (age class [6-18 months, 18-35 months and 36-59 months], *Plasmodium* infection, mosquito net use, distance between homestead and clinic, height-for-age z-score). We also conducted a pre-specified secondary analysis to assess the influence on effect estimates of excluding observations in a 14-day post-treatment prophylactic period.

To assess changes in intervention effect over time, we estimated effects on all events within the first 100 days versus the subsequent period. We arbitrarily defined a cut-point of 100 days because this period covered almost half of all episodes, and adjusted for baseline factors as described above. We similarly explored effects within the first 50 days.

In a secondary analysis we assessed intervention effects on time-to-first malaria episode using Kaplan-Meier analysis.

We conducted pre-specified subgroup analyses (all events; unadjusted) to explore to what extent the magnitude of treatment effects depended on age class, presence of parasitaemia, and nutritional status at baseline. To further explore whether interactions between iron deficiency and multi-nutrient interventions could be explained by age, we assessed effect differences between iron deficient and replete children in each age class separately.

Role of the funding source: The funder had no other role in the trial whatsoever, except for supplying finance and peer reviewing the original grant application. HV had full access to all the data in the study; takes responsibility for the integrity of the data and the accuracy of the data analysis; and had the final responsibility to submit this report for publication.

Results

Of 1029 screened children, 662 had height-for-age z scores ≤ –1.5 SD; of these, 612 were eligible and randomised. 20 children (3%) did not complete the trial: 3 died; 2 were withdrawn by parents; 15 emigrated from the area (**Figure 2)**. Another 2 children discontinued the intervention but were available for follow-up. Compliance was high (96%) and similar in all four groups.

Groups were similar in baseline characteristics except that there were slightly more boys and zinc-deficient children in the multi-nutrient group

Table 1. Baseline characteristics of study participants, by intervention group

	Zinc	Multi-nutrients without zinc	Multi-nutrients with zinc	Placebo
n	153	155	151	153
Sex M/F [n/n]	46%/54% [70/83]	56%/44% [87/68]	44%/56% [66/85]	50%/50% [76/77]
Age class				
6-17 months	24% [36]	23% [36]	24% [36]	24% [36]
18-35 months	36% [55]	36% [55]	34% [51]	35% [54]
36-59 months	41% [62]	41% [64]	42% [64]	41% [63]
Plasmodium infection [1]	43% [66]	41% [64]	44% [67]	44% [68]
Height-for-age, z-score	-2·36 ± 0·69	-2·50 ± 0·69	-2·39 ± 0·71	2·45 ± 0·69
Inflammation [2]	34% [52]	33% [51]	34% [51]	31% [47]
Zinc deficiency [3]				
All children	63% [97]	71% [110]	70% [105]	65% [100]
Without inflammation [4]	58% [59]	65% [68]	59% [59]	60% [64]
Haemoglobin concentration, g/L	101·8 ± 12·6	102·7 ± 12·8	103·8 ± 12·7	102.8 ± 12·7
Anaemia [5]	75% [114]	65% [100]	68% [103]	65% [100]
Iron deficiency [6]				
All children	16% [25]	18% [28]	20% [30]	19% [28]
Without inflammation [4]	23% [23]	24% [25]	24% [24]	24% [25]
Iron deficiency anaemia				
All children	12% [18]	12% [19]	15% [23]	14% [21]
Without inflammation [4]	16% [16]	16% [17]	18% [18]	18% [19]
Distance from homestead to dispensary, km [7]	3·66 ± 2·31	3·52 ± 2·06	3·54 ± 2·07	3·60 ± 3·38
Mosquito net use [8]	32% [48]	36% [55]	30% [45]	31% [46]

Mean ± SD, % [n] or median (25- and 75-percentiles) unless indicated otherwise.
[1] As indicated by a positive result for pLDH-based dipstick test (see text); [2] Plasma C-reactive protein concentration ≥ 8 mg/L; [3] Plasma zinc concentration < 9.9 μmol/L; [4] n=101, 104, 100 and 106, respectively (5 missing values for plasma ferritin concentration); [5] Haemoglobin concentration < 110 g/L; [6] Plasma ferritin concentration < 12μg/L (6 missing values); [7] Measured as the crow flies, based on global positioning data; [8] Data missing for 11 children.

(**Table 1**). The prevalence of zinc deficiency was 67% overall, and 60% in those without inflammation; this prevalence was dramatically reduced by zinc supplementation, whether given alone or with other micronutrients (**Table 2**).

Of 2,462 episodes of reported fever, 1,618 were accompanied by *Plasmodium* infection and were treated with anti-malarial drugs; 1,572 episodes classified as malaria: 1,499 (95%) due to *P. falciparum* and 73 (5%) due to other *Plasmodium* species. The incidence of malaria was 2.93 events/ child-year. There were 1,314, 1,119 and 263 episodes with densities exceeding 3,000, 10,000 and 100,000 parasites/μL, respectively. Thirty cases, occurring in 27 children, were referred to hospital: 5 for life-threatening disease; 25 lived far from the dispensary, and the clinician referred them as a precaution (haemoglobin concentrations < 60g/L without respiratory distress or dehydration: 18; other reasons: 7). There were no episodes of cerebral malaria.

When analyzing all episodes of malaria, we found no effect of zinc or multi-nutrients without zinc, whereas multi-nutrients with zinc seemed to increase the malaria rate (hazard ratio 1.14, 95% CI: 0.96-1.35; **Figure 3**). Similar results were obtained when excluding observations for 14 days post-treatment or when restricting analysis to *P. falciparum* cases (not shown). We found no evidence that zinc alone protected against episodes with parasite densities exceeding 3,000, 10,000 and 100,000 parasites/μL (hazard ratios: 0.96 [0.78-1.19], 0.93 (0.75-1.16) and 1.13 [0.75-1.68], respectively), or influenced the incidence or time-to-first episode of malaria (**Table 3**, **Figure 4**).

Supplementation with multi-nutrients, both with and without zinc, increased the incidence of malaria by one-third (Table 3), and reduced the time-to-first event (median: 54 days for both groups, as compared to 78 days for placebo; p=0.01 and 0.02, respectively; Tarone-Ware test); adjustment for baseline factors gave similar ratios (Table 3).

In the first 100 days of supplementation, we recorded 695 malaria episodes in 408 children. In this period, malaria rates in the groups receiving multi-nutrients without and with zinc exceeded those in the placebo group by 15% and 30%, respectively (Table 3). Corresponding estimates for the first 50 days were 28% (-3% to 70%) and 42% (6% to 91%).

Of 890 febrile episodes that did not classify as malaria cases, 827 (93%) were without *Plasmodium* infection. Zinc reduced the rate of non-malarial fever episodes by 21% (1%-37%), whilst multi-nutrients, with or without zinc, resulted in slightly lower reductions (**Figure 3**).

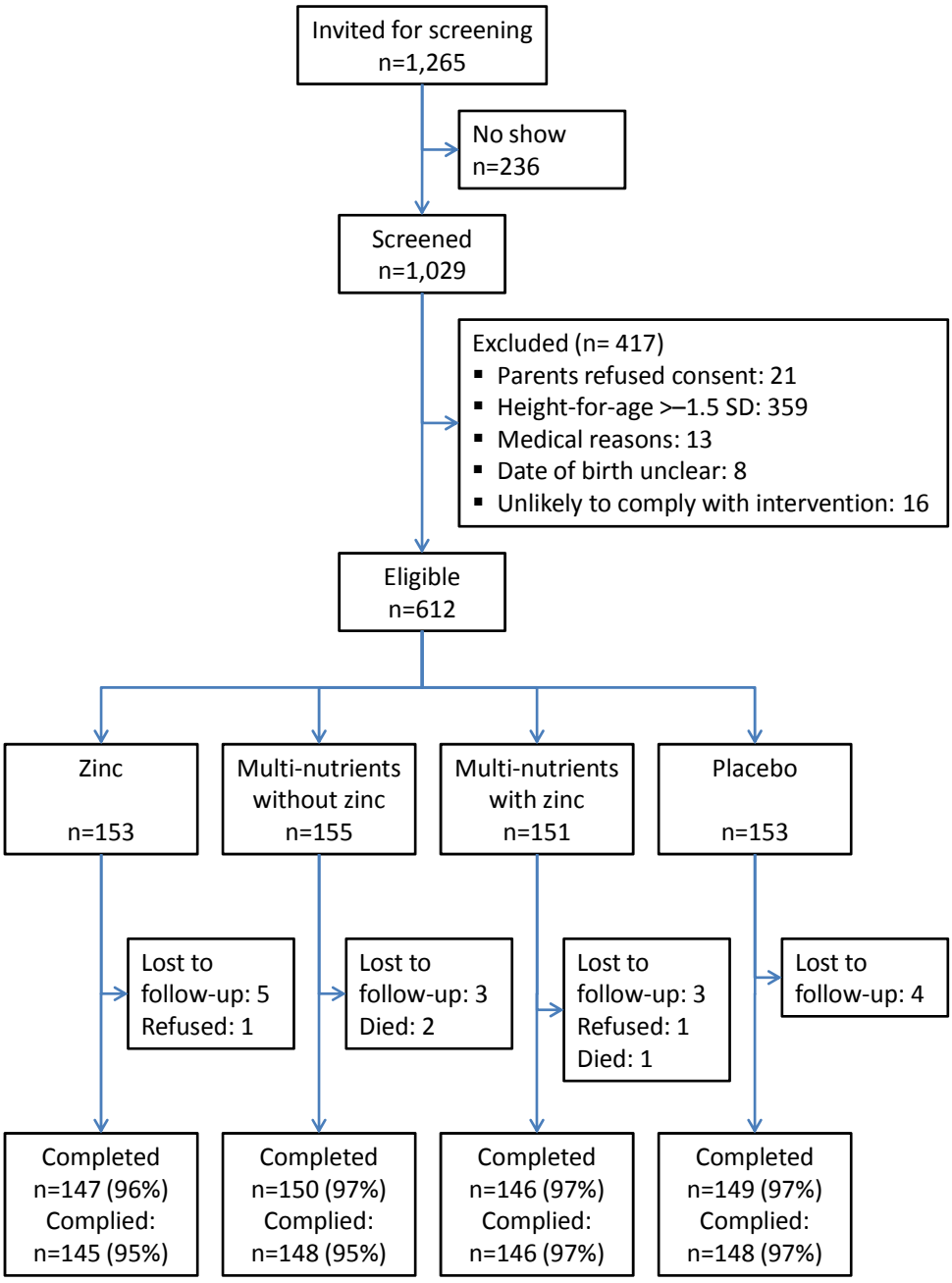

Figure 2. Study profile. Compliance was measured as the proportion of children who consumed > 95% of scheduled supplements.

Table 2. Intervention effects on inflammation and nutrient status at the second survey

	Zinc		Micronutrients without zinc		Micronutrients with zinc		Placebo
	Prevalence	Effect [1]	Prevalence	Effect [1]	Prevalence	Effect [1]	Prevalence
n [2]	149		151		148		150
Plasmodium infection [3]	33% [50]	5% (–5% to 15%)	34% [52]	6% (–4% to 16%)	33% [49]	5% (–5% to 15%)	28% [42]
Inflammation [4]	35% [54]	2% (-9% to 12%)	25% [38]	-10% (-20% to 1%)	33% [50]	-1% (-12% to 10%)	34% [52]
Zinc deficiency [5]							
All children	11% [16]	–52% (–60% to –42%)	57% [89]	–5% (–15% to 6%)	22% [33]	–40% (–50% to –30%)	62% [95]
Without inflammation	4% [4]	–58% (–67% to –46%)	47% [53]	–15% (–27% to –1%)	20% [19]	–43% (–54% to –29%)	62% [60]
Anaemia [6]	65% [100]	1% (–10% to 11%)	50% [[77]	–15% (-26 to –4%)	52% [79]	–12% (–23% to –1%)	65% [99]
Iron deficiency [7]							
All children	11% [17]	–2% (–9% to 6%)	1% [1]	–12% (–19% to –7%)	[0]	–13% (–19% to –8%)	13% [20]
Without inflammation	17% [16/95]	–3% (–14% to 8%)	[0/112]	–20% (–29% to –12%)	[0/98]	–20% (–29% to –12%)	20% [19/97]

Values between brackets indicate [n]

[1] Difference relative to placebo (95% CI) computed using Newcombe's method (Altman et al. 2000); [2] Differences between numbers reported and numbers randomised are due to drop-outs; percentages are computed with the number of randomised children in the denominator; [3] As indicated by a positive result for pLDH-based dipstick test (see text); [4] Plasma C-reactive protein concentration ≥ 8 mg/L; [5] Plasma zinc concentration < 9.9 μmol/L; [6] Haemoglobin concentration < 110 g/L; [7] Plasma ferritin concentration < 12μg/L.

Table 3. Intervention effects on the risk of uncomplicated malaria and severe disease

Event	Zinc		Multi-nutrients without zinc		Multi-nutrients with zinc		Placebo	
All episodes of malaria								
Incidence	2.89	[378/130.8]	2.95	[393/133.2]	3.26	[423/129.7]	2.87	[378/131.9]
HR [1]	0.99	[0.82-1.18]	1.04	[0.87-1.23]	1.14	[0.96-1.35]	1.0	(Reference)
HR, measured within first 100 days [2]	1.11	(0.89 to 1.37)	1.15	(0.94 to 1.41)	1.30	(1.06 to 1.61)	1.0	(Reference)
HR, measured after first 100 days [2]	0.94	(0.73 to 1.22)	0.95	(0.94 to 1.21)	1.03	(0.80 to 1.33)	1.0	(Reference)
First episode of malaria								
Incidence	2.71	[124/45.8]	3.27	[133/40.7]	3.37	[129/38.3]	2.52	[121/47.9]
Incidence difference	0.17	(–0.48 to 0.83)	0.74	(0.03 to 1.46)	0.84	(0.11 to 1.58)	1.0	(Reference)
Incidence ratio	1.08	(0.83 to 1.83)	1.29	(1.01 to 1.65)	1.33	(1.04 to 1.71)	1.0	(Reference)
HR [1]	1.12	(0.86 to 1.44)	1.38	(1.07 to 1.77)	1.35	(1.05 to 1.73)	1.0	(Reference)
Cumulative incidence at 224 days [3]	80%		85%		84%		79%	
Non-malarial fevers								
Incidence	1.57	(205/130.8)	1.67	(223/133.2)	1.61	(209/129.7)	1.92	(253/131.9)
HR [1]	0.79	(0.63-0.99)	0.83	(0.65-1.06)	0.85	(0.68-1.07)	1.0	(Reference)
All hospital admissions or deaths [4]								
Incidence	0.11	[14/130.8]	0.11	[15/133.2]	0.17	[22/129.7]	0.14	[19/131.9]
HR [1]	0.66	(0.33 to 1.36)	0.68	(0.31 to 1.53)	1.19	(0.64 to 2.24)	1.0	(Reference)

Numbers between brackets indicate [no. events/no. person-yr] or (95% CIs).
[1] Hazard ratio (HR), adjusted for age class (18-35 months and 36-59 months), presence of *Plasmodium* infection, mosquito net use (binary variable), distance between homestead and clinic (continuous variable) and height-for-age z-score (continuous variable) at baseline; [2] P-values for differences in effects within and after first 100 days: 0.27, 0.17 and 0.09 for zinc, multi-nutrients without zinc and multi-nutrients with zinc, respectively; [3] Corresponding to the minimum follow-up period for all children; [4] Excluding events due to trauma, poisoning or burns. (Includes 2 deaths that occurred without referral and 38 referrals that did not classify as malaria cases).

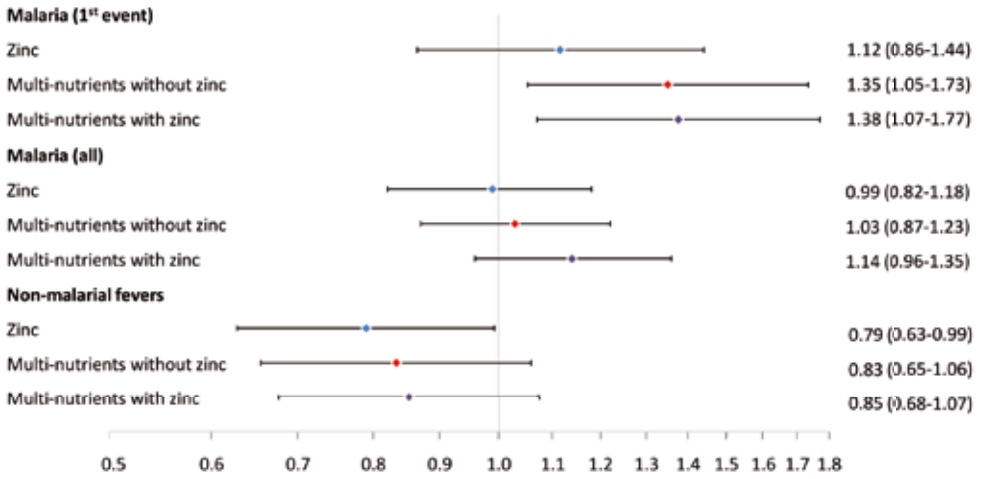

Figure 3. Intervention effects on hazard of malaria (1st episodes and all episodes; top and centre) and non-malarial fevers (all episodes; bottom), adjusted for age class (18-35 months and 36-59 months), *Plasmodium* infection status, distance between homestead and clinic (continuous variable), height-for-age z-score (continuous variable) and mosquito net use at baseline.

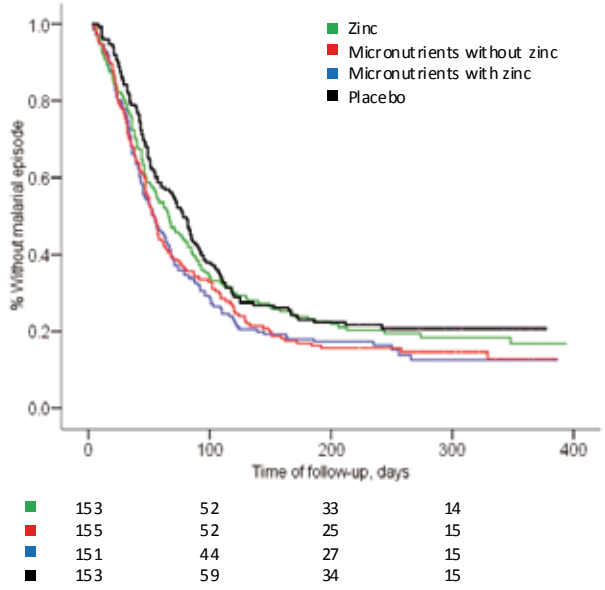

Figure 4. Intervention effects on time to first episode of malaria, as assessed by Kaplan-Meier analysis. Values indicate the number of children, by intervention group, who had remained free of malaria at 0, 100, 200 and 300 days. Pairwise group comparison with placebo yielded p-values of 0.32, 0.02 and 0.01 for zinc, multi-nutrients without zinc and multi-nutrients with zinc, respectively (Tarone-Ware test).

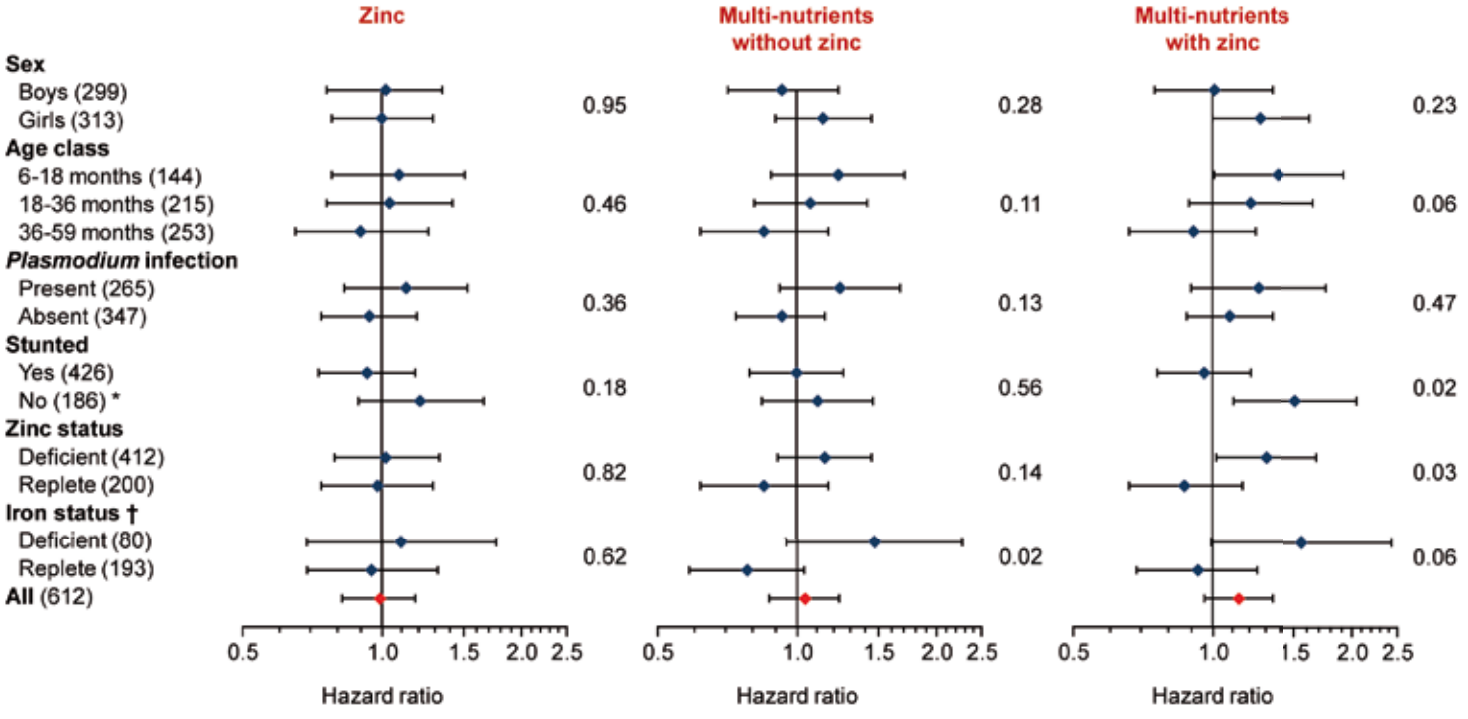

Figure 5. Subgroup analysis of intervention effects on the hazard of malaria (all episodes). Values between brackets indicate (n); values on the right indicate p-values for interaction tests. * Non-stunted: height-for-age z-score ≥ –2 and < –1.5; † Analysis restricted to children without inflammation or *Plasmodium* infection at baseline; analysis based on 265 events in children with iron deficiency and 542 events in their iron-replete peers

We found no evidence that baseline factors influenced the magnitude of the zinc effect. In contrast, the increased malaria rates due to multi-nutrients, whether with or without zinc, seemed more pronounced in younger children, and seemed mostly restricted to those who were zinc or iron deficient at baseline (**Figure 5**). Even when assessed in separate age classes, the increased effects of multi-nutrients on rates of malaria remained mostly and consistently restricted to children with iron deficiency at baseline (not shown). Among children who received multi-nutrients with zinc, the increased rate of malaria also seemed to be limited to the least stunted children.

Discussion

Despite a high prevalence of zinc deficiency, excellent compliance and few drop-outs, we found no evidence that preventive zinc supplementation, alone or with multi-nutrients, reduced rates of febrile attacks of malaria; there was strong evidence, however, that it protected against non-malarial febrile episodes. Multi-nutrient supplementation was associated with increased numbers of malaria episodes within the first 100 days but also seemed to reduce rates of non-malarial febrile episodes.

To increase specificity of malaria case definitions, a threshold parasitaemia is often used that is pre-determined by modelling fever risk as a function of density of asexual parasites (Schellenberg et al. 1994, Smith et al. 1994). Because densities can vary greatly within short time spans, ideally require leukocyte counts to be determined simultaneously (Delley et al. 2000, Koram and Molyneux 2007, O'Meara et al. 2007), and parasitaemia thresholds can vary with intervention group (O'Meara et al. 2007, Smith 2007), we favoured our approach of treating *Plasmodium*-infected participants at baseline. Even so, 32% of children had become infected but were symptom-free at the second survey. In such children, episodes of non-malarial fevers would have been misclassified as being malaria. Such misclassification may have increased with time as more children became asymptomatically infected, so that intervention effects on malarial fevers would have become increasingly mixed with protective effects that we measured against non-malarial fevers. This is consistent with hazard ratios on malaria measured after 100 days of follow-up being lower in all three micronutrient intervention groups than values observed within the first 100 days (Table 3), and the more pronounced effects measured in the analysis of first events. Thus estimates for the initial period may better reflect overall effect on malarial morbidity than estimates for the entire intervention period. Alternatively, intervention effects, particularly those of multi-nutrients, may have changed over time,

whereby a short-lived increase in risk would not be adequately reflected in the analysis of the overall time period.

Two previous trials indicated that zinc can protect against malaria. The Gambian trial (Bates et al. 1993) was not designed to assess this association, and episodes were recorded through routine clinic procedures. In Papua New Guinea, zinc reduced the incidence of febrile episodes with parasitaemia ≥ 9,200/μL and ≥ 100,000/μL by 38% (3%-60%) and 69% (36%-87%), respectively (Shankar et al. 2000). Both studies were smaller than our study (91 cases of *P. falciparum* malaria in Papua New Guinea, as compared to 1,499 in our study).

It seems unlikely that discrepancies with our findings are explained by differences in access to health care and a differential effect on rates of severe malaria: even with episodes with parasitaemia ≥ 100,000/μL, our confidence intervals (Table 3) are incompatible with the magnitude of the protective effect reported from Papua New Guinea. In addition, the overall and malaria-specific mortality reduction found in Pemba (7% and 10%, respectively) (Sazawal et al. 2007), seems inconsistent with the 69% reduction in episodes with parasitaemia ≥ 100,000/μL that was reported from Papua New Guinea. The intensity of malaria transmission in Papua New Guinea was much lower than in Burkina Faso, Pemba or our study, raising the question whether zinc only affords protection in populations with low acquired immunity. The absence of an age-dependent decrease in efficacy of zinc supplementation in our study (Figure 4) makes this unlikely. Thus the divergent results from Papua New Guinea are most likely due to unknown differences in host, parasite or environmental factors that predict the response to zinc.

When results from all trials are considered together, there is no evidence that zinc interventions can reduce the burden of malaria in Africa. In most African settings, however, any fever is treated with anti-malarial drugs, whether due to malaria or other causes (WHO 2010). Through its protective effect on non-malarial fevers, preventive zinc supplementation may reduce unnecessary anti-malarial therapy, particularly in settings with low malaria transmission. This is important in view of emerging *P. falciparum* resistance to artemisinin (Dondorp et al. 2009). Our results, and findings of protection afforded by zinc against diarrhoea and respiratory infections (IZiNCG 2009), should encourage efforts to increase the intake of zinc in deficient populations.

Our findings strongly suggest that multi-nutrient supplementation and home fortification can be unsafe in malaria-endemic areas even in conditions with excellent access to health care and appropriate treatment. The adverse effect of multi-nutrients was probably due at least in part to iron (Oppenheimer

2001, Sazawal et al. 2006, WHO 2007). Safety may be improved by modifying the formulation of supplements, in particular by reducing the iron dose, but multi-nutrients cannot be recommended until this has been demonstrated.

Based on a subgroup analysis of the Pemba sub-study with 162 adverse events (hospitals admissions or deaths), WHO now advocates a policy to restrict universal iron supplementation to individuals with iron deficiency (WHO 2007). Our subgroup analyses, based on 807 episodes of malaria (Figure 4), do not support this strategy, but rather suggest that effects of iron and other nutrients on malaria are more pronounced in iron-deficient children. Subgroup analyses are often over-interpreted, however, and should generally be considered exploratory (Assmann et al. 2000).

We found no evidence that zinc supplementation reduced the burden of malaria. However, it reduced the incidence of non-malarial febrile episodes. In malaria-endemic countries, caution should be taken when implementing multi-nutrient supplementation or home fortification as a public health measure.

Contributors: JV was responsible for data collection and analysis, administration and drafted the manuscript; PM assisted in statistical analyses; EJSJ, AEK and WTME assisted in data collection; RJK, AYD and DRAU were responsible for laboratory analyses in The Netherlands; RMO and HFJS provided supervisory support; HV was responsible for concept, design and supervision of all aspects of the study; HV and HFJS obtained funding. All authors participated in data interpretation and critical revision of the report for intellectual content; and provided final approval of the submitted version.

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Chapter 4

Effect of preventive supplementation with multi-nutrients including iron on malaria: a randomised trial among Tanzanian preschool children

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Summary

Background: There are concerns about the safety of supplementation with iron and other micronutrients in malaria-endemic areas. We assessed its effect on rates of malaria, using various case definitions.
Methods: Rural Tanzanian children aged 6–60 months and with height-for-age z-score < –1.5 SD (n=612) were randomly allocated to multi-nutrient supplements (with or without zinc) or placebo. Case definitions for malaria included episodes with asexual parasite densities > 10,000/μL or > 100,000/μL, or with haemoglobin concentration < 80 g/L. We compared rates using Cox regression, also to assess the influence on effects of age and iron status at baseline.
Findings: We found no effect of multi-nutrients on the overall rate of malaria episodes, regardless the case definition used. Subgroup analysis indicated, however, that this effect strongly depended on age and iron status at baseline; multi-nutrients increased malaria rates in the youngest children (HR 1.26; 95% CI: 0.97–1.64) and in those with iron deficiency (HR 1.53; 1.11–2.11), while supplementation had no evident effect among older and iron replete children (p-value for interaction with age and iron status: 0.02 and 0.007 respectively, for episodes with > 10,000 parasites/μL). For the effect on haemoglobin concentrations during malaria, an opposite age-dependent pattern was observed. Children aged 6-17 months were better able to maintain haemoglobin concentrations during malaria episodes (p-value for interaction with age: 0.008) when having received multi-nutrients.
Conclusions: Supplying multi-nutrients at levels normally provided through supplementation or home fortification cannot be universally recommended in malaria-endemic areas until its safety has been demonstrated. Our findings call into question the recommendation by the World Health Organization that iron supplements should be administered routinely to iron-deficient infants in settings with adequate access to anti-malarial treatment.

Introduction

Randomised trials in malaria-endemic areas have consistently shown that supplementation with iron, alone or in combination with other micronutrients, increases haemoglobin concentrations and reduces the prevalence of anaemia (Ekvall et al. 2000, Ouédraogo et al. 2008, Friis et al. 2003, Ojukwu et al. 2009). On the other hand, these nutrients can increase the incidence and severity of malaria episodes. In a large trial among children aged 1–35 months in Pemba, supplementation with iron and folic acid increased the incidence of serious adverse events by 12%, presumably due to malaria (Sazawal et al. 2006). Recently, we found that multi-nutrient supplementation in Tanzanian children aged 6–60 months increased malaria rates (Veenemans et al., submitted) but we did not assess effects on the severity of malaria.

During episodes of malaria, maintenance of haemoglobin concentration is critical for child survival. By increasing parasite propagation, supplementation may exacerbate haemolysis. Thus, gains in haemoglobin concentrations due to supplementation before febrile malaria attacks may be counteracted or outdone by more pronounced haemoglobin losses during such episodes. In a randomised trial in Papua New Guinean infants aged 6–12 months, the decrease in haemoglobin concentration associated with asymptomatic *Plasmodium* infection at two follow-up surveys seemed more pronounced among those receiving iron (Oppenheimer et al., 1986). By contrast, while supplementation with iron and folic acid increased rates of cerebral malaria in Pemba (Sazawal et al. 2006), it seemed to reduce the risk of hospitalisation with severe anemia, although the statistical evidence for this reduction was not strong. We have not found reports of randomised trials on the effect of preventive supplementation with iron or multi-nutrients on haemoglobin concentration during febrile episodes of uncomplicated malaria.

Subgroup analysis of data from a sub-study within the Pemba trial suggested that the increase in adverse events due to iron and folic acid is restricted to those who were iron-replete at baseline, and more pronounced in children aged 12–23 months than in their peers from other age groups. Such effect modification by iron status was not supported, however, by a recent meta-analysis of iron supplementation studies in malaria-endemic countries (Ojukwu et al. 2009). In our recent trial, subgroup analysis suggested that the effect of multi-nutrients on rates of malaria was larger in children aged 6–18 months than in older pre-school children (Veenemans et al., submitted). The absence of adequate protection in the youngest children and the gradual acquisition of protective immunity make an age-dependent effect plausible. In the present study, we assessed the effect of supplementation with multi-

nutrients on rates of malaria episodes with high parasite densities or with low haemoglobin concentrations, and on indicators of disease severity (including haemoglobin concentrations) during these episodes. We also explored to what extent the magnitude of intervention effects depended on age and iron status at baseline.

Methods

Study design: This study was part of a randomised efficacy trial with the primary aim to assess the effect of supplementation with zinc and other nutrients on malaria rates. Children received daily oral supplements with: a) zinc; b) multi-nutrients without zinc; c) both multi-nutrients and zinc ; or d) placebo. The multi-nutrient supplement included iron (18 mg as ferrous fumarate), folic acid (93.75 μg), vitamin A (450 μg, as retinyl palmitate), vitamin B_{12} (1.17 μg), vitamin B_1 (0.625 mg as thiamin nitrate), vitamin B_2 (0.55 mg riboflavin), copper (340 μg) and vitamin E (6.6 mg as RRR-α-tocopherol), all of which may have haematinic effects. Further details about the composition of the supplement, study design and intervention effects on malaria rates, will be reported elsewhere (Veenemans et al., submitted).

Study population and recruitment: The trial was conducted between February 2008 and March 2009 in a rural area in north-eastern Tanzania, with intense year-round transmission. All resident children listed in a pre-conducted census were invited for an information meeting and screening. They were eligible for randomisation when aged 6–59 months and with a height-for-age z-score below –1.5 SD. We excluded children with severe wasting (weight-for-age z-score < –3 SD), haemoglobin concentration < 70 g/L, signs of chronic illness, and those unlikely to remain permanently resident or comply with the supplementation for the duration of the trial, or whose parents or guardians refused consent.

At baseline, we recorded anthropometric measurements, collected venous blood in EDTA tubes, and conducted a rapid dipstick test to detect *Plasmodium* infection. Children with a positive test result were treated immediately with artemether-lumefantrine. We performed an automated blood examination (Sysmex KX21, Kobe, Japan) the same day, and stored plasma for subsequent analyses of biochemical indicators of iron status and inflammation in The Netherlands. An aliquot of fresh blood was used to detect *Plasmodium* infection by microscopy. The location of the child's homestead was determined using a global positioning system.

Randomisation and intervention: We used permuted blocks with random size (4 or 8) within 6 strata defined by the presence of *Plasmodium* infection and age class (6–17 months, 18–35 months and 36–60 months). The allocation sequence was generated a priori by a person not involved in the field work, using a table of random numbers, and the intervention (indicated by colour code) was enclosed in consecutively numbered opaque envelopes, separated in 6 batches for the strata mentioned above. At the end of each screening day, the names of eligible children were listed by screening number and each name was randomly allocated to an intervention by drawing the next envelope from a box that corresponded to the age- and malaria- specific stratum for that child.

Supplements, in the form of powder in colour-coded capsules, were contained in blister packs, and administered orally after suspending capsule contents in clean water or breastmilk. Supplementation was performed by local community volunteers, who daily reported about compliance to field staff at the research dispensary.

