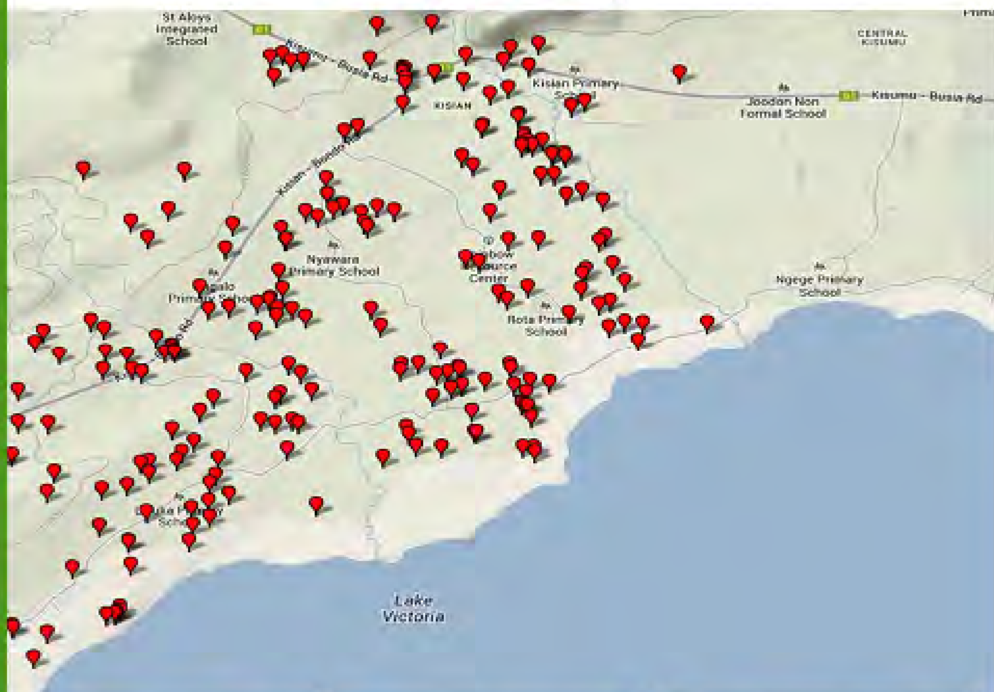
Safety and efficacy of iron supplementation in pregnant Kenyan Women

Martin N. Mwangi



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Martin N. Mwangi, 2014

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Safety and efficacy of iron supplementation in pregnant Kenyan women

Martin Ndegwa Mwangi

Thesis

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To Laura, John, and Dad

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Chapter 1

General introduction

Almost two billion people are currently anaemic globally. Most live in developing countries such as Kenya, where anaemia, iron deficiency, and malaria co-exist. Malaria infection and iron deficiency are the main causes of anaemia in malaria endemic regions of the world(Fleming, 1982)(Clara Menendez et al., 1997)(Schellenberg et al., 1999). Severe anaemia is increasingly recognized as an important manifestation of severe malaria in young children(Newton et al., 1997)(R. W. Snow et al., 1994). The global prevalence of anaemia among pregnant women is 41.8%(Peña-Rosas, De-Regil, Dowswell, & Viteri, 2012).
Malaria and anaemia remain inextricably linked with each other, and with poverty. Anaemia is an almost inevitable consequence of malarial infection(Roberts, Casals-Pascual, & Weatherall, 2005). The highest malaria and anaemia rates are seen in countries with the highest rates of extreme poverty. Pregnant women and children under five years are usually the highest risk group possibly because they have limited protective immunity.(Mor & Cardenas, 2010; Stirrat, 1994)

Since the British doctor Ronald Ross received the 1902 Nobel Prize in medicine for his work in malaria, more people have died from malaria than all world wars and terrorist attacks combined. This is in spite of the fact that the French chemists Pierre Joseph Pelletier and Joseph Bienaimé Caventou made quinine available from as early as 1820. According to the WHO, there were about 219 million cases of malaria and an estimated 660,000 deaths in 2010. Africa is the most affected continent: approximately 80% of cases and 90% of deaths are estimated to occur in the WHO African Region. However, although 40% of the world population is exposed to malaria parasites, malaria mortality fell 26% worldwide between 2000 and 2010. In the WHO African Region, the decrease was 33%. (WHO; World Malaria Report, 2013)

In the recent past, a lot of research has been directed towards understanding the biological question whether iron deficiency in the host is detrimental or beneficial. The question has been whether provision of iron to the host via the various intervention vehicles such as supplements, sprinkles, fortified foods etc. causes a harmful effect to the host or not e.g. by exacerbating malaria infection. In the Pemba study carried out by Sazawal et al (2006) in Zanzibar and involving more than 32,000 children, it was found that supplementation with iron and folic acid increased severe morbidity and mortality. Similar observations in malaria-prone developing countries were reported by others (Sazawal et al., 2006)(Smith,

Hendrickse, Harrison, Hayes, & Greenwood, 1989)(Metz, 2007). However, such an effect was not observed in developing countries that do not have high incidences of malaria(Tielsch et al., 2006)(Nacher et al., 2003). Current international guidelines on iron supplementation are faced with uncertainties due to the observed increase in morbidity and mortality rates especially in malaria endemic regions.

There have been major efforts by the global health research community to solve the mystery of the Pemba study. The WHO and the United Nations Children's Fund (UNICEF) released a joint statement in 2006 advising that in regions where the prevalence of malaria and other infectious diseases was high, iron and folic acid supplementation should only be administered to those who are anaemic, or who are at risk of becoming iron deficient(WHO/UNICEF, 2006). In 2007, the WHO convened a technical consultation group on prevention and control of iron deficiency in infants and young children in malaria endemic areas. It was determined that "universal 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"(WHO, 2007). The report emphasised the need to determine safe, effective, and sustainable methods to deliver iron.

A 2009 Cochrane review of 40 trials, involving 12,706 women, on routine antenatal iron or combination of iron with folic acid found insufficient data to determine if routine daily or intermittent iron or iron-folic acid supplementation in pregnancy improves health outcomes for women and babies(Peña-rosas & Viteri, 2009). This review was updated to compare the daily provision of iron supplements alone or in combination with folic acid or other micronutrients with no intervention, placebo or versus the use of the same supplements but without iron (e.g. only folic acid) among pregnant women living in a variety of settings, including malaria-endemic areas(WHO, 2012a),(Peña-Rosas et al., 2012). The updated review had among others, malaria as an outcome. Although the review found no evidence that iron supplementation increases placental malaria, the quality of evidence presented was judged as low because there were few trials that assessed effects on malaria, some of the trials had high levels of attrition, and in various studies the method of allocation concealment was unclear(Peña-Rosas et al., 2012). Based on the updated review, the WHO issued revised iron supplementation guidelines in 2012 aimed at directing global policy on interventions against anaemia especially in malaria endemic regions.

However, iron supplementation in malaria endemic regions remains controversial among researchers, nutritionists, and programme implementers. The safety and efficacy of daily oral use of iron supplements by pregnant women, as a public health intervention is still not clearly established despite the efforts by the WHO. Although universal iron supplementation continues to be recommended for women during pregnancy and three months postpartum, recommendations that malaria surveillance, diagnosis, and treatment be ensured during implementation of these programmes have been difficult to implement in resource poor settings.

Iron

Iron is essential for biological systems such as haematopoiesis, immune function, oxygen delivery, neurological function, and physical development. However, it also has a critical role in pathogens such as bacteria, viruses, fungi, and protozoa(Drakesmith & Prentice, 2008). The body has a highly regulated system for iron transport and storage.(Dunn, Suryo Rahmanto, & Richardson, 2007) Benefits of iron interventions have been demonstrated widely.(Lozoff, n.d.; Clara Menendez et al., 1997, 2004; Richard et al., 2006)

Erythrocytes carry most of the body's iron. An adult human produces approximately 200 billion erythrocytes per day to replace an equal number of cells that that reach the end of their life span. The body maintains the concentration of iron at about 40 mg iron/kg in women and about 50 mg iron/kg in men.(Brittenham, 2000) Humans are incapable of excreting excess iron thus they regulate the total iron in their body by controlling iron absorption in the gastrointestinal tract. If iron stores increase, absorption decreases and vice versa.(Bothwell, Charlton, Cook, & Finch, 1979)

A term pregnancy and delivery results in a loss of 450 – 600 mg of iron (1,200 – 1500 mL of blood).(Bothwell et al., 1979) Pregnant women deposit 270mg of iron in the fetus while an additional 90 mg is contained in the cord and placenta. 150 mg of iron are lost during delivery and early post natal period.

Iron deficiency is an imbalance in total body iron that results when the supply of iron is less than the body requirements and losses.(Brittenham, 2000) There are three stages of iron deficiency: storage iron depletion, early functional iron deficiency, and established functional iron deficiency. Iron deficiency may cause anaemia, impaired behavioural development, and decreased work capacity. If severe, iron deficiency causes increased mortality during pregnancy, and infancy. In addition, iron deficiency can lead to clinically significant immune deficiency and infections in children (Cunningham-Rundles et al., 2005).

Iron metabolism

Human iron metabolism is the set of chemical reactions maintaining human homeostasis of iron. Ferritin and haemosiderin, which are found primarily in the liver, spleen, reticuloendothelial cells, and bone marrow, are the major iron-storage compounds in the body(Usha Ramakrishnan & Semba., 2008).

Intracellular iron is spread over three different pools: 1) the functional pool, where it is bound mostly to haemoglobin; 2) the storage pool, where it is bound to ferritin; and 3) the regulatory pool, where iron regulating proteins IRP1 (iron regulatory protein) and IRP2 regulate transcription and translation of iron binding proteins. Serum ferritin is a good indicator of iron status, although its expression is up-regulated by inflammatory cytokines

during periods of infection. Small amounts of iron are also found in plasma(Parveen Kumar & Clark., 1998).

Iron is transported in the plasma bound to transferrin, a β–globulin that is synthesized in the liver. Human beings normally have 40 – 50mg Fe/kg body weight(Kraemer & Zimmermann, 2007). About two-thirds of the total body iron is in the circulation as haemoglobin.

Absorption of iron from the diet

Iron is absorbed in the duodenum by enterocytes of the duodenal lining and the jejunum. It must be in its ferrous (Fe^{2+}) form in order to be absorbed. Heme iron transporter (HCP 1) transports heme iron, which undergoes endocytosis after which ferrous iron (Fe^{2+}) is liberated within the endosome or lysosome. Non-heme iron includes ferrous and ferric iron salts (Fe^{3+}). Ferric iron is reduced to ferrous iron by ascorbic acid in the lumen or by membrane ferrireductases that include duodenal cytochrome B (DCYTB)(McKie et al., 2001). Transport of non-heme iron (which is now in ferrous form) from the intestinal lumen into the enterocytes is facilitated by the divalent metal ion transporter 1 (DMT 1) mainly because the acid microclimate at the apical membrane provides an H^+ electrochemical gradient that drives Fe^{2+} transport into the enterocytes(Gunshin et al., 1997). Once inside the enterocyte, iron that is not directly transferred to the circulation is stored as ferritin and is eventually lost when the cell is exfoliated at the villus tip. At the basolateral membrane, iron transport to transferrin in the circulation is mediated by ferroportin 1, in association with hephaestin. Hepcidin, which is produced by the liver, binds to ferroportin 1 causing its internalization and degradation and decreasing iron transfer into the blood(Nemeth et al., 2004)(Nemeth & Ganz, 2006). It is regulated by iron levels and erythropoiesis. In turn, it regulates iron uptake by enterocytes and release of iron stores from macrophages and hepatocytes. Ferroportin 1 also mediates export of iron from other cells, including macrophages(Donovan et al., 2005).

The regulation of ferroportin is the main way of regulating the amount of iron circulating in the body. This is because though DCYTB and DMT1 are unique to iron transport across the duodenum, ferroportin is distributed throughout the body on all cells that store iron. Iron deficiency and hypoxia stimulate duodenal expression of DMT1, DCYTB and ferroportin and thereby increase iron absorption(McKie et al., 2001)(Collins, Franck, Kowdley, & Ghishan, 2005).

Senescent red blood cells are broken down by macrophages in the spleen, bone marrow, and liver. The iron extracted is returned to the circulation where it binds to transferrin.

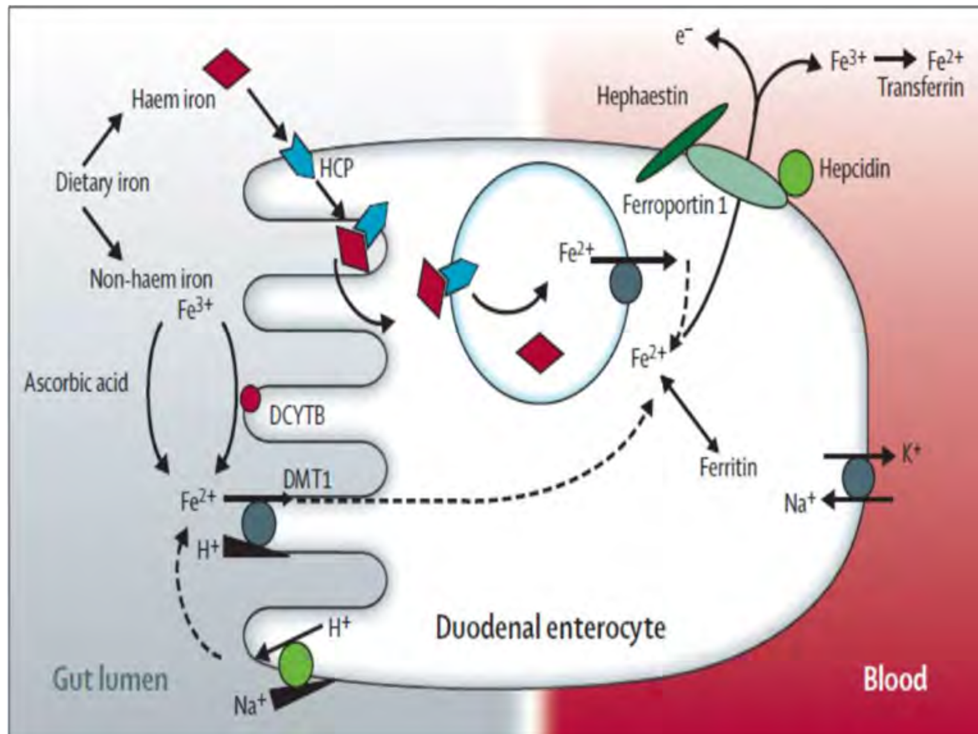

Figure 1: Regulation of intestinal iron uptake [Source: Zimmermann and Hurrell 2007]

Transferrin binds to specific transferrin receptors on erythroid precursors in the bone marrow. Iron deficiency increases iron transfer to the maximum by stimulating increased ferroportin expression on macrophages, hepatic synthesis of transferrin and expression of transferrin receptors in the bone marrow and other tissues.

Reasons for iron deficiency

1. High demand for iron beyond what the diet can supply
2. Increased iron loss usually through loss of blood
3. Nutritional deficiency
4. Inability to absorb iron because of damage to the intestinal lining e.g. in case of celiac sprue which severely reduce absorption surface area
5. Inflammation leading to hepcidin-induced restriction on iron release from enterocytes

Regulation of intracellular iron homeostasis

Hepcidin is the central regulator of iron homeostasis. It is a 25-amino-acid peptide hormone

produced by the liver. It regulates the export of iron from cells into plasma by controlling absorption from the intestine, export from macrophages, and release from body stores.(Nemeth & Ganz, 2006)(Ganz, 2007) This is done by controlling the entry of iron into plasma.

An increase in hepcidin synthesis causes a subsequent decrease in plasma iron and intestinal iron absorption. Hepcidin synthesis is increased by iron loading, inflammation, and infection and decreased by iron deficiency, and ineffective erythropoiesis.

Iron in circulation is tightly bound to transferrin. Body cells have receptors for transferrin–iron complexes on their surfaces. These receptors engulf and internalize both the protein and the iron attached to it. Once inside, the cell transfers the iron to ferritin, the internal iron storage molecule. The level of serum ferritin in the body is directly proportional to the amount of stored iron in the body. It can be determined using enzyme-linked immunosorbent assays (ELISAs) or two site immunoradiometric assays(Gibson & RS, 2005).

Iron absorption is influenced by dietary iron content, bioavailability of dietary iron, the amount of storage iron and the rate of erythrocyte production(Kraemer & Zimmermann, 2007). In developing countries, only 5% of dietary iron is normally absorbed from the average daily diet. Most of the iron in the body is obtained by recycling aged red blood cells in the reticuloendothelial system. Iron is lost through menstruation, sweat, urine, breast milk, shedding of skin cells and the mucosal lining of the gastrointestinal tract(Parveen Kumar & Clark., 1998). Thus people must continuously absorb iron. When iron loss exceeds iron absorption, the iron stores become depleted and the transferrin saturation in the blood then falls. If this drops to below 10%, then abnormal iron deficient erythropoiesis occur leading to microcytic anaemia.