Follow-up and case detection: A clinical officer was on duty at the research clinic 24 hours, 7 days per week. At recruitment, parents or guardians were requested to bring participating children to the dispensary immediately when detecting fever or any other illness.

For all children with reported fever, we collected blood by finger puncture to detect the presence of lactate dehydrogenase produced by live *P. falciparum* or other human *Plasmodium* species (CareStart™, G0121, Access Bio, Monmouth Jct, NJ). For those with a positive dipstick test result, we prepared two blood slides, and measured whole blood concentrations of haemoglobin and C-reactive protein using a portable photometer (HemoCue, Ängelholm, Sweden) and an immunoturbidimetric test (QuikRead, Orion Diagnostica, Espoo, Finland; detection limit: 8 mg/L), respectively. C-reactive protein is an acute phase protein; its plasma concentration increases within 6 hours after inflammatory stimulus, decreases rapidly with recovery, and reflects the severity of inflammation (Jaye and Waites 1997).

In children with confirmed malaria (case definition defined *a priori*; see below), we collected and stored an aliquot of plasma (EDTA Microtainer, Becton Dickinson, Franklin Lakes, NJ) in liquid nitrogen for subsequent measurement of histidine-rich protein-2 (HRP2) concentration. This protein is released into the plasma at schizont rupture, and its plasma concentration may more accurately represent total body parasite biomass, because it also reflects the presence of parasites that are sequestered by cytoadherence

to endothelial cells in the microvasculature of organs, so that they remain undetected with conventional microscopy. In patients with severe malaria admitted to hospital, plasma HRP2 concentrations better predict disease severity and death than parasite count determined by microscopy (Dondorp et al. 2005). Because the protein has a mean plasma elimination half-life exceeding 3.5 days, however, concentrations not only reflect current parasite load, but are also determined by the duration of the infection and preceding densities at the time of sample collection.

We conducted a second survey, following similar procedures as the baseline survey, at a median of 251 days (95% reference range: 191–296 days) after enrollment.

Artemether-lumefantrine (Novartis Pharma, Basel, Switzerland) was administered to any child with current *Plasmodium* infection upon enrolment, or with reported fever and a positive dipstick test result during the follow-up period.

Laboratory analyses: Peripheral blood parasite density was determined by microscopy; slides with results that were inconsistent with those from the dipstick test were read twice. Asexual *Plasmodium* parasites were counted against at least 200 leukocytes, and density, expressed per μL blood, was estimated using an assumed leukocyte density of 8,000/μL. For children with very high densities, parasites were counted per 2,000 erythrocytes, in which case we used the estimated erythrocyte count at the time of the episode to determine the number of parasites per μL. The erythrocyte concentration was estimated based on haemoglobin concentration measured by HemoCue meter, using a linear model describing the relationship between haemoglobin concentrations and erythrocyte counts as assessed during surveys.

Plasma concentrations of HRP2 were determined by enzyme-linked immunosorbent assay using a commercial kit (Cellabs, Sydney, New South Wales, Australia). Due to financial limitations, we assessed this indicator only in samples collected from children with a first episode of malaria. Samples were aliquoted by 2-, 10-, 25- and 50-fold dilution into microwells precoated with anti-HRP2 capture antibodies. Each plate included a positive and a negative control provided with the kit, and assay steps were performed according to the manufacturer's instructions. As a modification to the kit, we included serially diluted reference plasma on each plate, relative to which the concentration of HRP2 was determined for each plasma sample. The reference plasma consisted of a pooled sample collected from 50 children from the same study area with malaria, and was added by 10-, 20- ,40- ,80-

and 160-fold dilution to each plate. For samples that did not yield optical densities within the linear range of the reference curve, we repeated the assay at lower or higher dilutions as appropriate. HRP2 concentration in the reference plasma was estimated by comparison to a standard (also provided by the manufacturer of the kit) that was serially diluted in HRP2-negative human plasma, at decreasing concentrations (eight two-fold dilutions starting at 27.5 μg/L).

In plasma samples collected at baseline and the second survey, we measured concentrations of C-reactive protein and ferritin on a Beckman Coulter Unicel DxC880i system according to the manufacturer's instructions. Genotype for α^{+}-thalassaemia was determined as described earlier (Veenemans et al. 2008).

Definitions and statistical analysis: An episode of malaria was pre-defined as a positive result for the malaria dipstick test with any of the following: a) confirmed fever (axillary temperature ≥ 37.5 °C as measured by electronic thermometer), or b) reported but unconfirmed fever, in the presence of inflammation (whole blood C-reactive protein concentrations > 8 mg/L), separated by at least 14 days from a previous malaria episode. Iron deficiency was defined as plasma ferritin concentration < 12 μg/L; because inflammation can influence plasma ferritin concentrations independently of iron status, we also conducted analyses that were restricted to individuals without inflammation (C-reactive protein concentration < 8 mg/L). Anaemia was defined by haemoglobin concentration < 110 g/L (WHO, 2001).

All data were double-entered, cleaned and analyzed in SPSS (v15·0 for Windows, SPSS, Chicago, IL) and STATA (v11; College Station, Tx). For continuous outcome variables, we visually inspected distribution plots, and log transformed variables that were not normally distributed. We report arithmetic or geometric means (and 95% CIs) as appropriate. We expected no effect of zinc on haemoglobin concentrations, and in previous analysis we found no evidence of effects on malaria rates of zinc, either alone or with multi-nutrients. Thus, in the present analysis, we present marginal effects, whereby the pooled placebo and zinc groups are compared with the pooled groups receiving multi-nutrients.

We used Kaplan-Meier analysis and Cox regression models to assess intervention effects on rates of malaria with varying definitions to indicate disease severity (as defined above, episodes with parasite densities > 10,000/μL or > 100,000/μL, and episodes with haemoglobin concentrations < 80 g/L). In the Cox models, we used robust methods to account for correlation between episodes within children, and also assessed the influence of

adjustment for baseline factors that were prognostic for malaria (haemoglobin concentrations, *Plasmodium* infection, α^+-thalassaemia genotype, distance between homestead and research clinic, mosquito net use, and height-for-age z-scores) on effect estimates.

As indicators of severity, we assessed parasite density, *P. falciparum*-specific HRP2 concentration and C-reactive protein concentration and haemoglobin concentration. We used multivariate linear regression to assess crude effects of multi-nutrient supplementation on these indicators, with log transformation as appropriate. As with HRP2 concentration, we restricted our analysis of intervention effects on C-reactive protein concentration and parasite density to first episodes of malaria. For haemoglobin concentrations, we also explored intervention effects at the second episode to assess whether associations found were consistent with those found in the analysis of haemoglobin concentration at the first episode. In the analysis of the effect on haemoglobin concentration, we adjusted for standardised baseline haemoglobin concentration and time between randomisation and outcome measurement.

For all outcomes, we assessed the influence of adjustment for prognostic factors at baseline (see preceding paragraph), and assessed intervention effects with stratification by age class and iron status at baseline, and used multivariate regression models to test differences in effect between these subgroups.

Results

Of 612 randomised children, 592 (97%) completed the study: 3 died; 2 were withdrawn by parents; 15 emigrated from the study area (**Webfigure 1**). Another 2 children discontinued the intervention but were available for follow-up. Overall, 96% of all children took more than 95% of the scheduled supplements. Groups had similar baseline characteristics (**Table 1**).

Of 612 study participants, 507 (83 %) experienced at least one episode of malaria. The overall incidence of malaria episodes (1,572 malaria episodes in 526 child-years of observation, or 2.93 per child-year) declined with age (3.73, 3.49 and 2.19 among children aged 6–17 months, 18–35 months and 36–60 months, respectively). There were 1,119, 263 and 178 episodes with densities above 10,000, 100,000 parasites/μL or with haemoglobin concentrations below 80 g/L. Episodes with densities > 100,000/μL occurred most frequently among the youngest children (0.88, 0.52 and 0.16 episodes/child-year for the 3 age classes, respectively).

Table 1. Baseline characteristics of study participants, by intervention group

Characteristic	Without multi-nutrients	With multi-nutrients
n	306	306
Sex M/F [n/n]	48%/52% [146/160]	50%/50% [153/153]
Age	32·6 ± 15·7	32·4 ± 15·6
Plasmodium infection *	44% [134]	43% [131]
Haemoglobin concentration, g/L		
All children	102·3 ± 12·7	103·2 ± 12·7
6–17 months	97·2 ± 13·4 [72]	99·4 ± 13·2 [72]
18–35 months	101·0 ± 12·9 [109]	102·5 ± 13·0 [106]
36–59 months	106·4 ± 10·7 [125]	106·0 ± 11·7 [128]
Anaemia †	70% [214]	66% [203]
Inflammation ‡	32% [99]	33% [102]
Plasma ferritin concentration, μg/L §		
All children	37·5 (17.1 to 66.4) [304]	37·3 (15.4 to 73.6) [302]
No inflammation ¶	27·6 (12.6 to 53.9) [205]	24·9 (12.0 to 49.8) [201]
Iron deficiency ‖		
All children	17% [53]	19% [58]
No inflammation ¶	24% [49]	25% [50]
Distance from homestead to dispensary, km **	3·6 ± 2·3	3·5 ± 2·1
Mosquito net use ††	31% [94]	33% [100]
Anthropometric indices		
Height-for-age, z-score	–2·41 ± 0·69	–2·45 ± 0·70
Weight-for-height, z-score	–0·13 ± 0·83	–0·12 ± 0·84
Weight-for-age, z-score	–1·58 ± 0·74	–1·60 ± 0·76

Mean ± SD, % [n] or median (25- and 75-percentiles) unless indicated otherwise.

* As indicated by a positive result for pLDH-based dipstick test that detects the presence of lactate dehydrogenase of *P.falciparum* or other human *Plasmodium* species.

† Haemoglobin concentration < 110 g/L.

‡ With 6 missing values for plasma ferritin concentration.

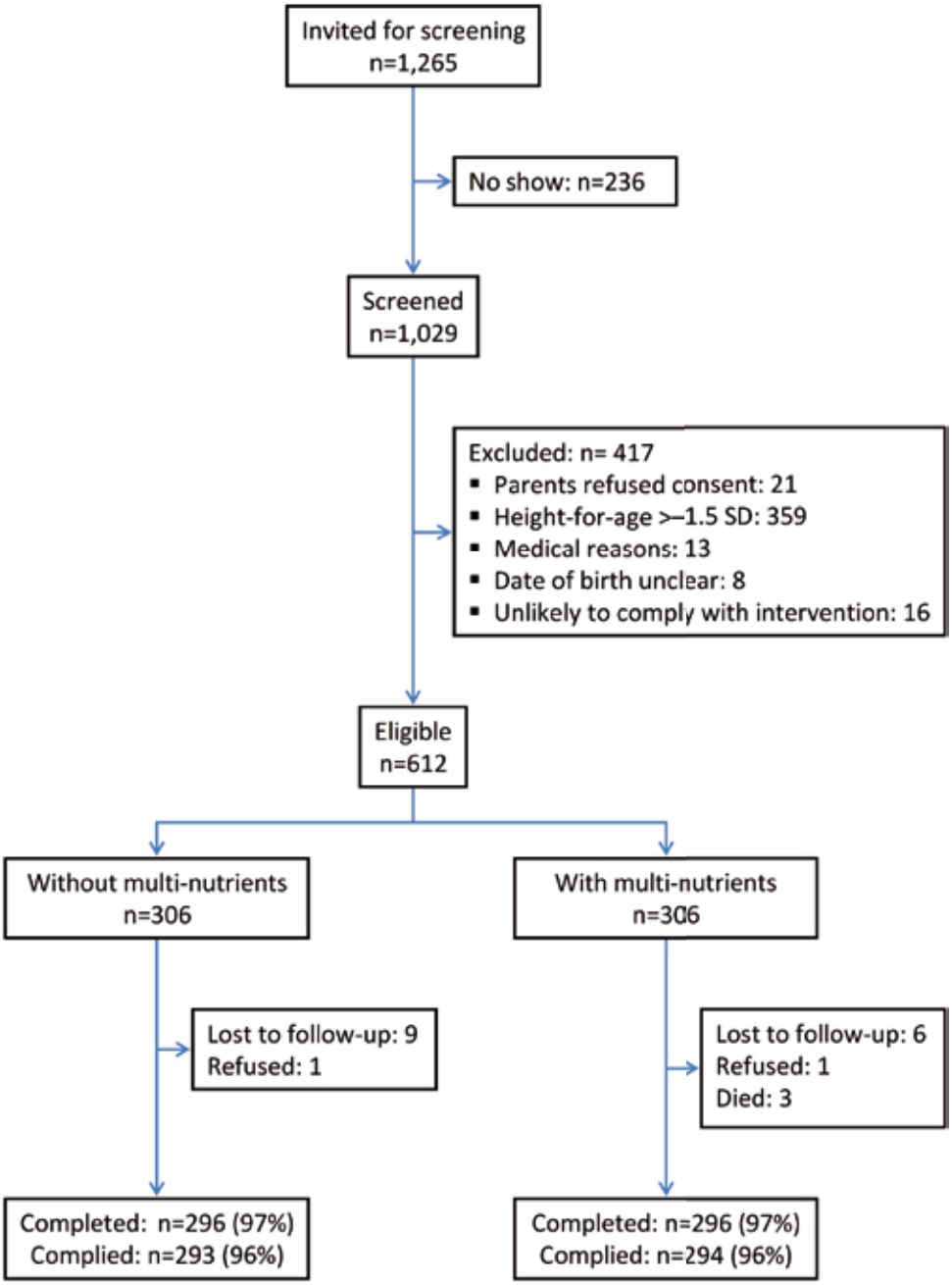
§ Plasma C-reactive protein concentration ≥ 8 mg/L.

¶ Analysis restricted to children without inflammation (n=101, 106, 103 and 104, respectively), with 5 missing values for ferritin.

‖ Iron deficiency: plasma ferritin concentration < 12μg/L.

** Measured as the crow flies, based on global positioning data.

†† Data missing for 11 children.



Webfigure 1. Flow chart
The identical number of participants in the groups with and without multi-nutrients is coincidental.

Assessment of the burden of iron deficiency: The prevalence of iron deficiency strongly declined with age (38%, 20% and 5% in children aged 6–17 months, 18–35 months and 36–60 months, respectively; p<0.001; corresponding values in those without inflammation: 45%, 28% and 7%; p<0.001). Analysis of haemoglobin concentrations at the second survey confirmed that children in the youngest age class responded most to multi-nutrient supplementation, with effects of 8.7 g/L (5.3 to 12.2 g/L), 2.5 g/L (–0.4 to 5.3 g/L) in children aged 6–17 months and 18–35 months, and no evident effect in children aged 36–60 months (**Figure 1, Webtable**). In children aged 6–17 months and 18–35 months, multi-nutrient supplementation reduced the prevalence of anaemia by 40% and 14%, respectively, whilst there was no evidence for such an effect in the oldest age class (Webtable).

Effect of multi-nutrients on malaria rates: Multi-nutrient supplementation led to decreased time to first episodes of malaria (**Webfigure 2**). We found no effect of multi-nutrient supplementation on the overall rate of episodes with densities > 100,000 parasites/μL, whether analysed for first events (HR, 95% CI: 0.93; 0.69–1.26), or for repeated events (1.07; 0.80–1.45). Stratification by age, however, showed that this effect strongly depended on age: among the youngest children, multi-nutrients increased rates by 55%, as compared to a decrease by 58% in the eldest children (p-value for interaction: 0.005; **Figure 2**). For episodes with parasite density > 10,000/μL, our data showed a similar age-dependent trend (p=0.02), although the contrast in effects between age classes was less pronounced (Figure 2). Adjustment for prognostic factors at baseline led to similar effect estimates (not shown).

When analyzing the effect of multi-nutrient on malaria with haemoglobin concentrations < 80 g/L, this trend seemed reversed (Figure 2).

Stratification by iron status at baseline indicated that the increase in malaria rates due to multi-nutrient supplementation was restricted to children with iron deficiency; there was no evidence that multi-nutrients increased malaria rates in their iron-replete peers (**Figure 3**). We found no evidence that iron deficiency was associated with protection against malaria among those receiving zinc or placebo (not shown).

Effect of multi-nutrients on indicators of malarial severity: There was no evidence that multi-nutrient supplementation affected parasite density, C-reactive protein concentration or HRP2 concentration at the time of first malaria episode (**Table 2**)**.** When stratified by age, however, multi-nutrients resulted in increased parasite densities in the youngest age class (p=0.02) whereas they seemed to slightly reduce densities in the eldest children

Webtable. Intervention effects on haemoglobin concentrations and indicators of iron status, inflammation and malaria at the second survey

	Without multi-nutrients		With multi-nutrients		Difference	
n	299		299			
Plasmodium infection *	31%	[92]	34%	[101]	3%	(–5% to 10%)
Inflammation †	35%	[106]	29%	[88]	–6%	(–13% to 2%)
Haemoglobin concentration ‡	103.8	[102.5 to 105.1]	107.0	[105.8 to 108.3]	2.9	[1·1 to 4·6)
Iron deficient	100.4	[97.5 to 103.3]	108.9	[106.4 to 111.5]	7.9	(4.2 to 11.6)
Iron replete	104.6	[103.1 to 106.1]	106.6	[105.2 to 108.1]	1.8	(–0.2 to 3.7)
Anaemia ¶	67%	[199]	52%	[156]	–14%	(–22% to 7%)
Age 6–17 months	89%	[62]	49%	[34]	–40%	(–25% to –52%)
Age 18–35 months	72%	[76]	57%	[58]	–14%	(–1% to –26%)
Age 36–59 months	49%	[61]	51%	[64]	2%	(–10% to 14%)
Ferritin concentration §						
All children	31.8	[28.7 to 35.3]	57.2	[52.6 to 62.1]	25.3	[18·3 to 33·4]
No inflammation	23.8	[21.1 to 26.8]	47.6	[43.7 to 51.8]	23.8	[17·4 to 31·2]
Iron deficiency						
All children	12%	[37/299]	0.3%	[1/299]	–12%	(–16% to –8%)
No inflammation	18%	[35/193]	0.5%	[1/211]	–18%	(–24% to –12%)

598 children were available for the second survey. Values represent percentages [n], or means [95% CI]
* As indicated by a positive result for pLDH-based dipstick test to detect lactate dehydrogenase of *P.falciparum* and other human *Plasmodium* species.
† Plasma C-reactive protein concentration ≥ 8 mg/L.
‡ In g/L; effects adjusted for standardised values for baseline haemoglobin.
§ In µg/L, geometric mean [95% CI]
¶ Haemoglobin concentration < 110 g/L

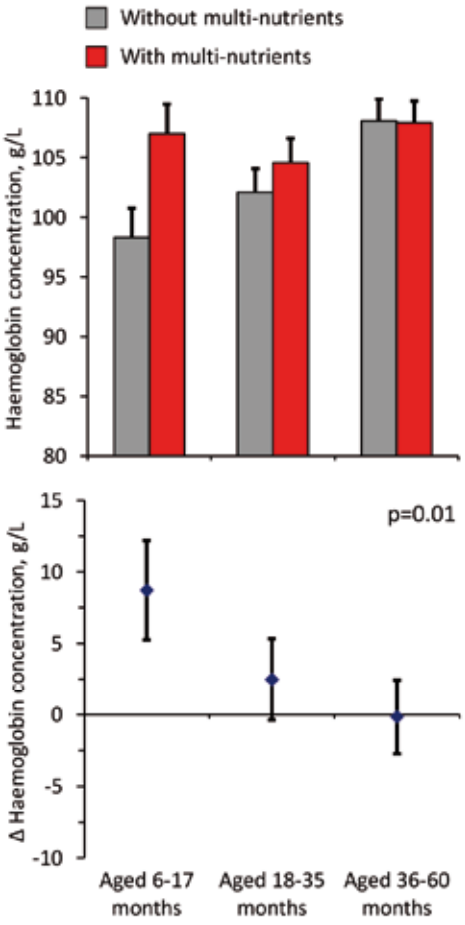
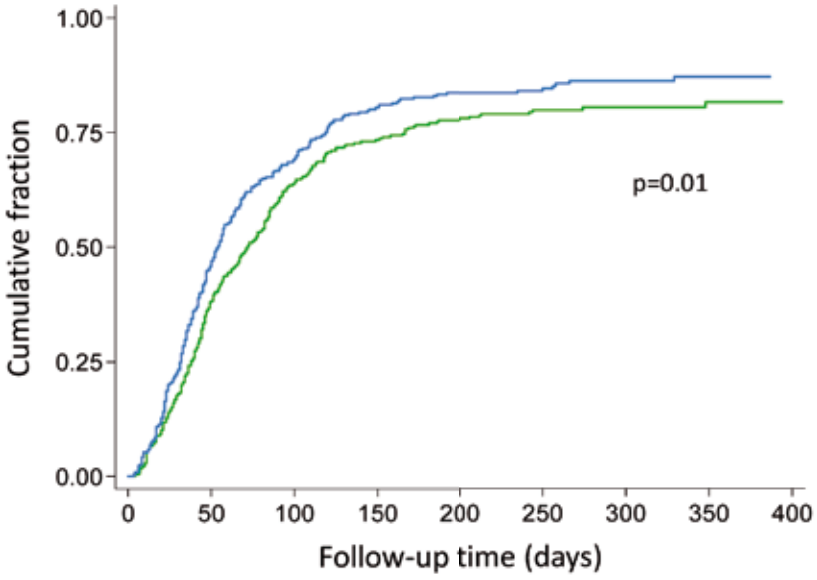


Figure 1. Effect of multi-nutrient supplementation on haemoglobin concentration at the second survey, by age class

Top panel: haemoglobin concentrations, by supplementation group and age class (n=598); bottom panel: effect of multi-nutrients on haemoglobin concentrations, by age class. Line bars indicate 95% CIs (only upper half of the interval indicated in top panel). The p-value for the difference in effect between age classes is indicated in the lower panel. All estimates are adjusted for standardized haemoglobin concentrations at baseline.



Number at risk

Without multi-nutrients	306	190	110	81	66	42	29	17	2
With multi-nutrients	306	166	95	61	51	30	23	11	2

Webfigure 2. Effect of multi-nutrient supplementation on time to first malaria episode
Episodes of malaria were as pre-specified (see text). Median time to event: 54 days and 72 days for groups with or without multi-nutrients, respectively; p-value for difference between groups obtained by Tarone-Ware test.

Table 2. Intervention effects on haemoglobin concentrations and indicators of severity during first malaria episode

	Without multi-nutrients		With multi-nutrients		Difference	
N	245		262			
Incidence, based on first episodes	2.61	(2.30 to 2.96)	3.32	(2.94 to 3.74)	0.71	(0.19–1.22)
Mean age at time of episode	33.7	(31.8 to 35.7)	33.1	(31.2 to 35.0)	–0.6	(–3.4 to 2.1)
Haemoglobin concentration, g/L *	99.6	(97.9 to 101.3)	99.1	(97.5 to 100.8)	–0.5	(–2.9 to 1.9)
Anaemia	75%	[183]	74%	[193]	–1%	(–12% to 10%)
Whole blood C-reactive protein concentration, mg/L †	66.3	(62.3 to 70.5)	66.2	(62.4 to 70.2)	0%	(–16% to 18%)
Parasite density, μ/L †	20,885	(18,278 to 23,862)	20,762	(18,295 to 23,561)	0%	(–15% to 17%)
Plasma HRP2 concentration, μg/L †	199	(177 to 223)	211	(187 to 238)	0%	(–11% to 18%)

Values represent means (95% CIs) or percentages [n] unless indicated otherwise.

* Estimates adjusted for standardised baseline haemoglobin concentrations.

† Estimates represent geometric mean and 95% confidence interval. Group differences indicate the percentage change due to multi-nutrient supplementation, as compared to the reference group. Values missing for 26, 11 and 1 children for parasite density, HRP2 concentration and C-reactive protein concentration, respectively.

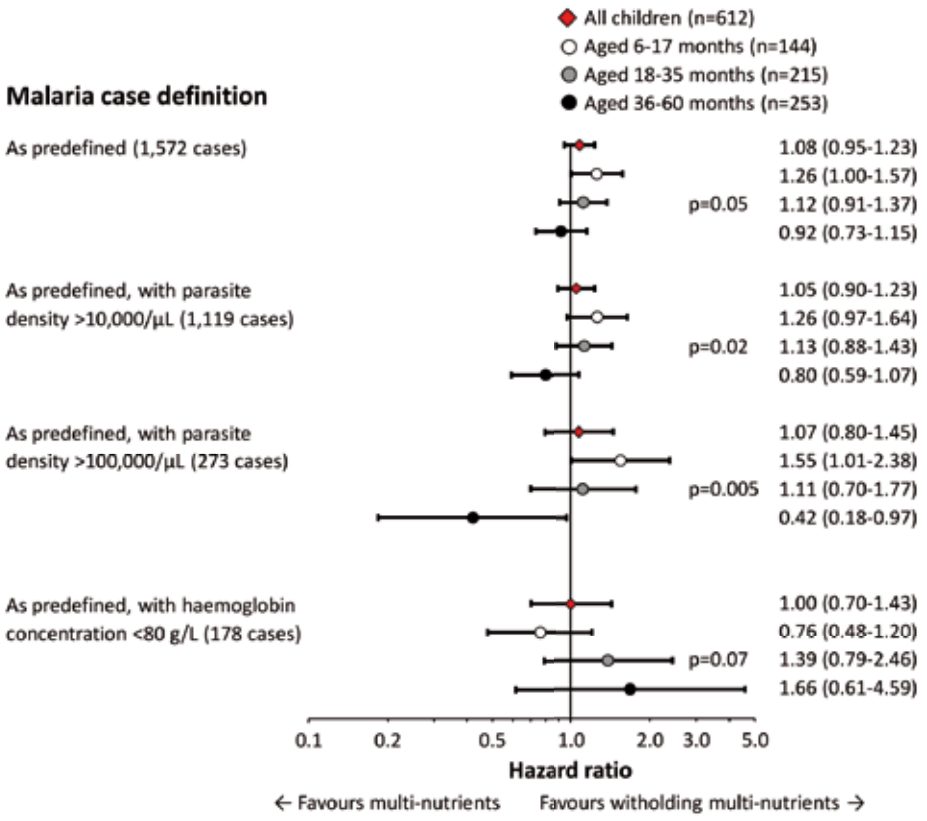


Figure 2. Effect of multi-nutrient supplementation on the rates of malaria, with case definitions of varying severity, by age class

Case definitions were either as predefined (see text), as predefined with densities > 10,000/μL or > 100,000 asexual parasites/μL, or with haemoglobin concentration below 80 g/L. Values on the right indicate crude hazard ratios (95% CIs); p-values for differences in effect between age classes (entered on an ordinal scale). Slide results were not available for 32 malaria cases; these were imputed as having densities below 10,000 parasites/μL. Adjustment for distance between homestead and dispensary, height-for-age z scores, mosquito net use and *Plasmodium* infection at baseline led to similar estimates (not shown).

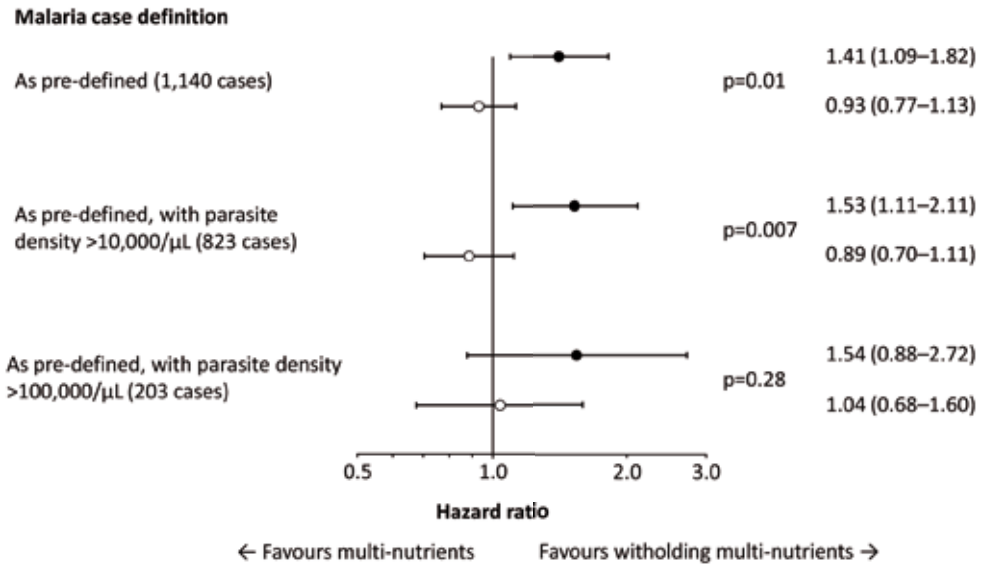


Figure 3. Effect of multi-nutrient supplementation on the rates of malaria, with case definitions of varying severity, by iron status at baseline

Iron status was defined as iron deficient (closed circles; plasma ferritin concentration < 12 μg/L) or iron replete (open circles; plasma ferritin concentration ≥ 12 μg/L, without inflammation). In the analysis, we excluded children in whom iron status was uncertain (plasma ferritin concentration ≥ 12 μg/L, with inflammation). P-values indicate differences in effect between iron-deficient and iron-replete children. For further explanation, see text and figure 2.

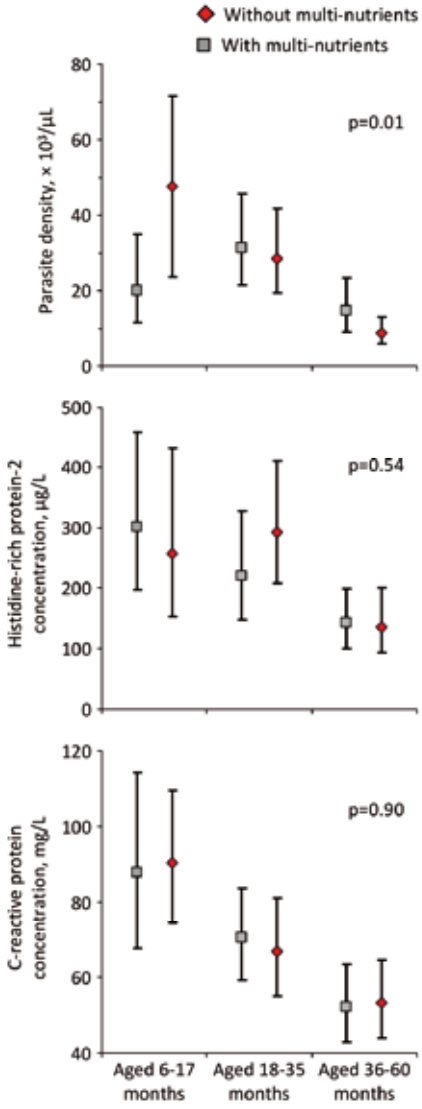


Figure 4. Intervention effects on indicators of severity of illness at the time of the first malaria episode, by age class

Estimates represent geometric means and 95% CIs, with p-values for the interaction between intervention and age. Analysis is based on 507 episodes, with 26, 11 and 1 missing values for parasite density, HRP2 concentration and C-reactive protein concentration, respectively.

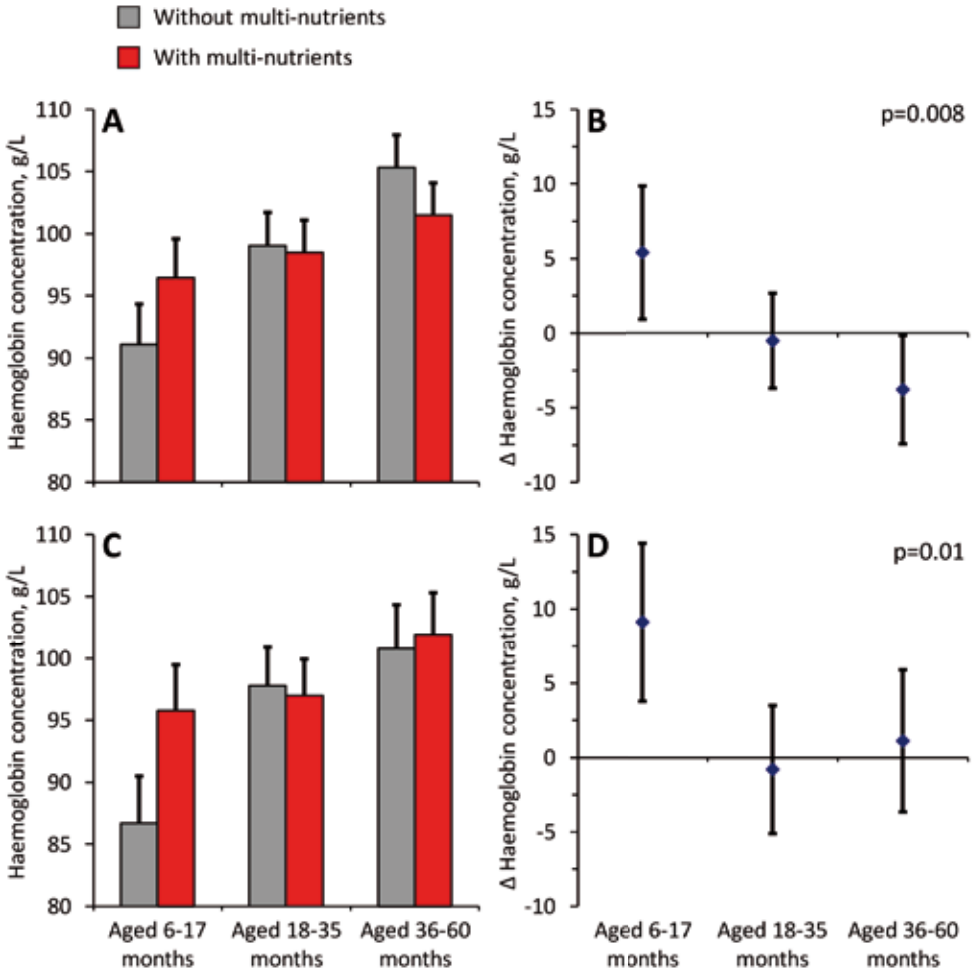


Figure 5. Effect of multi-nutrient supplementation on haemoglobin concentrations in children with malaria, by age class

Panels A, C: haemoglobin concentrations, by supplementation group and age class, at the time of first and second malaria episodes (505 and 381 cases, respectively); panels B, D: effect of multi-nutrients on haemoglobin concentrations, by age class, at the time of first and second episodes. Line bars indicate 95% CIs (only upper interval indicated in panels A and C). P-values for differences in effect between age classes are indicated in panel B and D. All estimates are adjusted for standardized haemoglobin concentrations at baseline and time between randomisation and outcome measurement; further adjustment for prognostic factors at baseline led to similar estimates (not shown).

(p=0.07; p-value for interaction: 0.01, **Figure 4**). Adjustment for prognostic factors at baseline resulted in virtually identical point estimates with slightly higher precision (not shown).

For the other indicators of severity (concentrations of C-reactive protein and HRP2) we also found a decrease with age (p<0.001 and 0.001 respectively), but no marked effect of multi-nutrients, whether analyzed with (Figure 4) or without stratification by age class (table 2).

We found no evidence that iron deficiency at baseline was associated with any of these indicators of malarial severity, or that it influenced the effect of multi-nutrient supplementation on these indicators (not shown).