Iron and immunity

Iron is intricately involved in both innate and adaptive immune responses to infection.(Weiss, 2002) Since almost all pathogenic microorganisms require iron for growth, the immediate response to infection is usually to withhold iron to invading pathogens. Increased hepcidin synthesis restricts delivery of iron to the plasma from macrophages, from intestinal absorption, and from hepatocyte stores.(Ward et al., 2011)

Many of the genes and proteins involved in iron homoeostasis play a vital role in controlling iron fluxes such that bacteria are prevented from utilising iron for growth.(Ward et al., 2011) Cells of the innate immune system, monocytes, macrophages, microglia and lymphocytes, are able to combat bacterial attacks by carefully controlling their iron fluxes, which are mediated by hepcidin and ferroportin. A variety of effector molecules, e.g. toll-like receptors, NF-kB, hypoxia factor-1, haem oxygenase, orchestrate the inflammatory response by mobilising a variety of cytokines, neurotrophic factors, chemokines, and

reactive oxygen and nitrogen species.(Ward et al., 2011) Imbalances in the host iron availability impair the host immune system.(Weiss, 2002)

The virulence of many bacteria is enhanced through exposure to iron.(Ratledge & Dover, 2000) Some bacteria acquire iron by secreting organic iron chelators called siderophores, by expressing surface receptors that interact with host iron-containing proteins, or both.

Anaemia

Anaemia is defined as a haemoglobin concentration below –2 standard deviations of the age- and sex-specific reference mean.(Usha Ramakrishnan & Semba., 2008) The cut-off values most commonly used to define anaemia are haemoglobin concentrations below 110 g/L for children under 5 years old and pregnant women, below 120 g/L for non-pregnant adult women, and below 130 g/L for adult men.

A WHO (2001) report outlined the main causes of anaemia as: dietary iron deficiency; infectious diseases such as malaria, hookworm infections, or schistosomiasis infections; deficiencies of key micronutrients such as folate, vitamin B12, or vitamin A; and inherited conditions that affect cell stability such as thalassaemia and sickle cell anaemia(WHO, 2001). The three main exogenous causes of anaemia, namely disease, blood loss and diet (Thurnham & Northrop-Clewes, 2007), are shown in figure 2.

Although there is great variation by region, young children and women of reproductive age are at greatest risk of anaemia, followed by the elderly and men. Anaemia may lead to: fatigue, headaches, faintness, breathlessness, angina of effort, intermittent limping due to weakness (claudication), palpitations, pallor (extreme paleness), tachycardia, systolic flow murmur, cardiac failure and rarely, papillo-edema and retinal haemorrhages after an acute bleed (Thurnham & Northrop-Clewes, 2007).

Very severe anaemia (haemoglobin < 50 gm/L) is associated with increased childhood and maternal mortality (Allen, 1997). In areas where severe anaemia (haemoglobin < 80 gm/L) is common, iron deficiency is usually one of multiple causes of anaemia (Brooker et al., 1999).

Iron deficiency anaemia (IDA)

Anaemia is the primary sign of iron deficiency(Kraemer & Zimmermann, 2007). Stored iron which is physiologically bound by ferritin molecules is usually almost entirely depleted before the development of IDA(Usha Ramakrishnan & Semba., 2008). The causes of IDA include: blood loss, increased demands such as growth and pregnancy, decreased absorption (e.g. postgastrectomy) and poor intake. IDA develops when there is inadequate iron for haemoglobin synthesis(Zimmermann & Hurrell, 2007). Normal levels of haemoglobin are maintained for as long as possible after the iron stores are depleted; latent iron deficiency is said to be present during this period.

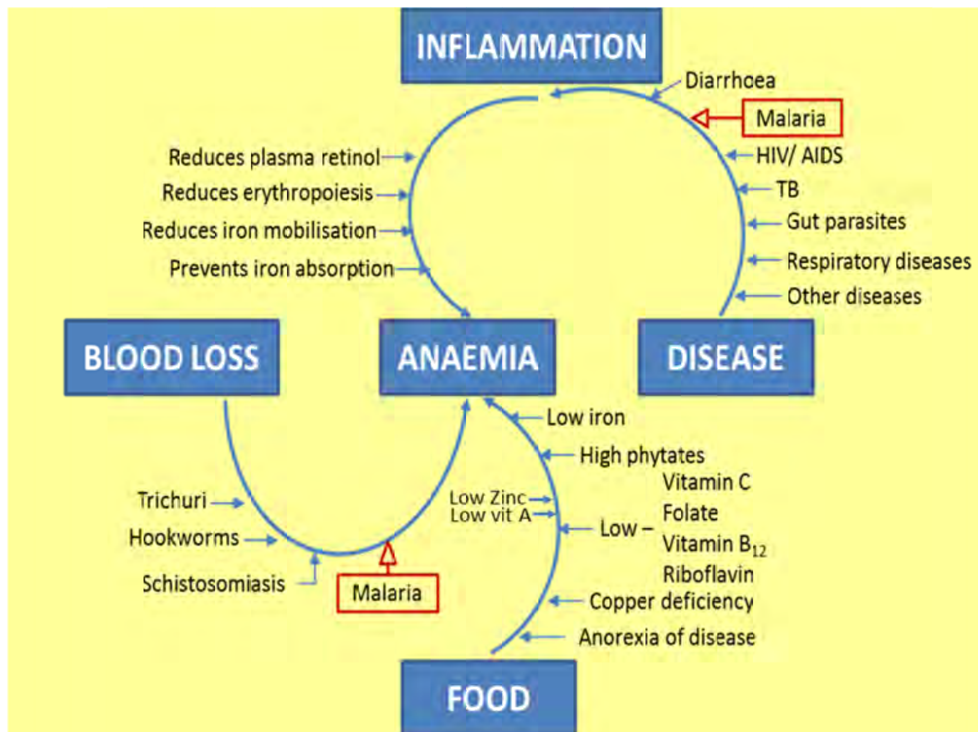

Figure 2: Exogenous factors contributing to anaemia. Adapted from: Thurnham and Northrop-Clewes 2007 in Nutritional Anaemia (Kraemer & Zimmermann, 2007)

The highest-risk groups for iron deficiency are preterm and low birth weight (LBW) infants, infants and children during periods of rapid growth, children consuming milk who have a sensitivity to cow's milk, premenopausal women, pregnant women, and individuals with nematode infections in the gastrointestinal tract(Semba Martin W; Piot, Peter, 2008). Low consumption of iron-containing foods and consumption of foods that interfere with iron absorption, such as phytates, also increase the risk of iron deficiency.

The correct management of iron deficiency anaemia is to find and treat the underlying cause, and to give iron to correct the anaemia and replace iron stores. The response to iron therapy can be monitored using the reticulocyte count and haemoglobin level with an expected rise in haemoglobin concentration of 1 g/ L per week. Iron deficiency anaemia can also be corrected with oral iron supplements. The preparation most commonly used is ferrous sulphate, from which iron is best absorbed when the patient is fasting. Iron stores are replaced much faster with parenteral iron than with oral iron, but the haematological response is not quicker. Oral iron should be given for long enough to correct the haemoglobin level and to replenish the iron stores. This can take six months. Failure of

response to oral iron may be due to lack of compliance, continuing haemorrhage, severe malabsorption or another cause of the anaemia e.g. malaria infection.

Although not all anaemias are caused by iron deficiency, in areas where the prevalence of anaemia exceeds 30–40%, most anaemia is caused largely by iron deficiency. This assumption may not hold in regions such as sub-Saharan Africa, where conditions such as thalassemia and infections such as malaria are endemic.

Anaemia caused by infectious diseases and inflammation

The infectious diseases that significantly cause anaemia are malaria, tuberculosis (TB) and HIV/AIDS. They act either individually or in combination and are most serious in developing countries.

Malaria frequently causes acquired haemolytic anaemia. The anaemia of malaria has several causes namely: rupture of parasitized red blood cells in tissue venules, destruction of parasitized and unparasitized red blood cells in the reticulo-endothelial system (especially the spleen), haemolysis due to the presence of malaria antigen, antibodies and marrow suppression(Anuraj H Shankar, 2008)(Graves & Gelband, 2006). In absence of treatment, this cycle of invasion and destruction of red blood cells is continuous thus making the person more anaemic.

Blood transfusion is indicated when there is acute intravascular haemolysis and when the haemoglobin concentration falls below critical values. It is effective in severely ill patients especially when more than 20% of red blood cells are parasitized(Moxham, 1994). Malaria not only causes blood loss leading to haemolysis but also causes inflammation leading to reduced iron absorption and mobilization in the gut(Kanjaksha & Kinjalka, 2007).

Inflammation accounts for a substantial percentage of anaemia especially in developing countries. The most common cause of iron deficiency anaemia worldwide is blood loss from the gastrointestinal tract resulting from hook worm infestation(Ong'echa et al., 2006). Studies from Zanzibar and Vietnam found that hookworm infestation can account for up to 40% of the iron deficiency anaemia in highly endemic areas. In such settings, the potential impact of deworming can be justified as part of the anaemia control program.

The current WHO Global Malaria Control strategy is focused on four goals (Semba Martin W; Piot, Peter, 2008):

1. Provide early diagnosis and prompt treatment,
2. Plan and implement selective and sustainable preventive measures, including vector control,
3. Provide early detection to contain or prevent epidemics, and
4. Strengthen local capacities in basic/applied research to permit the regular assessment of a country's malaria situation, in particular the ecological, social, and economic determinants of the disease.

Malaria

Malaria is an acute febrile illness characterized clinically by attacks of chills, fever and sweating as a consequence of asexual reproduction by species of *Plasmodium* within the red blood cells (RBC). A mosquito of the genus Anopheles transmits malaria.

There are approximately 515 million reported cases of malaria in the world per year, resulting in death of about 1-3 million people (R. Snow, Guerra, Noor, Myint, & Hay, 2005). Community-based intervention studies show that malaria may account for nearly half of the under-5 mortality in a large part of tropical Africa(B. M. Greenwood, 1991)(Alonso et al., 1991). A report on the global burden of disease indicates that malaria is responsible for 18% of all childhood deaths, 94% of which are in Africa [7, 18, 19].

Malaria is caused by five *Plasmodium* species: *P. vivax*, *P. ovale*, *P. falciparum*, *P. malariae* and P. knowlesi (Moxham, 1994). *P. vivax*, *P. ovale* and *P. malariae* are associated with morbidity but not major mortality while *P. falciparum* is associated with both morbidity and mortality (Graves P & H., 2006). P. knowlesi is a primate malaria parasite that is found in some locations in Southeast Asia.

The significance of malaria is region-specific e.g. nearly 90% of life-threatening *P. falciparum*-related disease continues to be in Africa, with the remaining 10% occurs primarily in Southeast Asia and India, followed by South America(Guinovart C, Navia MM, Tanner, & Alonso, 2006) (Bruce-Chwatt, 1988) (Krogstad, 1996). Studies have shown that malaria alone or acting in interaction with other diseases such as anaemia increases morbidity and mortality in a region (Fleming, 1982) (Schellenberg et al., 1999).

Clinical features and presentation

Disease processes in malaria result from the erythrocytic cycle of invasion and haemolysis leading to a fever, which is due to the release of pyrogens during schizont rupture. Anaemia is usually present and is largely the result of haemolysis (Alonso et al., 2004). Hemolysis liberates merozoites and releases several pyrogenic compounds from infected red blood cells. Febrile paroxysms, anaemia, splenomegaly and hepatomegaly are all signs of malaria (Anuraj H Shankar, 2008).

The parasite undergoes asexual reproduction in the human host and sexual reproduction in the mosquito. It is transmitted to humans as a sporozoite in the saliva of an infected female anopheline mosquito (Gardiner, Fayer, & Dubey, 1988). Sporozoites enter the venous circulation through the capillary beds and invade liver cells within minutes. Over the next 5–15 days, the sporozoite replicates to produce about 40,000 daughter parasites, called merozoites. In the case of *P. vivax* and *P. ovale*, dormant forms known as hypnozoites sometimes develop in the liver cells, remaining viable for up to 50 years (Krotoski, Collins, & Bray, 1982). When released from liver cells, merozoites invade erythrocytes and subsequently differentiate into trophozoites, which consume intracellular haemoglobin and give rise to 6–24 daughter merozoites. The red cell eventually ruptures, releasing these merozoites to invade new erythrocytes and perpetuate the cycle (Parveen Kumar & Clark., 1998) (Moxham, 1994) (Gardiner et al., 1988).

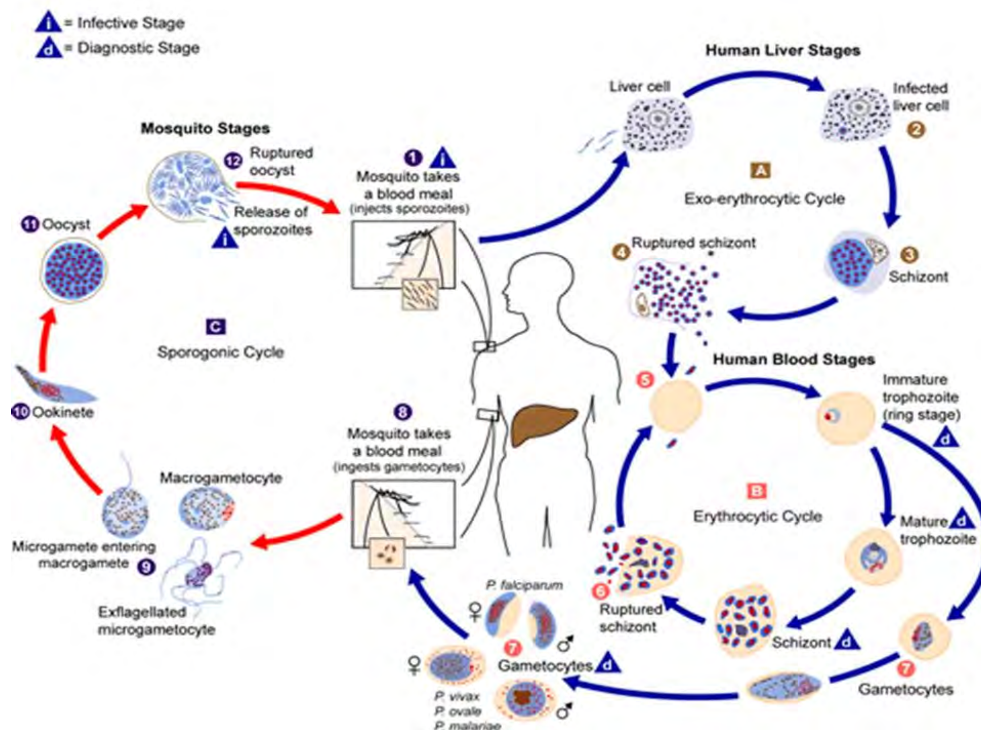

Figure 3: Life cycle of *Plasmodium* (Source: CDC 2006)

Vivax, *ovale* and malariae parasites invade 1-2% of red blood cells at most. *P. falciparum* parasites can invade higher proportions of red blood cells, accounting for the severity of disease and high mortality. *P. vivax* and *P. ovale* form hypnozoites within liver cells, which may cause relapsing malaria up to 2 – 3 years after infection. *P. falciparum* parasites have no hypnozoite form and so the infection is cured when parasites are cleared from the blood by treatment. *P. malariae* parasites lack the hypnozoite stage but can cause reappearance of parasitaemia (parasites in peripheral red blood cells) up to 20 or more years after infection (Schwartz, 1992).

The current WHO Global Malaria Control Strategy is being pursued through advances in malaria vaccine development and provision of insecticide treated bed nets (ITNs) and malaria treatment kits. Several trials (Binka et al., 1996) (Nevill et al., 1996)(D'Alessandro et al., 1997) demonstrated that ITNs are very effective in reducing morbidity and mortality. Meta-analysis of these trials indicated overall reductions in malaria morbidity by 48% and mortality by 20–40% (Lengeler, Armstrong-Schellenberg, D'Alessandro, Binka, & Cattani, 1998) (Lengeler, 1998). There is progress in the fight against malaria and it is projected to be the worlds' least cause of death by the year 2030 (WHO, 2008) (United Nations, 2008).

Iron and malaria infection

There are complex links between body iron status, iron supplementation and susceptibility to malaria and other infections. Malaria causes anaemia through suppression of erythropoiesis, increasing gross iron deficiency and causing mal-distribution of iron in the body (A M Prentice, Ghattas, Doherty, & Cox, 2007)

The causes of anaemia in malaria are multifactorial. They include obligatory destruction of red cells at merogony, accelerated destruction of non-parasitised red cells (major contributor in anaemia of severe malaria), bone marrow dysfunction that can persist for weeks, shortened red cell survival and increased splenic clearance. Massive gastrointestinal haemorrhage can also contribute to the anaemia of malaria.

According to Prentice et al (2007), malaria-induced destruction of infected and non-infected red blood cells both stresses and impedes the capacity of reticuloendothelial macrophages to recycle iron back to the bone marrow. Intravascular rupture of parasitized red cells, macrophagal phagocytosis of both parasitized and unparasitized red cells, and hypersplenism all contribute to the pathophysiology of the anaemia associated particularly with acute malaria (A M Prentice et al., 2007).

Iron can be sequestered and trapped in reticuloendothelial macrophages as a result of both chronic and acute malaria and can present a picture of iron-deficient erythropoiesis associated with normal or increased bone marrow iron (A M Prentice et al., 2007).

Observational studies linking iron status and malaria

Early studies in famine environments showed that malaria increased when iron rich diets were introduced (Murray, Murray, Murray, & Murray, 1978)(Murray, Murray, Murray, & Murray, 1975). Since then, the association between iron status and susceptibility to malaria in moderately malnourished populations has been investigated in various observational settings.