Effect of multi-nutrients on haemoglobin concentration during malaria episodes: We found no evidence that multi-nutrient supplementation affected haemoglobin concentrations or the proportion of children with anaemia at the first malaria episode (Table 2). Analysis by age class, however, showed that multi-nutrient supplementation increased haemoglobin concentrations by 5.3 g/L (0.8 to 9.8 g/L) in children aged 6–18 months, as compared to –0.7 g/L (–4.5 to 3.0 g/L) and –3.9 g/L (–7.5 to –0.2 g/L) in children aged 18–35 months and 36–59 months, respectively (difference in effect relative to that in the youngest class: –5.9 g/L [–11.7 to –0.1 g/L] and –9.2 g/L [–15.0 to –3.4 g/L]; p-value for differences in effect between age classes: 0.008). Adjustment for time between start of intervention and episode led to virtually identical estimates (**Figure 5**). Similar patterns were observed for the intervention effects on haemoglobin concentrations during the second malaria episode; the effect of multi-nutrients depended on age (p-value for interaction: 0.01), with children in the youngest age class gaining 9.1 g/L (5.3 to 14.4 g/L; Figure 4), compared to no evident effect in children ≥ 18 months.

When stratifying by iron status at baseline, we found that multi-nutrients increased hemoglobin concentrations at the time of the first malaria episode by 6.5 g/L (1.2 to 11.9 g/L) among children with iron deficiency at baseline, whereas no effect was apparent in their peers who were iron replete (–2.1 g/L, –4.7 to 0.5 g/L; difference in effects: 8.6 g/L, 2.7 to 14.6 g/L; p-value for interaction=0.004).

Discussion

Although multi-nutrient supplementation reduced the time to the first malaria episode, we found no evidence that it influenced overall rates of

malaria, regardless of the case definition used, or that it increased the severity of malaria as assessed by concentrations HRP2 or C-reactive protein. The effect of supplementation on overall malaria rates varied, however, with age class and iron status at baseline. Among children aged 6-17 months, multi-nutrients increased rates of malaria episodes, particularly of episodes with hyperparasitemia (> 100,000/ uL), while in older children (>35 months) they led to reduced rates of episodes with hyperparasitemia.

Nonetheless, the youngest children, who had a much higher prevalence of iron deficiency than their older peers, were better able to maintain their haemoglobin concentration when having received multi-nutrients, both at the time of the first and the second malaria episodes.

Contrasts between age-specific effects on malaria rates increased when defining episodes with higher parasite density cut-off values (Figure 2). To some extent, this may be because multi-nutrients led to increased parasite densities during malaria episodes (Figure 4), but an improved specificity of episodes with higher density cut-offs may also have played a role; while episodes with > 100,000 asexual parasites/μL are highly likely to be attributable to malaria (e.g. Bejon et al. 2007), the analysis based on episodes defined by lower density cut-off values will have included cases of non-malarial fever that were misclassified as being malaria because 32% of children had asymptomatically infected at the time of the 2nd survey. The resulting reduction in specificity of the case definition is likely to have biased measured intervention effects towards naught (O'Meara and Lang 2009). Either way, because our findings indicate that, among the youngest children, multi-nutrients increase rates of malaria with hyperparasitaemia, and probably also increase the rates of malaria episodes with lower densities, these findings strengthen earlier concerns about the safety of multi-nutrient supplementation in malaria-endemic areas (Sazawal et al. 2006, Veenemans et al. submitted), even in settings with excellent access to primary care.

In children aged 6–17 months, who had the highest prevalence of iron deficiency (45%), supplementation with multinutrients resulted in increased haemoglobin concentrations, as shown at the second survey (Figure 1). As a consequence, their initial haemoglobin concentrations just before malaria episodes were probably higher than in those without multi-nutrients, preventing anaemia from becoming as severe during malaria as in children who did not receive multi-nutrients (Figure 5). In older children, who had a lower prevalence of iron deficiency (7%), there was no obvious benefit of multi-nutrient supplementation during the second survey or during malaria episodes (Figures 1 and 5). An improvement of haemoglobin

concentrations by multi-nutrients in children with iron deficiency, resulting in the prevention of low haemoglobin concentrations during malaria episodes, may likewise explain the opposite trend in age-specific effects on episodes with haemoglobin concentrations < 80 g/L as compared to episodes with hyperparasitaemia (Figure 2). Thus our data suggest that iron supplementation can prevent episodes of malaria with severe anaemia. The critical issue remains, however, whether the benefits of higher hemoglobin concentrations during acute malaria and the expected reduced risk of severe malarial anemia on one hand outweigh the risk of potentially lethal disease manifestations associated with an increased frequency of malaria on the other hand.

We speculate that the iron in the multi-nutrients may have enhanced parasite proliferation specifically in the youngest children, because iron deficiency in this group leads to more efficient iron absorption and thus may lead to transient production of non-transferrin bound iron (Hutchinson et al. 2004, Baron et al. 2008, Dresow et al. 2008) which may act as a nutritional source and favour the proliferation of *Plasmodium* parasites (WHO 2007). Evidence in support of this hypothesised mechanism is, however, lacking. The absence of adequate protective immunity and the higher dose of iron per kilogram of bodyweight may have further contributed to the observed increase in rates in this subgroup. In children aged 35–60 months, the decrease in the rate of episodes with hyperparasitaemia due to multi-nutrients may indicate an immune-enhancing potential of supplementation, consistent with the idea that most micronutrients are essential for innate and acquired immunity and particularly for appropriate T cell responses to infections (Maggini et al. 2007, Duriancik et al. 2010). Such immunity is acquired with repeated or chronic exposure to infection, and plays a minor role in younger children.

Subgroup analysis should generally be interpreted with caution (Sun et al. 2010). On one hand, we did not specify interactions between multi-nutrient supplementation and iron status *a priori*, and the direction of the subgroup effect is in contrast with the findings previously obtained in the Pemba substudy with iron and folic acid (Sazawal et al. 2006). On the other hand, the difference in effects of multi-nutrients between children with and without iron deficiency was large (hazard ratios for episodes with parasitaemia > 10,000/μL: 1.53 versus 0.89), the statistical evidence for effect modification was strong (p=0.007), and it is biologically plausible that the effect of multi-nutrient supplementation on malaria rates depends on iron deficiency (and immune status) at baseline.

In their report of the findings from the Pemba sub-study, Sazawal et al. (2006) were cautious in their interpretation of the subgroup effect, stating that their results suggested that supplementation with iron and folic acid is beneficial in children with iron deficiency, but unsafe in those who are iron-replete. Based on these findings, an expert group convened by WHO recommended that iron supplements should be administered routinely to iron-deficient infants in settings with adequate access to anti-malarial treatment (WHO 2007). Unfortunately our findings challenge this recommendation. In the Pemba substudy, iron deficiency was defined by elevated molar ratios of zinc protoporphyrin and haem, while plasma ferritin concentration (that was used in our study) is probably a more specific indicator of iron deficiency.

In conclusion, subgroup analysis suggests that the effect of multi-nutrients on malaria varies strongly with age and iron status. In children aged 0.5 to 1.5 years, and those with iron deficiency, supplementation with multi-nutrients may be unsafe; although these children were better able to maintain their haemoglobin concentration during malaria attacks when having received multi-nutrients, they also had an increased risk of malaria, particularly of episodes with hyperparasitemia (> 100,000/μL). Supplying multi-nutrients at levels normally provided through supplementation or home fortification, even when targeting deficient children in settings with adequate access to care, cannot be recommended in malaria-endemic areas until its safety has been demonstrated.

Contributors: JV was responsible for data collection and analysis, administration and drafted the manuscript; LS, NI, ACvdH and LCdeB assisted in data collection; AYD and RJK were responsible for laboratory analyses in The Netherlands; RMO and HFJS provided supervisory support; HV was responsible for concept, design and supervision of all aspects of the study; HV and HFJS obtained funding. All authors participated in data interpretation and critical revision of the report for intellectual content; and provided final approval of the submitted version.

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Chapter 5

Effect of preventive supplementation with zinc and other micronutrients on non-malarial morbidity in rural Tanzanian preschool children: a randomised trial

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Summary

Background: The efficacy of preventive zinc supplementation against diarrhea and respiratory illness may depend on population-specific factors. Simultaneous supplementation with multiple micronutrients may be required to overcome the lack of response that can be found when zinc is given alone.
Objective: We aimed to assess the effect of supplementation with zinc and multiple micronutrients on diarrhea and other causes of non-malarial morbidity.
Design: Rural Tanzanian children (n=612) aged 6–60 months and with height-for-age z-score < –1.5 SD were randomized to daily supplementation with zinc (10 mg) alone, multi-nutrients without zinc, multi-nutrients with zinc, or placebo. During follow-up, we recorded morbidity episodes. The study was registered with ClinicalTrials.gov (NCT00623857).
Results: We found no evidence that concurrent supplementation with multi-nutrients influenced the magnitude of the effect of zinc on rates of diarrhea, respiratory illness, fever without localizing signs, or other illness. Zinc supplementation reduced the hazard of diarrhea by 24% (4%–40%). By contrast, multi-nutrients seemed to increase this rate (HR; 95% CI: 1.19; 0.94–1.50), particularly in children with asymptomatic *Giardia* infection at baseline (2.03; 1.24–3.32). Zinc also protected against episodes of fever without localizing signs (0.75; 0.57–0.96), but we found no evidence that it reduced the overall number of clinic visits.
Conclusion: We found no evidence that the efficacy of zinc supplements in reducing diarrhea rates is enhanced by concurrent supplementation with other micronutrients. By reducing rates of fever without localizing signs, supplementation with zinc may reduce inappropriate drug use with anti-malarial medications and antibiotics.

Introduction

There are no policy recommendations for preventive zinc interventions in developing countries, despite evidence from a recent meta-analysis of trials, mostly conducted in Asia and Latin America, that it can reduce the overall rates of diarrhea and respiratory illnesses by 20% and 14%, and rates of mortality by 6%. Results have varied between trials, however, probably due to heterogeneity in study populations with respect to factors that determine the response to zinc (Brown et al. 2009a).

Zinc deficiency often coexists with other nutritional deficiencies, and simultaneous supplementation of multiple micronutrients may be required to overcome the lack of response that can be found when zinc is given alone (Shrimpton et al. 2005). In a recent review of steps to be taken to translate research into zinc intervention programs, the International Zinc Nutrition Consultative Group urged the implementation of studies to assess the efficacy of zinc in combination with other micronutrients (Brown et al. 2009b).

Relatively few trials have been conducted in Africa, where the etiology of disease and environmental factors such as diet and micronutrient deficiencies may be different than in Asia and Latin America.

In this study, we aimed to assess the effect of supplementation with zinc and multiple micronutrients on diarrhea and other causes of non-malarial morbidity in young, rural Tanzanian children. In addition, we explored baseline factors that determined the magnitude of the morbidity response to such supplementation.

Subjects and methods

Study area: The study was part of a trial with the primary aim to assess the effects of supplementation with zinc and other micronutrients on malaria rates. Details of study design and effects on malaria rates will be reported elsewhere (Veenemans et al. submitted). The trial was performed in four villages (Ngojoro, Kwangwe, Bondo and Kwadoya) near Segera (S 05°23.050, E 038°34.745) in Handeni District, north-eastern Tanzania. This rural area is highly endemic for malaria, and access to health care is limited, with no primary care facilities in the study area except the research dispensary that was constructed specifically for this study. The vast majority of families live in self-constructed clay houses, few with adjacent pit-latrines. Most households are self-sustaining and the dietary intake of the children restricted to maize

and beans, with very low intake from animal products. This food contains a high concentration of phytates that limits the absorption of several trace elements, including zinc.

Recruitment: Data were collected between February 2008 and March 2009. All resident children aged 6–60 months were invited for screening. Venous blood and fresh stool samples were collected, anthropometric measurements recorded in duplicate, and children were examined by a clinical officer. Children were eligible when having a height-for-age z-score ≤ –1.5, no signs severe chronic diseases, weight-for-height z-score > –3 SD, and hemoglobin concentration > 70 g/L. Children whose parents refused consent or intended to move outside the study area during the follow-up period were excluded.

Randomization and interventions: Children were randomized within 6 strata defined by malaria infection (binary) and age class (6–17 months, 18–35 months and 36–60 months) and randomly permuted blocks with size randomly selected of 4 or 8. Children received daily supplements with either zinc alone (10mg as gluconate), multi-nutrients without zinc, zinc combined with multi-nutrients or placebo. The levels of magnesium and vitamin C in the multi-nutrient supplement were below upper limits that were based on osmotic diarrhea and related gastrointestinal disturbances as critical endpoints (WHO/FAO 2004); further details about the composition of the multi-nutrient supplement are shown in **Annex 3**. Supplements were packed as transparent blister-strips, each containing 15 capsules with powder that was similar in taste and appearance for all 4 intervention groups. Capsules were color-coded to reduce the chance that children would receive the wrong supplement. The color code was not disclosed to the researchers until after the database had been finalized.

Follow-up and case detection: Supplementation was performed by community volunteers, each supervising approximately 20 children. Supplements were taken out of capsules, mixed with water and administered early in the morning separate from the meal. All volunteers reported daily to the research dispensary, and project staff followed up immediately in case a child had missed a daily supplementation dose. Parents or guardians were requested to bring study children to the dispensary when they noticed fever or when their child was otherwise unwell. A clinical officer was on permanent duty, and assessed sick children according to a standardized form. In case of reported cough or difficult breathing, the clinical officer looked for signs of respiratory distress and counted breathing frequency twice, each time for one full minute. All children received first-line treatment free of charge.

When indicated, they were referred and provided with transport to the nearest district hospital. Parents could withdraw consent at any time.

Supplementation and follow-up continued until 12 March 2009, when the trial was stopped for all children simultaneously.

Outcome definitions: We used the following case definitions: 1) *Diarrhea*: guardian-reported loose or watery stools, with episodes separated by at least 48 h. To increase the specificity of the case definition we also defined cases as episodes as defined above with > 3 loose or watery stools during a 24-h period; 2) *Acute lower respiratory infections (ALRI)*: reported cough with respiratory rate exceeding age-specific cut-off values (> 50/min or > 40/min for children aged 6–12 mo and 12–60 mo, respectively (WHO 2000); 3) *Severe pneumonia*: ALRI as defined above with one or more danger signs (lower chest in drawing, nasal flaring, grunting or head nodding) (WHO 2000); 4) *Any respiratory illness*: guardian-reported cough or difficult breathing; 5) *Fever without localizing signs*: guardian reported fever that was not accompanied by cough, diarrhea or other localizing signs and with a negative result for a malaria dipstick test; 6) *Any other illness*: guardian-reported illness with symptoms involving skin, ears, eyes and abscesses, but excluding trauma or burns. Malaria was defined by a positive result for the malaria dipstick test in children with guardian-reported fever and any of the following: a) confirmed fever (axillary temperature ≥ 37.5°C), or b) unconfirmed fever with inflammation (whole blood C-reactive protein concentrations ≥ 8 mg/L), separated by at least 14 days from a previous malaria episode. The definitions provided above are not always mutually exclusive. In those cases when illness episodes fulfilled the criteria for more than one definition, they contributed to the analyses of all of these case definitions. Children who attended the dispensary for invited follow-up visits were only counted as new episode if presenting with new symptoms.

Laboratory procedures: Venous blood samples were collected in tubes with EDTA and centrifuged within 2 h after collection. An aliquot was analyzed the same day by hematology analyzer (Sysmex KX21, Kobe, Japan). As a measure of inflammation, we measured whole blood C-reactive protein concentration in the field by immunoturbidimetric assay (QuikRead, Orion Diagnostica, Espoo, Finland). Plasma concentrations of zinc were determined by inductively-coupled plasma-mass spectrometry. Plasma concentrations of C-reactive protein and ferritin were measured in The Netherlands on a Beckman Coulter Unicel DxC880i system. Stool samples collected during the surveys were preserved in sodium acetate acetic acid formalin, stored at 4 °C and tested for *Giardia intestinalis* parasites by enzyme-linked imunosorbent

assay (ProSpecT *Giardia* Microplate Assay, ProSpecT Giardia Microplate Assay, Oxoid, Basingstoke, UK). This test has a sensitivity and specificity of 93% and 100%, respectively, as compared to detection by either microscopy or the EIA in at least one of two sequential stool samples from individual subjects (Mank et al. 1997).

Statistical analysis: All data were double-entered, cleaned and analyzed in SPSS (v15·0 for Windows, SPSS, Chicago, IL) and STATA (v11; College Station, Tx). Height-for-age z-scores were calculated using Epi Info (version 3.3.2; http://www.cdc.gov/epiinfo); differences in proportions were calculated using CIA.(Altman et al. 2000) Being stunted was defined as height-for-age z scores < –2 SD.

We report incidence rates, and used a Cox proportional hazard model to assess intervention effects as crude and adjusted hazard ratios. We explored effects on diarrhea and respiratory illness by analyzing cases with and without fever separately. We conducted subgroup analyses to explore potential effect-modification by age classes (as defined by the strata used for randomization), being stunted, and the presence of *G. intestinalis* infection at baseline. In this analysis, we pooled children aged 18–35 mo and 16–60 mo because there were only 44 cases in the oldest age class. We explored to what extent adjustment for factors that were predictive for morbidity (age class, stunting, distance to the dispensary [≥ or < 4 km, which is close to the median], and mosquito net use) influenced estimates of the intervention effects.

Results

The trial profile is shown in **Figure 1**. Baseline characteristics were similar between intervention groups (**Table 1**). The prevalence of zinc deficiency was high (67%). *Giardia* infection was detected in 192 children (31%), and 426 children (70%) were stunted.

There were 3,268 clinic visits during the study period, of which 2,462 (75%) were accompanied by guardian-reported fever and 1,572 (48%) classified as malaria. Among the non-malarial fever cases, 658 were accompanied by diarrhea, cough, or other localizing signs, either alone or in combination, while 232 fever cases were without localizing signs (**Table 2**). For 223 children (36%), parents or caretakers reported at least one episode of diarrhea. A total of 390 diarrhea episodes were recorded, with an incidence rate of 0.74/ child-year. There were 1,333 episodes of reported cough, but only few (181)

Table 1. Baseline characteristics of study participants, by intervention group

	Zinc	Multi-nutrients without zinc	Multi-nutrients with zinc	Placebo
n	153	155	151	153
Sex M/F [n/n]	46%/54% [70/83]	56%/44% [87/68]	44%/56% [66/85]	50%/50% [76/77]
Age	32.5 ± 15.4	32.2 ± 15.7	32.5 ± 15.5	32.7 ± 16.1
Plasmodium infection [1]	43% [66]	41% [64]	44% [67]	44% [68]
Height-for-age, z-score	–2·36 ± 0·69	–2·50 ± 0·69	–2·39 ± 0·71	–2·45 ± 0·69
Inflammation [2]	34% [52]	33% [51]	34% [51]	31% [47]
Giardia intestinalis infection [3]	33% [50]	35% [54]	28% [42]	30% [46]
Zinc deficiency [4]	63% [97]	71% [110]	70% [105]	65% [100]
Iron deficiency [5]	23% [23]	24% [25]	24% [24]	24% [25]
Hemoglobin concentration, g/L	101·8 ± 12·6	102·7 ± 12·8	103·8 ± 12·7	102.8 ± 12·7
Distance from homestead to dispensary, km [6]	3·66 ± 2·31	3·52 ± 2·06	3·54 ± 2·07	3·60 ± 3·38
Mosquito net use [7]	32% [48]	36% [55]	30% [45]	31% [46]

Mean ± SD, % [n] unless indicated otherwise.

1 As indicated by a positive result for pLDH-based dipstick test (see text).

2 Plasma C-reactive protein concentration ≥ 8 mg/L.

3 Stool samples could not be analysed for 54 children (with 13, 14, 15 and 12 missing values in the 4 intervention groups). Prevalence calculated with total group numbers in denominator.

4 Plasma zinc concentration < 9.9 μmol/L.

5 Plasma ferritin concentration < 12μg/L (6 missing values); restricted to children without inflammation at baseline (n=101, 104, 100 and 106, respectively).

6 Measured as the crow flies, based on global positioning data.

7 Data missing for 11 children.

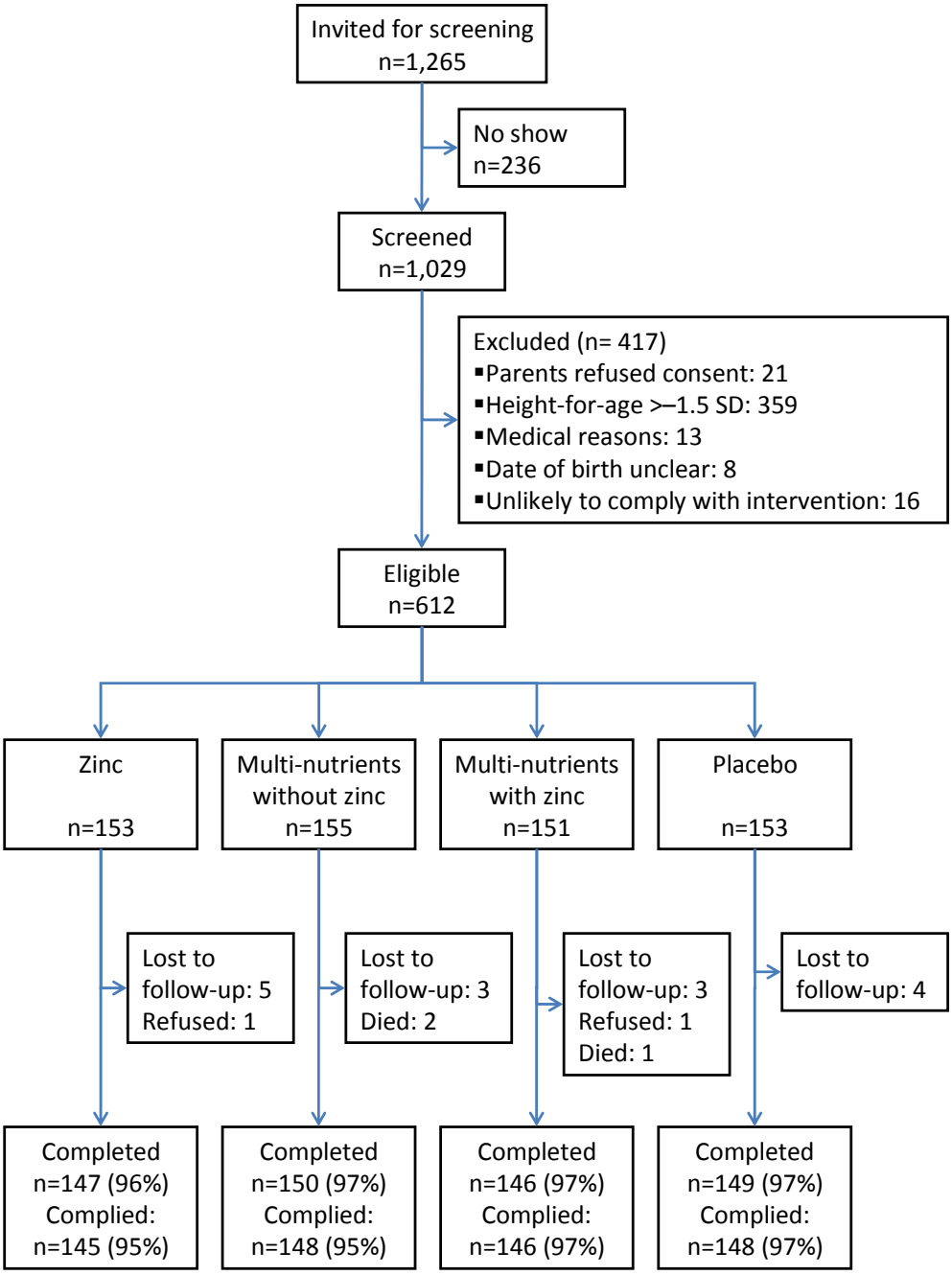

Figure 1. Trial profile

Table 2. Incidence rates for various illnesses, stratified by the presence of fever

	All	Fever reported	No fever reported
Total observation time, child-years	526	526	526
All clinic visits	6.2 (3,268)	4.7 (2,462)	1.5 (806)
Diarrhea			
Guardian-reported episodes	0.74 (390)	0.47 (248) [1]	0.27 (142)
> 3 loose stools/24h	0.44 (233)	0.41 (216)	0.03 (17)
Respiratory illness			
Cough	2.5 (1,333)	1.9 (998) [1]	0.64 (334)
Cough with fast breathing	0.34 (181)	0.28 (147) [1]	0.06 (34)
Severe pneumonia	0.05 (24)	0.04 (20)	0.01 (4)
Other illnesses [2]	1.4 (744)	0.68 (3,566) [1]	0.74 (388)
Fever without localizing signs [3]	0.44 (232)	NA	NA
Malaria	3.0 (1,572)	NA	NA
Hospital referrals	0.13 (68)	0.13 (68)	0.00 (0)

Values indicate the total number of episodes per child-year observed. Numbers between brackets indicate the number of episodes.
NA: Not applicable (fever is part of the case definition)

[1] Among these, the following number of cases was also classified as malaria: reported diarrhea: 137 (35%); reported diarrhea with ≥ 3 loose stools/24h: 69 (30%); reported cough: 491 (36%); other: 166 (22%).

[2] Includes symptoms of skin, ears, and eyes and abscesses; excluding trauma or burns.

[3] Cases classified as fever without localizing signs are not included in any of the other categories.

fulfilled criteria of ALRI as established by the World Health Organization (WHO 2000). The number of severe pneumonia cases (24) was insufficient for meaningful analysis. There were 744 visits for other reasons, mainly abscesses and symptoms involving skin, ears, and eyes.

Upon examination of interaction effects (**Table 3**), we found no evidence that concurrent supplementation with multi-nutrients influenced the magnitude of the effect of zinc on rates of diarrhea, respiratory illness, fever without localizing signs, or other illness. Thus in the remainder of this report, we will present marginal effects whereby the effect of multi-nutrients is assessed by comparing the pooled groups receiving placebo or zinc with the pooled groups receiving multi-nutrients, and the effect of zinc is assessed by comparing the pooled groups receiving no zinc (with or without multi-nutrients) and the pooled groups receiving zinc (with or without multi-nutrients).

Zinc supplementation reduced the rate of diarrhea by 24% (95% CI: 4% to 40%) (**Figure 2**), while multi-nutrients seemed to increase rates by 19% (–6% to 50%). We found similar effects when restricting the analysis to cases of diarrhea with ≥ 3 loose stools/24h (Figure 2). The protective effect appeared more evident for cases of diarrhea that were accompanied by fever (HR 0.73 [0.55-0.98]) than for those not accompanied by fever (HR 0.80 [0.55-1.16]), but the difference in effect was small.

There was no evident effect of either intervention on episodes of respiratory illness, whether defined as reported cough or as cough with fast breathing, or on episodes of other illnesses (Figure 2). By contrast, zinc reduced the rates of fever without localizing signs by 25% (4%—43%; adjusted for age class and distance between homestead and research clinic.

Age was strongly predictive for all morbidity outcomes, but there was no evidence that age class influenced the magnitude of the effect of the interventions for any of the outcomes considered (not shown). We found weak evidence that the zinc-induced reduction in diarrhea rates was more pronounced in stunted children (0.66; 0.49–0.90) than in those with a lesser degree of stunting (0.95; 0.62–1.45; interaction effect: 0.68 (0.41–1.15); **Figure 3;** upper panel, left).

The effect of multi-nutrient supplementation on diarrhea depended on the presence of *Giardia* infection at baseline (Figure 3; upper panel): multi-nutrient supplementation doubled the rate of diarrhea among those with *Giardia* at baseline (2.03; 1.24–3.32), whereas it had no obvious effect

Table 3. Incidence of non-malarial morbidity, by treatment group.

	Zinc	Multi-nutrients without zinc	Multi-nutrients with zinc	Placebo	Interaction effect [1]	p
Child-years at risk	130.8	133.2	129.7	131.9		
Diarrhea						
All reported episodes	0.62 (81)	0.92 (123)	0.67 (87)	0.75 (99)	0.88 (0.52–1.51)	0.65
> 3 loose stools/24h	0.35 (46)	0.56 (74)	0.42 (55)	0.44 (58)	0.96 (0.51–1.80)	0.89
Respiratory Illness						
Cough	2.5 (322)	2.5 (333)	2.7 (354)	2.5 (324)	1.09 (0.80–1.48)	0.57
Cough with fast breathing	0.34 (45)	0.32 (43)	0.39 (50)	0.33 (43)	1.13 (0.57–2.24)	0.72
Other	1.5 (195)	1.4 (186)	1.3 (169)	1.5 (194)	0.92 (0.62–1.37)	0.68
Fever without localizing signs	0.39 (51)	0.45 (60)	0.37 (48)	0.55 (73)	1.17 (0.66–2.06)	0.60

Values indicate the total number of episodes per child-year observed. Numbers between brackets indicate the number of cases.

[1] Measured in a Cox proportional hazard model as the hazard ratio associated with the interaction term between zinc and multi-nutrients.

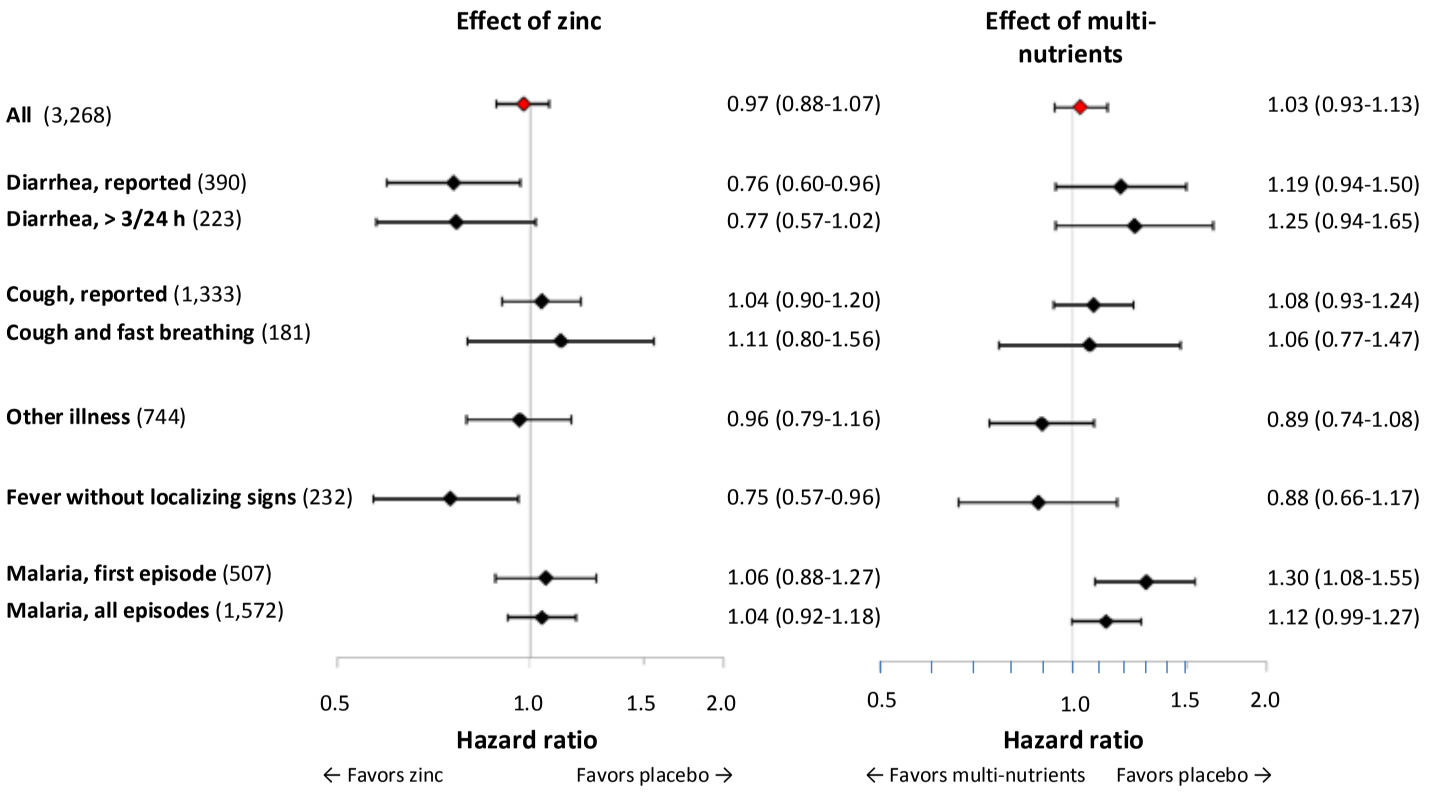

Figure 2. Effect of supplementation with zinc (left panel) or multi-nutrients (right panel) on selected morbidity outcomes
Effects are indicated by hazard ratios. Values between brackets (left) indicate the number of episodes for each illness definition. Horizontal line bars indicate 95% CIs. Estimates are adjusted for age class (indicated by dummies) and distance between homestead and research dispensary (< or ≥ 4 km); further adjustment for mosquito net use, sex, plasma zinc concentration, iron status or inflammation at baseline did not markedly change the effect estimates.

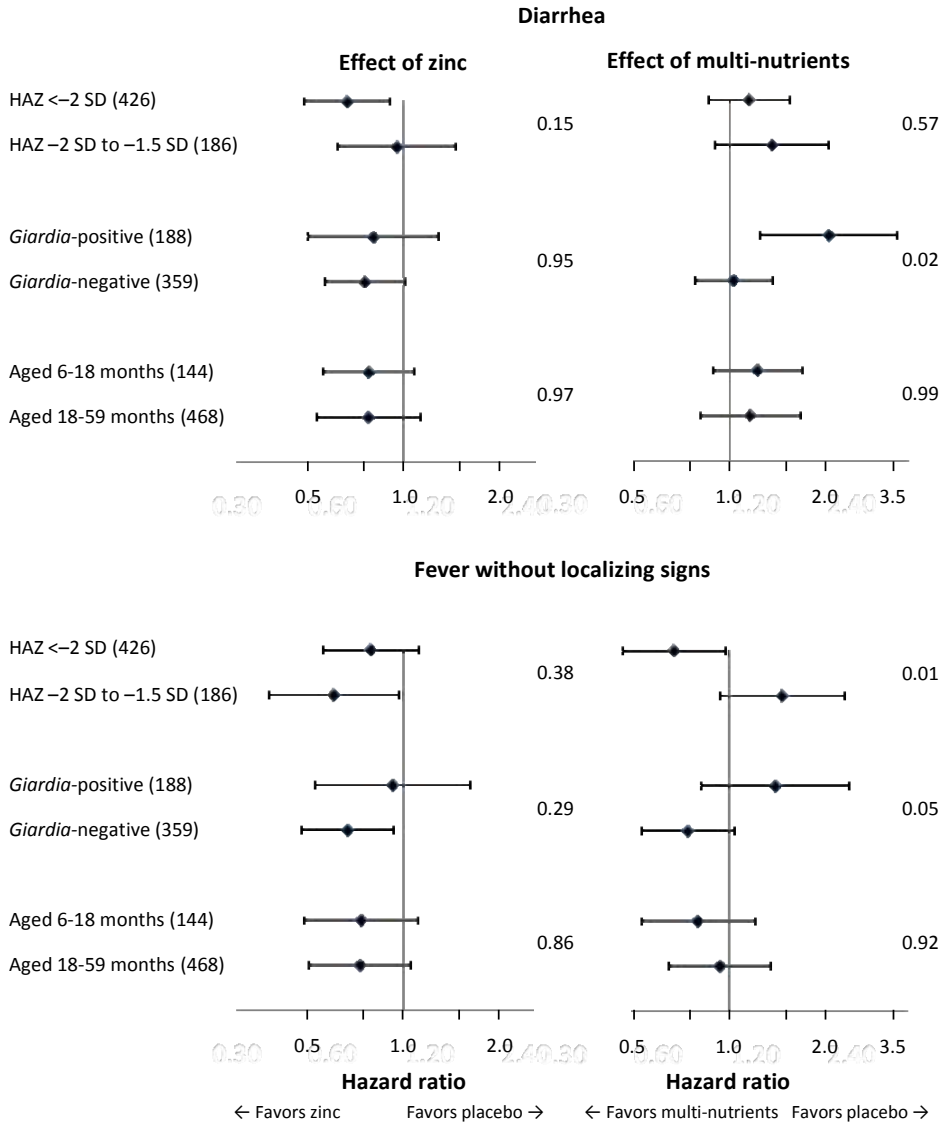

Figure 3. Subgroup analysis of effects of supplementation with zinc or multi-nutrients on diarrhea (top panels) and fever without localizing signs (bottom panels).
Values between brackets indicate the number of children in each subgroup. Horizontal line bars indicate 95% CIs. Effects are adjusted for age class, being stunted and distance (< or ≥ 4 km) unless these factors were used to define subgroups. Values on the right indicate p-values for interaction tests. Because the presence of *Giardia* infection could not be assessed in 54 children due to missing stool samples, the analysis is based on fewer cases of diarrhea (361) and fever without localizing signs (216) than reported in the text.

among those without *Giardia* infection (1.03; 0.78–1.36). This difference in intervention effect between *Giardia*-positive and *Giardia*-negative children was independent of age and stunting, and unlikely to have occurred by chance (interaction effect: 1.98; 1.13–3.47; p=0.02).