High serum ferritin was shown to be associated with high susceptibility to malaria (R. W. Snow et al., 1991)(Alice M. Nyakeriga Jeffrey R. Dorfman, Neal D. Alexander, Rune Back, Moses Kortok, Alex K. Chemtai, Kevin Marsh, Thomas N. Williams et al., 2004). These studies present a conflicting picture in which higher iron status was associated positively (R. W. Snow et al., 1991)(Inocent et al., 2008)(Alice M. Nyakeriga Jeffrey R. Dorfman, Neal D. Alexander, Rune Back, Moses Kortok, Alex K. Chemtai, Kevin Marsh, Thomas N. Williams et al., 2004)(S J Oppenheimer, Macfarlane, Moody, Bunari, & Hendrickse, 1986), negatively (Shipton, 2004), or neutrally to risk of malaria.

Table 1: Observational studies linking iron status and malaria

Source	Study design	Findings
Oppenheimer et al. 1986 (S J Oppenheimer & Cashin, 1986)	Birth–12 mo, subanalysis of placebo group of a trial of intramuscular iron dextran, Papua New Guinea, *n* = 212	Infants with Hb > 13.7 g/dL and Hb > 15.7 g/dL at birth were 2–3 times more likely to have a malaria-positive slide at 12 mo* (This population has high rates of single deletion α-thalassemia, which causes anaemia and protects against malaria and is therefore a potential confounder)
Snow et al. 1991 (R. W. Snow, Byass, Shenton, & Greenwood, 1991)	1–8 yr, observational study of premalaria season iron status and subsequent malaria morbidity, Gambia, *n* = 317	Susceptibility to malaria was not correlated with preseason serum iron, serum iron-binding capacity, or serum ferritin. Children who had a clinical attack of malaria with high levels of parasitaemia tended to have higher mean serum ferritin levels at baseline*
Nyakeriga et al. 2004 (Alice M. Nyakeriga Jeffrey R. Dorfman, Neal D. Alexander, Rune Back, Moses Kortok, Alex K. Chemtai, Kevin Marsh, Thomas N. Williams et al., 2004)	8 mo–8 yr, 2 cross-sectional surveys, Kenya, *n* = 234	Incidence of clinical malaria was lower in iron-deficient children (IRR = 0.7)* IRR of malaria was associated with plasma ferritin* Iron status markers were associated with malaria- specific IgGs* No difference in parasite density in patients with incident malaria between iron-deficient and non-iron-deficient groups
Inocent et al. 2008 (Inocent, Marceline, Bertrand, & Honore, 2008)	(0 – 60 months), prospective, *n=163*	41.7% and 63.2% of malaria patients were serum iron and Hb deficient respectively. The rates of SI, TS, Hb, HTC, MCV and MCH were significantly lower in malarial than in controls (P<0.01). Malaria negatively affects iron status.

IRR, incidence-rate ratio; IgG, immunoglobulin; TS, Transferrin saturation; SI, Serum iron; HTC, haematocrit; MCH, mean cell haemoglobin. *Significant differences, as reported by the authors.

Effect of iron supplementation on malaria infection

Iron supplementation is widely used in efforts to prevent anaemia. Increases in adverse malarial outcomes have been seen in studies of intramuscular (S J Oppenheimer, Gibson, et al., 1986) and parenteral (Byles & Dsa, 1970) iron administration.

Studies in East Africa (Murray et al., 1978) (Murray et al., 1975) (Keusch & Farthing,

1986) have provided evidence that there is an increased risk of serious infections (e.g. malaria and tuberculosis) when iron supplements are given to humans. These reports have been supported by research on animals (Keusch & Farthing, 1986). Other studies, however, report non-significant effects of iron supplementation on malaria outcomes (Berger et al., 2000) (M. R. Desai et al., 2003)(Nwanyanwu et al., 1996) (Verhoef et al., 2002). Various authors have carried out meta-analyses and reviews of studies on the effect of iron supplementation on malaria infection. From the reviews, mixed observations are seen on the effect of iron supplementation on malaria infection and though the meta-analysis by the different authors has used different randomised controlled trials to a large extent, their conclusions are consistent.

Of 15 studies reviewed by Prentice et al, (2007), six showed no effect of iron supplements on malaria risk, three found an increased frequency of malaria attacks in the iron-supplemented groups and six found non-significant increases in malaria rates. It is important to note that all but one of the studies that found non-significant increases in malaria outcomes provided access to health care facilities or active follow-up and treatment of malaria incident cases. The detrimental effects of iron supplementation may have been curtailed by concurrent effective treatment of malaria infections (Andrew M Prentice, 2008). Studies of iron supplementation of populations of anaemic children in malarious areas have reported both an improved haemoglobin response and decreased prevalence of severe anaemia(Clara Menendez et al., 1997) (M. R. Desai et al., 2003) (Verhoef et al., 2002) (Massaga et al., 2003). In their study in Kenya, Verhoef et al (2002) showed that iron supplementation (twice a week at 6 mg per kg bodyweight) gives substantial health benefits that may outweigh the associated risk of adverse effects caused by malaria (Verhoef et al., 2002).

Table 2: Effect of iron supplementation on malaria infection

First author	Study design	Findings
Shankar A.H. (A H Shankar, 2000)	Meta-analysis of 13 RCTs of the effect of iron supplementation on malaria and iron status	RR for clinical malaria attack = 1.1 (NS) RR for slide positive for *Plasmodium falciparum* = 1.17* Absolute increase in infection rate = 5.7% (NS) RR for spleen enlargement = 1.12 (NS) Mean increase in Hb = 1.25 g/dL RR for anaemia = 0.5*
Oppenheimer S.T. (Stephen J Oppenheimer, 2001)	Review of controlled intervention studies of iron supplementation	Oral iron therapy increased clinical malaria in 5 out of 9 studies No studies of iron therapy in malarious areas showed benefits on infectious morbidity

		Does not report on effect on iron status
Gera T. (Gera & Sachdev, 2002)	Systematic review of 28 RCTs of the effect of iron supplementation or fortification in children on infectious illness including 8 studies of the effect of iron supplementation on malaria)	Pooled OR for malaria-positive smear = 1.43,* but OR when adjusted for baseline malaria smear = 1.24 (NS) (The treatment effect increased by 2.89 times per unit increase in baseline malaria positivity; this supports the argument that iron supplementation of children with pre-existing malaria increases their risk of remaining malaria positive)
Prentice A.M. (A M Prentice et al., 2007)	Review of 15 RCTs of iron supplementation in children	Three trials show significant increase in malaria Six trials show non – significant increase Six trials show neutral findings

* Significant differences, as reported by the authors.
RCT, randomized, controlled trial; RR, relative risk; NS, not significant; haemoglobin, Hb; OR, odds ratio.

In the Pemba study, children aged 1-35 months were assigned to daily oral supplementation with: iron (12.5 mg) and folic acid (50 µg), iron, folic acid and zinc, or placebo. The primary endpoints were all-cause mortality and admission to hospital. Those who received iron and folic acid with or without zinc were 12% (95% CI 2-23, p=0.02) more likely to die or need treatment in hospital for an adverse event and 11% (1-23%, p=0.03) more likely to be admitted to hospital. There were also 15% (-7 to 41, p=0.19) more deaths in these groups (Sazawal et al., 2006). The results of the Pemba study raised new concerns about possible serious side effects of iron supplementation in populations where the aetiology of anaemia is not solely attributable to iron deficiency. However, since the design of the study was such that there was not a group that received iron without folic acid, it was not possible to attribute the deleterious effect of the supplement to one or another of the components (Sazawal et al., 2006). Iron supplementation is now advised to be withheld until the malaria treatment schedule is complete, both because iron may inhibit treatment and because the absorption of oral iron is blocked by the inflammatory response (A M Prentice et al., 2007).

There is a need to elucidate our understanding of the interactions between iron supplementation and infections. There is evidence to suggest that the apparent detrimental effect of iron supplementation may vary according to levels of antecedent iron status, the presence of genetic haemoglobin disorders and glucose-6-phosphate dehydrogenase (G6PD) deficiency, and other host genetic variants, such as haptoglobin polymorphisms (A M Prentice et al., 2007).

Effect of malaria on folate status

Malaria infection may induce folate deficiency especially in areas where both malaria and malnutrition are endemic. In Nigeria, serum folate concentration fell more rapidly during pregnancy in women not receiving antimalarial prophylaxis than in women protected against malaria (Fleming, Hendrickse, & Allan, 1968). In another study, Fleming, (1981) found that protection against malaria alone largely prevented severe anaemia in pregnancy even without addition of folate supplements (Fleming, 1981). American marines in Vietnam showed folate-deficient megaloblastic anaemia associated with *P. falciparum* and *P. vivax* malaria (Strickland & Kostinas, 1970). The cause of the folate deficiency is multifactorial and it includes inadequate intake, malabsorption, haemolysis and use of antimalarial drugs. It is thus difficult to determine the role of malaria infection in the genesis of folate deficiency.

Iron, folate and vitamin B12 have important roles in erythropoiesis. The majority of nutrition-related anaemias can be attributed to deficiency of one of these nutrients (Hoffbrand & Herbert, 1999). Iron requirements of erythroid cells during haemoglobin synthesis are much greater than that of all other cell types. In addition, folate and vitamin B12 are both required for the extensive DNA synthesis that accompanies the production of hundreds of billions of new erythrocytes each day.

Iron loss and/or deficiency will cause anaemia especially because of retarded RBC production rates characterized by smaller, less haemoglobinised erythrocytes (Bohnsack & Hirschi, 2004). In iron deficiency, decreased synthesis of heme results in decreased protein translation, especially of globins. This decreased protein translation in the iron-deficient erythroid cells results in impaired reticulocyte production and smaller, less haemoglobinised reticulocytes, leading to microcytic anaemia. During folate-deficient erythropoiesis, the folate-deficient erythroblasts surviving to the late stages produce fewer but larger reticulocytes, leading to macrocytic anaemia. The resultant anaemia induces EPO production, which decreases the apoptosis in the EPO-dependent cells relative to normal erythropoiesis. Erythroblasts require folate and vitamin B12 for proliferation during their differentiation. The deficiency of folate or vitamin B12 will inhibit purine and thymidylate syntheses, impair DNA synthesis and cause erythroblast apoptosis. This can result in anaemia due to ineffective erythropoiesis.

Folic acid is frequently prescribed for children with malaria, on the grounds that nutritional folate deficiency may compromise the enhanced erythropoiesis required to restore haemoglobin after an episode of haemolytic anaemia (Hensbroek et al., 1995).

Although some studies have supported the findings of the Pemba study (Smith et al., 1989) (Metz, 2007), developing countries that do not have high incidences of malaria did not observe the same findings (Tielsch et al., 2006) (Nacher et al., 2003). It is thus possible that there are interactions between iron, folate and malaria infection. However, malarial anaemia is responsible for the greatest amount of malaria-related morbidity and mortality ("WHO expert committee on malaria," 2000) and is the most common type of anaemia in infants in highly endemic areas (Clara Menendez et al., 1997) (Abdulla et al., 2001).

The Improved Nutrition through Staple foods for Africa (INSTAPA) project

The studies presented in this thesis were undertaken as part of the INSTAPA project (www.instapa.org). This project was financed by the 7th framework programme of the European Commission. Research scientists from Europe, Africa, and the United States of America undertook joint research in an endeavour to break the vicious cycle of poverty, malnutrition, and mortality currently afflicting Africa.

The project planners recognized that poor nutritional status and limited financial resources often compromise individual welfare in the developing world. In combination with a commonly high concurrent disease load, a self-perpetuating cycle of poverty, malnutrition, and mortality arises. This is illustrated in the figure below.

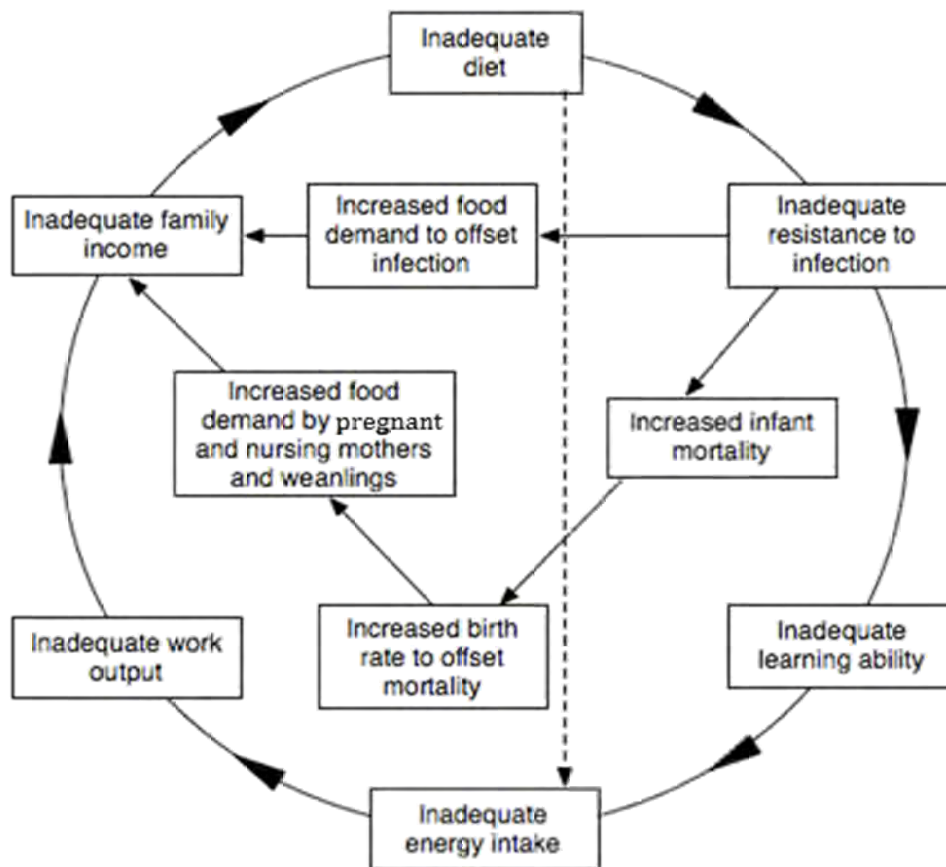

Figure 4: The vicious cycle of poverty, malnutrition, and poverty (adapted from www.instapa.org)

The nutritional quality of diets in developing countries in terms of micronutrient adequacy was identified as a major source of 'fuel' for the cycle above. About one-quarter of the world's population is anaemic, mainly due to iron deficiency. Because iron and zinc are found in many of the same foods, high rates of iron deficiency in sub-Saharan Africa suggest widespread occurrence of zinc deficiency in the same populations. Vitamin A deficiency affects an estimated 127 million preschool-age children and 7 million pregnant women. Approximately every third child with vitamin A deficiency lives in sub-Saharan Africa. The major causes of these micronutrient deficiencies comprise low intake due to food scarcity, unavailability of micronutrient-rich foods, poverty, or poor dietary habits; high demands that arise from growth spurts during infancy and adolescence, pregnancy, or increased blood loss as occurs with pronounced menstruation or infestation from parasites; and low bioavailability.

The vision of the INSTAPA consortium was to improve the dietary quality of most of the highly consumed foods in Africa. This would result in long-term health effects, which would be a major step towards the realization of the Millennium Development Goals (MDGs), set for 2015. It was decided that ways to improve the micronutrient content of the major staple foods in Africa would be investigated. The identified staple foods were millet, sorghum, maize, and cassava.

The target groups of the interventions within the consortium were women and children as these groups comprise the most vulnerable populations in African societies. The consortium focussed on three micronutrients; iron, zinc, and vitamin A. Various strategies aimed at boosting the micronutrient content of the identified foods with iron, zinc and vitamin A were investigated. This included interventions during cultivation, harvesting or post-harvest processing e.g. bio-fortification and post-harvest fortification.

The specific objectives of the INSTAPA project were:

1. To evaluate the genetic potential of cassava, maize, millet, and sorghum for increased content of provitamin A, iron and zinc and for reduced content of anti-nutrients
2. To determine the efficacy of fortified and biofortified cereal-based foods on nutritional status and health
3. To develop improved (traditional) processing methods of millet, sorghum and maize enhancing iron, zinc and vitamin A bioavailability and/or reducing inhibitory effects of anti-nutrient factors for improvement of micronutrient status of young children and women
4. To determine the safety and efficacy of iron-fortified maize to alleviate anaemia in malaria-endemic areas in sub-Saharan Africa
5. To determine the effect of maize-based complementary foods fortified with iron and zinc on cognitive and psychomotor development of young children

The strategic objectives of the project were:

1. To contribute significantly to the improvement of the dietary quality of very young children living in resource poor areas of developing countries resulting in long-term health effects and a major step towards the Millennium Development Goals set for 2015
2. To establish an international leading role of the consortium in the fields of biofortification, fortification and processing to increase the supply of bioavailable micronutrients from African staple foods by bringing together scientific and technological excellence
3. Capacity building in knowledge and skills to solve micronutrient deficiencies in sub-Saharan Africa and Europe
4. Communication within and beyond the consortium regarding the ethical and scientific issues of concern to the agricultural and health sectors as well as consumers, enabling African populations to define and choose diets for optimal health for their children.

In order to design suitable interventions to the identified issues, the INSTAPA consortium adopted a simple yet robust approach. Seven work packages comprising of one or more partners were tasked with specific objectives aimed at delivering a set of outcomes. These were:

1. Work package 1: Management and coordination of all other work packages
2. Work package 2: Bio-fortification
3. Work package 3: Post-harvest fortification
4. Work package 4: Post-harvest processing
5. **Work package 5: Safety of food-based interventions** (tasked with implementing the prenatal iron and malaria study – *this thesis*)
6. Work package 6: Cognitive development
7. Work package 7: Capacity building and dissemination

The project framework was complex and the participating partners (research institutions) were many. Below is the list of all the partners (*partners who were tasked with WP5 are in bold*).

1. **Wageningen University (WU), Netherlands**
2. Swiss Federal Institute of Technology (ETH), Switzerland
3. Institut de Recherche pour le Développement (IRD), France
4. **London School of Hygiene and Tropical Medicine (LSHTM), UK**
5. University of Kwazulu-Natal (UNSA), South-Africa
6. Université d'Abomey Calavi (UAC), Benin
7. International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Mali
8. **University of Nairobi (UNK), Kenya**
9. Centre National de la Recherche Scientifique et Technologique (DTA), Burkina Faso
10. International Food Policy Research Institute / Harvest Plus (IFPRI/HP), USA
11. International Institute for Tropical Agriculture (IITA), Nigeria
12. **Maseno University (MU), Kenya –** was later seconded to WP5 but was not in the initial list of partners.

Aims and outline of the thesis

The long-term aim of this study was to improve the health of pregnant women through iron interventions that are safe and efficacious. The new flour fortification legislation in Kenya now ensures that pregnant women receive iron through a combination of fortified foods and universal iron supplementation. In view of the findings of the Pemba trial, the current study aimed to assess whether the iron intake resulting from this policy is safe. We hypothesized that flour fortification alone would reduce the risk of *Plasmodium* infection in pregnant women as compared to combined supplementation and flour fortification.

The **general objective** of the study was to assess the effects of combined fortification of whole meal maize flour with iron (NaFeEDTA) and iron supplementation on safety indicators, and on iron status, in pregnant women.

The specific objectives were as follows:

1. To compare the presence of malarial infection in parturient women who received a combination of iron-fortified foods with iron supplements versus iron-fortified foods only; **(chapter 2)**
2. To assess intervention effects on the maternal prevalence of iron deficiency anaemia at 1 month after delivery; **(chapter 2)**
3. To assess intervention effects on neonatal iron stores at 1 month of age; **(chapter 2)**
4. To assess the diagnostic utility of Zinc protoporphyrin in diagnosing iron deficiency in malaria endemic regions; **(chapter 3)**
5. To identify baseline factors that are prognostic for the NTBI response to consumption of a single iron supplement; **(chapter 4)**
6. To determine the factors that predict *Plasmodium* infection in pregnancy; **(chapter 5)**
7. To identify factors associated with birth weight; **(chapter 6)**
8. To to develop a methodology to predict cases of low birth weight, using a single prognostic score that is based on prognostic variables collected at the second trimester of pregnancy; **(chapter 6)**
9. To develop methods for community-based flour fortification with iron;
10. To assess intervention effects on maternal intestinal pathogens at 1 month after delivery.

We conducted a pilot survey prior to the start of the fieldwork. This was aimed at testing key laboratory protocols and procedures, developing skills in collecting maternal placental blood, and preparing for the trial by fostering participation with local health facilities and research institutions. On pre-specified days of the pilot survey, parturient women with uncomplicated pregnancies delivering in Siaya District Hospital, Kenya, were selected consecutively. For all women who provided written consent (n=33), samples of cord blood (6 mL) were obtained after careful cleaning and by direct sampling from the umbilical vein. Immediately upon expulsion of the placenta, we collected (maternal) placental blood by puncturing the intervillous space. In addition, placental biopsies were sectioned from near to the centre of each placenta, adjacent to the basal plate (Bulmer, Rasheed, Francis, Morrison, & Greenwoods, 1993; Bulmer, Rasheed, Morrison, Francis, & Greenwoods,

1993). These were stored in paraformaldehyde for future histological examination of tissue sections to detect *Plasmodium* infection. Because the pilot study was aimed at the development and testing of operational processes, our sample size was chosen arbitrarily, and a formal analysis of data was not carried out.

Study area and population characteristics

The fieldwork was conducted from September 2011 to April 2013 in Kenya, Nyanza province, South-West Kisumu, which is located 1350 meters above sea level. Figure 6 shows the study area.

Figure 5: Map of study area - South-West Kisumu (maps of Africa and Kenya shown within map; the red square on the map of Kenya shows the study area). The red place marks show the coordinates of the homes of study participants. GPS data visualized at www.GPSVisualizer.com. Map courtesy of Google ©2013.

The area consists mainly of poor subsistence farmers. The total population based on the study census conducted in April 2010 was 12,202 people. According to the Kenya Integrated Household Budget Survey (KIHBS-2010) the poverty rate in the area is 47.8% (CBS, 2007). The diet of the people in the study area is mostly based on maize and has a low content of animal products. Maize is normally milled in the local posho (hammer) mills and it may be pre-blended with millet, sorghum or cassava. Although some of the families living along Lake Victoria are engaged in fishing, fish is prohibitively expensive and thus not normally consumed in large quantities by the local community.

The prevalence of anaemia in the area is high (69%) (Ouma et al., 2007) and intestinal

schistosomiasis is common (Samuels et al., 2012). Furthermore, malaria is highly endemic and transmission is perennial and intense, with seasonal peaks occurring during the long and short rains. The mean annual rainfall ranges from 800 to 1600 mm and shows a bimodal pattern with long rains from March to June and short rains from October to December. The average temperature is 26°C and the relative humidity range is between 60 and 80%. A person living in this area is normally exposed to between 30 and 100 infectious mosquito bites per year (A. Noor et al., 2009), although this rate is reducing (A. Noor et al., 2009) (Ministry of Public Health and Sanitation (MOPHS) & Ministry of Medical Services, 2010).

There are only two health centres in the area: Ober Kamoth health centre (0°6’53.197’’N, 34°36’34.077’’E) and Rota dispensary (0°5’34.386’’N, 34°40’24.604’’E). Although either can offer maternal and child health care, antenatal care, immunization, and family planning services, only Ober Kamoth offered maternity services thus it was chosen as the study site.

Study design

The study was a double blind, randomised, placebo-controlled intervention trial with two parallel groups. The study was primarily designed as an explanatory trial i.e. it aimed at measuring potential effects when iron is administered under optimal, controlled conditions. The study population was relatively homogeneous, and the primary analysis was chiefly per protocol. To increase the relevance of trial findings for policy decisions, the interventions were provided under conditions of normal health care.

Within or close to the study area, the prevalence of malarial infection in parturient women has been extensively measured (Ayisi et al., 2003; Parise et al., 2003; A M van Eijk et al., 2004) (Parise et al., 2003) (Anna M Van Eijk et al., 2002; A M van Eijk et al., 2004, 2007; Anna M van Eijk et al., 2002, 2003, 2004). These studies guided sample size calculations of our study.

Pregnant women with a gestational age of 16-23 weeks (n=470) received fortified flour and were randomly allocated to daily supplements with iron or placebo. Flour was fortified to a target level of 20 mg iron as NaFeEDTA per kg flour (FFI & report, 2008) (WHO et al., 2009). The study (www.ClinicalTrials.gov: NCT01308112) received ethical clearance in Kenya and the UK. Written informed consent was sought from all study participants.

Pregnant women were recruited from communities in the study area. In a census that we conducted in the study area, we established that there were 2,806 women of childbearing age (15 – 45 years). According to the Kenya Demographic and Health Survey (KDHS) 2008-2009, fewer than 5% of women in this area make their first visit to an antenatal clinic in the first trimester of pregnancy (Kenya National Bureau of Statistics (KNBS) & ICF Macro, 2010). Elaborate methods to detect early pregnancies, before or at 20 weeks of gestation, were thus developed and deployed (see Annex II). Women within the study

communities were asked to report to the research clinic when having missed their monthly period twice consecutively, or when otherwise suspecting that they were pregnant. In addition, locally trained community health workers visited each homestead monthly to detect pregnancies early.

Methods used to detect pregnancies included direct observation and questioning of possible cases, urine testing, and ultrasonography. Married women of reproductive age (15 – 45 years) were questioned about the date of their last menstrual period. Unmarried women of reproductive age were requested to present themselves at the research clinic for a free urine-based pregnancy test (Clearblue, Unipath Ltd., Bedford, UK) once every three months. The date of the last menstrual period (married women) and the outcome of the pregnancy test (unmarried women) was entered into a pre-designed pregnancy detection database, which contained the area population data. For married women, the database automatically compared the new menstrual date with the previous menstrual date and calculated the elapsed period. If suspected pregnant, the entry would be highlighted and a community health worker would be sent to request the woman to come for a free pregnancy test. For all pregnant women, gestational age was established by obstetric ultrasound (Aloka SSD-900 ultrasound system®, Hitachi Aloka Medical Ltd., Reeuwijk, Netherlands). The study clinicians handled the reproductive health information of all the women confidentially.

Women who were suspected pregnant were requested to give written informed consent for screening. A study questionnaire was then administered and medical examination carried out. Ultrasonography was carried out to confirm pregnancy, establish gestation age, and to establish whether the pregnancy was singleton, multiple etc. Homestead consent was sought from the participant's homestead members. The head of the homestead and women of childbearing age living in the homestead were invited for a group meeting, where field staff informed them of the study goals, objectives and procedures. They were asked to consent to fortification of their grain with iron. The head of the homestead signed the consent forms on behalf of the homestead members. A homestead was excluded if written consent was not given or if the pregnant woman was found ineligible due to other reasons.

To prevent severe anaemia, all women received chemotherapy for infections by Schistosoma spp. (praziquantel) and geohelminths (albendazole) at a gestation age of 13 to 20 weeks and as per recommendations of the WHO (WHO, 2002) (WHO/FAO, 2006) . Written informed consent for HIV testing was sought from all the pregnant women on the second visit to the research clinic.

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Chapter 2

Antenatal iron supplementation and *Plasmodium* infection in Kenyan women: a randomised trial

Abstract

Background: Whereas coverage of antenatal iron supplementation is low and benefits are uncertain, there is evidence that it can increase the burden of malaria. We aimed to measure the effect of iron supplementation during pregnancy on maternal *Plasmodium* infection assessed at delivery, birthweight, gestational duration, fetal growth and maternal and infant iron status.

Methods: 470 rural Kenyan women with singleton pregnancies, gestational age 13─23 weeks and hemoglobin concentration ≥90g/L were randomized to supervised daily supplementation with iron (60mg as ferrous fumarate) or placebo until 1 month postpartum. To prevent severe anemia, both groups additionally received 5.7mg iron/day through flour fortification. Intermittent preventive treatment against malaria was given as usual. *Plasmodium* infection was assessed at birth by dipstick tests, PCR and histological examination of placental biopsies.

Results: We found no effect on *Plasmodium* infection risk (both intervention groups: 45%; difference, 95%CI: 0%, ─9% to 9%). Iron supplementation increased birthweight by 143g (95%CI: 58─228g) and reduced the prevalence of low birthweight (<2,500g) by 65% (95%CI: 13%─86%). The effect on birthweight was restricted to a prespecified group of women who were initially iron-deficient (250g; p-interaction=0.008), and seemed achieved mostly through better fetal growth. Iron supplementation resulted in improved maternal iron status at 1 month postpartum, and enhanced neonatal iron stores.

Conclusions: In a mixed population that included women with anemia and iron deficiency at baseline, iron supplementation produced major gains in birth weight, with no apparent effect on *Plasmodium* infection. Coverage of universal antenatal iron supplementation must be increased.

Submitted for publication

Introduction

Although the World Health Organization (WHO) has recommended universal iron supplementation throughout pregnancy since 1959 (WHO, 1959), its delivery and adherence have been notably poor in developing countries. (Galloway & McGuire, 1994),(Mason, Lotfi, Dalmiya, Sethuraman, & Deitchler, 2001) Based on the association between anaemia and maternal mortality, iron deficiency in pregnancy has been estimated to cause 591,000 perinatal deaths and 115,000 maternal deaths worldwide. (Stoltzfus RJ, Mullany, & Black, 2004) These estimates were based on observational data, however, and attribution of causality is problematic because women who are anaemic are also likely to suffer from other disorders that cause maternal and perinatal deaths. A meta-analysis of randomised trials found no evidence of benefits of iron supplementation in terms of reduced adverse maternal and neonatal outcomes (low birth weight, delayed development, preterm birth, infection, postpartum haemorrhage). (Peña-rosas & Viteri, 2009)

Added to these uncertainties, a randomised trial among children (Sazawal et al., 2006) reinforced earlier concerns that iron interventions can increase rates of malaria and other infectious diseases. (Stephen J Oppenheimer, 2001) In response to this trial, a WHO expert group recommended against universal iron supplementation in children living in malaria-endemic areas, (WHO, 2004), (WHO, 2007) but antenatal iron supplementation continues to be recommended, despite reports from observational studies that iron deficiency is associated with protection against *Plasmodium* infection in placental blood.(Kabyemela, Fried, Kurtis, Mutabingwa, & Duffy, 2008), (Danquah, Bedu-Addo, & Mockenhaupt, 2008), (Edward L Senga, Harper, Koshy, Kazembe, & Brabin, 2011) In a randomised trial, iron supplementation was not accompanied by increased susceptibility to *Plasmodium* infection in Gambian women; (C. Menendez et al., 1994) however, participation was restricted to multigravidae and the study design had several limitations. (Peña-rosas & Viteri, 2009)

We aimed to measure the effect of daily supplementation with iron during pregnancy on maternal *Plasmodium* infection assessed at delivery. We anticipated that this effect may be influenced by iron status, gravidity, age and HIV infection at baseline. We also assessed intervention effects on birth weight, gestational duration and fetal growth at delivery, and maternal and neonatal iron status at 1 month post-partum.

Subjects and Methods

Area and population

We conducted the fieldwork from October 2011 to April 2013 in the administrative areas of Ojolla, Kanyawegi, Osiri and Rota Sub-Locations, Nyanza Province, Kenya. The area is located along the shores of Lake Victoria, at around 1,234m above sea level. The population consists mainly of poor families from the Luo tribe who rely on subsistence farming, with a diet mostly based on maize and with low intake of animal products.

Sorghum, millet, and cassava are also consumed albeit in lower quantities. Local people typically bring these grains regularly and in small quantities (0.5─2 kg) to village-based (posho) mills, where they are ground whole or de-hulled into flour. Malaria is highly endemic and transmission is perennial. Local residents are exposed to 30─100 infectious mosquito bites per year, (A. Noor et al., 2009) although this rate has reduced in recent years.(A. Noor et al., 2009), (Ministry of Public Health and Sanitation (MOPHS) & Ministry of Medical Services, 2010)

Anaemia (69%) (Ouma et al., 2007) is highly prevalent among pregnant women while intestinal schistosomiasis is highly prevalent in school-aged children. (Samuels et al., 2012) Fewer than 5% of women in these districts make their first visit to an antenatal clinic in the first trimester of pregnancy. (Kenya National Bureau of Statistics (KNBS) & ICF Macro, 2010) In Nyanza Province, 55% of deliveries take place at home and 1% en route to health facilities. (Kenya National Bureau of Statistics (KNBS) & ICF Macro, 2010) Because this estimate also includes urban residents, however, who deliver much less frequently at home than rural residents (national estimates: 25% versus 65%, respectively); (Kenya National Bureau of Statistics (KNBS) & ICF Macro, 2010) it probably is a gross underestimate of the percentage of home deliveries in our study area. As per national and international guidelines,(Ministry of Public Health and Sanitation, 2008), (Ministry of Public Health and Sanitation (MOPHS) & Ministry of Medical Services, 2010) pregnant women should receive daily supplementation with iron (60 mg as ferrous sulphate from first month of pregnancy or first antenatal contact; if 6 months duration cannot be achieved in pregnancy, supplementation should continue during the postpartum period for 6 months, or the dose increased to 120 mg iron; treatment of anaemia also requires the dose to be increased to 120 mg iron) and intermittent preventive treatment for malaria with 2-3 single doses of sulfadoxine-pyrimethamine (the first dose after quickening and subsequent doses at intervals of at least one month), (Ministry of Public Health and Sanitation (MOPHS) & Ministry of Medical Services, 2010) with delivery for both free of charge through antenatal services.

Although national coverage of iron supplementation increased notably between 2003 and 2008, only 54% of women in Nyanza Province reported taking iron tablets or syrup for < 60 days during their last pregnancy, whilst 33% did not take any iron supplements. (Kenya National Bureau of Statistics (KNBS) & ICF Macro, 2010) Despite reports of reduced in vivo efficacy of sulfadoxine-pyrimethamine, (Iriemenam et al., 2012) intermittent preventive treatment with this combination drug remains efficacious in preventing the adverse consequences of *Plasmodium* infection on maternal and fetal outcomes. (WHO, 2012c) Coverage nonetheless remains low: in 2008-2009, 17% of women in Nyanza Province received at least two doses of sulfadoxine-pyrimethamine during pregnancy, and 35% received at least one dose. (Kenya National Bureau of Statistics (KNBS) & ICF Macro, 2010)
At the time of our study, Ober Kamoth and Rota dispensaries were the only health centres

in the study area. We established Ober Kamoth as our study research clinic, upgraded the maternity ward to allow deliveries, installed an obstetric ultrasound instrument and trained local health staff in determination of gestational age. The study received clearance from ethical review committees in Kenya (Kenyatta National Hospital/University of Nairobi) and England (London School of Hygiene and Tropical Medicine), and was registered at ClinicalTrials.gov (NCT01308112).