With regards to the effects of multi-nutrients on fever without localizing signs, patterns observed in the subgroup analysis largely resembled those observed for diarrhea (Figure 3; lower panel). Both *Giardia* infection and being stunted at baseline determined the magnitude of the effect of multi-nutrients on disease rates. Multi-nutrients resulted in increased rates (1.39; 0.82–2.36) in children with *Giardia* infection and decreased rates in those without (0.74; 0.53–1.03) (interaction effect: 1.86; 1.00–3.47). In stunted children, however, multi-nutrients decreased rates (0.67; 0.46–0.97), whereas in children with a lesser degree of stunting, multi-nutrients seemed to increase rates (1.46; 0.93–2.28; interaction effect: 0.47; 0.26–0.83).

Lastly, multi-nutrients also increased the rate of reported cough among stunted children, but not so in their less stunted counterparts (HR: 1.34; 1.06–1.69 versus 0.98; 0.82–1.17; p-value for interaction: 0.05). When analyzing effects on ALRI, a similar pattern was seen (1.41; 0.79–2.52 versus 0.99; 0.65–1.50 among stunted and less stunted children respectively), but the number of cases was lower and thus the statistical evidence for this interaction weaker (p=0.33).

Discussion

We found no evidence that the effect of zinc supplementation on any of the morbidity outcomes assessed was influenced by concurrent supplementation with other micronutrients. Daily supplementation with zinc reduced the rate of fever without localizing signs by approximately one-quarter. By contrast, supplementation with multi-nutrients seemed to increase the rate of diarrhea, mostly so in children with *Giardia* infection at baseline.

The effect of zinc on diarrhea found in our study is consistent with the 20% reduction reported in a recent systematic review (Brown et al. 2009a). Contrary to the findings from this meta-analysis (Brown et al. 2009a), however, we found no evidence that this protection depended on age class, and we found only weak support that it depended on the initial degree of stunting. The incidence of diarrhea in our study was comparatively low, however, and the number of cases may have been too low to show such effect modification. For the same reason, we may have failed to show an effect of either zinc or

multi-nutrients on rates of respiratory illnesses.

Multi-nutrient supplementation seemed to increase diarrhea rates by 25%, whereby the subgroup analysis suggested that it more than doubled this rate among children with *Giardia* infection at baseline. This adds to findings, based on data from the same trial, that multi-nutrients increase the rate of first malaria attacks by 30% (8%-55%; figure 2), and the rate of all malaria episodes among the children aged 6-17 months and those with iron deficiency by 26% (0%-57%) and 41% (9%-82%), respectively (Veenemans et al. submitted).

Two other trials that investigated the added benefit of multi-nutrients in addition to zinc in preventing episodes of diarrhea also failed to show an advantage of combined supplementation above supplementation with zinc alone. In Peruvian preschool children, zinc alone tended to reduce the risk of diarrhea and respiratory illness, whereas supplementation with multi-nutrients including zinc tended to increase this risk (Penny et al. 2004). In South African children, there was no evidence that supplementation with multi-nutrients including zinc was more efficacious than zinc alone in reducing the burden of diarrhea or respiratory illnesses (Chhagan et al. 2009, Luabeya et al. 2007). Taken together, these findings do not support and even caution against multi-nutrient supplementation in areas that are endemic for malaria and other infectious diseases.

Giardia infection at baseline influenced the effect of multi-nutrients on rates of fever without localizing signs in a similar pattern as the effect on diarrhea, even when adjusting for age and distance between homestead and research clinic. Although our definition of fever cases without localizing signs excluded cases of diarrhea, this similarity in effect patterns (including the fact that zinc protected against both outcomes) suggests that many of these fever episodes may have been due to enteric infections. Further studies are needed to establish whether *Giardia* infection caused this effect modification or whether it was a marker of an unknown factor that may have played such a role, and to identify the mechanisms involved.

Our study design did not allow us to identify single nutrients that may have caused the increase in diarrhea associated with the multi-nutrient supplement among children with *Giardia*. At the doses provided, it is unlikely that magnesium or vitamin C caused osmotic diarrhea; we also found no evidence, which otherwise might have been expected, that the increased diarrhea rates were larger in the youngest children who received a larger dose per body weight that older children (Figure 3). Whilst most micronutrients seem to protect against diarrhea (Long et al. 2007), supplementation with

iron may increase the diarrhea incidence (Gera and Sachev 2002), possibly by enhancing proliferation and virulence of enteric pathogens, or facilitating pathogen invasion by increasing permeability of the small intestine (Foster et al. 2001, Nchito et al. 2006). Iron may also influence or impair the immune responses to pathogens (Long et al. 2007). *In vitro* studies using Caco-2 cells suggest that copper may also impair intestinal barrier function by enhancing paracellular permeability (Ferruzza et al. 1999, Liu and Chen 2004).

We found no evidence that the addition of vitamins and other minerals to zinc supplements is helpful in preventing morbidity; in fact, it may increase the diarrhea rates in specific subgroups. This study adds support that zinc supplementation reduces the risk of diarrhea in African children and, although it did not reduce the overall number of clinic visits, it may reduce inappropriate use of anti-malarial drugs and antibiotics by reducing rates of fever without localizing signs. Our results should therefore encourage efforts to increase the intake of zinc in vulnerable populations.

Contributors: JV was responsible for data collection and analysis, administration and drafted the manuscript; LRAS assisted in data collection; TGM advised on design; DRAU, RMO and HFJS provided supervisory support; AYD and RJK provided laboratory support in The Netherlands; HV was responsible for concept, design and supervision of all aspects of the study; HV and HFJS obtained funding. All authors participated in data interpretation and critical revision of the report for intellectual content; and provided final approval of the submitted version.

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Chapter 6

Protection against diarrhea and fever without localizing signs associated with asymptomatic *Giardia intestinalis* infection is lost with multi-nutrient supplementation: a prospective study among rural Tanzanian children

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Summary

Background: Asymptomatic infections with *Giardia intestinalis* are common among children in developing countries, and its role as pathogen in such settings has been questioned. We recently showed in an intervention trial among rural Tanzanian children that multi-nutrient supplementation increased diarrhea rates among children who tested positive, but not among those who tested negative at baseline. In the current paper we describe to what extent *Giardia* influenced the risk of diarrhea and fever without localizing signs, and how the infection influences the effect of the intervention on nutritional status.

Methods: Data were collected in the context of a randomized placebo-controlled efficacy trial with 2x2 factorial design assessing the effects of zinc and/or multi-micronutrients on morbidity (n=612; height-for-age z-score < –1.5 SD). Outcomes measures were episodes of diarrhea (any reported, or with > 3 stools in the last 24 h) and fever without localizing signs. *Giardia* was detected in stool by enzyme-linked immunosorbent assay.

Findings: Among children who did not receive multi-nutrients, asymptomatic *Giardia* infection was associated with a substantial reduction in the rate of diarrhea (HR 0.32; 0.15–0.66) and fever without localizing signs (HR 0.56; 0.36–0.87), whereas no such effect was observed among children who received multi-nutrients (p-values for interaction 0.03 for both outcomes). This interaction was independent of age, height-for-age z-scores and distance to the research dispensary.

Conclusion: Although causality of the *Giardia* associated reduction in morbidity cannot be established, the data show that multi-nutrient supplements neutralise this protection and are thus likely to influence the proliferation or virulence of *Giardia* or associated intestinal pathogens.

Introduction

In developed countries, *Giardia intestinalis* (syn. *G. duodenalis, G. lamblia*) causes diarrhea while the prevalence of infections in the general population usually does not exceed 5% (Marshall et al. 1997). In developing countries, however, asymptomatic infections are much more common, with prevalence values in pediatric populations typically being around 30% (Mason and Patterson 1987, Fraser et al. 1997, Siwila et al. 2010), and reports on their association with diarrhea are inconsistent. Some reported an association with persistent (Bhandari et al. 1999, Newman et al. 2001) but not acute diarrhea (Bhandari et al. 1999, Gascon et al. 2000, Fraser et al. 2000, Newman et al. 2001, Hollm-Delgado et al. 2009), whereas other studies found that *Giardia* infection was associated with protection against acute diarrhea (Fraser et al. 1997, Bilenko et al. 2004, Albert et al. 1999, Haque et al. 2009).

Because the role of *Giardia* as diarrhea-causing agent is controversial and re-infection can occur rapidly, it has been recommended that children with asymptomatic infection should not be treated in highly endemic areas (Gilman et al. 1988, Sullivan et al. 1988). This notion is challenged, however, by findings from surveys (Loewenson et al. 1986, Muniz-Jungqueira and Oliveira Queiróz 2002, Sackey et al. 2003, Al-Mekhlafi et al. 2005, Ettehad et al. 2010) and a prospective cohort study (Prado et al. 2005) suggesting that such infections may impair linear growth, presumably by reducing intake and causing malabsorption of nutrients. In addition, in a prospective cohort study, it was found that episodes of *Giardia* with diarrhea but not diarrhea itself were associated with impaired cognition, perhaps because infection can lead to deficiencies of zinc and other micronutrients that have been associated with deficits in cognitive development (Berkman et al. 2002).

In a community-based trial in Tanzanian preschool children, we found that multi-nutrient supplementation resulted in increased rates of diarrhea and fever without localizing signs (Veenemans et al., submitted). In the current study, we aim to explore the influence of asymptomatic *Giardia* infection at baseline on the effect of multi-nutrient supplementation, to compare rates of diarrhea and fever without localizing signs in children with and without *Giardia* infection, and to assess the influence of micronutrient supplementation on these rates. In addition, we explore the influence of *Giardia* infection on the response of nutritional indicators to multi-nutrient supplementation.

Methods

Study population: This study was part of a randomized placebo-controlled trial in children aged 6–60 months, with the primary aim to assess the effect of supplementation with zinc and other micronutrients on malaria rates. It was conducted in a rural area in Handeni District, Northern Tanzania that is highly endemic for malaria. In a pilot survey among children aged 6–72 months in 2006 (n=304), we found a high prevalence of *Giardia intestinalis* (30%; assessed by microscopic examination of a single stool sample per child), and only few cases of *Ascaris lumbricoides, Trichuris trichiura* or *Schistosoma intestinalis* (3%, 5% and 0%, respectively) (unpublished results). Residents in the area virtually all comprise poor farmer families engaged in subsistence farming, with oranges being produced seasonally as cash crops. Families are living in self constructed clay houses, with very few having pit latrines. Water for drinking and household use is collected from central shallow wells. Few people boil drinking water. Access to health care was limited until we constructed a research clinic at a central location in the study area, which provided free primary care to study participants.

The study was approved by ethical review committees in The Netherlands and Tanzania; parents or guardians gave written consent.

Design: Details about study design will be published elsewhere. In brief, between February and August 2008, we recruited all resident children aged 6–60 months, and excluded those with height-for-age z scores > –1.5 SD, weight-for-age z-score < –3 SD, haemoglobin concentration < 70 g/L and with signs of severe or chronic disease, until attaining the target number (n=600) (**Webfigure**).

The trial had a 2×2 factorial design with children receiving either multi-nutrients with zinc (**Webtable**), multi-nutrients without zinc, zinc alone (10 mg), or placebo. The levels of magnesium and vitamin C in the multi-nutrient supplement were below the upper limits that were based on osmotic diarrhea and related gastrointestinal disturbances as critical endpoints (WHO/FAO 2004). Supplements were color-coded and administered daily by community volunteers.

At baseline, we collected venous blood in tubes suitable for trace element analyses (Becton-Dickinson, Franklin Lakes, NJ) and a fresh stool sample for each child in a vial that was pre-filled with sodium acetate-acetic acid-formalin (SAF) and stored in a refrigerator immediately after collection. A second vial with unfixed feces was stored in liquid nitrogen (–196°C) for

subsequent genotyping. We computed anthropometric indices as the average of two recordings, taken on consecutive days.

At recruitment, we asked parents or guardians to bring their children to the research clinic if they noticed any signs of illness. A clinical officer was on 24h-duty and collected medical information on standardised forms that included a section on diarrhea. A second survey, at 251 days (median; 95% reference range: 191–296 days) after enrolment, followed similar procedures as the baseline survey. Follow-up continued for all children until March 2009, when the study ended for all children simultaneously.

Laboratory analyses: Stool samples were analyzed for the presence of *Giardia*-specific antigen by enzyme immunoassay (ProSpecT *Giardia* Microplate Assay, Oxoid, Basingstoke, UK). This test has a sensitivity and specificity of 93% and 100%, respectively, as compared to detection by either microscopy or the EIA in at least one of two sequential stool samples from individual subjects (Mank et al. 1997). Methods for measuring plasma indicators have been described elsewhere (Veenemans et al. submitted).

Statistical analyses: Cases of diarrhea were defined as: a) all dispensary visits for parent- or guardian-reported loose or watery stools, with episodes being separated by at least 48 h of being without symptoms; or b) similar episodes with ≥ 3 loose or watery stools per 24-h period. Fever without localizing signs was defined as cases with reported fever that did not classify as malaria and were not accompanied by cough, diarrhea or other localizing signs. Thus cases of diarrhea and fever without localizing signs were mutually exclusive.

Data were analyzed using SPSS (v15·0 for Windows, SPSS, Chicago, IL, USA) and STATA (v11; College Station, Tx, USA). We report incidence rates and assessed group differences by Kaplan-Meier analysis with Tarone-Ware test. Differences in the association between *Giardia* and morbidity outcomes between intervention groups were assessed by analysis within intervention strata, and directly by Cox regression analysis that included dummies for intervention groups and interaction terms. Cross-over between groups, whereby infected children became infection-free and vice versa, may dilute a potential effect of *Giardia* over time. For this reason, we restricted our primary analysis to first episodes, because an analysis of all events is probably more susceptible to such dilution of effect. However, because a substantial number of children experienced recurrent events and analysis of all events may better reflect total disease burden, we repeated these analyses based on all events, with robust estimates of the standard error to account for correlation between episodes within children. We explored potential confounding by adjusting

for factors that were previously found to be prognostic for diarrhea (age, distance and height-for-age z scores) (Veenemans et al, submitted).

We also used multivariate linear regression analysis with interaction terms to assess to what extent the effect of zinc and multi-nutrient supplementation (either alone or combined) on indicators of nutritional status depended on *Giardia* infection.

Results

The study profile is shown in **Figure 1**. *G. intestinalis* was detected in 192 children (31%). We failed to obtain fresh stool samples for 54 children at baseline and for 50 children during the second survey, when 20 children were lost to follow-up. Baseline characteristics are presented in **Table 1**. Children with *Giardia* infection were on average 3.3 months older than their uninfected peers, resided somewhat closer to the dispensary, had a lower prevalence of inflammation, marginally higher hemoglobin concentrations, as well as marginally lower plasma concentrations of soluble transferrin receptor and folate. All other biochemical indicators of nutritional status were similar, and we found no evidence that *Giardia* was associated with symptoms as reported by the mother. The percentage of children who received antibiotic or anti-malarial treatment at baseline was similar in both groups.

At the time of the second survey, 43% of children with *Giardia* infection at baseline no longer carried the parasite, while 23% of children who tested negative at baseline had become infected (Figure 1).

The incidence of first episodes of diarrhoea was almost 50% lower among children with *Giardia* at baseline than among those without (**Table 2**).There was no evidence that the association between *Giardia* infection and diarrhea differed between children receiving placebo or zinc (in both groups the infection was associated with a protection), or between children receiving multi-nutrients with or without zinc (no association in either group; F**igure 2**); we therefore combined children who received multi-nutrients with or without zinc (henceforth referred to as 'with multi-nutrients'), as well as their peers who received zinc or placebo ('without multi-nutrients') as two separate groups.

Thus analyzed, *Giardia* infection was associated with a substantial reduction in time to first diarrhea episode with ≥ 3 watery stools/24h ($p<0.001$), but only so among children without multi-nutrients, whereas no association

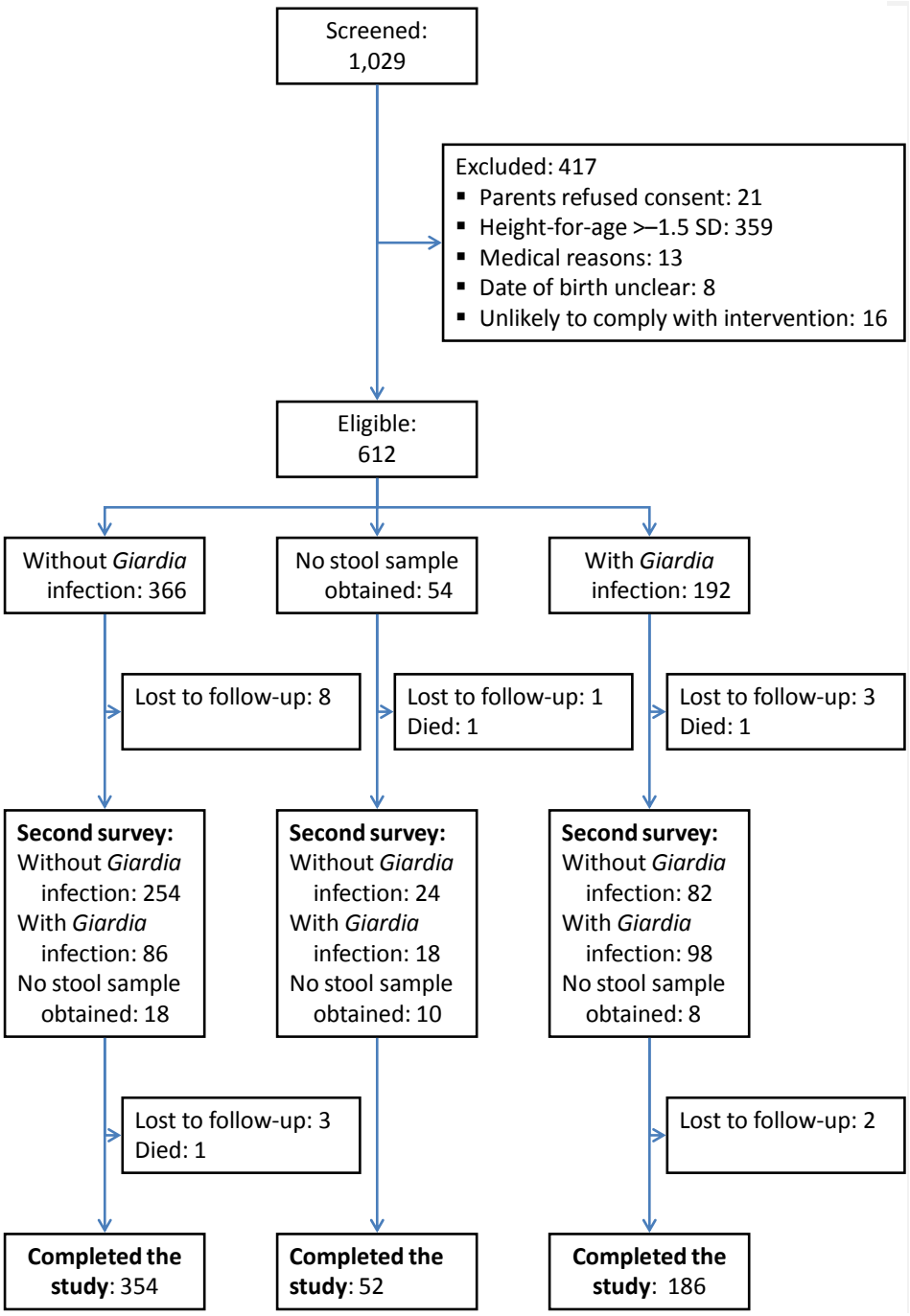

Figure 1. Study profile

Table 1. Baseline characteristics, by Giardia intestinalis infection status at baseline

Characteristic	*Giardia*-positive	*Giardia*-negative	p
n	**192**	**366**	
Age, [mean]	35.0 ±14.5	31.7 ±16.1	0.02
6–17 mo	16% (31)	27% (97)	
18–35 mo	35% (68)	33% (122)	
36–60 mo	48% (93)	40% (147)	
Sex, boys:girls (n:n)	53%:47% (102:90)	46%:54% (168:198)	0.06
Distance between homestead and research dispensary, km	3.1 (1.8)	3.7 (2.3)	0.01
Mosquito net use	31% (117)	33% (59)	0.43
Anthropometric indices			
Height-for-age z-score, SD	–2.44 ± 0.75	–2.40 ± 0.67	0.49
Weight-for-height z-score, SD	–0.17 ± 0.80	–0,07 ± 0.83	0.20
P. falciparum antigenemia (n) [1]	44% (84)	43% (157)	0.46
Inflammation [2]	24% (46)	37% (134)	0.001
Biochemical indicators of nutritional status			
Hemoglobin concentration, g/L	104.8 ± 12.1	101.7 ± 12.6	0.005
Anemia [3]	65% (125)	71% (260)	0.09
Plasma zinc concentration, mmol/L [4]	9.0 ± 2.4	9.0 ± 2.3	0.86
Plasma ferritin concentration, µg/L [5,6]	31.1 (26.8–36.1)	35.1 (32.0–40.4)	0.14
Iron deficiency [5]	20% (38)	17% (60)	0.17
Plasma sTfR concentration, mg/L [5]	2.4 (2.2–2.5)	2.6 (2.5–2.7)	0.02
Plasma cobalamin concentration, pmol/L [5]	347 (326–369)	333 (318–350)	0.33
Plasma folate concentration, nmol/L [5]	7.1 (6.5–7.7)	8.6 (7.9–9.2)	0.002
Symptoms reported at clinical assessment			
Sick (according to mother)	20% (39)	23% (84)	0.27
Fever now or in the last 24 h	16% (30)	19% (68)	0.43
Sick according to clinical officer	5% (10)	6% (22)	0.44
Abdominal pain/discomfort	24% (46)	31% (112)	0.06
Diarrhea	10% (19)	12% (42)	0.34
Vomiting	3% (5)	4% (13)	0.37
Lack of appetite	12% (23)	11% (40)	0.49
Treatment provided at baseline			
Metronidazole	2% (3)	3% (10)	0.29
Mebendazole	10% (19)	8% (29)	0.26
Beta-lactam antibiotics	12% (22)	10% (36)	0.32
Artemether-lumefantrine	44% (84)	43% (157)	0.46

sTfR: soluble transferrin receptor. Values indicate mean ± SD unless indicated otherwise. For 54 children, infection status for *Giardia intestinalis* was unknown because fresh stool samples could not be obtained.
[1] As determined by pLDH test; children with a positive test result were treated with artemether-lumefantrin, regardless of the presence or absence of malaria symptoms (see text); [2] Inflammation: whole blood C-reactive protein concentration > 8 mg/L; [3] Anemia: hemoglobin concentration < 110 g/L; [4] Iron deficiency: plasma ferritin concentration < 12 µg/L (6 missing values); [5] Geometric mean (95% confidence interval)

Table 2. Incidence rates of diarrhoea or fever of unknown origin by baseline Giardia test result by Giardia intestinalis infection status at baseline and intervention group [1]

	Giardia-positive	*Giardia*-negative	Rate ratio	p
Analysis of first episodes				
Any reported diarrhea (223) [2]				
All	0.43 [58/135.6]	0.68 [147/217.6]	0.63 [0.46–0.86]	
Without multi-nutrients	0.29 [21/72.5]	0.72 [79/109.7]	0.40 [0.24–0.66]	
With multi-nutrients	0.43 [37/63.1]	0.68 [68/107.9]	0.93 [0.60–1.41]	0.01
Episodes with > 3/24 h (157) [2]				
All	0.24 [36/149.3]	0.45 [109/244.7]	0.54 [0.36–0.79]	
Without multi-nutrients	0.11 [9/79.7]	0.46 [57/123.2]	0.24 [0.11–0.50]	
With multi-nutrients	0.39 [27/69.6]	0.43 [52/121.4]	0.91 [0.55–1.47]	0.003
Fever without localizing signs (172) [2]				
All	0.43 [49/144.8]	0.68 [110/255.2]	0.78 [0.55–1.11]	
Without multi-nutrients	0.31 [23/74.1]	0.48 [60/124.0]	0.64 [0.37–1.05]	
With multi-nutrients	0.37 [26/76.6]	0.38 [50/131.2]	0.97 [0.58–1.58]	0.25
Analysis of all episodes				
Any reported diarrhea (390)				
All	0.55 [95/172.1]	0.85 [266/311.9]	0.66 [0.49–0.89]	
Without multi-nutrients	0.35 [30/85.3]	0.88 [138/157.2]	0.41 [0.25–0.67]	
With multi-nutrients	0.75 [65/83.8]	0.82 [128/154.7]	0.93 [0.63–1.36]	0.01
Episodes with > 3/24 h (223)				
All	0.30 [51/172.1]	0.53 [164/311.9]	0.58 [0.39–0.84]	
Without multi-nutrients	0.16 [14/85.3]	0.52 [82/157.2]	0.32 [0.15–0.66]	
With multi-nutrients	0.43 [37/83.8]	0.53 [82/154.7]	0.83 [0.53–1.30]	0.03
Fever without localizing signs (232)				
All	0.38 [66/172.1]	0.48 [150/311.9]	0.79 [0.57–1.10]	
Without multi-nutrients	0.32 [27/85.3]	0.57 [89/157.2]	0.56 [0.36–0.87]	
With multi-nutrients	0.45 [39/83.8]	0.39 [61/154.7]	1.13 [0.71–1.80]	0.03

Rate ratios indicate crude incidence ratios for first or only events, and crude hazard ratios for recurrent events. p-values based on tests for differences between rate ratios in children with and without multi-nutrients (based on Cox proportional hazards model that included interaction term between multi-nutrients and *Giardia*)
[1] Numbers in intervention groups: without multi-nutrients: n =306 (96 *Giardia*-positive, 185 negative, 25 missing); with multinutrients: n=306 (96 *Giardia*-positive, 181 negative, 29 missing); [2] 19,12 and 13 cases of reported diarrhea, diarrhea with ≥ 3 loose stools/24h and fever without localizing signs occured in children for whom *Giardia* status was unknown

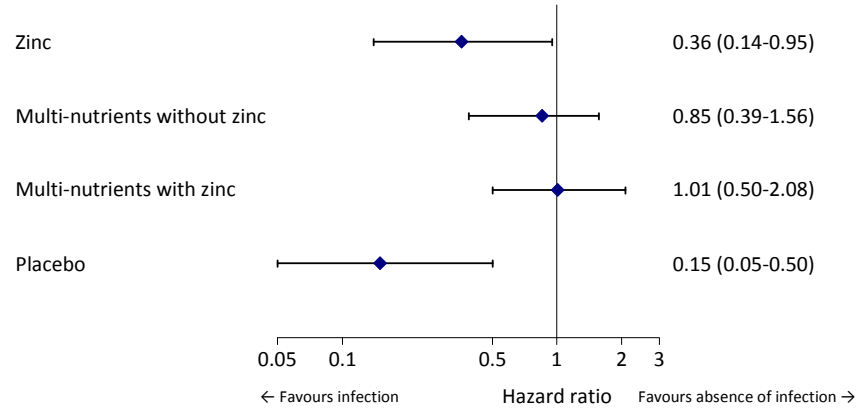

Figure 2. Association between *Giardia* and rates of diarrheal episodes with ≥ 3 watery stools/24h, by intervention group
Estimates indicate crude incidence ratios of first or only episodes, with 95% confidence intervals.

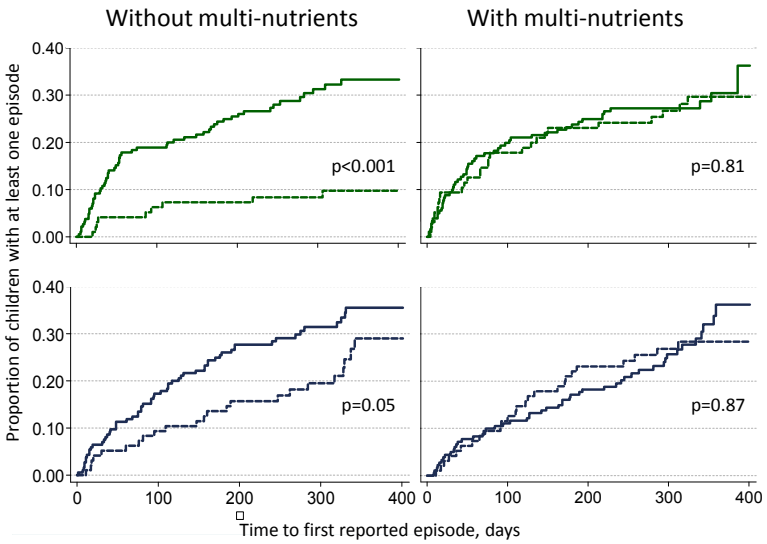

Figure 3. Association between *Giardia* infection and time to first episode of diarrhea with ≥ 3 watery stools/24-h (upper panels; 157 cases) or fever without localizing signs (lower panels; 172 cases), by intervention group.
Panels on the left and right: children without and with multi-nutrients, respectively; solid and dashed lines: children without and with *Giardia intestinalis* infection at baseline, respectively. Values indicate p-values for group differences (Tarone-Ware test). Hazard ratios for interaction between *Giardia* and multi-nutrient supplementation were 3.55 (1.52–8.24) and 1.50 (0.76–2.92) for diarrhea and fever without localizing signs, respectively (Cox proportional hazards model).

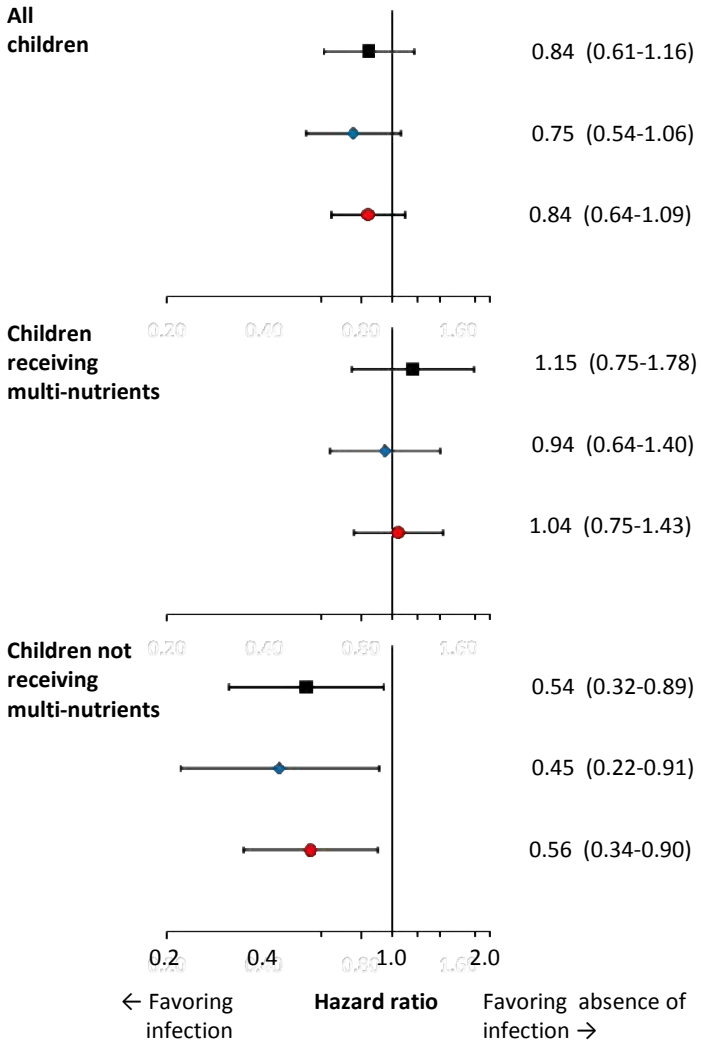

Figure 4. Associations between *Giardia* infection and diarrhea, and between *Giardia* and fever without localizing signs, assessed in all children (upper panel) or by intervention group (lower two panels).

Black square: fever without localizing signs; blue diamond: reported diarrhea, ≥ 3 watery stools per day; red circle: reported diarrhea. Hazard ratios (95% CIs) were calculated using multivariate Cox regression, adjusted for age (continuous), height-for-age z-score (continuous) and distance between homestead and research clinic (< or ≥ 4 km. Further adjustment for sex, zinc deficiency, mosquito net use, weight-for-height z scores and inflammation at baseline led to virtually identical estimates; not shown).