Study type

The study was a double-blinded, randomised, placebo-controlled intervention trial with two parallel groups of pregnant women receiving daily supplementation with and without iron. To prevent severe anaemia, all women were dewormed before the start of the intervention, and received a small dose of iron through flour fortification during the intervention.

Recruitment

Based on community censuses that we conducted in April-May 2010 and February-March 2011, and that was updated based on monthly reports by community health workers (henceforth referred to as volunteers), we listed all women aged 15–45 years in the study area. Meetings were held with local authorities, key persons and volunteers to discuss the aims and procedures of the study. As agreed in these meetings, the volunteers visited all homesteads to inform its members about the study aims and to enrol women in a pregnancy surveillance programme. Women who were married or living together were asked to record their menstrual period on a calendar, and to voluntarily report to research staff when having missed their monthly period twice consecutively, or when otherwise suspecting that they were pregnant. In addition, the volunteers visited each woman monthly at her homestead to discuss their menstrual periods. Women who reported the start of their menstrual period to have occurred 10 weeks earlier were invited to the research clinic for pregnancy testing. Because we suspected that reports of menstrual period might be unreliable in women who had never been married, divorced, separated or widowed, we invited them to the research clinic for pregnancy testing every 12 weeks. Women who were pregnant or who had delivered were excluded from pregnancy monitoring for the first 3 months of the neonatal period. Pregnancy tests were subsequently administered at 12-weekly intervals until menstrual periods recurred; thereafter, pregnancy detection procedures were as described above. Volunteers reported monthly to the research team, and data were entered into a database for pregnancy monitoring and tracking.

When women attended the research clinic for pregnancy testing, we informed them again about study aims and procedures, and asked them to sign an informed consent form. Vital data and obstetric history were recorded in a standard questionnaire, a medical examination carried out, and a rapid test (Clearblue, Swiss Precision Diagnostics, Bedford, UK) was administered to detect human chorionic gonadotropin as a pregnancy marker in urine. Women were examined by obstetric ultrasonography (Aloka SSD-900, Hitachi Medical

Systems, Reeuwijk, Netherlands) to confirm pregnancy; to estimate gestation age (counted from first day of last menstrual period) from fetal crown-rump length, biparietal diameter, and head circumference; and to assess multiplicity of pregnancy. As per recommendations of the World Health Organization, (WHO, 2002) all eligible women received preventive chemotherapy with praziquantel against Schistosoma infections, and albendazole against geohelminth infections. They were asked to return to research clinic within 14─21 days to further assess eligibility.

Within 48 h of the screening visit described in the preceding paragraph, field staff visited the homestead of eligible women to inform homestead members about study aims and procedures, to ask the head of the household to sign a form on behalf of the entire homestead indicating consent for their flour to be fortified. From the moment that this consent had been obtained, we fortified flour with iron for the pregnant woman and all her household members at local posho mills, until she left the study.

We trained the operators of 45 posho millers in the study area on fortification procedures; three other mills in the area were excluded because they had porous sieves that prevented efficient homogenisation of fortificant in the flour. Research staff diluted premix containing highly concentrated NaFeEDTA (Fortitech, Gadstrup, Denmark) with white maize flour using standardized protocols. The resulting preblend was packed in 10g sachets and given weekly to millers, who kept them in closed plastic, opaque containers. Millers were not given any financial incentives but they were visited weekly to ensure their adherence to standard procedures. A registered member of a participating homestead brought grain to the mill. The mill operator sprinkled the contents of the sachets onto the grain in the feed hopper just before it was milled, at a target dose of one sachet (20mg iron as NaFeEDTA) per kg flour, as per WHO recommendations for populations with an average per capita consumption of wholemeal flour of 150-300g/day. (WHO et al., 2009) Arbitrarily selected samples of fortified flour were kept in sealed opaque containers for subsequent determination of fortificant iron content. Flour consumption was measured by weighed intake studies in 72 arbitrarily selected pregnant women.

At the second visit to the research clinic, women were counselled and asked for consent for HIV testing. They were medically examined and a venous blood sample was collected in a tube with K2EDTA (Becton Dickinson, Nairobi, Kenya; catalogue 367863). Haemoglobin concentration was measured by photometer (HemoCue 301, Ängelholm, Sweden). The ratio of zinc protoporphyrin:haem (ZPP:H) was measured in whole blood and washed erythrocytes by haematofluorometer.

Washed erythrocytes (120 µl) were stored in a DNA-stabilizing buffer (AS1, Qiagen, Valencia, CA, USA) with PBS, first at 2-8 °C and later at –80°C, for subsequent DNA extraction and detection of *P. falciparum* infection by PCR test. Plasma samples were stored for subsequent detection of *Plasmodium* antigens and HIV antibodies by rapid dipstick tests. Additional plasma samples were stored in liquid nitrogen (-196 °C) in the

field and at −80 °C during transport and storage in The Netherlands for subsequent assessment of iron markers (concentrations of ferritin, soluble transferrin receptor), other nutritional markers (concentrations of cobalamin and folate) and inflammation markers (concentrations of C-reactive protein and α1-acid glycoprotein).

Eligibility for randomisation

Women were included when aged 15-45 years; consent had been obtained; likely to be available for study until 1 month after delivery and planning to deliver in the pre-designated health facility. They were excluded when having obvious mental retardation or a metabolic disorder, a medical history of sickle cell anaemia, epilepsy, diabetes, an obstetric history suggestive of eclampsia or pre-eclampsia; carrying multiples; gestational age at the second visit was below 13 weeks or exceeded 23 weeks; homestead members had not provided consent; no venous blood sample was collected, haemoglobin concentration was <90 g/L. Recruitment continued until the target sample size (n=450) had been attained.

Randomisation and blinding

To allow masking the organoleptic properties of supplemental iron, the supplements were pre-packed as opaque capsules that were identical in appearance except that the shell for each type (iron or placebo) was into two colours (blue and dark green for iron, white and buff for placebo). The code linking each colour to the type of supplement was kept in sealed envelopes and was not available to the research team until after the fieldwork was completed, preliminary statistical analyses were done to describe baseline and end-of-intervention characteristics, and a statistical analysis plan was finalised.

One of us (HV) not involved in the fieldwork used tables of random numbers to produce a list of sequential numbers linked with each of these four colours. The numbers were individually written on sealed, opaque envelopes, and the corresponding colour was written on a paper slip that was inserted in each envelope. Fieldworkers who allocated treatment were not allowed to open these envelopes until it was formally established that the woman was eligible for randomisation and registered in a central randomisation log. Each participating pregnant woman was then allocated in order of enrolment to the colour indicated in the next available envelope.

The supplements were contained in blister packs of 10 capsules each. Each blister pack contained capsules of one of the four colours and had no other marks or writings on it. These procedures ensured that participants and field staff, including outcome assessors, were blinded to intervention throughout the trial until the data analysis.

Interventions

Women received daily supplements with either iron (60 mg as ferrous fumarate; Dr. Paul Lohmann, Emmertal, Germany; catalogue 500005025200) or placebo, with starch as filler.

We used ferrous fumarate out of concerns that ferrous sulphate, because of its hygroscopic properties, could react with the gelatin shells of capsules, eventually making them brittle. Bioavailability of iron from ferrous fumarate and ferrous sulphate is similar. (Harrington et al., 2011) The field team ensured that women swallowed the first dose in the presence of a volunteer and research assistant who were assigned during follow-up to that woman. Thereafter, supplementation was supervised daily by these research assistants at the homestead of the women or a place nearby. Women were advised not to take any other supplements during the remainder of the pregnancy. The research assistant recorded compliance and reasons for non-compliance on pre-designed forms and submitted these reports weekly to field research staff, who maintained these data into an electronic database and followed up immediately as needed. The volunteers helped to solve small problems such as tracking of women who were absent or missing.

Follow-up

As part of the consent procedure, participating women were advised to attend regular health services to receive antenatal care as usual or for medical care when becoming ill during the intervention period, except that they were instructed to not take supplements with iron and/or folic acid supplied by these services or from other sources. They were asked to ensure that all diagnoses, treatments and drugs administered were recorded in booklets that they were handed by the routine health services for antenatal care. As pre-agreed, we asked each woman for this booklet when she completed the intervention period, and entered relevant information into a study database.

In preparation of deliveries that might take place before the woman reached the research clinic, we supplied the household of each participating women with coolers and ice packs, as well as delivery kits, approximately 3 weeks before she was due to deliver. Research assistants had been trained in advance in the collection and cold storage of blood samples and placentas. All participants were asked, however, to contact the volunteer or research assistant as soon as they went into labour, so that the field team could be alerted by mobile phone. An obstetric nurse was stand-by day and night to assist in the delivery and was dispatched immediately by ambulance to bring the woman to the research health centre. Complicated cases were referred and brought to Kisumu Provincial Teaching and Referral Hospital at a distance of 18 km from the research clinic. A laboratory technician was stand-by to collect biological samples at delivery. In case of deliveries took place at home, samples were collected as soon as possible but at the latest within two hours after delivery.

At delivery, we recorded date of birth, neonatal data (gender, anthropometric data) and place of delivery (home or hospital). Maternal blood (6 mL) was collected by venipuncture within 1 hour after delivery. Maternal placental blood (3 mL) was collected by puncturing the intervillous space of the placenta with a thick needle. (Othoro et al., 2006) Neonate blood (6 mL) was obtained after careful cleaning of the umbilical cord and by direct sampling from the umbilical vein. All blood samples were collected in tubes with K2EDTA

(Becton Dickinson). In addition, placental biopsies were collected and stored in 40% formaldehyde solution with PBS at pH 7.2 for subsequent histological examination.

At delivery, we also asked each woman for her clinic attendance booklet, and extracted information about the number of doses received for intermittent preventive treatment against malaria, and whether or not they had received supplements with iron and/or folic acid during antenatal clinics.

All participating women received therapeutic courses of artemether-lumefantrin,10,15 praziquantel and albendazole9 immediately after delivery to avoid inflammation-induced effects on plasma iron markers at one month post-partum. We preferred potential benefits of the intervention to be assessed at that time point rather than at delivery because plasma iron markers may change rapidly perinatally independently of iron status. Thus follow-up and daily supplementation continued for one month after delivery, using procedures as described in the preceding paragraphs, and then we collected maternal blood (6 mL) by venipuncture in a K2EDTA tube (Becton-Dickinson) and peripheral blood from neonates (400-600 μL) by heel puncture in a heparin tube (Becton Dickinson, catalogue 365986). Plasma specimens collected during follow-up were stored as described for the baseline survey.

Laboratory measurements

The ZPP:H ratio was measured for each sample in duplicate using a haematofluorometer (Model ZPP 206D, Aviv Biomedical, Lakewood NJ, USA). Control samples at low, medium, and high levels (AVIV, catalogue 9999-40839) were run after every 30 readings while two level (low, high) calibration samples (AVIV, catalogue 9999-112562) were run twice yearly.

Plasmodium antigenaemia was assayed by rapid dipstick tests (Access Bio Inc, Somerset, NJ, USA; CareStart, catalogue G0151 and G0171) that can detect *P. falciparum*-specific histidine-rich protein-2 (HRP2), *Plasmodium* lactate dehydrogenase (pLDH) specific to either *P. falciparum* or to non-*falciparum* species, i.e. *P. ovale*, *P. malariae* or *P. vivax*. Whereas HRP2-based tests detects current or recent *P. falciparum* infection, pLDH-based tests only indicate current infection.(Makler, Piper, & Milhous, 1998; Moody, 2002; Piper et al., 1999)

DNA was extracted from erythrocytes (Qiagen whole blood DNA isolation protocol according to the manufacturer's instructions except that DNA was eluted in 50 µl nuclease-free water) and stored at –20°C until PCR analysis. *P. falciparum*-specific DNA was detected as per protocol(Hermsen et al., 2001) with the following modifications: we added an internal amplification control detecting the human household gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase), using a Texas Red-labeled probe and GAPDH-specific primers: Fwd: 5' GAA-GGT-GAA-GGT-CGG-AGT-C 3'; Rev: 5'

GAA-GAT-GGT-GAT-GGG-ATT-TC 3’; Probe: 5’ Texas Red CAA-GCT-TCC-CGT-TCT-CAG-CC HBQ1 -3’. Amplification reactions were performed in a volume of 25 µl, a total of 2.5 µl 1x Qiagen buffer, 6.5 mM MgCl2, 250 µM dNTPs, 400 nM of each *P. falciparum* 18S primer, 100 nM *P. falciparum* 18S probe, 40 nM of each GAPDH primer, 100 nM GAPDH probe, 0.625 U HotstarTaq polymerase (Qiagen), and 2.5µl sample DNA. Biolegio (Nijmegen, The Netherlands) provided all primers and probes. Amplification comprised of 10 minutes at 95°C, followed by 45 20-second cycles at 95°C and 1 minute at 60°C. In each PCR run, water controls, no-template controls, high and low positive controls were all included in duplicate. The high and low positive controls contain a known amount of *P. falciparum*; thus, the Ct values of these controls were used to determine the PCR amplification efficacy. The Ct values of these controls have a range between 2 SD (calculated by measuring the controls 12 times over several days) and the Ct values of the controls were required to fall within this range.

Tissue sections of placental biopsies were examined histologically to detect *Plasmodium* infection.(Bulmer, Rasheed, Francis, et al., 1993),(Bulmer, Rasheed, Morrison, et al., 1993)

HIV infection was assessed by rapid antibody tests (Alere™, Waltham, Massachusetts, USA); a positive result was confirmed using other antibody tests (Unigold; Trinity Biotech, Co Wicklow, Ireland and/or Bioline; Pantech, Umhlanga, Kwazulu-Natal, South Africa).

Plasma concentrations of ferritin, soluble transferrin receptor (sTfR), transferrin, C-reactive protein, and α1-acid glycoprotein were measured on a Beckman Coulter UniCel DxC 880i analyser as per manufacturer’s instructions.

Fortificant iron content in flour samples were analysed in duplicate, based on the principle that solubilized ferrous iron reacts with orthophenanthroline, a chromogenic reagent, in the presence of hydroxylamine hydrochloride (reducing agent), resulting in a pink-coloured complex. Flour samples (10 g) were mixed with 30 mL water to dissolve organic constituents. After shaking, centrifugation and filtration (Acrodisc LC-13 mm syringe filter, 0.45 µm PVDF membrane), we added solutions of 0.5 mL hydroxylammonium chloride (500 g/L), 5 ml sodium acetate (4 M) and 2.5 mL ortho-phenthroline (2.5 g/L) to 15 g of the supernatant, and made up to 50 mL with water. Iron concentrations were determined spectrophotometrically at an absorbance of 510 nm.

Outcomes

The primary outcome was defined as past or present maternal *Plasmodium* infection assessed at parturition, regardless of species, as indicated by one or more positive results for the presence of pLDH or HRP2 in plasma (dipstick tests), *Plasmodium* pigment upon histological examination of placental biopsies), or *P. falciparum* DNA (PCR test).

Secondary outcomes were: patent *Plasmodium* infection (similarly defined as in primary

outcome, but restricted to results from dipstick tests and biopsy histology); sub-patent *Plasmodium* infection (similarly as in primary outcome, but restricted to results from PCR tests); current or recent *Plasmodium* infection (similarly as in primary outcome, but restricted to results from dipstick tests and PCR tests); birth weight; gestational age at delivery; fetal weight for gestational age; and maternal and neonatal iron status at one month after delivery (as assessed by haemoglobin concentrations, iron deficiency anaemia, and iron stores).

Statistical analysis

We do not report sample size calculations because we interpreted a completed study; all information about precision is indicated by the 95% CIs of our effect estimates.

Data cleaning and analysis was done using SPSS 21 (SPSS, Chicago, USA) and CIA 2.2.0 (http://www.som.soton.ac.uk/research/sites/cia/). Analyses were done according to a statistical analysis plan that was finalised before the treatment allocation code was revealed.

Haemoglobin concentration was calculated as the mean of duplicate measurements or median values if more than two recordings were available. Fetal weight-for-gestational-age z-scores were derived using estimated mean birth weight at 40 weeks of gestation for Kenyan children as a reference, (Mikolajczyk et al., 2011)and with a corresponding SD value of 12.8 g (estimate obtained from the population presented in this paper).

We used the following definitions: anaemia: haemoglobin concentration <110 g/L (WHO, 2011a); iron deficiency: plasma ferritin concentration <15 µg/L (WHO, 2011b); iron-replete: plasma ferritin concentration ≥15 µg/L in the absence of inflammation; iron status uncertain: plasma ferritin concentration ≥15 µg/L in the presence of inflammation; iron deficiency anaemia: anaemia co-existing with iron deficiency; inflammation: plasma concentrations of C-reactive protein (CRP) >10 mg/L (Nielsen, Bek, Rasmussen, Qvist, & Tobiassen, 1990) and/or α1-acid glycoprotein >1 g/L (Filteau et al., 1993); gravidity: the number of times a woman reports to have been pregnant, regardless of the outcome of these pregnancies, with twins and other multiple births counted as 1, and including the current pregnancy; fetal death: occurred before birth; neonatal death: death of a live-born infant within the first 7 days of life (early neonatal death), at 7–28 days of life (late neonatal death); small-for-gestational age: birth weight < 10-percentile of fetal weight at a particular gestational age; compliance: the proportion of women who consumed >90% of scheduled supplements.