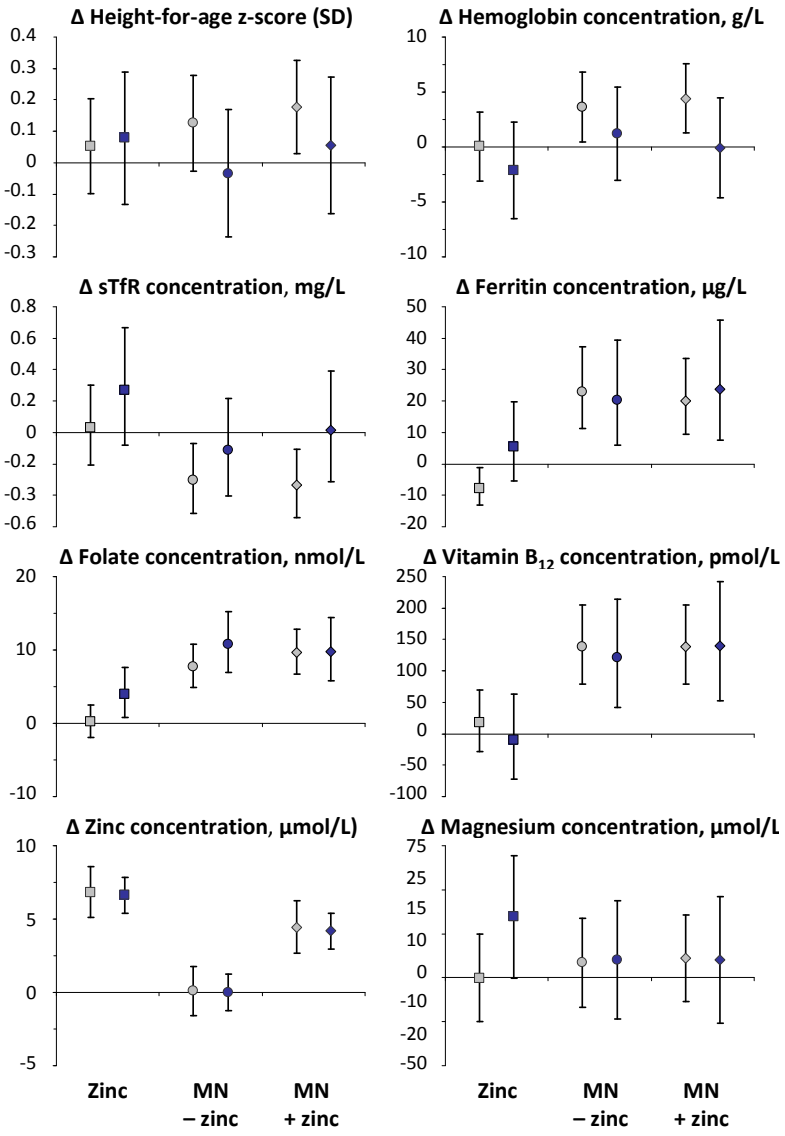

Figure 5. Effect of multi-nutrient supplementation on indicators of nutritional status, by *Giardia intestinalis* infection status at baseline.
sTfR: soluble transferrin receptor; MN: multi-nutrients. Grey and blue: without and with *Giardia* infection, respectively. Dependent variables were log-transformed as appropriate, and expressed in natural units by exponentiation of estimates resulting from the analysis. For each nutritional status indicator investigated, effects are adjusted for the same indicator measured at baseline. Line bars indicate 95% CIs.

was found between *Giardia* infection and diarrhea in those receiving multi-nutrients (**Figure 3**; top panels).

Adjustment for age and distance to the dispensary led to smaller but still substantial associations between *Giardia* infection and diarrhea (all events), whilst interaction effects between infection and the multi-nutrient intervention remained virtually unchanged (**Figure 4).** Further adjustment for baseline factors previously found to be prognostic for diarrhea (height-for-age z-scores, sex, inflammation and use of mosquito nets) led to similar effect estimates (not shown). We also explored the association between *Giardia* infection and diarrhea within age classes in children without multi-nutrients; although the numbers of cases within these strata was low, all estimates pointed towards a protective association (HR: 0.36 [0.13 to 1.01], 0.81 [0.43 to 1.55], 0.19 [0.04 to 0.84] in children aged 6–17 months, 18–35 months and 36–60 months, respectively).

Similar patterns were seen for episodes of fever without localizing signs: *Giardia* infection was associated with a reduction in the time to first episodes of such fevers among those receiving zinc or placebo, but not among those receiving multi-nutrients. Adjusted estimates of hazard ratios (including all events) are shown in figure 3.

The effect of the multi-nutrients on height-for-age z-scores, hemoglobin concentrations and plasma transferrin receptor concentrations measured at the second survey tended to be greater in children without *Giardia* infection at baseline, whereas supplements seemed to have little effect in those who tested positive at baseline (**Figure 5**). The overall effects were rather small, and statistical evidence for differences in effect between children with and without *Giardia* was weak (p-values for interaction between *Giardia* and multi-nutrients: 0.13 [height-for-age z-scores], 0.24 [hemoglobin concentrations] and 0.32 [plasma soluble transferrin receptor concentrations]). Adjustment for age led to similar conclusions (not shown). For other indicators of nutritional status (plasma concentrations of zinc, magnesium, cobalamin, folate and ferritin), there was no evidence that *Giardia* infection influenced the effect of supplementation.

Discussion

Giardia intestinalis infection at baseline was associated with a marked reduction in the rates of subsequent diarrhea among children receiving zinc or placebo, but not in those receiving multi-nutrients. Multi-nutrient

supplementation among children with *Giardia* infection resulted in disease rates similar to those found in uninfected children. Similar patterns were observed for cases of fever without localizing signs.

Substantial cross-over occurred between groups in the course of the study, and this may lead to underestimates of differences between children with and without *Giardia* infection. Our Kaplan-Meier analysis indicates, however, that the protective association occurred almost from the start of the follow-up period, when presumably few cross-over cases had occurred.

Our study is limited by the observational nature of our data, which does not allow a conclusion that the protection observed was caused by *Giardia* infection. Although this association was still present after adjustment for age and other potentially confounding factors, we cannot exclude the possibility that children with *Giardia* infection differed from their uninfected peers in other unmeasured characteristics that are prognostic for diarrhea (e.g. sanitation, or previous or current exposure to other gastro-enteric pathogens). We did not measure breastfeeding behavior, but it is unlikely that this could have explained the protective association found against diarrhea in children not receiving multi-nutrients: even in older children (aged 36–60 months), *Giardia* infection was associated with a reduction in hazard rates by 81% (16% to 96%).

Our findings support the view that the parasite is not an important cause of diarrhea in our study population. *G. intestinalis* comprises various genotypes, and its prevalence and its association with diarrheal symptoms seems to vary with geographic areas (Ward 2009). A recent study showed that *Giardia* infection was associated with protection against diarrhea, whereas *G. intestinalis* assemblage A was associated with acute diarrhea (Haque et al. 2009). Thus, due to variation in genotypes and environmental factors, our findings may not apply to other populations, and further research is also needed to determine *G. intestinalis* genotype in this population.

It is not inconceivable that *Giardia* infection protects against diarrhea, for example by competing with or suppressing other enteric pathogens, or by inducing changes in mucosal immunity (e.g. Ljungstrom et al. 1985, Bilenko et al. 2004). Chronic or repeated exposure to non-pathogenic *Giardia* genotypes may have induced immunity against more pathogenic genotypes. This cannot fully explain the protective effect observed, however, because the magnitude of the protective association found probably exceeds the *Giardia*-attributable fraction of diarrhea.

Giardia infection may also be a marker of an unknown factor (e.g. previous exposure to other pathogens) that leads to protection against both diarrhea and fever without localizing signs. Whatever the cause, *Giardia*-associated protection was no longer present when giving multi-nutrients. This interaction is supported by the magnitude of the subgroup effect, the consistency in the protective effect being observed in groups receiving placebo and zinc and with respect to two mutually exclusive but probably related outcomes (diarrhoea and fever without localising signs), whilst the probability that the association is due to chance seems low.

Further studies are needed to evaluate how supplemental micronutrients influence the composition, proliferation and pathogenicity of intestinal biota, and the interaction of these biota with their host. Iron deserves special attention in view of findings that it can enhance the virulence and invasion of *Salmonella enteritidis* (Foster et al. 2001), whilst a recent study suggest that supplementation with bovine lactoferrin, an iron binding-protein, reduced the prevalence of *Giardia* among in Peruvian preschool children (Ochoa et al. 2008).

Our study findings do not support treatment of *Giardia* infections in symptom-free children, and question the benefit of providing multi-nutrient supplements in populations frequently exposed to diarrheal diseases.

In conclusion, *Giardia* infection at baseline was associated with a marked reduction in the rates of subsequent diarrhea. Our data suggest that it is a marker for the response in diarrhea to multi-nutrient supplements.

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Chapter 7

α^+-Thalassaemia protects against anaemia associated with asymptomatic malaria: evidence from community-based surveys in Tanzania and Kenya

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Summary

Background: Hospital-based studies have found that α^+-thalassemia is associated with a marked protection against admission with severe, life-threatening falciparum malaria. Evidence suggests that α^+-thalassemia does not prevent infection or high parasite densities, but rather limits progression to severe disease, in particular severe malarial anemia. However, evidence of a protective effect against anemia due to mild or asymptomatic infections is inconclusive.

Methods: In two community-based surveys in Kenya and Tanzania among afebrile children (0.5 to 8 years, n=801) we assessed to what extent α^+-thalassemia influences the effect of asymptomatic *P. falciparum* infections on hemoglobin concentrations.

Results: The effect of infection on hemoglobin concentrations was most pronounced in children with inflammation. Among these children, *P. falciparum* was associated with a reduction of hemoglobin concentration by 21.8 g/L in those with a normal genotype, as compared to only 16.7 g/L (difference, 95% CI: 17.2 g/L, 8.3 to 26.2 g/L) and 4.6 g/L (5.1 g/L, 1.0 to 11.1g/L) in children with hetero- and homozygous α^+-thalassemia, respectively. In children without inflammation, the effect of infection was smaller and similar between genotypes.

Conclusions: α^+-thalassemia limits the decline in hemoglobin concentration that is associated with afebrile infections. This protective effect depends on the presence of inflammation.

Introduction

α^+-thalassemia is highly prevalent in sub-Saharan Africa, Asia and Melanesia. Heterozygosis is characterized by the commonly deletional loss of one of the duplicated α^+-globin genes ($-\alpha/\alpha\alpha$) and slight hematologic changes, whereas homozygotes ($-\alpha/-\alpha$) generally have mild microcytic anemia (Williams et al. 1996, Mockenhaupt et al. 1999, Weatherall and Clegg 2001). Case-control studies have consistently shown that α^+-thalassemia protects against severe, life-threatening malaria requiring hospital admission (Mockenhaupt et al. 2004, Allen et al. 1997, Williams et al. 2005, May et al. 2007). This protective effect is most pronounced in homozygotes, and acts primarily against severe malarial anemia (Allen et al. 1997, Wambua et al. 2006, May et al. 2007). No study has found evidence of a reduced incidence of uncomplicated malaria episodes in α^+-thalassemic children (Wambua et al. 2006, Williams et al. 1996, 2005). Similarly, none of the above studies found an association between α^+-thalassemia and parasite prevalence or density. Although the mechanisms of protection are largely unknown, these findings suggest that α^+-thalassemia prevents disease progression through mechanisms other than limiting parasite replication, and that the protection by α^+-thalassemia is limited to severe manifestations of disease (Williams 2006, Pasvol 2006).

Severe malarial anemia can develop rapidly as a result of hemolysis during acute malaria episodes (Ekvall et al. 2001, Ekvall 2003), but may also be the cumulative result of smaller reductions in hemoglobin concentration during repeated episodes of uncomplicated malaria or impaired erythropoiesis during chronic asymptomatic infections (Casals-Pascual and Roberts 2006). The notion that severe malarial anemia can develop gradually is supported by observations from community-based surveys and hospital studies that many cases occur with only mild or no symptoms, no reported history of fever, and relatively low parasite densities (Marsh et al. 1995, Menendez et al. 1997, Biemba et al. 2000, Mockenhaupt 2004, Koram et al. 2005, Ong'chega et al. 2006), suggesting that adaptation to low hemoglobin concentrations has taken place over time.

Recently, it has been hypothesized that the protection by α^+-thalassemia is restricted to severe malarial anemia resulting from acute hemolysis, while conferring no protection against gradual reductions in hemoglobin concentrations during chronic or repeated infections (May et al. 2007). This was supported by findings from a large cohort study among Kenyan children, which found no evidence that α^+-thalassemia influences the degree of anemia associated with episodes of uncomplicated falciparum malaria (Wambua et al. 2006).

By contrast, based on a birth cohort study in Melanesia, Oppenheimer et al. suggested that malarial anemia is relatively mild in children with α^+-thalassemia, although this effect was detected only at the age of 6 months and was no longer present at 12 months (Oppenheimer et al. 1987). In non-hospitalized Nigerian children, malaria-associated anemia seemed less pronounced in heterozygous than in normal children and was virtually absent in homozygotes, although the difference in effect of infection on hemoglobin concentration was not statistically significant (Mockenhaupt et al. 1999). Similarly, a study in Ghana among predominantly asymptomatic pregnant women showed that the effect of *P. falciparum* infection on hemoglobin concentration was less pronounced in women with α^+-thalassemia than in their counterparts with a normal genotype (Mockenhaupt et al. 2000).

We hypothesized that α^+-thalassemia exerts its protective effect against severe malarial anemia by preventing the gradual decline in hemoglobin concentrations during milder or asymptomatic *P. falciparum* infections. The present study aimed to investigate to what extent α^+-thalassemia influences the degree of anemia associated with *P. falciparum* infection among afebrile children recruited during community-based surveys.

Materials and methods

Study area and population: We used data from two community-based surveys: The first survey was conducted in a lowland area in northern Tanzania, around Segera and Kwedizinga wards in Handeni District, in May–July 2006. The second survey was conducted in Marafa, the hinterland of Malindi district, in the coastal lowlands of Kenya, in May 2004, as a baseline assessment for an intervention trial (details are reported elsewhere; Andang'o et al. 2007). Malaria transmission is intense and perennial in both areas, with virtually all infections due to *P. falciparum*. In both areas poor farmer families predominate, with limited access to health care. The study was approved by the responsible Ethics Review Committees in The Netherlands, Kenya and Tanzania; informed consent was obtained from community leaders and local government officials, and from parents or guardians.

Sampling methods and eligibility criteria: For the Tanzanian survey, children were randomly selected from a census list that included all resident children aged 6–72 months in the study area. Children were eligible to participate in the survey when they had no signs of severe febrile disease or severe malnutrition at the time of assessment. Not all selected children participated; non-participating children were on average younger than their

participating peers. To avoid bias on replacement, we re-sampled children from within the same age category as the non-participants (age categories: 6–18 months, 18–36 months and 36–72 months) until 304 participating children were obtained.

From 325 children selected, 21 did not participate in the study: 2 children died between the time of the census and the start of the study; 3 children were temporarily absent; for 10 children, the parents refused consent; 4 children did not show up for unknown reasons; and 2 children were not eligible because they were sick and referred to the hospital on the day of recruitment. The Kenyan survey (n=516) was conducted in four schools, among all children who were enrolled in nursery or the first year of primary school. Three weeks before the baseline assessment of the trial, 528 children were screened for eligibility. Seven children with hemoglobin concentrations below 70 g/L, and five who were expected to be non-compliant or absent during the subsequent intervention period were excluded from participation.

Field procedures: All children were examined by a clinical officer, who also measured axillary temperature. Anthropometric measurements were collected from all children. Venous blood was collected in containers with sodium heparin as anticoagulant (Becton-Dickinson, Franklin Lakes, NJ). Children were treated free of charge for common childhood infections and anemia according to guidelines of the Kenyan and Tanzanian Ministries of Health.

Laboratory procedures: In Tanzania, parasitemia was detected by rapid immunochromatographic assay (Optimal, Flow Inc., Portland, OR). Contrary to most other types of rapid dipstick tests, this test detects *P. falciparum*-specific lactate dehydrogenase (pLDH) which is only produced by live parasites. The test result becomes negative rapidly after parasite clearance (Makler et al. 1998, Piper et al. 1999). In Kenya, parasitemia was detected by conventional microscopy.

Whole blood hemoglobin concentrations were measured by hematology analyzers (Beckman Coulter, Fullerton, CA; and KX-21, Sysmex Corporation, Kobe, Japan; for samples collected from Tanzania and Kenya, respectively).

Plasma was separated by centrifugation, transferred to cryotubes and stored in liquid nitrogen. We preserved red cells, including the buffy coats (90 μL), in microcentrifuge tubes containing a DNA-stabilizing buffer (AS1, Qiagen, Valencia, CA) at 4 °C, for subsequent α^+-thalassemia genotyping. Plasma concentrations of ferritin and C-reactive protein were measured as

indicators of iron status and inflammation, on a Behring nephelometer (BN-prospec, Dade-Behring, Marburg, Germany) in The Netherlands (Meander Medical Centre, Amersfoort). DNA was extracted using the QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany) according to instructions by the manufacturer. The $-\alpha^{3.7}$ deletion type of α^{+}-thalassemia was determined by polymerase chain reaction assays, as described by Lui et al. (2000). Other types of α^{+}-thalassemia, including the $-\alpha^{4.2}$ type, occur only sporadically in Africa (Weatherall and Clegg 2001, Ekvall 2003).

Statistical analyses: We used the following definitions: current infection: the presence of at least one asexual *P. falciparum* parasite upon microscopic examination of blood smears from Kenyan children, or a positive result for the pLDH test in blood collected from Tanzanian children; inflammation: plasma C-reactive protein concentrations > 10 mg/L (Gabay and Kushner 1999); anemia: hemoglobin concentrations < 110 g/L and < 115 g/L for children aged < 5 years and ≥ 5 years, respectively; iron deficiency: plasma ferritin concentration < 12 μg/L and < 15 μg/L for children aged < 5 years and ≥ 5 years, respectively (WHO 2001). Because it is difficult to interpret estimates of iron deficiency in a population with high prevalence of malaria and inflammation (both affect plasma ferritin concentrations independently of iron status), we restricted this analysis to children without inflammation.

All data were entered in a dedicated Microsoft Access database, and cleaned and analyzed using SPSS (vs 13.0 for Windows; SPSS Inc., Chicago, IL). Anthropometric z-scores were calculated using Epi Info software (version 3.3.2; www.cdc.gov/epiinfo). For normally distributed variables, we calculated means, SDs and 95% CIs. For variables that were not normally distributed, we calculated the geometric mean. We used the normal approximation of the binary distribution to obtain prevalence differences and their corresponding 95% CIs. For variables that could not be normalized by log-transformation, we assessed group differences by Mann-Whitney or Kruskal-Wallis tests.

We assessed the effect of *P. falciparum* infection on hemoglobin concentrations in a multivariate linear regression model that adjusted for age, weight-for-height z-score, iron deficiency, and study site. In this model, we evaluated whether the effect of *P. falciparum* infection depended on genotype, by examining interaction terms (dummies for α^{+}-thalassemia genotype × infection status). We did not adjust for the presence of inflammation because we considered that the effect of infection on hemoglobin concentration may at least in part be mediated through inflammation. Thus, we repeated the above analysis whereby we stratified children with *P. falciparum* infection

by inflammation status. The groups thus formed were mutually exclusive, and were compared to the reference group of children without malaria, and without inflammation. For each of the groups, we directly evaluated whether the effect of infection on hemoglobin concentration depended on genotype by multivariate analysis.

Results

In Kenya and Tanzania, ten and nine children, respectively, were febrile at the time of examination and excluded from the analysis. The characteristics of the remaining 801 children are shown in **Table 1**. Eight Tanzanian children were afebrile but reported sick by the clinical officer (five were weak without localized signs but with a history of fever, one had signs of an upper respiratory tract infection, two had signs of a gastro-intestinal infection and one was completing a quinine course for malaria). Mothers of the Tanzanian children reported the following symptoms at the time of examination: history of fever in the last 24 h (23%), cough (43%), vomiting or diarrhea (16%), ear problems (5 %), skin lesions (19%). For Kenyan children, none of the children included in the study was reported sick by the clinical officer but mothers were questioned about the history of illness in the previous 2 weeks. The following symptoms were reported: history of fever (8%), cough (18%), gastro-intestinal symptoms (11%), ear problem (1%) and skin problem (5%).

The Tanzanian children were younger and their hemoglobin concentrations were lower as compared to the children in Kenya. There were no children with a hemoglobin concentration below 50 g/L. In Kenya and Tanzania, the prevalence of *P. falciparum* infection was similar, but the prevalence of inflammation was higher in Tanzania (78/295; 26%) than in Kenya (35/506; 7%). Children with *P. falciparum* infection had a higher prevalence of inflammation than those without infection (21% vs 8%; difference, 95% CI: 13 %, 9% to 18%), and higher plasma concentrations of C-reactive protein ($p<0.001$; Mann-Whitney test). We found no evidence that parasite density was different between the study sites ($p=0.11$, Mann-Whitney test), but the frequency of inflammation among children with *P. falciparum* infection was lower in the children from Kenya (9%; 25/265) than in those from Tanzania (43%; 58/134). In the following paragraphs, data are presented from the 2 study sites combined unless specified otherwise.

For 47 children, α-globin genotyping results were not available because samples were unclearly labeled or because of technical problems. Among the remaining children, 12% were homozygous, 46% were heterozygous

Table 1. Characteristics of study population, by genotype and study site

	$\alpha^{+3.7}$-genotype			
	Normal	Heterozygote	Homozygote	Undetermined
Tanzanian and Kenya combined, % (n)	42.0 (317)	45.8 (345)	12.2 (92)	47
Tanzania, % (n)	62.1 (169)	33.5 (91)	4.4 (12)	(23)
Age, years	2.7 (1.6)	2.9 (1.5)	2.3 (2.0)	2.6 (1.6)
Hemoglobin concentration, g/L	105.3 (17.2)	107.6 (17.1)	94.2 (14.3)	104.3 (19.2)
Anemia, % (n) [a]	55.6 (94)	48.4 (44)	100 (12)	52.2 (12)
Plasma ferritin concentration, µg/L [b,c]	19.7	22.0	21.5	13.8
Iron deficiency, % (n) [a,b,d]	13.2 (16)	16.7 (12)	16.7 (1)	22.2 (4)
Inflammation, % (n) [a]	28.4 (48)	20.9 (19)	50.0 (6)	21.7 (5)
P. falciparum infection, % (n) [a]	49.1 (83)	39.6 (36)	50.0 (6)	39.1 (9)
Height-for-age z-score	–1.76 (1.27)	–1.64 (1.23)	–1.57 (0.89)	–1.41 (0.87)
Kenya, % (n)	30.7 (148)	52.7 (254)	16.6 (80)	(24)
Age, years	5.9 (1.5)	5.9 (1.5)	5.8 (1.5)	5.9 (1.3)
Hemoglobin concentration, g/L	113.6 (11.8)	111.9 (10.3)	104.8 (11.47)	114.2 (8.12)
Anemia, % (n) [a]	39.9 (59)	59.4 (151)	80.0(64)	45.8 (11)
Plasma ferritin concentration, µg/L [c]	23.8	22.6	27.3	21.3
Iron deficiency, % (n) [a,d]	16.8 (23)	17.8 (43)	5.6 (4)	23.5 (5)
Inflammation, % (n) [a]	7.4 (11)	5.1 (13)	10 (8)	12.5 (3)
P. falciparum infection, % (n)	48.6 (72)	53.5 (136)	56.3 (45)	50 (12)
Height-for-age z-score	–0.77 (0.72)	–0.76 (0.72)	–0.85 (0.72)	–0.58 (0.59)

Mean (SD) unless otherwise indicated.
[a] Definitions as specified in the text; [b] n=90 for heterozygotes due 1 missing value for plasma ferritin concentration; [c] Geometric mean; [d] Restricted to children without inflammation

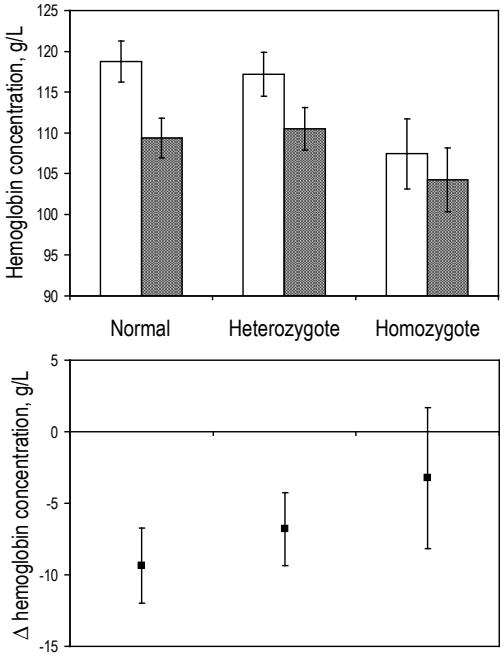

Figure 1. Associations between hemoglobin concentration and malarial infection, by α^+-globin genotype.
Footnote: Hemoglobin concentrations indicated are group means (upper panel) or differences in means (lower panel) as obtained by multivariate regression analysis that adjusted for standardized age and height-for-age z-score, iron deficiency and study site. White bars: without *P. falciparum* infection; black bars: with *P. falciparum* infection. Line bars indicate 95% CIs

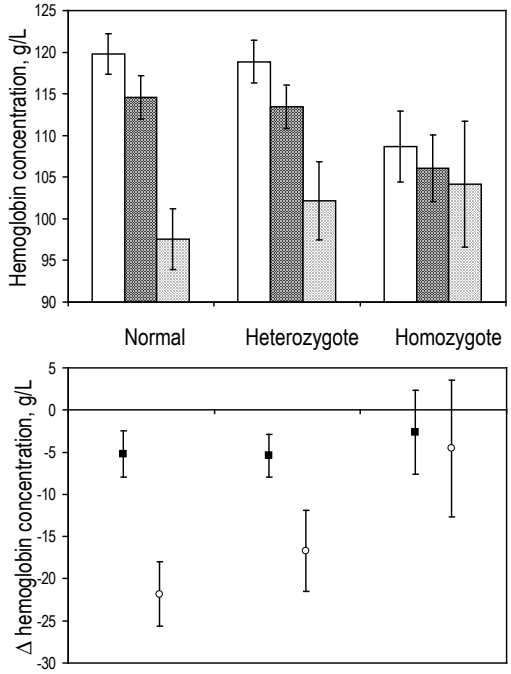

Figure 2. Hemoglobin concentration in groups defined by malarial infection, inflammation and α^+-globin genotype.

Footnote: Hemoglobin concentrations indicated are group means (upper panel) or differences in means (lower panel) as obtained by multivariate regression analysis, adjusted for standardized age and height-for-age z-score, iron deficiency and study site. White bars: without *P. falciparum* infection and without inflammation (plasma C-reactive protein concentrations > 10 mg/L); black bars: with *P. falciparum* infection but without inflammation; grey bars: without *P. falciparum* infection and without inflammation. There were insufficient children with inflammation but without *P. falciparum* infection for meaningful analysis; these data were therefore left out. Line bars indicate 95% CIs.

and 42% had a normal genotype. Compared to their Tanzanian peers, the children in Kenya had a higher prevalence of both hetero- and homozygous α^+-thalassemia. There was no evidence that parasite density was associated with genotype (p=0.67; Kruskal-Wallis test).

In children with normal, heterozygous and homozygous genotypes, *P. falciparum* infection was associated with reductions in hemoglobin concentration of 9.3 g/L, 6.7 g/L and 3.2 g/L, respectively (**Figure 1**). Compared to the effect observed in children with a normal genotype, this reduction appeared smaller in heterozygotes (difference, 95 % CI: 2.6 g/L, –1.0 g/L to 6.3 g/L) and was clearly smaller homozygotes (6.1 g/L, 0.5 g/L to 11.7 g/L). This pattern of protection by α^+-thalassemia was seen consistently within the populations from both study sites, although it seemed less pronounced in the Kenyan children (data not shown).

Compared to uninfected children without inflammation, children in whom *P. falciparum* infection was accompanied by inflammation generally had larger reductions in hemoglobin than those with infection but without inflammation (**Figure 2**). In children without inflammation, *P. falciparum* infection was associated with only small reductions in hemoglobin concentration, whereby the effects were similar across α-globin genotypes. By contrast, in infected children with inflammation, the *P. falciparum*-associated reduction in hemoglobin concentration strongly depended on genotype; in children with a normal genotype this reduction was 21.8 g/L (18.0 to 25.6 g/L), as compared to 16.7 g/L (11.9 to 21.9 g/L) in heterozygotes, and only 4.6 g/L (–3.5 to 12.7 g/L) in homozygotes. The difference in effect was 5.1 g/L (–1.0 to 11.1 g/L) and 17.2 g/L (8.3 to 26.2 g/L), for heterozygotes and homozygotes respectively. These estimates were adjusted for study site, age, height-for-age z-scores and iron deficiency. There were only 27 children with inflammation but without malaria. Among these children, we found no evidence of a protection by thalassemia, but the estimates in this group were imprecise (data not shown).

Among children without infection and without inflammation, homozygotes had lower hemoglobin concentrations than those with a normal genotype (difference in means: –11.1 g/L, –7.0 g/L to –15.2 g/L) (figure 2). The opposite was observed among children with *P. falciparum* infection and with inflammation: among these children, homozygotes tended to have higher hemoglobin concentrations than those with a normal genotype (6.2 g/L, 95% CI: –1.8 g/L to 14.2 g/L).

Reanalysis of these data by study site led to similar results and conclusions,

although the estimates obtained were less precise due to smaller numbers Both in Tanzania and Kenya, the *P. falciparum*-associated hemoglobin reduction in the presence of inflammation, was significantly smaller in homozygous children than in those with a normal genotype (difference in effects, 95% CI: 24.2 g/L, 2.8 g/L to 45.7 g/L and 13.6 g/L, 2.6 g/L to 24.5 g/L, for children from Tanzania and Kenya, respectively).

As a last step, we evaluated plasma concentrations of C-reactive protein among children with *P. falciparum* infection by genotype. In the presence of *P. falciparum* infection, inflammation occurred less frequently in heterozygous children than in their counterparts with a normal genotype (14% versus 29%; difference, 95% CI: 15%, 10% to 20%). Likewise, plasma C-reactive protein concentrations were lower in these children than in those with a normal genotype (p=0.005; Mann-Whitney test). Among homozygotes, inflammation occurred less frequently than in children with a normal genotype (18% versus 29%; difference, 95% CI: 11%, 2% to 20%), but the group was small and we found no evidence of a difference in C-reactive protein concentrations (p=0.63; Mann-Whitney test).

Discussion

The data from our survey among afebrile children from communities with intense malaria transmission support our hypothesis that α^+-thalassemia protects against the decline in hemoglobin concentration associated with mild or asymptomatic *P. falciparum* infections. The protective effect was more pronounced in homozygotes than in heterozygotes, and was observed when infection was accompanied by inflammation. In children without inflammation, *P. falciparum* was associated with only small reductions of hemoglobin concentrations that were similar across genotypes.

Children in Kenya were older and may have had higher degrees of protective immunity against malaria than their peers in Tanzania. They also may have differed in other, unmeasured characteristics. However, reanalysis of the data presented in Figures 1–2 by study site yielded similar results. In addition, the estimates were obtained from a multivariate analysis that adjusted for study site, and several other factors that may have been associated with anemia. Thus it is unlikely that our results can be explained by differences in population characteristics between the study sites. Because data were collected cross-sectionally we have no insight in the dynamics of the decline in hemoglobin concentrations, or the preceding duration of infection at the point of measurement. We have no reason to assume, however, that the

duration of infection was different between the genotype groups, and we do not think that the cross-sectional design has affected the validity of our results.

Our data show that protection by α^+-thalassemia is not confined to the severe forms of malarial anemia, as suggested by others (Wambua et al. 2006, May et al. 2007). A recent large study at the Kenyan coast reported that both in children recruited from community-based surveys ('steady state') and in those with uncomplicated malaria, the reductions in hemoglobin concentrations due α^+-thalassemia were similar, suggesting no interaction between *P. falciparum* infection and α^+-thalassemia during uncomplicated disease episodes. Among children admitted to the hospital with severe malaria, however, heterozygotes and homozygotes had higher mean hemoglobin concentrations than children with normal genotype (Wambua et al. 2006). This suggested that protection by α^+-thalassemia mainly prevents the decline in hemoglobin concentration during the progression from episodes of uncomplicated malaria to severe malarial anemia, and does not play a role during milder infections. It should be noted, however, that the group of children in 'steady state' probably included both children with and without (asymptomatic) malaria infection, and that their infection status was not considered in the analysis. Moreover, children in this group were on average five years older than patients with uncomplicated malaria. By contrast, our findings show that α^+-thalassemia already protects against malaria-associated anemia in children with *P. falciparum* infections that are accompanied by inflammation but without febrile illness. This is compatible with our hypothesis that α^+-thalassemia protects against severe malarial anemia by preventing the gradual decline in hemoglobin concentration during repeated or chronic infections.

Because the protective effect was observed particularly when the infection was accompanied by inflammation, our findings suggest that even in a spectrum of relatively mild infections, there may be differences in severity between children with and without thalassemia. This was supported by the finding that plasma concentrations of C-reactive protein were lower among heterozygotes and also seemed lower among homozygotes. In addition to having smaller declines in hemoglobin concentration when infection is accompanied by inflammation, children with α^+-thalassemia may also be less likely to develop inflammation when infected. Similarly, among predominantly asymptomatic pregnant Ghanaian women with *P. falciparum* infection, α^+-thalassemia was associated with reduced frequency of inflammation (Mockenhaupt et al. 2001). In the same women, the effect of *P. falciparum* infection on hemoglobin concentration was less pronounced

among those with α^+-thalassemia than among their counterparts with a normal genotype (Mockenhaupt et al. 2000).

In endemic areas, many children have *P.falciparum* infection with inflammation but without symptoms (Hurt et al. 1994, Verhoef et al. 2002). However, increased concentrations of C-reactive protein among infected children can not always be attributed to parasitemia (Hurt et al. 1994). Thus the protection by α^+-thalassemia among children with *P. falciparum* infection and inflammation, as observed in this study, may not be specific for malaria. In inflammatory processes, cytokines such as TNF-α, IL-1, IL-6 and IFN-γ limit the supply of iron to the erythron through the action of hepcidin (Nemeth et al. 2006), and limit the proliferation of erythroid progenitor cells by suppressing the production of and responsiveness to erythropoietin (Weiss et al. 2005). Thus, even when inflammation is not due to *P. falciparum* infection, it may exacerbate anemia by interfering with the host's ability to effectively compensate for the loss of red blood cells due to malaria. Several studies have suggested that α^+-thalassemia may restrict the consequences of inflammatory processes in general. In a study in Papua New Guinea, α^+-thalassemia not only reduced hospitalizations due to severe malaria, but also and to a similar extent reduced the risk of hospitalization due to other infectious diseases (Allen et al. 1997). Similarly, other observations suggest that α^+-thalassemia also protects against severe non-malarial anemia (Mockenhaupt et al. 2004, Pasvol 2006). Our sample size was insufficient to evaluate the protective effect among children with inflammation but without parasitemia. Moreover, such analysis would have been difficult to interpret because inflammation may be milder in those children, and also because bone marrow suppression and anemia may persist after the infection has been cleared (Camacho et al. 1998, Kurtzhals et al. 2003, Helleberg et al. 2005). The question whether α^+-thalassemia protects against anemia during inflammatory processes other than malaria therefore remains unanswered. Similarly, the exact mechanism whereby α^+-thalassemia might modify the inflammatory response remains unresolved.

In conclusion, our data show that the protective effect of α^+-thalassemia against malaria-associated anemia is not confined to severe malaria cases, but is already present in mild, predominantly asymptomatic *P. falciparum* infections. The protective effect is evident only when the infection is accompanied by inflammation. This observation, in combination with the finding that the protection by α^+-thalassemia seems to be restricted to the anemic forms of disease, may give further direction in the search for the protective mechanisms in α^+-thalassemia.

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Chapter 8

Effect of α^+-Thalassaemia on episodes of fever due to malaria and other causes: a community-based cohort study in Tanzania

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Summary

Background: It is controversial to what degree α^+-thalassemia protects against episodes of uncomplicated malaria and febrile disease due to infections other than *Plasmodium*.

Methods: In a community-based cohort study among pre-school Tanzanian children aged 6-60 months (n= 612), we compared rates of fevers due to malaria and other causes between children with homo-or heterozygous α^+-thalassemia and those with a normal genotype, using a Cox proportional hazards model that included multiple events per child.

Results: The overall incidence of uncomplicated malaria was 2.93 (1572/526 child-years), and we found no evidence of group differences between the genotypes (hazard ratios 0.93; 95% CI: 0.82-1.06 and 0.91; 0.73-1.14 for hetero- and homozygotes respectively, adjusted for baseline factors that were predictive for outcome). However, the effect of α^+-thalassemia strongly depended on age; α^+-thalassemic children below 18 months experienced episodes more frequently than those with a normal genotype (1.30; 1.02-1.65 and 1.15; 0.80-1.65 for hetero- and homozygotes respectively), whereas among older children we observed protective effects (0.80; 0.68-0.95 and 0.78; 0.60-1.03; p-value for interaction 0.001 and 0.10 for hetero- and homozygotes respectively). No effect was observed on non-malarial febrile episodes.

Conclusions: α^+-thalassemia influences the risk of uncomplicated malaria in an age-dependent manner, whereby an initial increase of febrile episodes among the youngest children is followed by a protection at older age. This suggests that the acquisition of protective immunity is more efficient among α^+-thalassemic children, and contributes important information to the existing epidemiological evidence that directs the search of protective mechanisms involved.

Introduction

α^+-thalassemia is a common genetic trait in malaria-endemic areas in sub-Saharan Africa, Asia and Melanesia. Normal individuals have 4 genes coding for α-globulin (duplicate genes located on each chromosome 16) while hetero- and homozygous individuals have only 3 ($-\alpha/\alpha\alpha$) or 2 ($-\alpha/-\alpha$) functional genes. Consequently, children with α^+-thalassemia produce fewer α-globin chains, resulting in some reduction in hemoglobin concentrations but no other manifestations. In fact, in areas that are highly endemic for malaria, they may be protected against the decline in hemoglobin concentration that is associated with asymptomatic *Plasmodium* infection (Veenemans et al. 2008).

While case-control studies (Allen et al. 1997, Mockenhaupt et al. 2004, May et al. 2007) and two cohort studies (Williams et al. 2005a, Wambua et al. 2006) have consistently shown that α^+-thalassemia is associated with reduced risks of severe malaria, reports on its effect on uncomplicated malaria are inconsistent. A study in Vanuatu showed that, among children aged below 5 years, homozygous (but not heterozygous) α^+-thalassemia was paradoxically associated with an increased incidence of uncomplicated malaria due to both *Plasmodium vivax* and *P. falciparum* (incidence ratio [95% CI]: 2.2[1.3–2.7] and 1.6[1.0–2.6], respectively); among children aged 5–9 years, however, there was no evidence of such an association (Williams et al. 1996). The authors suggested that early exposure to the more benign *P. vivax* induced cross-species protection against *P. falciparum*. Subsequent studies in Africa found no effect (Allen et al. 1993, Williams et al. 2005b, Wambua et al. 2006) or protection (Enevold et al. 2008) by α^+-thalassemia against uncomplicated malaria due to *P. falciparum*.

In a prospective case-control study in Papua New Guinean children, α^+-thalassemia was also strongly protective against hospitalization for disease episodes caused by infections other than malaria (Allen et al. 1997).

We conducted a community-based cohort study among preschool children to assess effects of α^+-thalassemia on the rate of fevers due to malaria and fever due to other causes, and evaluated to what extent this effect depended on age.

Methods

Study area and population: The study was conducted in February 2008–March 2009 in 4 rural villages in Handeni District, northeastern Tanzania. People originally belonged to the Wazigua and Wabondei tribes, but settlement of migrant plantation workers in the past has resulted in a mixture of many tribes with different origins. Plantations have long since closed, and the population, residing in scattered hamlets in the area, is mainly comprised of poor farmer families engaged in subsistence farming. Transmission of malaria (more than 95% due to *Plasmodium falciparum)* is intense and perennial, with peaks during the rainy seasons and annual inoculation rates of 35–400 per year (Ellman et al. 1998). Access to primary health care is limited: apart from several local traditional healers the research dispensary was the only health facility in the area. Artemether-lumefantrine was the first-line treatment for uncomplicated malaria, and available for free only in public health care facilities but not in local shops; accordingly, home treatment with efficacious anti-malarial drugs probably occurred only sporadically.

Study design: This prospective cohort study was part of a double-blind randomized trial aimed at measuring the effects of preventive supplementation with zinc and other multi-nutrients on the incidence of malaria (Veenemans et al. submitted). During the follow-up period, children daily visited a centre close to their homestead where micronutrient supplements were administered by community volunteers. Malaria morbidity was assessed by passive case detection; parents were encouraged to bring their children to the research dispensary in case of fever or other signs of illness.

Ethical approval was provided by the review committees in The Netherlands and Tanzania. Individual written consent was obtained from parents or guardians.

Recruitment: Before recruitment, which took place between February and August 2008, we conducted a census and registered all resident children aged below 60 months. Parents and guardians were invited to an information seminar and subsequent 2-day screening procedure to determine eligibility. A medical examination was performed by a clinical officer, anthropometric indices were calculated as the average of two recordings taken at consecutive days, and venous blood samples were collected. An aliquot of blood was centrifuged immediately after collection, and a 90 μL red cell pellet with the buffy coat was mixed with 90 μL phosphate-buffered saline and 180 μL a DNA stabilizing buffer (AS1; Qiagen, Hilden, Germany) and stored at 4 °C

for subsequent genotyping. Plasma was stored in liquid nitrogen. A second aliquot of whole blood was examined by hematology analyzer (Sysmex KX21, Kobe, Japan) the same day.

Children were eligible to be randomized when aged 6-59 months, and with height-for-age z-score <-1.5SD. We excluded children with weight-for-age z-score <–3SD, hemoglobin concentration <70 g/L, or signs of severe or chronic disease, those unlikely to comply with interventions, or whose parents/guardians refused consent. Eligible children were randomly allocated to receive daily supplements with zinc alone, zinc in combination with other micronutrients, micronutrients alone, or placebo. We used block randomisation within 6 strata defined by *Plasmodium* infection and age class (6-17 months, 18-35 months and 36-60 months). Further details about allocation procedure, composition of the supplements and intervention effects will be reported elsewhere. All children with *Plasmodium* infection were treated with artemether-lumefantrine (Novartis Pharma, Basel, Switzerland) upon enrollment.

Follow-up: During the follow-up period, a clinical officer was on permanent (24 h) duty at the research clinic. For any child reporting with fever (axillary temperature ≥ 37.5 °C) or a history of fever according to the guardian, a finger-prick blood sample was collected to detect the presence of malaria parasites. For all children with a positive dipstick we prepared two blood slides and measured whole blood concentrations of haemoglobin and C-reactive protein. Children were treated free of charge, and referred to the district hospital when indicated.

Laboratory procedures:
In samples collected during the baseline survey and from sick children, we used a dipstick test (pLDH rapid dipstick test, CareStart™, Access Bio, Monmouth Jct, USA) to detect the presence of parasite-specific lactate dehydrogenase (*P. falciparum* and other *Plasmodium* species). This test has a sensitivity of 96% for blood samples with > 50 parasites/μL (Piper et al. 1999). Blood films were prepared using standard methods. For slides of sick children, parasites were counted against at least 200 leukocytes, and parasite density was calculated assuming a leukocyte count of 8000/μL. At least 500 leukocytes were counted before a slide was considered negative. When densities were very high, parasites were counted per 2,000 erythrocytes, in which case we used the estimated erythrocyte count at the time of the episode to determine the number of parasites per μL blood. The erythrocyte density was estimated based on the haemoglobin concentration measured at the time of the episode, using a linear model based on survey data that described the

relation between haemoglobin concentration and erythrocyte count.

Within 6 months after collection, DNA was isolated from whole blood samples and the $-\alpha^{3,7}$ deletion type of α^{+}-thalassemia was determined by polymerase chain reaction (Liu et al. 2000). This type of deletion is the most common form of α^{+}-thalassemia in Africa (Williams 2006), with prevalence values often exceeding 50% in eastern Africa (Williams 2006, Enevold et al. 2008, Veenemans et al. 2008); other types of thalassaemia are rare.

Whole-blood concentrations of hemoglobin and C-reactive protein were measured in the field using a photometer (HemoCue, Ängelholm, Sweden) and a immunoturbidimetric test (QuikRead, Orion Diagnostica, Espoo, Finland), respectively. Plasma concentrations of C-reactive protein and ferritin for survey samples were measured in The Netherlands (Meander Medical Centre, Amersfoort) on a Beckman Coulter Unicel DxC880i system according to the manufacturer's instructions. Plasma concentrations of *P. falciparum* specific histidine-rich protein-2 (HRP2) in samples collected during the first malaria episode were measured using a commercial enzyme-linked immunosorbent assay kit (Malaria Ag Celisa; Cellabs, Brookvale NSW, Australia). This protein is released into the plasma at schizont rupture, and its plasma concentration may more accurately represent total body parasite biomass, because it also reflects the presence of sequestered parasites that remain largely undetected with conventional microscopy (Dondorp et al. 2005).

Statistical analysis: All data were double-entered and analyses were performed using SPSS (v15·0 for Windows, SPSS, Chicago, IL), CIA (v2.1.2) (Altman et al. 2000) and STATA (v11; College Station, Tx) Anthropometric indices were calculated using Epi Info software (version 3.3.2; http://www.cdc.gov/epiinfo).

The primary outcome, an episode of malaria, was pre-defined as a guardian-reported history of fever accompanied by either an axillary temperature ≥ 37,5 °C or inflammation (whole blood C-reactive protein concentration ≥ 8 g/L), plus a positive result for the pLDH rapid dipstick test. We assumed that children were protected against new malaria infections for 14 days after treatment with artemether-lumefantrine, so any recurrent symptoms during this period were assumed to be part of the initial episode and were not counted as separate episodes**.** Additional outcomes were hospital admissions or death due to infection-related causes (combined end-point), non-malarial febrile episodes (defined as any episode of reported fever that did not classify as malaria, separated by at least 2 days), and the severity of malaria episodes

as indicated by parasite density, hemoglobin concentrations, concentration of whole blood C-reactive protein and plasma HRP2.

In the primary analysis we compared disease rates between genotypes using Cox regression with robust estimates of the standard error to account for multiple episodes within children). We explored to what extent adjustment for baseline factors presumed or shown to be prognostic for malaria (age class, mosquito net use, *Plasmodium* infection, distance between homestead and clinic, and height-for-age z-score and intervention) influenced the estimates. Because we anticipated that the effect of α^+-thalassemia would depend on age, we conducted a stratified analysis within each of the three age classes used for randomization, and assessed interaction using a Cox regression model. We calculated incidence rates and rate ratios for first or only episodes, but used Cox regression model to obtain adjusted hazard ratios. Continuous variables were log-transformed as appropriate to obtain normal distributions, and associations were assessed by ANOVA and multiple linear regression analysis.

Results

Of 1,029 children screened, 417 were not eligible, mostly because their height-for-age score exceeded –1.5 SD, and 612 children were enrolled (**Figure 1**). During follow-up, 20 of 612 (3%) children were lost (3 died; 2 withdrawn by parents; 15 moved away).

Of 612 children included for follow-up, 304 (50%) had a normal genotype, 41% (251) were heterozygote, 9% (55) were homozygous, whilst DNA could not be amplified for 2 children. *Plasmodium* infection was detected in 265 children (43%), virtually all due to *P. falciparum* (261), with similar prevalence values among the genotypes. Homozygotes had lower hemoglobin concentrations and height-for-age z-scores, seemed slightly younger, appeared to have a higher prevalence of iron deficiency, and used mosquito nets less frequently than those with a normal genotype. They also less frequently received zinc supplements, with or without multi-nutrients (**Table 1)**. No major differences were found between children with heterozygous and normal genotype in factors that were prognostic for malaria. Hemoglobin concentrations were lower among children with α^+-thalassemia.

Of 2,462 episodes of reported fever, 1,618 had a positive dipstick test result, and 1,572 episodes were classified as malaria cases that occurred in a total follow-up time 526 child-years. The incidence of malaria was 2.93 per child-year at

Table 1. Baseline characteristics of study participants and distribution of malaria prognostic factors, by genotype

	Normal (αα/αα)	Heterozygote (-α/αα)	p	Homozygote (-α/-α)	p
n	304 (50%)	251 (41%)		55 (9%)	
Sex, M/F [n/n]	50%/50% [151/153]	46%/54% [116/135]	0.44	54 %/46% [30/25]	0.56
Age, months	33.4±15.9	32.0±15.2	0.27	29.9±16.0	0.12
Age class			0.65		0.08
6-17 months	21% [64]	24% [61]		35% [19]	
18-35 months	36% [108]	35% [87]		33% [18]	
36-59 months	41% [132]	41% [103]		42% [18]	
Plasmodium infection *	44% [134]	43% [107]	0.80	42% [23]	0.77
Anemia ¶	62% [189]	71% [178]	0.03	87% [48]	<0.001
Hemoglobin concentrations, g/L	104.7±12.5	102.2±12.4	0.02	94.9±12.8	<0.001
Without *Plasmodium* infection	106.7±12.6	104.8±11.6	0.14	95.3±13.9	<0.001
With *Plasmodium* infection	102.0±11.8	98.8±12.5	0.04	94.3±11.3	0.005
Inflammation †	32% [96]	33% [82]	0.99	38% [21]	0.62
Mosquito net use ††	34% [101]	33% [82]	0.92	18% [10]	0.03
Height-for-age z-score	-2.38±0.72	-2.43±0.64	0.28	-2.63±0.75	0.01
Distance from homestead to dispensary, km **	3·56±2·21	3·65±2·28	0.62	3·36±1·84	0.54
Intervention					
Placebo	26% [78]	22% [54]		38% [21]	
Zinc	25% [76]	27% [67]		18% [10]	
Multi-nutrients without zinc	23% [71]	27% [68]		27% [15]	
Multi-nutrients with zinc	26% [79]	25% [62]		16% [9]	
Iron deficiency ‖					
All children	15% [46]	20% [49]	0.17	26% [14]	0.08
Without inflammation (406) §	22% [45/205]	25% [41/167]	0.80	35% [12/34]	0.13

Mean ± SD, % [n] or median (25- and 75-percentiles) unless indicated otherwise. P-values for differences relative to the reference group of children with normal genotype were obtained by Pearson Chi-Square test (age class), Fischer's Exact test (anemia, *Plasmodium* infection, inflammation, mosquito net use, iron deficiency), or Students t-test (continuous variables). Because interventions were randomly allocated, no p-values are provided for intervention groups.

¶ Hemoglobin concentration < 110 g/L

* As indicated by a positive result for pLDH-based dipstick test (see text).

† Plasma C-reactive protein concentration ≥ 8 mg/L

†† Data missing for 11 children.

** Measured as the crow flies, based on global positioning data.

‖ Plasma ferritin concentration <12 μg/L (6 missing values).

§ Restricted to children plasma C-reactive protein concentration ≥ 8 mg/L

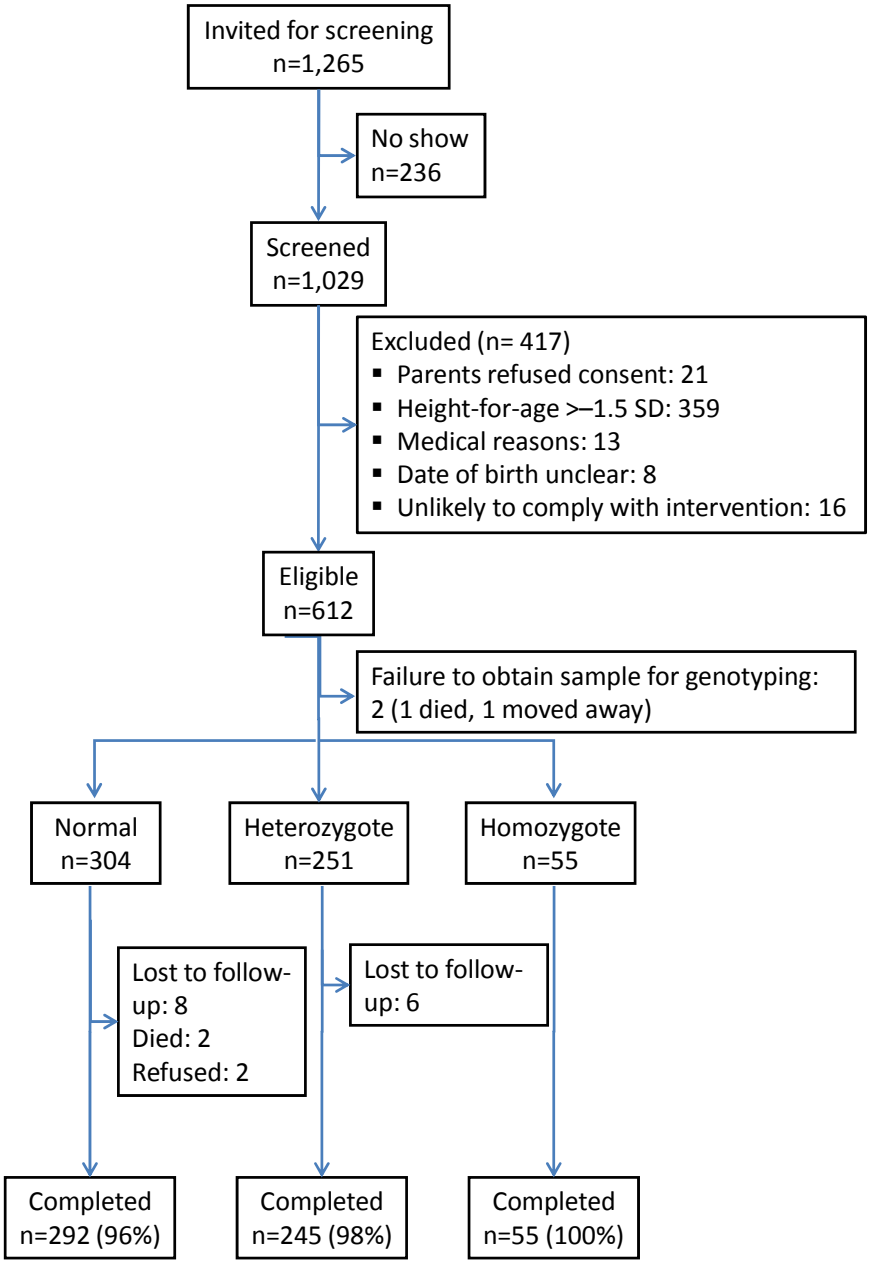


Figure 1. Study profile.

Table 2. Rates of uncomplicated malaria, non-malarial febrile episodes and severe events (hospital admission or death), by genotype

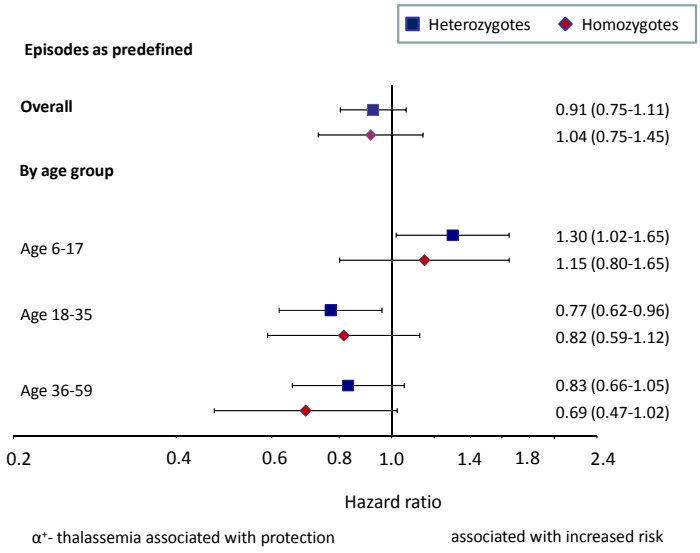
Event	Normal ($\alpha\alpha/\alpha\alpha$)	Heterozygotes ($-\alpha/\alpha\alpha$)	Homozygotes ($-\alpha/-\alpha$)
All episodes of malaria			
Incidence	3.10 (812/262)	2.89 (622/215)	2.83 (136/48)
Hazard ratio, crude	1.00 Reference	0.93 [0.80-1.06]	0.92 [0.73-1.14]
Hazard ratio, adjusted †	1.00 Reference	0.93 [0.82-1.06]	0.91 [0.73-1.14]
Episodes with parasitemia >5,000/μL (1,249)	1.00 Reference	0.94 [0.81-1.10]	0.89 [0.67-1.17]
Episodes with parasitemia >10,000/μL (1,119)	1.00 Reference	0.95 [0.81-1.11]	0.91 [0.68-1.22]
Episodes with parasitemia >100,000/μL (263)	1.00 Reference	0.94 [0.69-1.29]	0.98 [0.57-1.69]
Episodes with haemoglobin concentration <80 g/L (178)	1.00 Reference	1.25 [0.86-1.84]	2.65 [1.71-4.10]
1st episodes of malaria			
Incidence	3.08 (257/84)	2.66 (199/75)	3.44 (49/14)
Incidence rate ratio	1.00 Reference	0.86 [0.71-1.04]	1.12 [0.81-1.52]
Hazard ratio, crude	1.00 Reference	0.90 [0.75-1.09]	1.10 [0.81-1.49]
Hazard ratio, adjusted †	1.00 Reference	0.89 [0.74-1.08]	1.06 [[0.78-1.44]
All episodes of non-malarial fever			
Incidence	1.64 (431/262)	1.72 (370/215)	1.82 (87/48)
Hazard ratio, crude	1.00 Reference	1.04 [0.85-1.28]	1.10 [0.82-1.49]
Hazard ratio, adjusted ¶	1.00 Reference	1.00 [0.84-1.19]	0.98 [0.73-1.36]
All hospital admissions or deaths §			
Incidence	0.15 (39/262)	0.10 (21/215)	0.19 (9/48)
Hazard ratio, crude	1.00 Reference	0.66 [0.38-1.14]	1.26 [0.56-2.89]
Hazard ratio, adjusted ¶	1.00 Reference	0.57 [0.33-0.98]	0.89 [0.36-2.24]

Values between brackets indicate (cases), (cases/child-year) or [95% CIs].

† Adjusted for experimental intervention (indicated by dummies), mosquito net use, distance between homestead and research clinic, height-for-age z-score and *Plasmodium* infection at baseline. There was no evidence of an interaction between genotype and experimental intervention.

¶ Adjusted for age class, experimental intervention (dummies), distance between homestead and research clinic, height-for-age z-score and *Plasmodium* infection at baseline.

§ Hospital admissions or deaths (of which two occurred outside hospital) for infection-related causes, excluding events due to trauma, poisoning or burns (1 case occurred in a child from whom genotyping was unavailable).



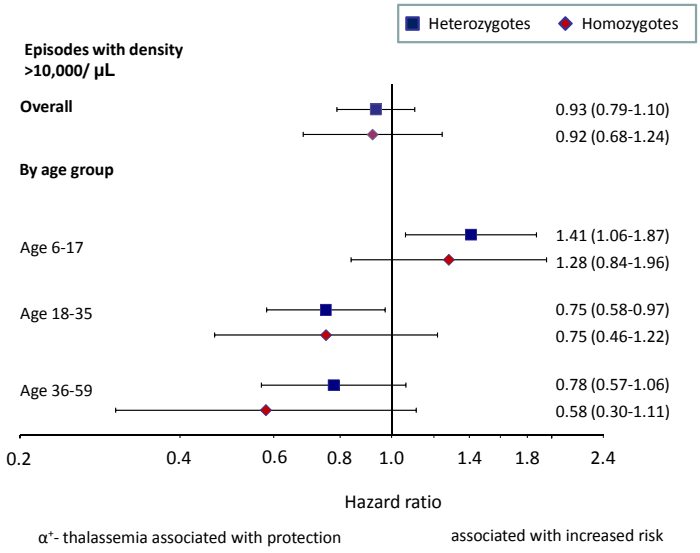


Figure 2. Association between α^+-thalassemia and malaria rates, overall and by age class. Top panel: episodes as predefined; bottom panel: episodes as predefined, but with densities of asexual parasites >10,000/µL.Line bars and values between brackets indicate 95% CIs. Estimates adjusted for baseline *Plasmodium* infection status, distance between homestead and clinic (continuous variable), height-for-age z-score (continuous variable), mosquito net use and experimental intervention produced similar estimates; adjusted p-values for difference in hazard ratios between children < 18 months or ≥ 18 months: 0.006 and 0.18 for hetero-and homozygotes, respectively. Bottom panel: adjusted p-values for difference in hazard ratios between children < 18 months or ≥ 18 months: 0.002 and 0.07 for hetero-and homozygotes, respectively.

risk; 507 children (83%) experienced at least one malaria episode, recurrent episodes occurred in 395 (63%) of children. The remaining 890 episodes were classified as non-febrile malaria episodes; the incidence of such cases was 1.69/child-year. Hospital-referral for infection-related causes occurred in 68 cases, of which 30 were malaria (19 cases with hemoglobin concentration < 60 g/L, all except one without signs of heart failure or respiratory distress). Malaria incidence decreased with age class (3.73, 3.48 and 2.19 episodes/year for age classes 6–17, 18–35 and 36–59 months respectively; p<0.001).

Rates of uncomplicated malaria were similar among the three genotypes (**Table 2, Figure 2**), whether analyzed with or without adjustment for mosquito net use, *Plasmodium* infection at baseline, distance to the research clinic, stunting and experimental intervention. Stratification by age-class**,** however, showed that heterozygotes had increased malaria attack rates when aged below 18 months (hazard ratio: 1.30 [1.02–1.65]), whereas they were protected against malaria when aged older than 18 months (Figure 2; hazard ratio for all children ≥18 months combined: 0.80 [95% CI: 0.68-0.95]; p-value for effect difference between age classes: 0.001). A similar pattern occurred in homozygotes, even though estimates were less precise due to the smaller number of cases (hazard ratio for all children ≥18 months combined: 0.78; 0.60-1.03; p-value for the effect difference between age classes: 0.10). Restriction of the analysis to malaria cases with densities above 5,000 or 10,000 parasites/μL resulted in similar overall estimates (**Table 2**), and also revealed similar age-dependent patterns with and without adjustment for baseline factors prognostic for malaria (data shown for cut-off 10,000 in Figure 2, bottom; p-values for interaction tests between genotype and age: 0.001 and 0.05, for hetero-and homozygotes respectively). There was no evidence that the magnitude of the effects of thalassemia on malaria rates were influenced by the intervention (p=0.94 and 0.95 for interaction with hetero- and homozygotes respectively).

We found no evidence that α^+-thalassemia was associated with a change in time to first event, or with the rate of relatively severe malaria episodes (with densities above 100,000 parasites/μL or hemoglobin concentrations < 80 g/L; Table 2). In fact, the rate of episodes with hemoglobin concentrations < 80 g/L was highest among homozygotes and lowest among children with a normal genotype (regardless of age), whereby the overall incidence of these episodes strongly decreased with age (0.82, 0.33 and 0.09 episodes/child-year, for age classes 6–17, 18–35 and 36–59 months, respectively).

When genotype was not taken into account, parasite densities during the first malaria episode declined with age (p<0.001; **Figure 3**). In children

aged below 18 months, α^+-thalassaemia seemed associated with increased parasite densities (Figure 3), although the statistical evidence for such an association was weak (p-value for the difference between genotypes: 0.18). For homozygotes, the association between genotype and density seemed to depend on age (p=0.04), while for heterozygotes there was no such evidence (p=0.78). α^+-thalassaemia was not found to be associated with parasite density in older age groups. Other indicators of severity (plasma concentrations of C-reactive protein or HRP2) were comparable between genotypes, whether analyzed for all age classes combined or separately (data not shown).

The declines in average hemoglobin concentrations between baseline and the first malaria episode were only minor and not substantially different in hetero- or homozygotes (-4.5 g/L and -2.1 g/L) from values observed in children with a normal genotype (-3.0 g/L).

Lastly, the rate of non-malaria febrile episodes was similar for all genotypes (Table 2); stratification by age did not indicate differences in age-specific effects (not shown).

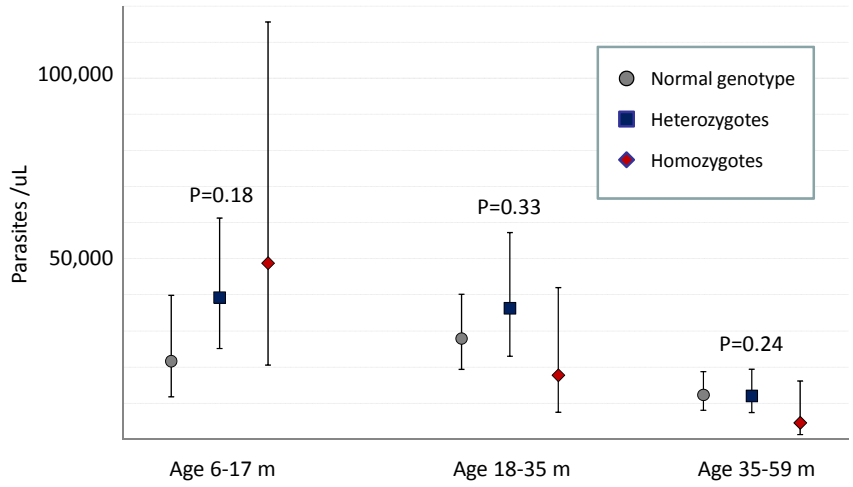


Figure 3. Association between α^+-thalassemia and parasite density during first episode of malaria, by age class. Markers and line bars indicate geometric means and 95% CIs, respectively; p-values for differences between genotypes within age classes obtained by ANOVA)

Discussion

In this cohort of Tanzanian children, α^+-thalassemia was associated with increased rates of malaria in children aged < 18 months, but with protection against malaria in older children. This effect appeared specific for malaria; we found no evidence that it applied to febrile episodes due to other causes. We did not find α^+-thalassemia to be associated with the severity of malaria episodes as measured by hemoglobin concentrations and other indicators.

Although we treated all *Plasmodium*-infected children at baseline, the specificity of our case definition may have gradually decreased with time, as more children became asymptomatically infected. Similar results were obtained, however, when restricting the analysis to cases with parasite densities > 10,000/μL. Because this case definition is more specific for detecting true malaria cases, it is unlikely that a low specificity affected the validity of our conclusions.

Our results contribute to evidence that predisposition to malaria due to *P. falciparum* in both heterozygotes and homozygotes for α^+-thalassemia early in life results in protection against malaria due to the same species at older age. In an area adjacent to ours, α^+-thalassemia was found to be associated with protection against malaria in children aged 6 months to 20 years (Enevold et al. 2008). This protection seemed more pronounced among children aged > 5 years, but the analysis was based on 50 episodes (41 among children aged < 5 years) and had insufficient precision to adequately assess age-specific effects in early life.

In Vanuatu (Williams et al. 1996), the incidence of malaria due to *P. falciparum* and *P. vivax* was increased in children aged < 5 years with homozygous α^+-thalassemia relative to children with normal genotype, but the study found no evidence of protection among either hetero- or homozygotes in children aged 5-9 years. Contrary to our findings, the incidence rates in heterozygotes and children with normal genotype were similar, regardless of whether the analysis was stratified by age or not. Our estimates are more precise, however, due to the larger number of malaria cases in our study (622 and 812 in children with heterozygote and normal genotypes, respectively, versus 159 and 304 in Vanuatu).

The mechanism underlying the protection is thus probably based on the earlier acquisition of protective immunity in children with thalassemia. The age that forms a turning point between increased risk and protection will hence depend on transmission intensity, which may at least in part explain

differences between studies; under conditions of intense transmission, such as in our study, this turning point would be attained earlier in life than in conditions of less intense transmission, such as encountered elsewhere in Tanzania (Enevold et al. 2008) and in Vanuatu (Williams et al. 1996).

It has been put forward that *Plasmodium* parasites preferentially invade reticulocytes. Thus reticulocysis, induced by thalassemia-associated ineffective erythropoiesis, would favor proliferation of *Plasmodium* parasites (Weatherall et al. 2001). However, we did not find strong support that thalassemia was associated with increased parasite densities in young children, and neither did any previous study. In addition, a recent study found no evidence that reticulocyte counts were increased in individuals with α^+-thalassemia (Krugner et al. 2006).

Others hypothesized that increased parasite-induced surface expression of neo-antigens on thalassemic erythrocytes results in enhanced binding of IgG antibody and more rapid clearance of parasitized erythrocytes in the spleen (Luzzi et al. 1991). Clearance in the spleen may be further enhanced by a reduced red cell deformability of thalassemic cells (Dondorp, 1999). In children aged 6-18 months, in whom protection by maternal antibodies has waned but acquired immunity is still low and parasite replication only partly restrained by an effective circulating antibody repertoire, such increased antigen presentation in the spleen may result in a more rapid development of symptoms.

Our finding that α^+-thalassemia is associated with an increased frequency of malaria episodes in children aged 6-18 months may seem contradicting with reports from hospital-based studies that α^+-thalassemia protects against severe malarial anemia (Wambua et al. 2006, May et al. 2007), which has the highest incidence in the same age range (Snow et al. 1997). An increase in fever rate may, however, not necessarily translate to an increased risk of severe malaria anemia if the decline in haemoglobin during these attacks is halted or sufficiently slowed down to before reaching a critical level that leads to admission. A potential mechanism for such phenomenon has recently been proposed (Fowkes et al. 2008). In thalassemia, the total amount of hemoglobin is divided over erythrocytes that are disproportionately increased in numbers but reduced in size and hemoglobin content. Thus, an equal proportion of erythrocytes being destroyed by malaria parasites results in a smaller hemoglobin reduction in individuals with α^+-thalassamia than in their peers with normal genotype.

When analyzing the decline in hemoglobin concentration between baseline

and the first malaria episode, we did not find evidence of such protection. It should be noted, however, that the average decline in hemoglobin concentration during malaria episodes was relatively small, and that very few episodes (19) occurred whereby hemoglobin concentrations dropped below 60 g/L. This is probably due to good access to primary care and prompt effective treatment, and hematological gains due to the nutrient interventions. By contrast, in a pilot survey in the same area in 2006, we found that children with α^+-thalassemia were protected against the decline in hemoglobin concentration associated with mild and asymptomatic infections (Veenemans et al. 2007). At that time, there were no first-line health facilities in the area, and artemether-lumefantrine was not available through public facilities. Thus this setting was probably better comparable to the circumstances under which α^+-thalassemia has been providing a survival advantage in the past.

In pre-school Tanzanian children living in an area of intense transmission, effects of α^+-thalassemia on malaria rates were age-dependent: it was associated with increased rates in children aged <18 months as opposed to decreased rates in older children. We did not find evidence that α^+-thalassemia was associated with the severity of malaria episodes.

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Chapter 9

Discussion

This chapter aims to assist in the interpretation of key findings for policy makers and fellow researchers. This discussion will be presented in the light of three of the specific objectives presented in the introductory chapter of this thesis:

1. To assess the effect of supplementation with zinc, alone or in combination with other nutrients, on the rates of malaria (primary objective);
2. To assess intervention effects on rates of other common diseases (diarrhoea, respiratory infections, non-malarial febrile illness);
3. To identify factors that determine the magnitude of the effect of the interventions.

The last specific objective dealing with α^+-thalassaemia will not be further discussed beyond the relevant chapters of this thesis. I will focus here on lessons about study design and analysis that are critical in addressing the objectives formulated above and which are pertinent to the study described in this thesis.

1. Design of malaria intervention trials

1.1. Malaria case definition

In our trial, we administered a therapeutic course of artemether-lumefantrine to all *Plasmodium*-infected children at baseline, with the aim to clear asexual parasitaemia and thus to increase the specificity of our case definition for malaria episodes. There is limited experience with this approach. In several efficacy malaria vaccine trials, all participants were pre-treated with either sulfadoxine-pyrimethamine (Alonso et al. 1994, D'Alessandro et al. 1995) or quinine-doxycycline (Sherwood et al. 1996). In another malaria vaccine trial in Thailand, participants at baseline received treatment with artesunate/mefloquine or with chloroquine when infected with *P. falciparum* or *P. vivax*, respectively (Nosten et al. 1996).