The full analysis population included all subjects who were randomised and who a) received at least one dose of the experimental supplements (iron or placebo); b) *Plasmodium* infection status could be ascertained from blood or placental samples collected at birth or within 48 h after birth. The per protocol population consisted of the full analysis set with restriction to participants who a) consumed >90% of scheduled supplements; and

b) had singleton pregnancies.

Our primary analysis was restricted to the per protocol population, and concerned the difference between intervention groups in proportions of women with *Plasmodium* infection. Confounding was assessed by comparing odds ratios with and without adjustment for baseline factors known (gravidity [primigravidae, secundigravidae, multigravidae]; (M. Desai et al., 2007) maternal age [< 20 years and ≥ 20 years]; (M. Desai et al., 2007), (Tako et al., 2005) HIV infection (Kuile et al., 2004)) or suspected (*Plasmodium* infection status; iron status, entered as haemoglobin concentration [continuous, centred around the mean] and plasma ferritin concentration (<12 μg/L or ≥ 12 μg/L); gestational age [continuous, centred around the mean]) to be prognostic for the primary outcome. We conducted an exploratory analysis on the full analysis set, and used stratified and direct multivariate analyses to explore the influence of predefined baseline factors (gravidity; (Nosten, ter Kuile, Maelankirri, Decludt, & White, 1991), (A. M. Greenwood, Armstrong, Byass, Snow, & Greenwood, 1992) maternal age; (Espinoza, Hidalgo, & Chedraui, 2005), (Leenstra et al., 2003), (Marques et al., 2005), (S J Rogerson et al., 2000), (Walker-abbey et al., 2005) HIV infection; (M. Desai et al., 2007) iron status, indicated by anaemia and iron deficiency) on the magnitude of the intervention effect. P-values for interactions with binary outcomes were calculated by likelihood ratio tests. We similarly assessed to what extent the intervention effect is influenced by intermittent preventive treatment during the intervention period.

We used multiple linear regression analysis to assess intervention effects on birth weight, gestational age at delivery, and maternal and infant haemoglobin concentration at 30 days post-partum. We conducted these analyses with and without adjustment for baseline haemoglobin concentration; in the analysis of the haemoglobin response to intervention, we also accounted for inflammation at the end survey. We used stratified analysis and multivariate analysis to assess effect modification by pre-specified baseline factors (iron status, gravidity, maternal age, *Plasmodium* infection, HIV infection), and by intermittent preventive treatment use.

Results

Of 2,015 women invited for screening, 470 (23%) were eligible and randomised to intervention **(Figure 1 & Table 1)**. Of these, 434 (92%) were included in the full analysis set, and 430 (92%) in the per protocol analysis.One woman died as a result of postpartum haemorrhage, and another at 2 weeks postpartum due to pneumonia and cardiac arrest. Adverse maternal, foetal and neonatal events are summarised in Figure 1 and **table 2**.

Of all deliveries, 415 (88.3%) took place at the research clinic, 38 (8.1%) in referral hospitals and 17 (3.6%) at home. Placental biopsies were incorrectly preserved and lost for 85 (18.1%) women, whilst 42 (8.9%) placentae were missing or poorly preserved because

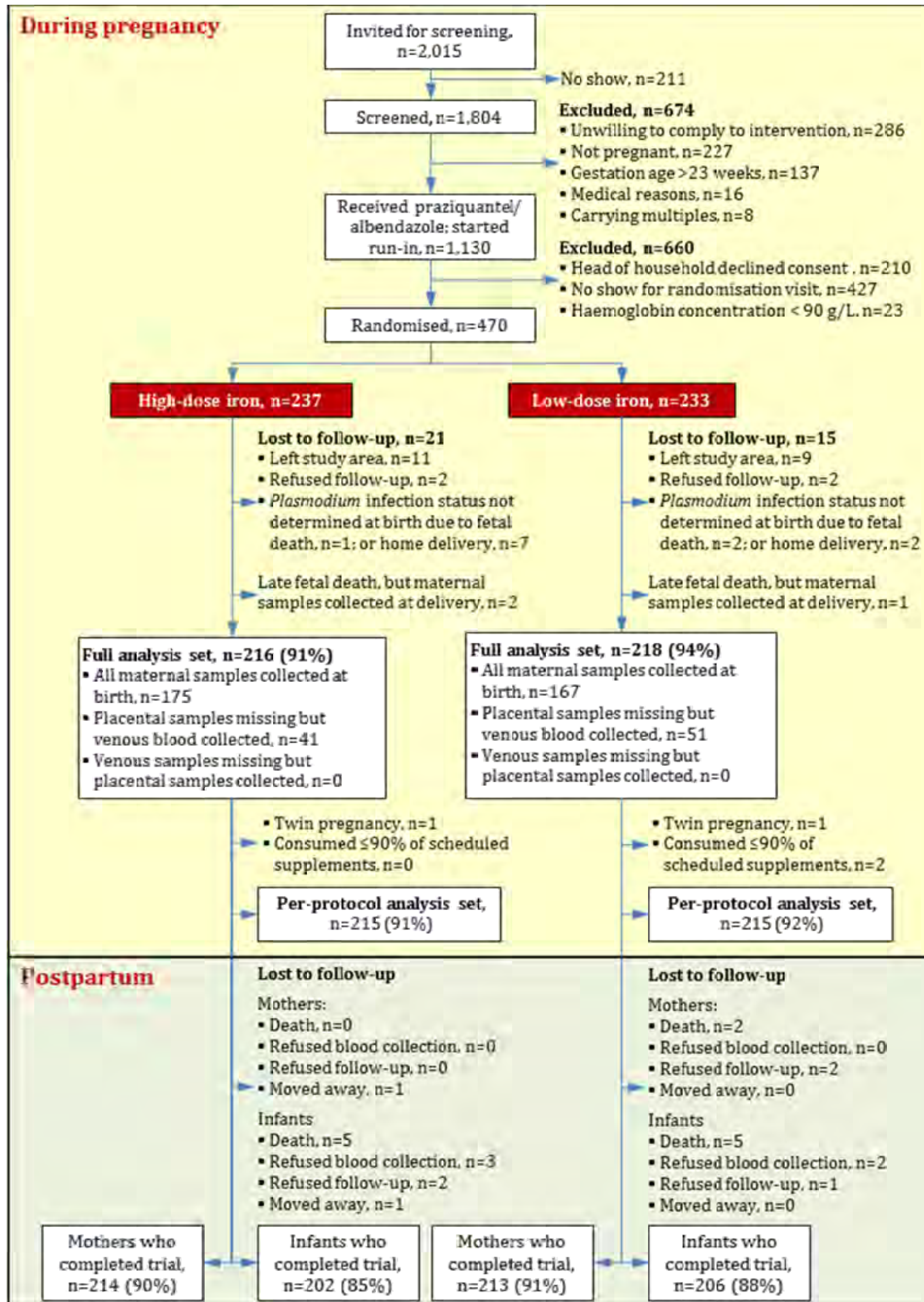


Figure 1: Participant flow

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

Characteristics	High-dose iron group		Low-dose iron group	
	Per protocol analysis set	Lost to follow-up or excluded	Per protocol analysis set	Lost to follow-up or excluded
n	215	22	212	21
Height, cm	162.5 (5.9)	161.7 (3.9)	162.6 (6.7)	160.9 (7.2)
Weight, kg	58.0 (7.5)	59.8 (8.7)	57.4 (7.5)	58.7 (8.5)
Body mass index, kg/m^2	22.0 (2.7)	22.9 (3.2)	21.7 (2.6)	22.7 (2.8)
Marital status				
Married or living together	190 [88.4%]	14 [63.6%]	171 [80.7%]	13 [61.9%]
Divorced or separated	7 [3.3%]	1 [4.5%]	9 [4.2%]	1 [4.8%]
Never married	18 [8.4%]	7 [31.8%]	32 [15.1%]	7 [33.3%]
Age, years	24.0 [9.0]	20.5 [7.0]	24.0 [9.0]	21.0 [10.0]
Gestational age, weeks [1]	17.4 [4.0]	18.0 [4.0]	17.3 [4.0]	18.4 [4.0]
Gravidity				
Primigravidae	27 [12.6%]	10 [45.5%]	40 [18.9%]	8 [38.1%]
Secundigravidae	53 [24.7%]	4 [18.2%]	32 [15.1%]	3 [14.3%]
Multigravidae	135 [62.8%]	8 [36.4%]	140 [66.0%]	10 [47.6%]
***Plasmodium* infection**				
Any *Plasmodium* spp.[2], by any dipstick or PCR	78 [36.3%]	11 [50.0%]	80 [37.7%]	6 [28.6%]
***P. falciparum*, by HRP2- or LDH-based dipstick, or PCR**	77 [35.8%]	11 [50.0%]	80 [37.7%]	6 [28.6%]
Current or recent *P. falciparum* infection, by either HRP2- or LDH-based dipstick	39 [18.1%]	8 [36.4%]	43 [20.3%]	1 [4.8%]
***P. falciparum*, by PCR**	70 [32.6%]	11 [50.0%]	76 [35.8%]	6 [28.6%]
HIV infection	46 [21.4%]	2 [9.1%][5]	47 [22.2%]	4 [19.0%]
Plasma CRP concentration, mg/L	4.0 [7.3]	9.8 [25.6]	4.3 [8.5]	2.5 [10.1]
Plasma AGP concentration, g/L	0.8 (0.3)	1.0 (0.4)	0.8 (0.3)	0.7 (0.3)
Inflammation [3]				
Plasma CRP concentration ≥10 mg/L	50 [23.3%]	11 [50.0%]	58 [27.4%]	7 [33.3%]
Plasma AGP *concentration* ≥1.0 g/L	35 [16.3%]	8 [36.4%]	38 [17.9%]	4 [19.0%]
Plasma CRP concentration ≥10 mg/L or AGP ≥1.0 g/L	63 [29.3%]	13 [59.1%]	69 [32.5%]	7 [33.3%]
Hb concentration, g/L	113.4 (10.7)	118.2 (11.7)	112.2 (11.9)	115.1 (12.1)

Anemia, Hb concentration <110g/L	77 [35.8%]	5 [22.7%]	87 [41.0%]	6 [28.6%]
Plasma ferritin concentration, µg/L	13.7 [21.0]	15.7 [41.1]	13.8 [20.4]	17.0 [21.9]
Plasma sTfR concentration, mg/L	1.8 [1.1]	2.3 [0.8]	2.0 [1.2]	1.8 [0.8]
Plasma transferrin concentration, g/L	3.1 (0.6)	3.0 (0.4)	3.1 (0.5)	3.0 (0.5)
Iron deficiency, plasma ferritin concentration <15µg/L				
All women	115 [53.5%]	11 [50.0%]	112 [52.8%]	10 [47.6%]
Those with CRP concentration <10 mg/L	94/165 [57.0%]	7/11 [63.6%]	87/154 [56.5%]	9/14 [64.3%]
Those with AGP concentration <1.0g/L	105/180 [58.3%]	9/14 [64.3%]	97/174 [55.7%]	9/17 [52.9%]
Those with concentrations of CRP <10mg/L or AGP <1.0 g/L	109/152 [71.7%]	8/9 [88.9%]	100/143 [69.9%]	1/14 [7.1%]
Whole blood ZPP:heme ratio, µmol/mol	89.5 [53.0]	85.3 [28.1]	90.8 [62.8]	86.0 [39.3]
Erythrocyte ZPP:heme ratio, µmol/mol	37.5 [44.5]	34.3 [38.8]	36.0 [57.8]	32.5 [35.5]
Erythrocyte FEP concentration, µg/dl [4]	22.2 [31.1]	15.0 [15.5]	19.4 [31.9]	16.2 [60.3]

Mean (SD), Median [IQR], n [%] or n/n [%]
AGP: α_1-acid glycoprotein; CRP: C-reactive protein; FEP: free erythrocyte protoporphyrin; HRP2: *P. falciparum*-specific histidine-rich protein-2); LDH: *P. falciparum*-specific lactate dehydrogenase; sTfR: soluble transferrin receptor; ZPP: zinc protoporphyrin
[1] All women except one were in the 2nd trimester of pregnancy; [2] Only one participant (high-dose iron group) had infection by a *Plasmodium* species other than *P. falciparum*; [3] Only one participant (high-dose iron group) had current fever defined as axillary temperature ≥ 37.5 °C; [4] One missing value in the per protocol analysis dataset; [5] HIV status of two participants was not determined

delivery took place at home or in referral hospitals. Two mothers refused consent for neonatal blood collection at 1-month post-partum. Samples from two babies in the control group were inadequate to perform all the biochemical tests.

Analysis of flour samples showed an average iron content of 18.0 mg/kg (SD: 2.2 mg/kg). Pregnant women had an average daily intake of flour and fortificant iron of 286 g and 5.7 mg, respectively.

Table 2: Frequency of adverse events in mothers and children

Adverse events	High–dose iron group	Low–dose iron group
Maternal events		
Maternal death	0	2 [1]
Hospital admissions	1	4
Pre-eclampsia	0	1
Atonic uterine contraction	0	1
General sickness (self-reported)	4	3
Malaria	1	1
Pneumonia	1	0
Meningitis	1	0
Breech delivery	1	0
Total	9	12
Fetal and neonatal events		
Fetal death	3	3
Early neonatal death [2]	2	2
Late neonatal death [3]	2	2
Infant death after 28 days of life	1	1
Neural tube defect	0	2
Neonatal sepsis	1	1
Total	9	11

[1] One woman died due to postpartum hemorrhage, and another at 2weeks postpartum due to pneumonia and cardiac arrest. [2] Early neonatal death: death of a live-born infant within the first week of life; [3] Late neonatal death: death of a live-born infant at 7□28 days of life

Except for some mild imbalance in marital status and gravidity, intervention groups were similar regarding baseline characteristics (Table 1). Compliance in the high-dose and low-dose iron groups was 100% and 99.1%, respectively. Records from clinic attendance booklets indicated that women in high-dose iron and low-dose iron groups were similar regarding the percentage who received iron supplements from external sources in addition to supplements administered by the study (9.3% versus 9.9%), and who received insecticide-treated bed nets (15.2% versus 15.9%).

PP: Per protocol; High-dose iron: daily supplementation with iron (60 mg as ferrous fumarate) plus fortified flour throughout the intervention period; low-dose iron: fortified flour throughout the intervention period. Percentages reported between parentheses are calculated with the number randomised to intervention as the denominator

Maternal outcomes at delivery: We found no effect of iron on *Plasmodium* infection, however defined **(Table 3)**; crude and adjusted odds ratios were 1.05 and 0.97, respectively, indicating no evidence for confounding by baseline factors (gravidity, HIV infection status, maternal age, gestation age, *Plasmodium* infection, haemoglobin concentration, plasma ferritin concentration, marital status). There was weak evidence that

iron increased the risk of *Plasmodium* infection in women who were anaemic or iron deficient at baseline **(Figure 2).**

Iron supplementation increased haemoglobin concentrations by 9.4 g/L, reduced the prevalence of anaemia by 29.8% and reduced ZPP:haem ratios in whole blood and erythrocytes (Table 2).

Neonatal outcomes at delivery: Iron supplementation increased birth weight by 143 g (Table 2), and reduced the prevalence of low birth weight by 65% (3.1% versus 8.8%; ratio, 95%CI: 0.35, 14.1─87.2). The effect of iron on birth weight seemed larger in women with iron deficiency at baseline than in their peers who were initially iron-replete (249 g versus ─14 g; difference, 95% CI: 263 g, 136□362 g; Figure 2).

Iron increased foetal-weight z-score at delivery by 0.27 SD, and neonatal length by 1.0 cm. There was no evidence that iron increased gestational age at delivery, but there was weak evidence that it reduced the proportion of neonates that were born prematurely by 6.3% (Table 2). We did not find an effect of iron on neonate iron markers (haemoglobin concentration, ZPP:haem ratios).

Outcomes at 1 month postpartum: Iron supplementation improved maternal iron status at 1 month postpartum, as shown by increased haemoglobin concentrations plasma ferritin concentrations, as well as reductions in the prevalence of anaemia, iron deficiency and plasma transferrin receptor concentration **(Table 4)**.

In the analysis of haemoglobin concentration, adjustment for baseline haemoglobin, ferritin concentration, maternal age, gravidity, marital status, and HIV infection status, as well as for time between delivery and blood collection one month post-partum, led to similar effect estimates. Beneficial gains in iron status were greater in women with poor iron status at baseline. For example, iron supplementation increased haemoglobin concentration by 15.6 g/L in women who were initially anaemic, as compared to a gain of 3.7 g/L in those who were initially non-anaemic (Figure 2).