One reported disadvantage of pre-treating trial participants at baseline is that it can suppress new infections for some time (Coulibaly et al. 2002, Moorthy 2009, see also Annex 2). Thus in a malaria vaccine trial, pre-treatment with sulfadoxine-pyrimethamine resulted in a reduction of the number of infections and a corresponding loss of power to detect effects on the study end point, geometric mean parasite density (Genton et al. 2002). Because the drugs used for pre-treatment in our study have relatively short terminal elimination half-lives (artemether: 1 h, lumefantrine: 3 days; WHO 2010),

this drug combination produces a shorter post-treatment prophylactic effect, and may be more suitable than the relatively long-acting drugs that have been used in previous studies (terminal elimination half-lives: 4–9 days for sulfadoxine, 4 days for pyrimethamine, 15-23 hour for doxycycline, 21 days for mefloquine and 1-2 months for chloroquine; WHO 2010, FK/CvZ 2010).

We also selectively pre-treated children with *Plasmodium* infection at baseline (as opposed to treating all children), and we assessed the influence on effect estimates of censoring for a pre-specified period of 14 days following treatment. Such censoring resulted in similar effect estimates.

Another, more critical problem identified by our study is that the validity of the efficacy estimate is undermined by children becoming asymptomatically infected in the course of the intervention period. When finalising our statistical analysis plan, we were aware that this might constitute a problem but, in retrospect, we probably insufficiently appreciated that even small reductions in specificity of the case definition can lead to substantial bias. It would have been more appropriate to base the primary analysis on the effect on all episodes in a relatively short period (e.g. the first 100 days) after randomisation. Even though pre-treatment of infected children at baseline was only partially effective in increasing the specificity of our case definition, the alternative approach of defining cases based on fever thresholds of parasitaemia also suffers from severe shortcomings (Chapter 2). Further research is needed to resolve this issue. For the time being, it would seem prudent to combine these approaches, similarly to the analyses that I present in this thesis.

Additional research is also needed to replace conventional microscopy as the technique of choice to detect and quantify parasitaemia. Several new methods are available that may be more suitable for incorporation in malaria case definitions. First, fluorescence cytometry using DNA-selective stains allows examination of a larger volume of a blood specimen than microscopy, and offers the possibility to both precisely count malaria parasites and to accurately differentiate *P. falciparum* from other human malaria species (Shapiro and Mandy 2007, Shapiro and Perlmutter 2008). Second, because parasite counts by microscopic examination of peripheral blood poorly reflect the total body parasite biomass, it would be helpful to use markers that are specific for malaria-induced pathology. One such candidate marker is plasma concentration of histidine-rich protein-2 (HRP-2) (Dondorp et al. 2005). A key issue to be evaluated is to what extent plasma HRP2 concentrations can be used to distinguish between asymptomatic infections and febrile malaria episodes. There are virtually no data available from large

scale community-based studies, however, which describe plasma HRP2 concentrations in children with asymptomatic infection. One complicating factor is that plasma HRP-2 concentrations may persist and accumulate over time during infection, so that they do not necessarily reflect current but also recent parasite load. Hence, an alternative or perhaps complementary marker may be plasma concentration of *Plasmodium*-specific or *Plasmodium* species-specific lactate dehydrogenase (pLDH), a soluble enzyme that is expressed at high levels in blood-stage parasites. pLDH is released during lysis of parasitized erythrocytes and is rapidly eliminated following parasite clearance; its plasma concentrations therefore reflect current parasitaemia (Oduola et al. 1997, Piper et al. 1999, Moody et al. 2000, Singh et al. 2003, Hopkins et al. 2007).

1.2. Analysis of recurrent events vs first or only event

As discussed above, a gradual loss of specificity of the case definition probably explains the discrepancy between effect estimates obtained in the time to first event analysis and those obtained in the analysis of all events over the total follow-up period. On the other hand, multi-nutrients may have resulted in a transient increase in malaria rates, which was not detected when assessing effects on all events over the total follow-up time. A transient increase in rates may theoretically occur if episodes of malaria induced by multi-nutrients result in acquired immunity that prevents subsequent episodes. When planning our statistical analysis (Annex 2), we anticipated that our interventions would protect against malaria, and considered changes in the degree of protective efficacy less relevant for public health than an overall reduction in total burden of disease. For this reason, our primary analysis was based on all events over the total intervention period. In retrospect, however, if an intervention would lead to transiently increased rates of severe disease or death, then a time to first event analysis may be of greater public health importance than an analysis of all events.

2. Zinc interventions in Africa

2.1 What does this study add?

The trial reported in this thesis is the first to examine the potential benefit of combining zinc with vitamins and other minerals. With an analysis based on 1,572 cases, we believe it is by far the largest to date to assess the effect of nutrient interventions on malaria.

We found no evidence that concurrent supplementation with multi-nutrients influenced the magnitude of the effect of zinc on rates of malaria (Chapter

3) or diarrhoea (Chapter 5). The absence of an evident protection against malaria (Chapters 3 and 4) is consistent with the report from Burkina Faso (Muller et al. 2001).

2.2 Implications for public health policies and research

There has been frustratingly little programmatic progress for preventive zinc interventions. It is usually a body of evidence consisting of many studies, rather than a single trial, that changes medical or public health practice. The findings described in this thesis strengthen the case for scaling up zinc interventions in deficient populations by confirming its protective role against diarrhoea in African population of children (Chapter 5), by providing new evidence that it protects against episodes of fever without localizing signs (Chapter 3) and episodes of non-malarial fever accompanied by diarrhoea (Chapter 5). Such interventions can be implemented in Africa without concerns that it will cause adverse effects due to malaria (this thesis) or HIV infection (Bobat et al. 2005). The finding that zinc alone can achieve these benefits without addition of other micronutrients is of particular relevance for efforts to develop biofortified crops, because this approach must necessarily focus on single nutrients or at most a few nutrients (Chapter 1).

Our results do not preclude that zinc can protect against disease associated with severe malaria. There is increasing evidence that severe malaria may predispose to increased small bowel permeability (Wilairatana et al. 2007) and invasive bacterial disease (Mabey et al. 1987, Graham et al. 2000a, Calis et al. 2008, Berkley et al. 2009, Najm et al. 2010, Mackenzie et al. 2010), mainly due to enteric pathogens such as non-typhoidal *Salmonellae*, but also due to species normally found in the respiratory system such as *Haemophilus influenzae* b and *Streptococcus pneumonia* (Berkley et al. 1999, 2009, Graham et al. 2000b,c, Walsh et al. 2000, Bronzan et al. 2007, Nadjm et al. 2010, Chimalizeni et al. 2010). The reason for this predisposition is not known but may be due to increased availability of iron following haemolysis, or because intestinal permeability is increased in inflammatory conditions (Graham et al. 2000b, Bronzan et al. 2007, Graham 2008). Invasive bacteraemia contributes to the pathophysiology of severe malaria, and is associated with increased mortality (Berkley et al. 1999, Morpeth et al. 2009). Increased small bowel permeability may also be due to persistent mucosal enteropathy that is common in African children and characterized by villous atrophy, crypt hyperplasia and increased numbers of intra-epithelial lymphocytes (Campbell et al. 2003), and probably a consequence of repeated mucosal injury and inflammation (Lunn et al. 1991, Campbell et al. 2003).

By improving intestinal integrity and hence barrier function, zinc may

reduce susceptibility to invasive infection. Thus, one might expect it to have at least some effect in protecting against malaria-associated severe disease and death, consistent with the small reduction in mortality found in the zinc supplementation study in Pemba (Sazawal 2007). Therapeutic zinc supplementation can reduce the duration of acute and persistent diarrhoea (Haider and Bhutta 2009), suggesting that it may act fast. Thus a trial is warranted to assess possible benefits of zinc supplementation as adjunct therapy in children admitted to hospital with severe malaria.

3. Interventions with multi-nutrients in malaria-endemic settings

3.1 What does this study add?

Determinants of anaemia: In the study population described in this thesis, there was strong evidence of deficiencies in zinc, iron, folate and vitamin B_{12}, as shown by the effect of supplementation on biochemical indicators of nutritional status (Chapter 6, Figure 5). Iron deficiency occurred in one-quarter of children at baseline (Chapter 3, Table 1). At the second survey, iron deficiency was virtually absent in the two groups receiving multi-nutrients (Chapter 4, Webtable). Children aged 6-17 months had the highest prevalence of iron deficiency at baseline, and had the highest response in haemoglobin concentration to multi-nutrient supplementation (Chapter 4, text and Figure 1). Despite multi-nutrient supplementation, 50% of children remained anaemic at the second survey, which probably shows the importance of infections and infection-induced inflammation as determinants of anaemia.

Although malaria and iron deficiency are well-described causes of anaemia in rural African children, there have been few investigations of the contributions by other nutritional deficiencies. In our pilot study, we found that vitamin B_{12} deficiency is relatively rare (prevalence: 4%) but it is associated with marked reductions in haemoglobin concentration (Chapter 1). Vitamin A deficiency was more widespread (13%) but there was no evidence that it was independently associated with haemoglobin concentration in a multivariate model that also included other possible causes of anaemia. Although plasma folate concentration responded to supplementation with folic acid and other micronutrients (Chapter 6), baseline data of our trial showed no evidence that it was associated with haemoglobin concentrations. This corroborates a report from a case-control study in Malawi, in which deficiency of vitamin B_{12} but not of folate was associated with severe anaemia (Calis et al. 2008).

Based on the same study in Malawi, the authors report that iron deficiency was not a prominent cause of severe anaemia, because children with severe anaemia (haemoglobin concentration < 50 g/L) had higher odds of being iron deficient (as indicated by plasma markers as a measure for stainable iron stores in bone marrow slides) than apparently healthy control children. We believe that their suggestion that iron deficiency protects against severe anemia is inappropriate, because infection-induced inflammation results in increased amounts of stainable haemosiderin iron in macrophages of the monocyte phagocyte system (Cavill 2002). It is more plausible that infections and bacteraemia, which were more common in children with severe anaemia than their controls, caused the presence of iron stores, instead of the reverse. Significantly, the authors found that hookworm was associated with increased odds of severe anaemia, a relationship that is mediated mostly by iron deficiency**.** Moreover, the fact that supplementation with iron and multi-nutrients tended to decrease the rate of episodes with hemoglobin concentration below 80 g/L in both the Pemba study and in our trial, contradicts this notion.

Effects on malaria: We found strong evidence that, even in a setting with good access to care, multi-nutrient supplementation increases malaria rates, perhaps transiently, as shown by the increased rates of all episodes in the first 50 or 100 days of intervention, the reduced time to first episode, and the increased incidence of first episodes (Chapter 2). We suspect that this effect can be largely attributed to iron.

Subgroup analysis suggested that the effect on malaria rates varied with age class and iron status, and was most pronounced in children within the youngest age class (6-17 months) and those with iron deficiency at baseline. In older children, multi-nutrients seemed to decrease the rate of malaria episodes with hyperparasitaemia. We found an opposite trend of the effect of multi-nutrients with age when malaria was defined as episodes with haemoglobin concentrations < 80 g/L, presumably because children who were initially iron deficient were better able to maintain their haemoglobin concentration during malaria attacks when having received multi-nutrients (Chapter 4). A synthesis of selected findings reported in Chapters 3 and 4 of this thesis is provided in **Box 1**.

Box 1. Effects of haematinics on rates of malaria: synthesis of effects observed

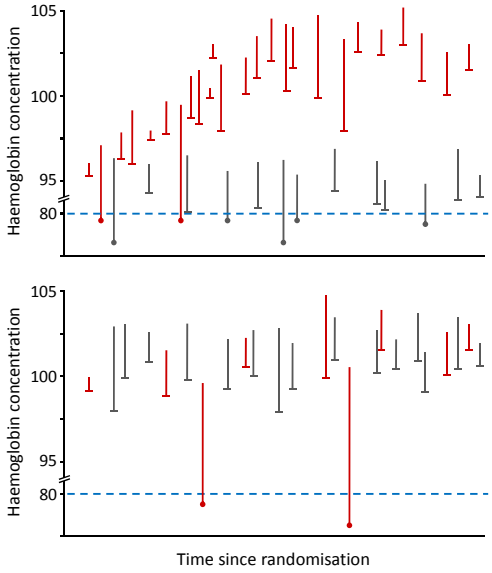

Figure 1. Simulation model with hypothetical data illustrating possible interactions between iron deficiency and iron supplementation on rates of malaria and rates of malaria associated with haemoglobin concentrations < 80 g/L
Line bars indicate haemoglobin concentrations just before or at the onset of febrile malaria episodes (upper end) and at the time of detection of episodes and/or initiation of chemotherapy (lower end). Red and grey bars represent events in children receiving iron supplements or placebo, respectively. Dashed blue lines indicate a threshold used to define malaria episodes with haemoglobin concentration < 80 g/L; such episodes are marked with a circle at the lower end of the bar. For further explanation: see text.

The model in **Figure 1** presents a synthesis of selected findings reported in Chapters 3 and 4 of this thesis, using simulated data from a fictive population of preschool children taking part in a trial comparing placebo versus supplementation with iron and other multi-nutrients. This model incorporates several phenomena that may explain our findings:

1. At randomisation, there is a subpopulation of children, mostly infants, with iron deficiency and low haemoglobin concentrations (upper panel) and a subpopulation of iron-replete children, mostly older, with haemoglobin concentrations that are higher (due to other causes of anaemia, these are still below reference values) (lower panel);

2. Over time, supplementation with micronutrients results in increased haemoglobin concentrations in children with iron deficiency but not in those who are iron-replete;
3. When presenting with febrile malaria, children with iron deficiency at baseline who received multi-nutrients have elevated haemoglobin concentrations group as compared to their peers with iron deficiency at baseline who received placebo;
4. Multi-nutrient supplementation results in increased rates of malaria episodes in children with iron deficiency at baseline but not in those who were iron-replete;
5. In children with iron deficiency, iron supplementation tends to result in decreased rates of malaria episodes with haemoglobin concentrations < 80 g/L but not in those who are iron-replete.

The direction of the subgroup effects measured in our trial (Chapter 4) is in contrast with the findings of the Pemba sub-study (Chapter 1). As correctly pointed out by others (Roth and Black 2010), the Pemba trial was unique in assessing effects on severe morbidity and death, which may be of greater public health relevance than trials such as ours that are based mostly on cases of uncomplicated malaria. In their report of the Pemba sub-study, however, Sazawal et al. (2006) were cautious in their interpretation of the subgroup effect, stating that their results suggested that supplementation with iron and folic acid is beneficial in children with iron deficiency, but unsafe in those who are iron-replete. The question arises whether this evidence is sufficient to support the WHO recommendation (WHO 2007) that iron supplements be administered routinely to iron-deficient infants under conditions with adequate access to anti-malarial treatment.

Examination shows that the evidence available meets only few criteria to make the claim of a subgroup effect credible (**Table 2**). The biological rationale for the interaction (Table 2, point 11) provided by the WHO Expert Consultation (WHO 2007) is that supplemental iron leads to the transient formation in plasma of non-transferrin bound iron (NTBI), but iron-deficient children may be protected from this effect because iron is removed more rapidly from the plasma due to their higher tissue requirements for iron. Indeed, a large bolus of supplemental ferrous salts taken in a single dose can lead to the transient formation in plasma of non-transferrin bound iron (NTBI) (Hutchinson et al. 2004, Baron et al. 2008, Dresow et al. 2008), which does not normally occur in healthy individuals (Esposito et al. 2002) and which may act as a nutritional source and favour the proliferation of blood

pathogens such as *Plasmodium* parasites (WHO 2007). There are also plausible mechanisms, however, to suggest that supplemental iron is more harmful in iron-deficient children than their iron-replete peers: because iron absorption is negatively associated with iron stores and is elevated in those with iron deficiency (Hunt 2003), one would expect NTBI production due to iron supplementation to be more pronounced in children with iron deficiency.

Table 2. Application of criteria [1] to evaluate the credibility of interaction of iron status at baseline on supplementation effect, as suggested by the Pemba sub-study (Sazawal et al. 2006)

Criterion		Answer	Credibility
Design			
1	Is the subgroup variable a characteristic measured at baseline or after randomisation?	At baseline	+
2	Is the effect suggested by comparisons within rather than between studies?	Yes	+
3	Was the hypothesis specified a priori?	Apparently not	–
4	Was the direction of the subgroup effect specified *a priori*?	Apparently not	–
5	Was the subgroup effect one of a small number of hypothesised effects tested?	Unknown	0
Analysis			
6	Does the interaction test suggest a low likelihood that chance explains the apparent subgroup effect?	Interaction test result not reported	–
7	Is the significant subgroup effect independent?	Not assessed [2]	0
Context			
8	Is the size of the subgroup effect large?	Yes	+
9	Is the interaction consistent across studies?	No	–[3]
10	Is the interaction consistent across closely related outcomes within the study?	Not assessed	–
11	Is there indirect evidence that supports the hypothesised interaction (biological rationale)?	Limited evidence [4]	0

+: high; –: low; 0: neutral
[1] Oxman and Guyatt 1992, Sun et al. 2010; [2] The supplementation effect also seemed to depend on the presence of anaemia at baseline. [3] Ac compared to evidence provided in Chapter 4 of this thesis. [4] See text.

Another issue is that the methods to detect iron deficiency differed between the Pemba study and our trial. We defined iron deficiency on the basis of plasma ferritin concentration while accounting for inflammation, which we

believe to provide a more valid indication than zinc protoporphyrin:haem (ZPP:H) ratio in whole blood, the marker used in the Pemba trial. To its credit, the ZPP:H ratio in whole blood may be less dependent on acute malaria than plasma ferritin concentration (Sazawal et al. 2006), and may react more slowly in response to inflammation, probably because of the time required for new erythrocytes with increased ZPP contents to enter the circulation and replace a substantial proportion of erythrocytes with normal ZPP contents. Nevertheless, *Plasmodium* infection may be associated with elevated ZPP:H ratios (Schneider et al. 1993, Stoltzfus et al. 2000), even in asymptomatic children (Veenemans and Verhoef, unpublished results). Plasma concentrations of bilirubin, a breakdown product of normal haem catabolism that is known to fluoresce in the same wavelength as ZPP (Buhrmann et al. 1978, Bartels et al. 1989) are increased under influence by haemolysis in febrile and asymptomatic malaria infections (Mishra et al. 2004, Iwalokun et al. 2006, Brasseur et al. 2007, Tagbor et al. 2008). In our trial, baseline *Plasmodium* infection, inflammation, young age, homozygous α^+-thalassemia and iron deficiency were all independently and significantly associated with increased ZPP:H ratios (Veenemans and Verhoef, unpublished results). Because it was logistically not feasible to measure ZPP:H ratios in fresh blood, these were measured in whole blood samples that had been stored in liquid nitrogen.

Thus, it seems likely that elevated ZPP:H ratios at baseline in the Pemba sub-study were co-determined to a large extent by current or recent *Plasmodium* infection, and hence had low specificity for detecting iron deficiency. Indeed, the prevalence of iron deficiency (75%) as reported by Sazawal et al. (2006) seems unrealistically high in view of lower prevalence estimates in similarly aged populations as reported in this thesis (28%–45%; Chapter 4) and elsewhere in eastern Africa (e.g. 45%–53%, Verhoef et al. 2001; 24%–38%, Desai et al. 2003). Consequently, ZPP:H ratios may have been a marker of frequent or chronic exposure to malaria and hence acquired immunity, rather than iron status. Such immunity may be further enhanced by iron supplements, in agreement with the protection against episodes with hyperparasitaemia that multi-nutrients seem to provide in older children (Chapter 4, Figure 2), explaining the beneficial effect of iron supplements in children with elevated ZPP:H ratios.

In our trial, examination of intervention effects within subgroups stratified by ZPP:H ratios shows that multi-nutrients appeared to increase malaria rates among children with ZPP:H ratios ≥ 80, similar to the effect observed among iron deficient children (RR 1.27 [95%CI: 1.01 to 1.61], 1.29 [0.94-1.71] and 1.53 [0.94-2.49]; for episodes as predefined and with densities > 10,000

and 100,000 parasites/μL respectively, with p-values for interaction 0.12, 0.10 and 0.12). Thus, our results do not support a strategy of using ZPP:H ratios as marker to target children expected to benefit from iron supplementation.

It may be argued that explanations can be found to reconcile the paradoxical findings of the subgroup analysis from our study and those from the Pemba study. One may reason that, in settings with good access to care, the eventual effect on mortality of multi-nutrient (or iron) supplementation in iron deficient children may be favorable despite an increase in parasite virulence and rates of uncomplicated malaria. For example, whilst early treatment of febrile episodes timely halts progression of uncomplicated episodes to cerebral malaria, supplementation may prevent deaths resulting from the (gradual) development of severe anaemia. We believe, however, that because such explanations are not directly substantiated by the available evidence, because they may not apply to all settings, and because alternative explanations for the discrepancy between the subgroup effects are possible, the current assumption that iron supplementation is safe when targeting iron deficient children in settings with adequate access to care is insufficiently justified.

3.2 Implications for public health policies and research

The recommendation by the World Health Organization that iron supplements should be administered routinely to iron-deficient infants in settings with adequate access to anti-malarial treatment should be reconsidered because the evidence that such intervention is safe in this subgroup is limited. In settings different from the Pemba study, and/or when using more specific markers of iron deficiency, the current WHO recommendation may actually be harmful.

In general, our study results underscore the need to thoroughly assess the safety of micronutrients interventions before they can be recommended for public health in malaria-endemic areas, even when targeting deficient subgroups. This is the case for supplementation and home fortification using different combinations or levels of micronutrients than studied so far, but also applies to food-based interventions.

Further research is needed to assess the safety of specific nutrients in such areas, and to identify and further characterise factors that determine the response to supplementation. Such studies will help to identify subpopulations that can benefit from these nutrients and other subpopulations in which they cause harm. Special consideration should be given to the influence of age, iron status and malaria exposure.

4. Interaction by *Giardia intestinalis* on the effect of multi-nutrients on diarrhoea

4.1 What does this study add?

Because *Giardia intestinalis* infection had been described as a possible cause of nutrient malabsorption, we originally decided to include its assessment with a view to assess its possible association with micronutrient status, and as a possible modifier of the effect of micronutrient supplementation. Contrary to our expectation, we found that it was associated with marked protection against diarrhoea in the groups receiving placebo and zinc but not in the two groups receiving multi-nutrients (Chapters 5 and 6). The protection was particularly strong in the placebo group: children with *Giardia* infection had 85% fewer diarrhoea episodes (95% CI: 50%-95%) than those without infection (Chapter 6, Figure 2). To our knowledge, a protective effect of this magnitude has not been described so far in other studies. It is important because diarrhoea is one of the main causes of child mortality in developing countries.

How much credibility can be given to this interaction? Our claim is strongly supported by the magnitude of the subgroup effect, the consistency in the protective effect being observed in groups receiving placebo and zinc and with respect to two mutually exclusive but probably related outcomes (diarrhoea and fever without localising signs), whilst the probability that the association is due to chance seems low. On the other hand, the interaction was identified *a posteriori*, and a plausible mechanism to explain the interaction is missing (**Table 3**). There is little support from other studies; this may be due, however, to variation in *Giardia* genotypes that may occur in between geographical areas. In aggregate, the evidence is weak.

4.2 Implications for public health policies and research

Our results do not support policies to control *Giardia* infection through community-based interventions. Further studies are needed to assess the association between *Giardia* infection and the burden of diarrhoea. Data may exist from past studies that can be helpful in this respect. Confirmation of a protective association would indicate that *Giardia* infection can possibly be used as a marker to predict the response to supplementation with multi-nutrients. On the other hand, *Giardia* infection may also be a marker of underlying factors that cause the protection observed; if so, identification of these factors could possibly lead to new interventions to control diarrhoea. Additional research recommendations are discussed in Chapter 6.

Table 3. Application of criteria [1] to evaluate the credibility of interaction of Giardia intestinalis infection at baseline on the effect of supplementation with multi-nutrients on rates of diarrhoea

Criterion		Answer	Credibility
Design			
1	Is the subgroup variable a characteristic measured at baseline or after randomisation?	At baseline	+
2	Is the effect suggested by comparisons within rather than between studies?	Yes	+
3	Was the hypothesis specified a priori?	No	–
4	Was the direction of the subgroup effect specified a priori?	No	–
5	Was the subgroup effect one of a small number of hypothesised effects tested?	Yes	+
Analysis			
6	Does the interaction test suggest a low likelihood that chance explains the apparent subgroup effect?	Yes	+
7	Is the significant subgroup effect independent?	Not applicable [2]	0
Context			
8	Is the size of the subgroup effect large?	Yes	+
9	Is the interaction consistent across studies?	Not applicable	–
10	Is the interaction consistent across closely related outcomes within the study?	Yes	+
11	Is there indirect evidence that supports the hypothesised interaction (biological rationale)?	No	–

+: high; –: low; 0: neutral
[1] Oxman and Guyatt 1992, Sun et al. 2010; [2] We did not identify other baseline factors that modified the effect supplementation with multi-nutrients on diarrhoea.

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Annex 1

Safety of iron-fortified foods in malaria-endemic areas

Hans Verhoef, Jacobien Veenemans

Am J Clin Nutr 2009;89:1949–50.

Sir,

Based on the finding that supplementation with iron and folic acid in malaria-endemic areas may cause an increased risk of hospitalization and death (Sazawal et al. 2006), an expert group convened by the World Health Organization (WHO) recommended that iron supplementation should be restricted in areas where malaria transmission is intense and infectious disease highly prevalent (WHO 2007). The group exempted industrial fortification with from these restrictions, under the assumption that the iron would be consumed in smaller amounts throughout the day and therefore absorbed more slowly. It also recommended that iron preparations added to food after cooking ('home fortification') should not be used in malaria-endemic areas because, when administered in a single meal, the dose of iron is still relatively high (WHO 2007).

To circumvent this problem, Barbara Troesch and colleagues (2009) studied the effects of consuming food that was home-fortified with micronutrient powders containing low amounts of highly bioavailable iron. We disagree with their conclusion that this approach may allow for effective, untargeted in-home fortification of complementary foods: it can be valid only if the adverse effects of iron observed by Sazawal et al. (2006) were due to an increased proliferation and invasion of enteric pathogens. It is indeed conceivable that iron leads to invasive non-typhoid *Salmonella* bacteremia, a complicating factor associated with death in African children with severe malarial anemia (Graham et al. 2000).

The WHO expert group considered an alternative explanation, however, that a large bolus of iron taken in a single dose leads to the transient formation in plasma of non-transferrin bound iron (WHO 2007), which may act as a nutritional source and favor the proliferation of pathogens such as malaria. If this were indeed the underlying mechanism, then the increased risk of adverse events can be avoided only by reducing the amount of iron absorbed.

The micronutrient powder containing 3 mg iron as NaFeEDTA investigated by Troesch et al. (2009) would in fact supply more absorbed iron than the original formulation of Sprinkles™, which contain 12.5 g iron as ferrous fumarate (0.28 mg as compared with < 0.21 mg; **Table 1**). Moreover, in African populations, this difference would probably be more than indicated than in Table 1, because iron absorption is exponentionally increased in individuals with iron deficiency (Hallberg 2001), as opposed to the volunteers investigated by Troesch et al. (2009), who were mostly healthy and iron replete.

Table. Amounts of iron absorbed through various iron interventions

	Native iron in food	Dose of iron ingested	Fractional iron absorption	Amount of iron absorbed, mg
Complementary food (60 g whole maize flour) fortified with 3 mg iron as NaFeEDTA [1]	0.75 mg	3 mg	7.39%	0.28
Complementary food (60 g whole maize flour) fortified with 3 mg iron as ferrous sulfate [1]	0.75 mg	3 mg	1.55%	0.06
Complementary food (60 g whole maize flour) fortified with 12.5 mg iron as ferrous fumarate [2]	0.75 mg	12.5 mg	< 1.55% [3]	< 0.21

Dose and formulation of iron [1] as investigated by Troesch et al. (2009) or [2] as in Sprinkles™ (http://www.sghi.org/);
[3] The fractional iron absorption is below 1.55% because it is known to be negatively associated with the dose of iron ingested (Hallberg 2001).

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Annex 2

Statistical analysis plan

Jacobien Veenemans, Hans Verhoef, Paul Milligan

This plan is based on the original proposal, and has been finalised before analyses of the intervention effects were performed.

To assess intervention effects on the primary outcome (episodes of malaria), and related endpoints, we will take the following steps (in sequential order):

1. Flow of trial participants and compliance to treatment, by treatment group

When describing the flow of participants, we will account for all individuals who were invited for screening. Compliance will be measured as the proportion of children who have consumed > 95% of scheduled supplements. In addition, intervention efficacy in improving plasma zinc concentration or height-for-age z-score will also be considered as indicative of good compliance. For individuals who were randomised to treatment but who stopped their participation before the scheduled end of intervention, we will provide reasons for their drop-out, if possible.

2. Description of baseline characteristics, by treatment group

Groups will be described regarding baseline characteristics. We will refrain from formal testing for group differences, because differences that do exist are necessarily caused by chance alone. In addition, minor imbalances at baseline are unlikely to be detected by statistical testing, but may still lead to confounding if the variable is strongly associated with outcome.

Baseline characteristics of each group will be described in the baseline table (prevalence, means ± SD, or median ± IQR, as appropriate).

3. Group descriptions for (primary) outcomes

The primary endpoint is an episode of uncomplicated malaria, defined as: *a)* reported febrile illness with axillary temperature ≥ 37.5 °C and a positive result for a pLDH dipstick test, OR *b)* reported febrile illness, with whole blood C-reactive protein concentration ≥ 8 mg/L and a positive result for a pLDH dipstick test.

For each intervention group, we will report the median survival time to 1st episode of uncomplicated malaria, and the cumulative incidence over the minimum follow-up period (224 days; i.e. the time period between 01 August 2008, the last recruitment, and 12 March 2009, the end date of the trial) as estimated by Kaplan Meier method, the total number of episodes

of uncomplicated malaria, the time at risk and the incidence rate (total number of episodes divided by time at risk). In addition, we will report the number of episodes of severe malaria and death (composite indicator) in each intervention group.

3.1 Comments on case definition:

Sensitivity: Our case definition is based on the detection of *Plasmodium* lactate dehydrogenase (pLDH) by immuno-chromatographic dipstick test (Makler et al. 1998, Piper et al. 1999). This test has high sensitivity (> 95%) for *P. falciparum*, but also for other *Plasmodium* spp., when used under field conditions (Cooke et al. 1999, Piper et al. 1999, Moody 2002, WHO 2009).

Specificity: To clear infection, and improve the specificity of our primary endpoint, all children with a positive result for a malaria dipstick test at baseline were given a 3-day course of anti-malarial drugs (artemether-lumefantrine). This drug combination is very efficacious in the treatment of uncomplicated malaria due to *Plasmodium falciparum* (Ogbonna and Uneke 2008) and also against infections caused by *P. vivax, P. malariae,* and *P. ovale* (White 2008). We therefore assume that a case as defined above is specific for an episode of malaria due to a newly acquired infection.

Nonetheless, the specificity of the case definition probably decreased with time since the last treatment: at the second survey (median follow-up time: 271 days or 8.5 months), we found that 32% of children were infected as assessed by pLDH test even if the overwhelming majority were symptom-free. In children who have become asymptomatically infected in the course of the intervention period, febrile illness and other symptoms may be due to another condition and the malaria parasites in their blood are merely coincidental, so that these cases would erroneously be considered as being malaria. On the other hand, we found that most malaria cases occurred in the first few months after randomization (e.g. at 61 days and 271 days, 50% and 83% of children, respectively, had experienced their first malarial episode). Thus we expect that our case definition has a high specificity and high positive predictive value to detect malaria, particularly in the first few months of the intervention; the gradual loss in specificity that may have developed over time probably will not cause substantial underestimation of intervention effects.

The use of threshold parasite density to define malaria cases: The use of a pre-specified threshold parasite density in peripheral blood has been advocated and is widely used to increase the specificity of case definitions for malaria in

endemic populations (Schellenberg et al. 1994, Smith et al. 2004). Although we may use this approach to explore to what extent it influences intervention effect, we decided not to do this in our primary analysis, for the following reasons:

1. The simple case definition used above (i.e. without threshold density) is probably quite specific;
2. Many assumptions must necessarily be made in establishing this threshold density: it is likely to depend on immunity, and therefore on age, nutritional status (e.g. intervention arm and duration) and numerous behavioural and environmental factors that influence exposure (Koram and Molyneux 2007);
3. Parasite density is only a weak predictor of mortality in falciparum malaria, because the circulating stages do not necessarily reflect the total body load of parasites: the latter also comprises the more pathogenic mature stages in parasitized erythrocytes that are usually sequestered by cytoadherence to endothelial cells in the microvasculature of organs (Dondorp et al. 2005). Cytoadherence may also explain the great variations in parasite density that have been observed within short periods of time (Koram and Molyneux 2007).
4. The parasite count by examination of blood films is subject to large variation, even with well-trained microscopists, and may not represent actual circulating parasite concentrations. This is particularly the case when the true parasite density is low.
    a. Counting 200 WBC is equivalent to counting a volume of approximately 0.025 μL (assuming an average WBC count of 8×10^6/mL). This is only 0.004 part of the total volume in the thick film. Counting such small volumes make the result of the count subject to great variation.
    b. Many parasites are washed off during the staining.
    c. In our trial, it was for practical reasons not possible to count WBCs in blood samples collected from sick children. Thus we would be restricted to using an assumed, fixed WBC count. Because malaria is known to suppress immunity and reduce WBC counts (McKenzie et al. 2005), this could lead to substantially biased (overestimated) parasite counts. On the other hand, many children may have had concurrent bacterial infections, with an accompanying increase in WBC count, which would lead to substantial underestimates of parasite density.

We will explore to what extent the estimates of intervention effects are modified by using various cut-off values for parasite density (1,000, 3,000 and 5,000 parasites per μL).

Plasma concentration of histidine-rich protein-2 may be a better 'signature' indicator for incorporation in malaria case definitions. This protein is found in the parasite cytoplasm or parasite food vacuole, but it is also found in the host erythrocyte cytoplasm and red cell membrane. At schizont rupture, sequestered falciparum parasites secrete histidine-rich protein-2 into the plasma. Plasma concentrations of histidine-rich protein-2 accurate indicate the total body parasite biomass in acute falciparum malaria, and can be measured with a commercially available enzyme-linked immunosorbent assay that is based on monoclonal-antibodies specific for *P. falciparum* (Cellabs, Sydney, NSW, Australia).

Severe malaria will be defined as haemoglobin concentration < 60 g/L with confirmed *P. falciparum* infection or *P. falciparum* antigenaemia, or fever with a positive pLDH dipstick test result and manifestations of neurological involvement (e.g. seizures).

4. Analysis of intervention effects

4.1 Primary analysis

The primary measure of intervention efficacy is calculated as the percentage reduction in the total number of malaria episodes, estimated as (100 x (1-hazard ratio)) relative to placebo, by using a Cox regression model that includes multiple episodes. A robust estimate of the standard error will be used to allow for dependence among multiple events within each person. The primary analysis is by intention-to-treat; in addition, we will conduct an exploratory per protocol analysis. In the primary analysis we will adjust for covariates that are prognostic for outcome. These covariates are divided into 2 groups: *a)* variables known from previous studies to be associated with outcome, and that will be used as covariates whether or not they are significantly associated with outcome in this study, and *b)* variables that we suspect to be associated with malaria risk. The latter (group b) will only be included if they are significantly associated with outcome, or if they substantially change the effect estimate.