Table 3: Effect of iron on maternal and neonatal outcomes at delivery, per protocol analysis

Indicator	High-dose iron	Low-dose iron	Difference or per cent change (95% CI)
Maternal outcomes			
n	215 [90.7%]	215 [92.3%]	
Plasmodium infection (see text for definitions)			
Patent and sub-patent infections (primary outcome)	96 [44.7%]	96 [44.7%]	0.0% (−9.3% to 9.3%)
Patent infections	84 [39.1%]	81 [37.7%]	1.4% (−7.7% to 10.5%)
Sub-patent infections	49 [22.8%]	52 [24.2%]	−1.4% (−9.4% to 6.6%)
Current or recent infection	60 [27.9%]	66 [30.7%]	−2.8% (−11.3% to 5.8%)
Hb concentration, g/L	120.7 (16.4)	111.6 (18.9)	9.1 (5.7 to 12.4)
High Hb concentration (>130) g/L	66 [30.7%]	37[1] [17.3%]	13.4% (5.3% to 21.3%)
Anemia (Hb concentration <110 g/L)	46 [21.4%]	108 [1] [50.5%]	− 29.1% (−37.3% to −20.1%)
Severe anemia (Hb concentration <70 g/L)	3 [1.4%]	1[1] [0.5%]	0.9% (−1.4% to 3.6%)
ZPP in maternal venous blood, µmol:mol heme	103.2 [63.0, 126.5]	155.6 [87.0, 207.5]	−33.7% (−40.1% to −26.5%)
ZPP in maternal erythrocytes, µmol:mol heme	27.7 [10.5, 43.0]	65.8 [25.0, 110.0]	−57.9% (−64.9% to −49.7%)
Neonatal outcomes			
n	220	218	
Sex, male:female	109:111 [49.5%, 50.5%]	109:109 [50.0%, 50.0%]	
Birthweight, g [2]	3,191 (441)	3,048 (410)	143 (58 to 228)
Low birthweight (<2,500 g)	6/194 [3.1%]	17/196 [8.7%]	−5.6% (−10.6% to −0.9%)
Fetal weight z-score at delivery, SD [3]	0.70 (1.17)	0.42 (1.11)	0.27 (0.04 to 0.50)
Small-for-gestational age	5/194 (2.6%)	8/195 (4.1%)	−1.5% (−5.6% to 2.3%)
Length, cm	50.6 (4.2)	49.6 (4.3)	1.0 (0.1 to 1.8)
Head circumference, cm	35.0 [33.5, 35.5]	34.6 [33.5, 35.5]	0.9% (−0.2% to 2.0%)
Gestational age at delivery, weeks	38.6 (1.9)	38.4 (1.9)	0.2 (−0.2 to 0.6)
Premature birth (< 37 weeks gestation)	32 [14.9%]	45 [20.9%]	−6.0% (−13.3% to 1.2%)
Hb concentration in cord blood, g/L	153.6 (21.5)	150.5 (21.1)	3.1 (−1.0 to 7.2)
ZPP in cord blood, µmol:mol heme	137.4 [113.0, 162.5]	139.5 [110.5, 170.0]	−1.5% (−7.4% to 4.7%)
ZPP in cord erythrocytes, µmol:mol heme	55.0 [42.0, 75.0]	55.7 [37.0, 79.5]	−1.3% (−10.5% to 8.9%)

Mean (SD), geometric mean [25-, 75-percentiles], n [%] or n/n [%].
[1]One missing value; [2] Excluding neonates whose weight could not be accurately measured because they were born in hospital, or whose weight was not measured within 24h after birth; thus n=194 and 196 for high-dose and low-dose iron groups, respectively. When including these infants, mean birthweights were 3,213 g (SD 456 g; n=209) and 3,060 g (439 g; n=207) for high-dose and low-dose iron groups, respectively; difference, 95% CI: 153 g, 67–240 g; [3] n=194 for high-dose iron group and n=196 for low-dose iron group.

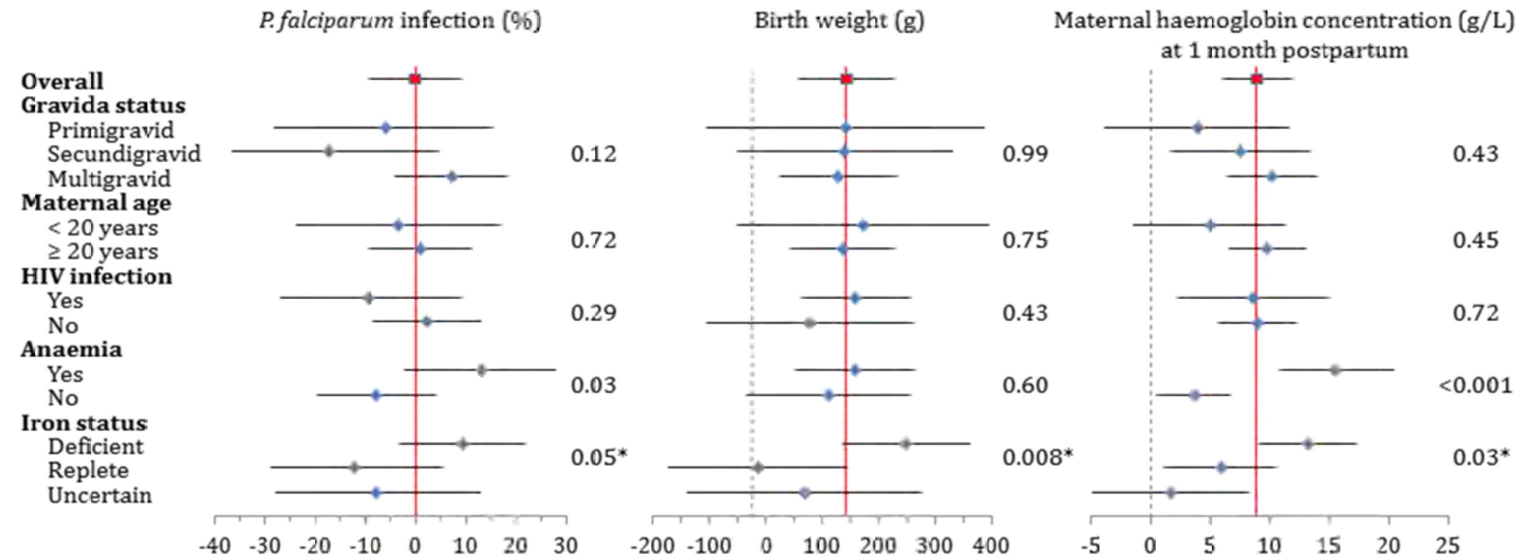
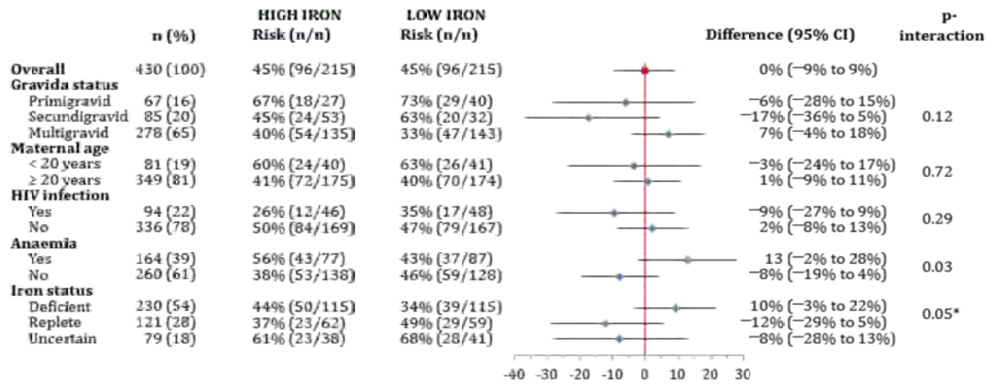


Figure 2: Effect of iron supplementation on selected outcomes, by subgroups
Red vertical lines indicate overall effect; dotted lines indicate no effect. Haemoglobin concentrations were missing at 1 month postpartum for two HIV-infected women.

Table 4: Effect of iron on maternal and neonatal iron status at 1 month post-partum

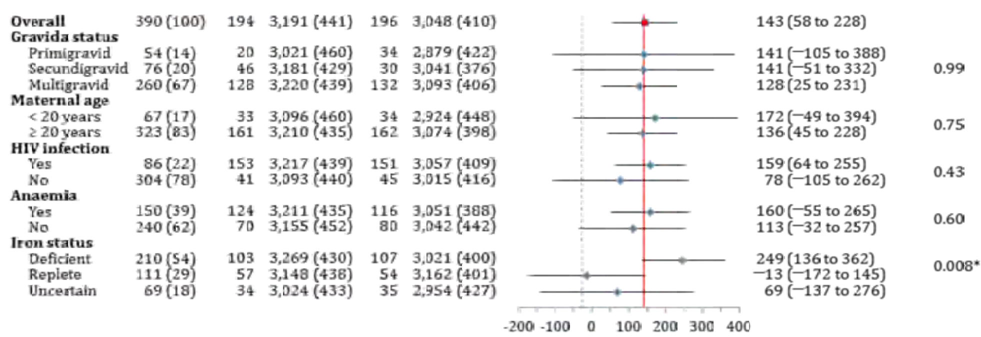
Indicator	High-dose iron	Low-dose iron	Difference or per cent change (95% CI)
Maternal outcomes			
n	214 [90.3%]	213 [91.4%]	
Hb concentration, g/L	129.0 (13.9)	120.1 (16.5)	8.9 (6.0 to 11.8)
Anemia (Hb concentration <120 g/L)	52/214 [24.3%]	96/213 [45.1%]	−20.8% (−29.3% to −11.8%)
Plasma ferritin concentration, µg/L	32.7 [19.5, 58.7]	14.6 [6.9, 29.8]	124.1% (87.3% to 168.1%)
Iron deficiency (plasma ferritin concentration <15 µg/L)			
All persons	40/214 [18.7%]	118/212 [55.7%]	−37.0% (−45.0% to −28.1%)
Those without inflammation [1]	26/133 [19.5%]	80/135 [59.3%]	−39.7% (−49.6% to −28.4%)
Iron deficiency anemia [2]	10/214 [4.7%]	46/212 [21.7%]	−17.0% (−23.4% to −10.8%)
Plasma transferrin concentration, g/L	2.65 (0.50)	3.07 (0.52)	−0.42 (−0.52 to −0.32)
Plasma transferrin receptor concentration, mg/L	1.78 [1.24, 2.32]	2.52 [1.72, 3.64]	−29.4% (−36.1% to −22.1%)
Neonatal outcomes			
n	202 [85.2%]	206 [88.4%]	−3.2%
Hb concentration, g/L	131.4 [111.5, 144.0]	129.6 [114.5, 147.5]	1.3% (−2.9% to 5.7%)
Plasma ferritin concentration, µg/L	163.0 [110.0, 268.9]	139.2 [93.2, 212.0]	17.1% (2.1% to 34.3%)
Plasma transferrin receptor concentration, mg/L	1.2 [1.0, 1.5]	1.3 [1.0, 1.5]	−4.9% (− 11.5% to 2.1%)
Current or recent *Plasmodium* infection	3/200 [1.5%]	3/197 [1.5%]	0.0% (− 0.03 to 0.03)

Mean (SD), geometric mean [25th, 75th percentiles], n [%] or n/n [%], *Geometric mean, **median
[1] Concentrations of C-reactive protein <10 mg/L or α_1-acid glycoprotein <1.0 g/L; [2]Presence of both anemia and iron deficiency;



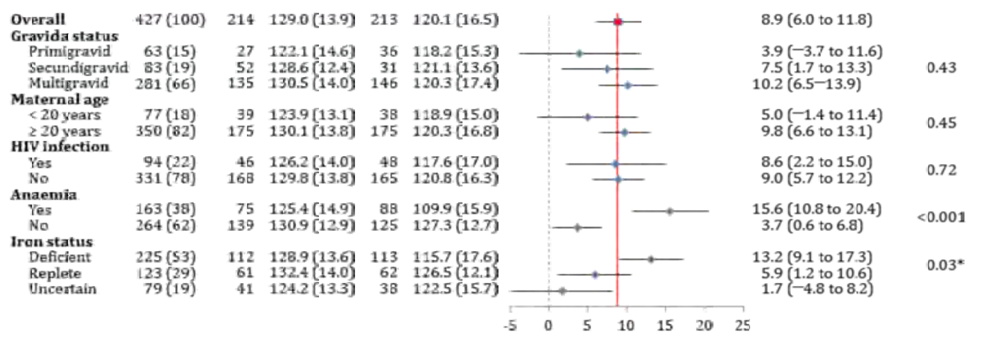
Outcome: Risk of *Plasmodium* infection

	n (%)	HIGH IRON Risk (n/n)	LOW IRON Risk (n/n)	Difference (95% CI)	p-interaction
Overall	430 (100)	45% (96/215)	45% (96/215)	0% (−9% to 9%)	
Gravida status					
Primigravid	67 (16)	67% (18/27)	73% (29/40)	−6% (−28% to 15%)	0.12
Secundigravid	85 (20)	45% (24/53)	63% (20/32)	−17% (−36% to 5%)	
Multigravid	278 (65)	40% (54/135)	33% (47/143)	7% (−4% to 18%)	
Maternal age					
< 20 years	81 (19)	60% (24/40)	63% (26/41)	−3% (−24% to 17%)	0.72
≥ 20 years	349 (81)	41% (72/175)	40% (70/174)	1% (−9% to 11%)	
HIV infection					
Yes	94 (22)	26% (12/46)	35% (17/48)	−9% (−27% to 9%)	0.29
No	336 (78)	50% (84/169)	47% (79/167)	2% (−8% to 13%)	
Anaemia					
Yes	164 (39)	56% (43/77)	43% (37/87)	13 (−2% to 28%)	0.03
No	260 (61)	38% (53/138)	46% (59/128)	−8% (−19% to 4%)	
Iron status					
Deficient	230 (54)	44% (50/115)	34% (39/115)	10% (−3% to 22%)	0.05*
Replete	121 (28)	37% (23/62)	49% (29/59)	−12% (−29% to 5%)	
Uncertain	79 (18)	61% (23/38)	68% (28/41)	−8% (−28% to 13%)	



Outcome: Birth weight (g)

	n (%)	HIGH IRON n	HIGH IRON mean (SD)	LOW IRON n	LOW IRON mean (SD)	Difference (95% CI)	p-interaction
Overall	390 (100)	194	3,191 (441)	196	3,048 (410)	143 (58 to 228)	
Gravida status							
Primigravid	54 (14)	20	3,021 (460)	34	2,879 (422)	141 (−105 to 388)	0.99
Secundigravid	76 (20)	46	3,181 (429)	30	3,041 (376)	141 (−51 to 332)	
Multigravid	260 (67)	128	3,220 (439)	132	3,093 (406)	128 (25 to 231)	
Maternal age							
< 20 years	67 (17)	33	3,096 (460)	34	2,924 (448)	172 (−49 to 394)	0.75
≥ 20 years	323 (83)	161	3,210 (435)	162	3,074 (398)	136 (45 to 228)	
HIV infection							
Yes	86 (22)	153	3,217 (439)	151	3,057 (409)	159 (64 to 255)	0.43
No	304 (78)	41	3,093 (440)	45	3,015 (416)	78 (−105 to 262)	
Anaemia							
Yes	150 (39)	124	3,211 (435)	116	3,051 (388)	160 (−55 to 265)	0.60
No	240 (62)	70	3,155 (452)	80	3,042 (442)	113 (−32 to 257)	
Iron status							
Deficient	210 (54)	103	3,269 (430)	107	3,021 (400)	249 (136 to 362)	0.008*
Replete	111 (29)	57	3,148 (438)	54	3,162 (401)	−13 (−172 to 145)	
Uncertain	69 (18)	34	3,024 (433)	35	2,954 (427)	69 (−137 to 276)	



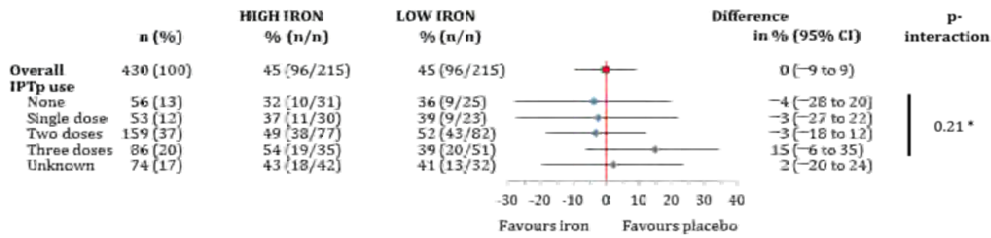
Outcome: Haemoglobin concentration (g/L) at 1 month postpartum

	n (%)	HIGH IRON n	HIGH IRON mean (SD)	LOW IRON n	LOW IRON mean (SD)	Difference (95% CI)	p-interaction
Overall	427 (100)	214	129.0 (13.9)	213	120.1 (16.5)	8.9 (6.0 to 11.8)	
Gravida status							
Primigravid	63 (15)	27	122.1 (14.6)	36	118.2 (15.3)	3.9 (−3.7 to 11.6)	0.43
Secundigravid	83 (19)	52	128.6 (12.4)	31	121.1 (13.6)	7.5 (1.7 to 13.3)	
Multigravid	281 (66)	135	130.5 (14.0)	146	120.3 (17.4)	10.2 (6.5–13.9)	
Maternal age							
< 20 years	77 (18)	39	123.9 (13.1)	38	118.9 (15.0)	5.0 (−1.4 to 11.4)	0.45
≥ 20 years	350 (82)	175	130.1 (13.8)	175	120.3 (16.8)	9.8 (6.6 to 13.1)	
HIV infection							
Yes	94 (22)	46	126.2 (14.0)	48	117.6 (17.0)	8.6 (2.2 to 15.0)	0.72
No	331 (78)	168	129.8 (13.8)	165	120.8 (16.3)	9.0 (5.7 to 12.2)	
Anaemia							
Yes	163 (38)	75	125.4 (14.9)	88	109.9 (15.9)	15.6 (10.8 to 20.4)	<0.001
No	264 (62)	139	130.9 (12.9)	125	127.3 (12.7)	3.7 (0.6 to 6.8)	
Iron status							
Deficient	225 (53)	112	128.9 (13.6)	113	115.7 (17.6)	13.2 (9.1 to 17.3)	0.03*
Replete	123 (29)	61	132.4 (14.0)	62	126.5 (12.1)	5.9 (1.2 to 10.6)	
Uncertain	79 (19)	41	124.2 (13.3)	38	122.5 (15.7)	1.7 (−4.8 to 8.2)	