Group a) includes:

a. Age class (6-17 months; 18-35 months; 35-60 months);
b. Exposure to malaria, indicated by malaria antigenaemia at baseline.

Group b) includes:

a. Distance to the dispensary (continuous)
b. Nutritional status (plasma concentrations of zinc and other nutrients; anthropometric indices, haemoglobin concentration) (continuous and dichotomized into deficient/replete, stunted/non-stunted, wasted/non-wasted, and anaemic/non-anaemic);

Effects will be reported as point estimates with corresponding 95% CIs.

Secondary analyses will include pairwise comparisons of the intervention groups with placebo, using the Kaplan Meier method (with p-values derived from Tarone-Ware test, based on 1st episodes), and measurement of the ratio of incidences (the total number of episodes divided by the time at risk in each group).

Summary

4.1	*Primary analysis*	Technique(s)
a.	Measure hazard ratios based on time-to-event analysis (including all events), adjusting for prognostic factors at baseline (see 4.1)	Cox PH regression model
4.2	*Secondary analysis*	
b.	Pairwise comparison of groups regarding time to event	Kaplan-Meier plots with p-value derived from Tarone-Ware test
c.	Measure incidence ratios and incidence differences, attributable fractions, and child-years of supplementation required to prevent an incident case of malaria	As described by Altman et al. 2000; Sahai and Khurshid 1996

5. Assessment of effect modification

5.1 Interaction between interventions

We anticipated that zinc and multi-nutrients can synergistically reduce malaria; on the other hand, our sample size is probably insufficient to show such effect modification even if it exists. Stratified analyses and Cox regression models will be used to evaluate possible interaction between interventions, i.e. the effect on the time to the recurrent malaria episodes of supplementation with zinc and other micronutrients is greater than the sum of the effects by either zinc alone or micronutrients other than zinc alone (including an interaction term in the Cox model). If there is evidence of such interaction, then the effect of zinc will be reported separately for children who received supplements with and without multi-nutrients. On the other

hand, if there is no evidence of such interaction, then marginal effects of zinc will reported with adjustment for the effect of supplementation with multi-nutrients.

5.2 Interactions between baseline factors and interventions: subgroup analyses
We will consider *a priori* the following baseline factors as potential modifiers of the effects of supplementation with zinc and/or multi-nutrients:

Potential effect modifier	**Indicator of:**	**Putative mechanism:**
Age class	Indicator of acquired immunity against malaria	The capacity of nutrients to boost immunity is likely to depend on the level of acquired immunity
P. falciparum parasitaemia	Indicator of exposure to malarial infection and acquired immunity	See above
Plasma zinc concentration	Considered the best available indicator of zinc status	The absorption and utilisation of supplemental zinc are increased in deficiencies of zinc and other micronutrients
Stunting	Indicator of zinc status	See above

Interactions will be assessed by stratified Kaplan-Meier analyses, and by multivariate Cox regression models with baseline factors either as categorical variables (age class, malaria antigenaemia) or continuous factors (plasma zinc concentration, height-for-age z-score) with non-linear terms, or analysed as categories, as appropriate. In these analyses, we will adjust for study design and baseline factors that are prognostic for outcome.

5.3 Interactions between interventions and time, or the number of episodes experienced
We hypothesize that children who received zinc and/or other micronutrients are better able to 'utilise' a malaria episode to develop protective immunity against subsequent symptomatic attacks. We will conduct an exploratory analysis to assess whether the intervention effect depends on (increases with) the number of episodes a child has experienced.

6. Analysis of recurrent events

6.1 Recrudescence versus reinfection
In the calculation of incidence of recurrent events, the total number of malaria episodes is counted, including multiple episodes in the same child.

Recurrent events can be caused by new infections or recrudescences. In our trial, we were not able distinguish between the two.

Because new infections are very rare in the first 14 days after treatment [1] (Mutabingwa 2005, WHO 2006), we considered all cases in which fever and parasitemia recurred or failed to resolve within 2 weeks as continuations of the same episode and not as new episodes. These cases were treated with the second-line drug, unless we had strong suspicion that children did not adhere to the initial treatment schedule of artemether-lumefantrine, in which case that treatment was repeated.

In the period of 2 weeks after treatment and beyond, we considered it important to capture all episodes, regardless whether they were due to a new infection or recrudescence.

We have tried to optimise compliance to treatment by giving the 1st dose at the dispensary under supervision by project staff, and by subsequent supervision by community-volunteers.

6.1 Censoring of observation time after chemotherapy

Assuming successful treatment with artemether-lumefantrine, children subsequently enter a period that they are not at risk of developing malaria. Thus, a number of days should be subtracted from the total observation time to account for this gap in time at risk, because failure to do so would result in an underestimate of the intervention effect.

The duration of this post-treatment prophylactic effect (PTP) varies between individuals. It is likely to depend on the pharmacokinetics of lumefantrine (which is eliminated much more slowly than its partner artemether; 3 days versus 1 h, respectively), the sensitivity of the parasites to the drugs, and the level of immunity of the individual concerned.

Based on data from our trial, we estimate that the PTP ranges between 20-30 days for the majority of children (**figure 1**). Data from most other trials suggest an almost absolute protection against reinfection during the 1st 14 days post-treatment, but a decrease in protection between 14-28 days (Mutabingwa 2005, WHO 2006).

[1] In an effectiveness trial conducted in 2002 in an area adjacent to ours, parasitological failure after treatment with artemether-lumefantrine was found in 1% and 21 % of children at day 14 and 28 respectively (Mutabingwa 2005). The majority among these represented new infections (0.3% and 18%, respectively), and recrudescences were very rare.

To avoid excluding cases from the analysis as would occur when censoring a period during which some children are no longer protected, using a conservative estimate of the PTP is probably most appropriate. Censoring observation time after treatment should therefore not exceed 14 days.

Some children presented within 14 days after the last treatment (see table below), with fever and a positive dipstick test result. There were, however, very few of such cases and we do not expect that excluding these cases will lead to bias.

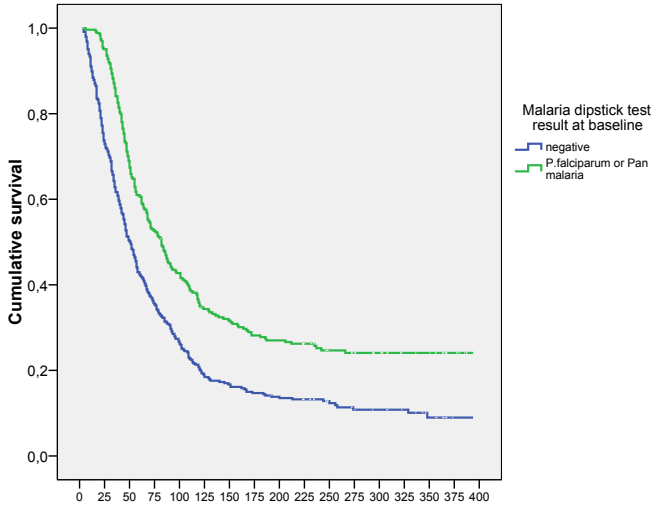

Figure 1. Kaplan-Meier curves showing time to first malaria episode (days), by infection status at baseline

Recurrent events within 2 weeks
1. After treatment for parasitaemia at baseline
▪ 6 children presented with fever and a positive dipstick within 14 days after baseline treatment. For 2 children, the attending clinician judged that these cases were probably malaria (based on additional features such as a considerable decline in haemoglobin concentration and the absence of another focus of fever). ▪ In the remaining 4 children, the symptoms were a-specific for malaria, the dipstick vaguely positive and the blood slide negative
2. After treatment for uncomplicated malaria
▪ Number of recurrent episodes of uncomplicated malaria in our trial (all episodes, excluding the 1st): 1134. ▪ 14 (1.2 %) followed within 14 days since previous treatment; ▪ For 10 of these children, we had serious doubts whether they actually adhered to the treatment schedule; ▪ The remaining 4 children came back on day 14, 14, 13 and 13 respectively. They seemed to have taken the appropriate doses, and treatment failure may have been caused by unusual pharmacokinetic properties of the individual child or by drug resistance.

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Annex 3

Composition of the multi-nutrient supplement

Hans Verhoef, Jacobien Veenemans

For practical reasons, children participating in the study daily received one standard dose of supplements, i.e. one capsule, regardless of age class. Target levels of individual nutrients in the supplement were set to supply at least 1 Recommended Daily Allowance (RDA) as determined for all children aged 1-3 years (FNB/IOM 1998, 2000, 2001), with exceptions for zinc, iron and vitamin C. One RDA represents the average daily dietary intake level that is considered sufficient to meet the nutrient requirement of 97.5% of healthy children in Canada or the USA. Because children also receive nutrients from food, this will result in a higher total intake of micronutrients than recommended for US children. The micronutrients needs of children from poor, rural African families are likely to be higher than those of their US peers, however, because of infection-induced increases in metabolic rate or infection-induced losses of micronutrients.

For vitamins, an overage was used to compensate for possible losses or degradation during storage due to environmental factors, and to ensure that the target level will be minimally achieved. Because we could not find guidance on the overage levels to be used, we assessed these levels rather arbitrarily on the basis of guesstimated losses due to environmental degradation.

The calculation of RDAs is based on estimates of fractional absorption for zinc and iron that are inappropriately low for African children from poor rural, families, who typically have diets with high contents of phytates that inhibit the absorption of these trace elements. In our trial, zinc was supplemented at 10 mg/day, with consideration that daily doses of 10-20 mg have been used repeatedly and successfully in many randomized controlled trials in children in the same age group without adverse effects (e.g. Bhandari et al 2002, Penny et al. 2004, Brooks et al. 2004, Castillo-Duran et al. 2001, Kikafunda et al. 1998, Muller et al. 2003, Sazawal et al. 1995, Roy 1991, Bhutta et al. 1999, Umeta et al. 2000). It is unlikely that daily supplementation with zinc at such a dose will adversely affect copper and iron absorption and metabolism (Fischer Walker et al. 2005).

The supplements also contained 18 mg iron/day, close to the recommended nutrient intake (RNI) for infants aged 6-12 months and set to supply on average 2 mg/kg body weight, daily supplementation levels recommended to prevent iron deficiency anaemia (2 mg/kg, WHO 2001). (The intakes of supplemental iron actually achieved were 1.7 mg/kg for children of all ages; for children aged 6-17 months, 18-35 months and 36-60 months, these intakes were 2.3 mg/kg, 1.7 mg/kg and 1.3 mg/kg, respectively.) There is no evidence of adverse effects at this level of iron supplementation on

biochemical zinc status, either whether given alone or in combination with zinc (Fischer Walker et al. 2005). For vitamin C, the RDA has been estimated as the amount required to provide antioxidant protection. Vitamin C was supplemented at levels above the RDA to optimise the absorption of iron. Even when absorption is doubled due to the high levels of vitamin C in the supplement (see below), the levels of supplemental iron would not exceed the Acceptable Intake (AI) or tolerable upper intake level (UL) for the age range included in this study (IOM 2001). For none of the nutrients included in the supplements would the levels of supplementation exceed the AI or UL for children within the age range in our study, even when assuming no losses due to environmental degradation.

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Table. 1. Target level and form of micronutrients to be supplemented

Active substance	Target level			Form	Infants 6-12 months		Children 1-3 years		Children 4-5 years	
	Declared	Overage [1]	Formulated		RDA/RNI [2]	UL	RDA/RNI [2]	UL	RDA/RNI [2]	UL
Vitamin A	300 µg RAE	50%	450 µg RAE [3]	All-*trans* retinyl acetate (powder)	500 µg	600 µg	300 µg	600 µg	400 µg	900 µg
Vitamin B_1	0.5 mg	25%	0.625 mg	Thiamin mononitrate	0.3 mg	ND	0.5 mg	ND	0.6 mg	ND
Vitamin B_2	0.5 mg	10%	0.55 mg	Riboflavin	0.4 mg	ND	0.5 mg	ND	0.6 mg	ND
Niacin	6 mg NE	10%	6.6 mg	Niacine	4 NE	ND	6 NE	10 mg	8 NE	15 mg
Vitamin B_6	0.5 mg	15%	0.575 mg	Pyridoxine	0.3 mg	ND	0.5 mg	30 mg	0.6 mg	40 mg
Folate	150 µg DFE	25%	93.75 µg [4]	Folic acid	80 µg	ND	150 µg	300 µg DFE	200 µg	400 µg DFE
Vitamin B_{12}	0.9 µg	30%	1.17 µg	Cyanocobalamid in mannitol	0.5 µg	ND	0.9 µg	ND	1.2 µg	ND
Vitamin C	50 mg	50%	75 mg	Purified L-ascorbic acid	50 mg	ND	15 mg	400 mg	25 mg	650 mg
Vitamin D	5 µg	35%	6.75 µg [5]	Vitamin D_3 (cholecalciferol)	5 µg	25 µg	5 µg	50 µg	5 µg	50 µg
Vitamin E	6 mg TE	10%	6.6 mg	RRR-⊛-tocopherol acetate	0.6 mg/kg bw	ND	6 mg	200 mg	7 mg	300 mg
Vitamin K	30 µg	50%	45 µg	Phylloquinone (vitamin K_1) 5%	2.5 µg	ND	30 µg	ND	55 µg	ND
Zinc	10 mg	0%	10 mg	Zinc as gluconate	8.4 mg	5 mg	8.3 mg	7 mg	9.6 mg	12 mg
Iron	18 mg	0%	18 mg	Ferrous fumarate	18.6 mg	40 mg	11.6 mg	40 mg	12.6 mg	40 mg
Iodine	90 µg	0%	90 µg	Potassium iodate	90 µg	ND	90 µg	200 µg	90 µg	300 µg
Copper	340 µg	0%	340 µg	Cupric gluconate	220 µg	ND	340 µg	1 mg	440 µg	3 mg
Selenium	20 µg	0%	20 µg	Sodium selenate	20 µg	60 µg	20 µg	90 µg	30 µg	150 µg
Magnesium	65 mg	0%	65 mg	Trimagnesium dicitrate anhydrous	75 mg	ND	80 mg	65 mg [6]	130 mg	110 mg [6]

RNI: Recommended Nutrient Intake as established by WHO/FAO (2001); UL: Tolerable Upper Intake Level as established by FNB/IOM (2001); RAE: retinol activity equivalents; NE: niacin equivalents; DFE: dietary folate equivalents; TE: ⊛-tocopherol equivalents; ND: Not derived

[1] Overage was calculated from declared amount (D) and formulated amount (F) as O=(F-D)*100/D; [2] Values indicated RDA except for iron and zinc, for which RNI values are provided; [3] Equivalent to 1,500 IU; [4] Based on IOM estimates that 0.5 µg folic acid taken on an empty stomach corresponds to 1 µg DFE; [5] Equivalent to 270 IU; [6] UL applies to supplementary magnesium.

Summary

Background: Zinc is important for innate and adaptive immune responses to infection. Preventive zinc supplementation has been shown to reduce the incidence of acute diarrhoea by 20%. Few trials have evaluated its effect against malaria. Because trial results for both outcomes are inconsistent, research priorities must shift from studies to measure efficacy to identifying factors that determine the magnitude of the effect of zinc supplementation. We hypothesized that protection by zinc supplementation depends on concomitant supplementation with other nutrients.

Objectives: Specific objectives were: a) to assess the effect of supplementation with zinc, alone or in combination with other nutrients, on the rates of malaria (primary objective); b) to assess intervention effects on rates of diarrhoea and other common diseases; c) to identify factors that determine the magnitude of the effect of the interventions. Our studies also provided an opportunity to assess effects of α^+-thalassaemia on malaria and malaria-associated anaemia. This haemoglobin disorder is highly prevalent in eastern Africa and that has recently been reported to protect against severe malaria.

Methods: In a highly malaria-endemic area in rural Tanzania, we randomised children (n=612) aged 6-60 months with height-for-age z-score ≤ –1.5 SD to daily supplementation with: a) zinc, vitamins and other mineral elements ('multi-nutrients'); b) zinc; c) multi-nutrients without zinc; or d) placebo. Those with *Plasmodium* infection at baseline were treated. Field staff and participants were blinded to treatment. Sick children were detected and evaluated in a research clinic. The primary outcome, an episode of malaria, was pre-defined as current *Plasmodium* antigenaemia in children with guardian-reported fever and any of the following: a) confirmed fever (axillary temperature ≥ 37.5 °C), or b) unconfirmed fever with inflammation (whole blood C-reactive protein concentrations ≥ 8 mg/L), separated by at least 14 days from a previous malaria episode.

Results: The primary analysis included 1,572 episodes of malaria and 526 child-years of observation. The prevalence of zinc deficiency (plasma zinc concentration < 9.9 μmol/mL) was 67% overall, and 60% in those without inflammation (plasma C-reactive protein concentration < 8 mg/L). This prevalence was dramatically reduced by zinc supplementation.

We found no evidence that concurrent supplementation with multi-nutrients influenced the magnitude of the effect of zinc on rates of malaria or diarrhoea, so that marginal effects will be presented in the remainder of this summary.

Although we found no evidence that zinc alone protected against malaria, it reduced rates of diarrhoea by 24% (95% CI: 4%–40%) and of episodes of fever without localising signs by 25% (4%–43%), two disorders with mutually exclusive case definitions.

We found no effect of multi-nutrients on the overall rate of malaria episodes, regardless the case definition used, but the effect estimate was likely underestimated by children becoming asymptomatically infected in the course of the intervention period. In the first 100 days of intervention, and in the analysis of first events, supplementation with multi-nutrients, with or without zinc, increased the hazard of malaria by one-third. In addition, subgroup analysis indicated that this effect depended strongly on age and iron status at baseline, with rates of episodes with parasite densities > 10,000 parasites/ μL increasing by 27 % (1%-61%) and 53% (11%–111%) in the youngest children (6-17 months) and in children with iron deficiency, whilst there was no evident effect in older children or those without iron deficiency (p-values for interaction: 0.02 and 0.007).

Despite the increase in malaria rates, the children who had the lowest haemoglobin concentrations during malaria (those aged 6-17 months) were better able to maintain their haemoglobin concentrations when having received multi-nutrients. Direct epidemiological evidence is lacking, however, if and under what conditions the higher haemoglobin concentrations during malaria (and expected reduced risk of death due to severe malarial anemia) outweigh the possible increase in other potentially lethal disease manifestations.

Multi-nutrient supplementation seemed to increase the rate of diarrhoea by 19% (–6% to 50%). Subgroup analysis indicated that this effect depended on *Giardia intestinalis* infection at baseline (p-values for interaction: 0.03): in those without multi-nutrients, infection was associated with a reduction in rates of diarrhoea by 68% (34%-85%), whilst there was no evidence for such protection in those receiving multi-nutrients. Similar effect modification was found for fever without localizing signs.

Of 612 children in the trial, 50% had normal genotype, whilst 41% and 9% were heterozygote and homozygous, respectively, for α+-thalassaemia. We found no evidence of group differences in malaria rates between genotypes. Subgroup analysis suggested, however, that the effect of α^{+}-thalassemia depended on age. Thus in children below 18 months, malaria rates were increased by 30% (2%–65%) in heterozygotes, whereas they were decreased by 20% (5%–32%) in older children (p-value for interaction: 0.001). Similar

patterns were found for homozygotes, even though estimates were less precise due the smaller numbers of children in this age class. Based on data from a pilot survey and a study in Kenya, we found that children with α+-thalassaemia (particularly homozygotes) were protected against the decline in haemoglobin concentration associated with mild to asymptomatic infections, particularly when these infections were accompanied by inflammation.

Interpretation and conclusions for policies: We found no evidence that addition of vitamins and other mineral elements increased the health benefits of zinc supplements. The beneficial effects of zinc described in this thesis strengthen the case for scaling up zinc interventions in deficient populations of African children, without concerns that it will cause adverse effects due to malaria.

Multi-nutrient supplementation may be unsafe in malaria-endemic areas, particularly in young children with iron deficiency. Thus the recommendation by the World Health Organization that iron supplements should be administered routinely to iron-deficient infants in settings with adequate access to anti-malarial treatment is insufficiently supported by evidence and should be reconsidered. Our results underscore that supplementation or home fortification, even when targeting deficient subgroups in settings with access to adequate primary care, should not be recommended in malaria-endemic areas until their safety has been demonstrated.

Samenvatting

Kinderen in ontwikkelingslanden hebben, vooral door een eenzijdig dieet, vaak een tekort aan vele voedingsstoffen. Van verscheidene van deze voedingsstoffen, o.a. van zink, wordt aangenomen dat zij een belangrijke rol spelen in de afweer tegen infecties. Inderdaad is in meerdere onderzoeken aangetoond dat het geven van zink het risico op diarree in jonge kinderen met 20% kan verlagen, en bovendien de frequentie en duur van luchtweginfecties beperkt. Ondanks deze voordelen komen beleidsrichtlijnen of programma's met het doel zinktekort op grote schaal te bestrijden nauwelijks van de grond.

Malaria is de belangrijkste kinderziekte op het platteland van Afrika; er wordt geschat dat er jaarlijk bijna 1 miljoen kinderen aan overlijden. In gebieden waar malaria veel voorkomt (hoog endemische gebieden) worden kinderen vanaf hun geboorte zo vaak blootgesteld dat hun afweersysteem de infectie op den duur onder controle kan houden. In de eerste 5 levensjaren is deze immuniteit echter nog niet compleet en gaan infecties vaak gepaard met koorts, of met ernstige ziekteverschijnselen, waaronder cerebrale malaria en ernstige bloedarmoede.

Het is onduidelijk of het geven van zink ook de frequentie en ernst van malaria aanvallen kan verminderen; hier zijn nog maar weinig onderzoeken naar gedaan, en met tegenstrijdige resultaten. Ook is er weinig bekend over de factoren die het effect van zink kunnen beinvloeden. Omdat kinderen met zinktekort vaak ook gebrek hebben aan andere voedingstoffen die essentieel zijn voor het immuunsysteem, veronderstelden wij dat de werkzaamheid van zink wordt verhoogd door gelijktijdig geven van andere mineralen en vitamines. Het in dit proefschrift beschreven onderzoek was primair opgezet om het effect van zink, al dan niet in combinatie met een 'cocktail' van andere voedingsstoffen (zie Annex 3) op het risico op malaria te evalueren. Het onderzoek vond plaats in een arm gebied op het Tanzaniaanse platteland, onder 612 jonge kinderen (0.5 tot 5 jaar), met een matige tot ernstige achterstand in lengtegroei. Deze kinderen kregen, gedurende minstens 7 maanden, dagelijks een van de volgende voedingssupplementen : 1) alleen zink; 2) zink in combinatie met een 'cocktail' van andere voedingsstoffen (hierna'multi-nutrienten' genoemd); 3) multi-nutrienten maar geen zink; 4) placebo. Kinderen werden willekeurig aan een van deze 4 groepen toegewezen (gerandomiseerd), en het was zowel voor de deelenemers als het studieteam onbekend welke combinatie de kinderen kregen (het onderzoek was geblindeerd). Aan de ouders van alle kinderen die aan de studie meededen werd gevraagd hun kind naar de kliniek te brengen wanneer zij merkten dat het koorts had, of zich anderszins onwel voelde. Bij ieder bezoek

aan de kliniek vanwege koorts werd middels een snelle diagnostische test (uitgevoerd met kleine druppel bloed) vastgesteld of het kind malaria had. Indien het resultaat van deze test positief was werd ook het aantal parasieten per microliter bloed bepaald.

In totaal traden er tijdens de studie 1,572 malaria-aanvallen op (veel kinderen hadden meerdere episodes). Uit metingen in bloedmonsters die aan het begin van de studie waren verzameld, bleek dat zinktekort voorkwam bij 67% van de kinderen, terwijl dit bij kinderen die zink hadden ontvangen aan het eind van de studie was teruggebracht tot 15%. Ondanks deze verbetering in zinkstatus vonden wij geen aanwijzingen dat er in de groep van kinderen die zink ontvingen minder malaria optrad (hoofdstuk 3). Ook bleek het effect van zink niet af te hangen van het gelijktijdig geven van multi-nutrienten. Wel hadden kinderen die zink ontvingen minder vaak koortsaanvallen die *niet* door malaria veroorzaakt werden (hoofdstuk 3), en minder vaak diarree (hoofdstuk 5), dan degenen die geen zink kregen.

Veel jonge Afrikaanse kinderen hebben ook bloedarmoede, een tekort aan hemoglobine (een eiwit -met daarin ijzer-bevattende heemmolekulen- dat verantwoordelijk is voor zuurstoftransport naar weefsels). De belangrijkste oorzaken zijn ijzergebrek, malaria, andere veelvoorkomende infecties, en bepaalde erfelijke aandoeningen zoals alpha-thalassemie. Bij al deze aandoeningen treedt een onvoldoende aanmaak van hemoglobine op; bij malaria is er daarnaast ook sprake van een afbraak van rode bloedcellen en hemoglobine, dat het voedingssubstraat van malariaparasieten vormt.

IJzer-bevattende voedingssupplementen verlagen het risico op bloedarmoede, en veelal werd aangenomen dat zij daarmee ook het risico op sterfte (tgv ernstige bloedarmoede) zouden kunnen verminderen. Ook in onze trial was ijzergebrek vrijwel afwezig nadat kinderen enkele maanden ijzer-bevattende supplementen hadden ontvangen (hoofdstuk 4). Omdat ziekteverwekkers echter ook baat hebben bij het ijzer in dergelijke supplementen, is het geven daarvan mogelijk niet altijd veilig. Dit geldt zeker wanneer het gaat om kinderen op van het platteland van Afrika, waar basisgezondheidszorg veelal afwezig is, en infecties (met name malaria) niet tijdig behandeld kunnen worden.

Uit ons onderzoek bleek dat het toedienen van multi-nutrienten, al dan niet met zink, leidde tot een 30% toename in de incidentie van malaria, berekend op basis van eerste episodes. Omdat de meeste kinderen meerdere malaria episodes doormaakten hebben wij ook gekeken naar het effect van de interventie op het totaal aantal malaria-episodes; in deze analyse vonden wij

eveneens een toename in malaria ten gevolge van multi-nutrienten van 30% in eerste 100 dagen van de interventie (hoofdstuk 4). Wij nemen aan dat deze toename toe te schrijven is aan het ijzer in de supplementen, maar een rol van andere micronutrienten kan niet worden uitgesloten.

Ook liet ons onderzoek zien dat het effect van multi-nutrienten sterk afhangt van leeftijd en ijzerstatus; de kans op het optreden van relatief ernstige malaria (een episode met meer dan 10,000 parasieten per microliter bloed) nam met ruim 50% toe, juist kinderen met ijzergebrek, terwijl de interventie geen effect had onder kinderen zonder ijzergebrek (hoofdstuk 4). De kans dat dit verschil in effect tussen kinderen met en zonder ijzergebrek aan toeval te wijten was, was slechts 0.007%. Ondanks de geobserveerde toename in de frequentie van malaria episodes, hadden kinderen wel een kleinere kans op (ernstige) bloedarmoede tijdens een malaria aanval wanneer zij de supplementen hadden gekregen.

De bevinding dat multi-nutrienten resulteerden in frequentere en/of ernstiger malaria episodes juist in kinderen met ijzergebrek is in contrast met de resultaten van een eerdere studie in Pemba, waar ijzer juist veilig leek te zijn in deze kinderen (in deze studie werd gekeken naar mortaliteit en ziekenhuisopname). Onze resultaten suggereren dat het geven van multi-nutrienten (incl. ijzer) niet altijd veilig is, zelfs aan kinderen met aangetoond ijzergebrek. Deze infomatie heeft mogelijk consequenties voor de huidige WHO richtlijnen, waarin wordt aanbevolen ijzersupplementen alleen te geven aan kinderen met aangetoond ijzergebrek (hoofdstuk 9).

Tenslotte vonden wij nog een andere aanwijzing dat het geven van multi-nutrienten aan kinderen in dit soort settings niet altijd gunstig hoeft te zijn: onder kinderen die bij het begin van de interventie waren geïnfecteerd met de darmparasiet *Giardia intestinalis,* resulteerde het geven van multi-nutrienten in verdubbeling in de frequentie van diarree (hoofdstuk 5). De aanwezigheid van de parasite bij baseline was overigens ge-associeerd met een substantiele bescherming tegen diarree (hoofdstuk 6).

Samenvattend, vonden wij geen aanwijzingen dat zinksupplementen het risico op malaria verminderen. De bevinding dat zink leidt tot een reductie in de frequentie van diarree en koortsepisodes die niet door malaria worden veroorzaakt, ondersteunen de noodzaak om succesvolle zinkinterventies te ontwikkelen voor populaties waar zinktekort vaak voorkomt.

Onze bevindingen benadrukken dat het geven van multi-nutrienten door middel van supplementen of ‘home- fortification’ aan kinderen in malaria-

endemische gebieden mogelijk onveilig is; het kan daarom niet worden aanbevolen totdat het tegendeel bewezen is. Op basis van onze gegevens lijkt zelfs het gericht geven van ijzersupplementen aan kinderen met ijzergebrek onveilig.

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The study was conducted under the auspices of the Kilimanjaro Christian Medical Centre, Moshi.

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as the father of my children, he cannot act as my co-supervisor.

Huub Savelkoul took over the role as my promoter from Professor Clive West, who received the terrible news of his imminent death on the very day that this research project got started. Clive indeed died shortly afterwards. He was a remarkable man, and instrumental in getting this project started.

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About the author

Jacobien Veenemans was born in 1974 in Laren (N.H), The Netherlands. During her study (medicine, at the University of Amsterdam), she did a research elective in Kenya, where she assisted in the implementation of a randomized trial to assess the effects of iron supplementation and intermittent malaria treatment on hemoglobin concentration and malaria morbidity among rural pre-school children (1998-1999).
During this period, she became interested in biomedical research. In the years following her interships (2002) she worked in the department of Internal Medicine in hospital "De Gelderse Vallei" in Ede, The Netherlands, and obtained funding for the project described in this thesis, in close collaboration with Hans Verhoef and Professor Clive West. The project officially started in November 2004. She became initially involved in T cell stimulation experiments and flow cytometry at the Cell Biology and Immunology Group at Wageningen University, The Netherlands. This experience kindled an interest in laboratory work, and her interest expanded from clinical into more fundamental biology.

During the implementation of the trial described in this thesis, she combined research and data collection with clinical supervision and management tasks, before returning to the Netherlands in August 2009, where she spent one year analysing data and writing the papers presented in this thesis. She intends to pursue a further career as medical microbiologist, eventually combining practical work related to patient care with applied research.

Other publications:

Mbugi EV, Meijerink M, *Veenemans J*, Jeurink PV, McCall M, Olomi RM, Shao JF, Chilongola JO, Verhoef H, Savelkoul HF. Effect of nutrient deficiencies on in vitro Th1 and Th2 cytokine response of peripheral blood mononuclear cells to Plasmodium falciparum infection. Malar J 2010;9:162.

Mbugi EV, Meijerink M, *Veenemans J*, Jeurink PV, McCall M, Olomi RM, Shao JF, Verhoef H, Savelkoul HF. Alterations in early cytokine-mediated immune responses to Plasmodium falciparum infection in Tanzanian children with mineral element deficiencies: a cross-sectional survey. Malar J 2010;9:130.

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Verhoef H, West CE, *Veenemans J*, Beguin Y, Kok FJ. Stunting may determine the severity of malaria-associated anemia in African children. Pediatrics 2002;110:e48.
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Verhoef H, *Veenemans J*, West CE. HIV-1 infection and malaria parasitaemia. Lancet 2001;357:232–33.

Training and Supervision Plan

The Basic Package

- WIAS Introduction Course, Wageningen, The Netherlands (2007)
- Philosophy and Ethics of Food Science and Technology, VLAG Graduate School, Wageningen, The Netherlands (2007)

Subtotal 3 credits

Scientific Exposure

International conferences

- Zinc, at home in the environment, IZA, Brussels (2005)
- Young Investigators Workshop, Istanbul (2007)
- 1st Micronutrient Forum, Istanbul (2007)
- 2nd Micronutrient Forum, Beijing (2009)

Seminars and Workshops

- WIAS Science day (2005)
- Workshop MACH project: preliminary results pilot study, planning randomised trial (2006)
- BD Biosciences, science event (2007)
- Workshop MACH project: results of a randomised trial (2009)
- Technical workshop on nutrition trials in rural Africa (2010)

Presentations

- Zinc and human health; from research to programs. Presentation at European conference organized by the International Zinc Association, Antwerp, Belgium (2005)
- Effects of supplementation of zinc and other micronutrients on the health, development and well-being of African children, Poster at opening Biotechnology Laboratory, Moshi (2005)
- Effects of zinc supplementation on malaria: an overview of the evidence. Moshi, Tanzania (2005)
- H.pylori and iron deficiency. KCMC, Moshi, Tanzania (2005)
- Lot Quality Assurance Sampling: background and application. Presentation for MSc students, Moshi, Tanzania (2005)
- Micronutrients and malaria. Poster at WIAS Science day, Wageningen (2007)
- Effects of zinc deficiency on the cellular immunity against malaria**.** Poster at European and Developing Countries Clinical Trials Partnership (EDCTP) 4th Annual Forum, Ouagadougou, Burkina Faso (2007)
- Factors associated with hemoglobin concentrations in Tanzanian children: a cross-sectional study. Poster at 2nd Micronutrient Forum meeting, Beijing, China (2009)
- Hypomagnesemia: prevalence and its associations with hypocalcemia, zinc deficiency and Giardia intestinalis in rural Tanzanian children. Poster at 2nd Micronutrient Forum meeting, Beijing, China (2009)

Subtotal Scientific Exposure 14,4 credits

In-Depth Studies

- Fish Immunology Workshop, Wageningen, The Netherlands (2005
- Course Clinical Immunology, Eijkman Graduate School, Utrecht, The Netherlands (2007)
- Course Infectionbiology, Eijkman Graduate School, Utrecht, The Netherlands (2007)
- Ecophysiology of the gastro-intestinal tract, Wageningen (2007)
- Design and analysis of randomized trials, Moshi, Tanzania (2005-2006)
- Statistics course, WIAS, Wageningen (2009)
- Journal club meetings, Moshi/Muheza, Tanzania (2005-2009)

Subtotal 11,8 credits

Professional Skills Support Courses

- Theoretical training on alpha-thalassemia genotyping at Biotechnology Laboratory, Moshi, Tanzania (2005)
- Course on scientific writing, by the editor of the Journal of Tropical Medicine and International Health. Moshi, Tanzania (2006)
- Course ELISA: basic understanding and trouble shooting, Wageningen, The Netherlands (2007)
- Course Flow cytometry, Becton Dickinson, Wageningen (2007)
- Training Good Clinical Practice, Moshi/Muheza, Tanzania (2008)

Subtotal 4,8 credits

Research Skills Training

- Preparing PhD proposal and Statistical Analysis Plan (2005, 2009)
- Giardia detection by ELISA, Public Health Laboratory, Haarlem, The Netherlands (2007)

Subtotal 4,0 credits

Supervising theses

- 10 major MSc theses (2005-2010)
- 1 minor MSC thesis (2010)

Subtotal 21,5 credits

Total: 59,2 credits [1]

Herewith the WIAS Graduate School declares that the PhD candidate has complied with the educational requirements set by the educational Committee of WIAS.

[1] A credit point represents a study load of 28 hours

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