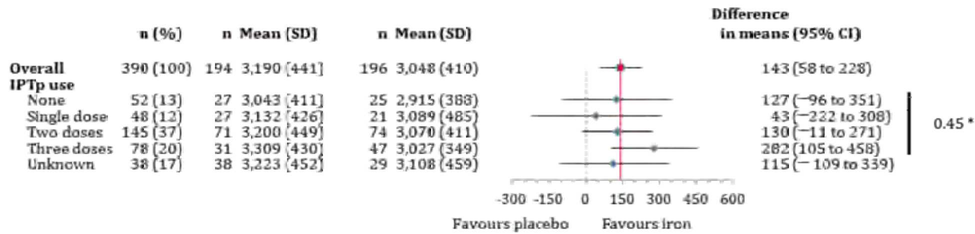
* Analysis restricted to those who were iron deficient or iron replete

Supplementary Figure 1: Effect of iron on key outcomes, by subgroups

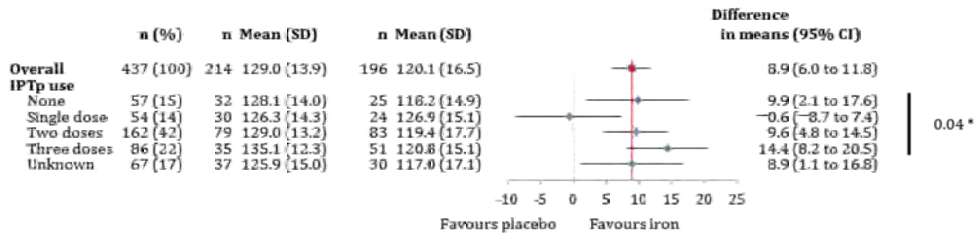
Outcome: *Plasmodium* infection assessed at delivery



Outcome: birth weight (g)



Outcome: maternal haemoglobin concentration (g/L) at 1 month postpartum



* Analysis excluding those whose IPTp use was unknown

Supplementary Figure 2: Effect of iron on key outcomes, by IPTp use

Discussion

Overall, we found no effect of daily iron supplementation in pregnancy on maternal *Plasmodium* infection. However, iron supplementation increased birth weight by 143g, and reduced the prevalence of low birth weight by 65%. There was no evidence that the gains in birth weight depended on gravidity, maternal age, HIV infection, anaemia and IPTp use, suggesting that all subgroups thus defined benefitted from iron. In a pre-specified subgroup analysis, however, there was strong statistical support that the effect on birth weight depended on initial iron status. In women who were initially deficient, iron increased birth weight by 249g despite an apparent increase in *P. falciparum* infection by 10%. Iron led to

improved maternal and neonatal iron stores at 1 month postpartum.

Although *P. falciparum* infection in pregnancy is usually asymptomatic, it may cause adverse birth outcomes (reduced birth weight, intrauterine growth retardation, preterm delivery, increased neonatal mortality) and adverse maternal outcomes (increased risk of severe anaemia and death). (M. Desai et al., 2007) We found no effect of iron on *Plasmodium* infection, however, regardless of pre-specified case definitions, and the upper limit of the 95% CI was consistent with a 9% increase only in the cumulative incidence of infection (Table 1; primary outcome).

A unique aspect of this study is the ethical licence, created by the concern about the potential risk of malaria, to assess the benefits of iron supplementation in a mixed population that included women with anaemia and iron deficiency at baseline. Recent meta-analyses, (Peña-Rosas et al., 2012), (Vucic et al., 2013), (Haider et al., 2013) published after we started our trial, found no (Vucic et al., 2013) or only small effects (31g, 95%CI: 6–56g, (Peña-Rosas et al., 2012) and 40.8g, 0.97–80.6g) (Haider et al., 2013) of antenatal iron supplementation on birth weight. All but three of the studies (Preziosi et al., 1997), (Christian et al., 2003), (Zeng et al., 2008) reviewed, however, excluded women who were initially iron deficient or anaemic, and most reported good iron status at baseline. Our data show no evident effect on birth weight of iron supplementation in iron-replete women (Figure 3). Pena-Rosas et al. could not conduct subgroup analysis by initial anaemia status, because there was not a single woman who was reported to be initially anaemic in the studies reviewed. (Peña-Rosas et al., 2012)

The large benefit on birth weight in our study may also be explained by a superior compliance compared to all studies reviewed. (Peña-Rosas et al., 2012), (Vucic et al., 2013), (Haider et al., 2013) Whereas we observed daily throughout the intervention period if women swallowed supplements, supervision was unclear in other studies, or contact with participants was limited to repeated visits or phone calls in the period until delivery. Although several studies used capsules or coated tablets, (Cogswell, Parvanta, Ickes, Yip, & Brittenham, 2003), (Zeng et al., 2008), (Falahi, Akbari, Ebrahimzade, & Gargari) iron was mostly given as tablets with ferrous salts, which produce a strong and unpleasant taste that may have discouraged adherence. Attrition was high in many studies, although it is unclear whether this occurred selectively in the iron groups. Although supplement use was assessed in most studies by tablet counts or self-reports, such methods tend to overestimate adherence. (Cramer, 1989), (Olivieri, Matsui, Hermann, & Koren, 1991), (Shalansky, Levy, & Ignaszewski, 2004), (Grosset, Bone, Reid, & Grosset, 2006) With few exceptions, however, adherence was not reported or poor.

The gain in birth weight seemed achieved mostly through improved fetal growth (as evident by an increased fetal weight-for-gestational-age and body length), and perhaps to some extent by a reduction in premature births. Consistent with and supportive of our finding of

the selective effect of iron on birth weight, the haemoglobin response to iron was larger in women who were initially iron deficient as compared to those who were iron replete.

Low birthweight is the primary determinant of neonatal and post-neonatal mortality, and is believed to be an important cause of neonatal morbidity, inhibited growth and cognitive development, and chronic diseases later in life.

Our finding that infants whose mothers received iron had elevated plasma ferritin concentrations at 1 month post-partum adds to growing evidence, (Scholl, 2011) so far confirmed only in a single trial, (Preziosi et al., 1997) that antenatal iron supplementation improves neonatal iron stores, thus delaying the age at which iron deficiency is likely to develop during infancy.

The main limitation of our study is that *Plasmodium* infection was determined in samples collected at delivery, which may not have captured all infections experienced during the intervention period. To avoid ethical dilemmas, we did not collect blood samples between enrolment and delivery, and referred women for antenatal care after enrolment to the regular health services, where they received standard service. Our inability to assess infection in this period was compensated by histological assessment of *Plasmodium* pigment in placental biopsies, (Bulmer, Rasheed, Francis, et al., 1993; Bulmer, Rasheed, Morrison, et al., 1993) which probably captures the vast majority of past infections.
Our results cannot be extrapolated to children, for whom there is substantial evidence that iron supplementation can increase malaria rates. (Andrew M Prentice & Cox, 2012)

Concluding remarks

Antenatal iron supplementation leads to large improvements of birth weight, fetal growth and infant iron stores, with potentially immense benefits for infant survival and health that should outweigh any possible concerns about risks of malaria. Scaling up universal iron supplementation in pregnancy in developing countries is likely to generate major public health gains.

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Chapter 3

Diagnostic utility of zinc protoporphyrin to detect iron deficiency in Kenyan pregnant women

Abstract

Zinc protoporphyrin (ZPP) indicates iron-deficient erythropoiesis and is a screening marker of iron deficiency (ID) in pregnant women and children. There are concerns about gross discrepancies of reported prevalence estimates for ID obtained by ZPP and circulating ferritin concentrations.

We used baseline samples collected from a randomized controlled trial involving 470 rural Kenyan women with singleton pregnancies, gestational age 13─23 weeks and haemoglobin concentration ≥ 90 g/L, to examine associations between ZPP and *Plasmodium* infection, HIV, and α+-thalassemia. We assessed the diagnostic utility of ZPP (measured in whole blood and erythrocytes) and Erythrocyte Protoporphyrin(EP), either alone or in combination with haemoglobin, in detecting ID (plasma ferritin <15µg/L).

Individually, whole blood ZPP (WB-ZPP), erythrocyte ZPP (E-ZPP) and EP had limited ability to discriminate between women with and without ID. Combining each of these markers with haemoglobin had no additional diagnostic value. Conventional cutpoints for WB-ZPP (>70 µmol/mol heme) resulted in gross estimates of the prevalence of ID. E-ZPP may have limited value to rule out ID when used for screening in conditions with a low prevalence (e.g. 10%). ZPP is of unreliable diagnostic utility when discriminating between pregnant women with and without ID. ZPP measurements in washed erythrocytes are best.

Submitted for publication

Introduction

Zinc protoporphyrin (ZPP) indicates iron-deficient erythropoiesis. It can be measured instantly and at low assay cost using portable hematofluorometers, and it has been used as a screening marker to manage iron deficiency in children and pregnant women, (NCCLS, 1996), (WHO/CDC, 2007) with the advantage that values remain stable throughout gestation in women with adequate iron nutrition. (Ron B. Schifman, Thomasson, & Evers, 1987), (Romslo, Haram, Sagen, & Augensen, 1983), (Harthoorn-Lasthuizen, Lindemans, & Langenhuijsen, 2000) ZPP has also been recommended to be used in combination with haemoglobin concentration in surveys to assess population iron status. (NCCLS, 1996) However, we have concerns about gross discrepancies of reported prevalence estimates for iron deficiency obtained by ZPP and circulating ferritin concentrations. For example, in Kenyan children, this prevalence was 80% versus 41% (after correction for inflammation) for ZPP >80 µmol/mol heme and ferritin concentration <12 µg/L, respectively,(Grant et al., 2012) whilst corresponding values were 56% versus 40% for Tanzanian children without *Plasmodium* infection. (Rebecca J. Stoltzfus et al., 1997)

Non-heme protoporphyrin includes both ZPP and free erythrocyte protoporphyrin (FEP, i.e. the metal-free form that occurs naturally in erythrocytes). If the supply of iron is insufficient or when iron utilization is impaired (e.g. anemia of chronic disease), zinc is used in the biosynthetic pathway of heme instead of iron, resulting in the formation of ZPP. Thus increased ZPP concentrations in whole blood or erythrocytes reflect depleted iron stores and a decrease in circulating iron in the bone marrow. (NCCLS, 1996) (WHO/CDC, 2007) Contrary to heme, ZPP and FEP fluoresce at 555-780 nm when exited at 408 nm. Because the intensity of the fluorescent signal is proportional to the molar ratio of ZPP to haemoglobin, hematofluorometer measurements should theoretically not be influenced by volume of the blood sample, pregnancy-induced hemodilution or whether it is determined in fresh whole blood or in erythrocytes. In practice, however, measurement in washed erythrocytes yields more valid results because washing removes haemoglobin breakdown products such as bilirubin or other serum constituents (e.g. riboflavin) that fluoresce at the same wavelength as the porphyrins. (Hastka, Lasserre, Schwarzbeck, Strauch, & Hehlmann, 1992)

In addition to being raised in iron deficiency, ZPP can be elevated by other factors causing an inadequate supply of iron to erythroblasts (inflammation), increased erythropoiesis (hemolysis, sickle cell anemia, thalassemia), or disturbances in the heme synthetic pathway (lead poisoning). (NCCLS, 1996)

ZPP is the predominant form of non-heme protoporphyrin in normal erythrocytes. (WHO/CDC, 2007)In many disorders, however, the ratio of ZPP and FEP is highly variable because of the high FEP content in reticulocytes. Using acid extraction, chelated zinc can be liberated from ZPP, yielding a larger pool of metal-free erythrocyte protoporphyrin (henceforth referred to as erythrocyte protoporphyrin, EP). (NCCLS, 1996)

We conducted a study in pregnant women, with the aim to examine associations between ZPP and disorders that are common in Africa (*Plasmodium* infection, HIV infection, α+-thalassemia). In addition, we assessed the diagnostic utility of ZPP (measured in whole blood and erythrocytes) and EP, either alone or in combination with haemoglobin concentration, in detecting iron deficiency defined as plasma ferritin <15 μg/L.

Subjects and Methods

Study population: For this study, we used samples collected at baseline for a randomized controlled trial to investigate the safety and efficacy of iron supplementation in Kenyan pregnant women (Chapter 2). The study (www.ClinicalTrials.gov:NCT01308112) received ethical clearance in Kenya and England; written consent was obtained from all participating women. Fieldwork was conducted from October 2011 to October 2012 in a rural area highly endemic for malaria in western Kenya. A surveillance system was set up to detect pregnancies in the late stage of the first trimester to early stage of second trimester. Pregnancy was confirmed and gestational age was determined by urine test and ultrasound examination respectively. Immediately upon confirmation of pregnancy, women received preventive chemotherapy with albendazole and praziquantel against helminth infections and intestinal schistosomiasis to prevent severe anaemia.

At the second visit to the research clinic, 14─21 days after the initial visit, we collected a venous blood sample to measure haemoglobin concentrations (HemoCue301, Ängelholm, Sweden), and ZPP in whole blood and washed erythrocytes (both in duplicate; Aviv 206D, Lakewood NJ, USA). Erythrocytes were stored in DNA-stabilizing buffer (AS1, Qiagen, Valencia, CA) and plasma in liquid nitrogen and dry ice until analysis.

Women were included when aged 15─45 years; consent had been obtained; likely to be available for study until 1 month after delivery and planning to deliver in the pre-designated health facility. They were excluded when having obvious mental retardation or a metabolic disorder, a medical history of sickle cell anemia, epilepsy, diabetes, an obstetric history suggestive of eclampsia or pre-eclampsia; carrying multiples; gestational age at the second visit was <13weeks or >23weeks; no venous blood was collected, or haemoglobin concentration was <90g/L.

To wash erythrocytes, blood samples were centrifuged (8min, 600xg), plasma was removed and replaced with an equal volume of phosphate buffered saline (Medicago, Uppsala, Sweden; catalogue no. 09-2051-100) in 100 mL deionized water. Following renewed centrifugation (8min, 600×g), the supernatant and buffy coat were discarded. ZPP was measured in the washed erythrocytes.

Washed erythrocytes (20μL) were transferred to 2mL cryotubes prefilled with 0.3mL solution 0.9% saline and 50% Celite (Sigma-Aldrich, catalogue 525235, St. Louis, MO). Aliquots were stored in liquid nitrogen and dry ice until analysis for EP concentration in

The Netherlands.

Laboratory analyses: ZPP content was measured by hematofluorometer (Aviv 206D, Lakewood Township, NJ, USA). Control samples at low, medium, and high levels (AVIV) were run after every 30 readings while two level calibration (Aviv; low, high) samples were run twice per year. To measure EP, washed erythrocytes (20µL) were transferred to 2mL cryotubes prefilled with 0.3mL solution 0.9% saline and 50% Celite (Sigma-Aldrich, catalogue 525235, St. Louis, MO). Protoporphyrins were extracted and separated from heme as described (Piomelli, 1977) and determined quantitatively by a spectrofluorometer using a protoporphyrin IX standard (Sigma-Aldrich, catalogue 282820).

We measured iron markers (plasma concentrations of ferritin, soluble transferrin receptor, and transferrin), inflammation markers (plasma concentrations of C-reactive protein [CRP] and α1-acid glycoprotein [AGP]), vitamins (plasma concentrations of folate and vitamin B12) and a marker of hemolysis (plasma lactate dehydrogenase concentration) on a Beckman Coulter UniCel DxC 880i analyzer as per manufacturer's instructions.

Plasmodium antigenaemia was assayed by dipstick tests (Access Bio Inc., Somerset, NJ, USA; CareStart, catalogue G0151 and G0171) that can detect *P. falciparum*-specific histidine-rich protein-2 (HRP2), *Plasmodium* lactate dehydrogenase (pLDH) specific to either *P. falciparum* or to non-*falciparum* species, i.e. *P. ovale*, *P. malariae* or *P. vivax*. Whereas HRP2-based tests detects current or recent *P. falciparum* infection, pLDH-based tests only indicate current infection.(Makler et al., 1998)(Piper et al., 1999)(Moody, 2002) HIV infection was assayed using antibody tests (Alere, Waltham, MA; confirmed by Unigold; Trinity Biotech, Bray, Ireland and/or Bioline; Pantech, Umhlanga, South Africa).

We determined α+-thalassaemia genotype by polymerase chain reaction;(Veenemans et al., 2008)(Veenemans, Jansen, et al., 2011) because of practical reasons, we could perform this analysis only in the first 213 successively recruited women.

Definitions: We used the following definitions: anemia: haemoglobin concentration <110 g/L;(WHO, 2011a) iron deficiency: plasma ferritin concentration <15 µg/L;(WHO, 2011b) inflammation: plasma concentrations of CRP >10 mg/L(Nielsen et al., 1990) and/or AGP >1 g/L;(Filteau et al., 1993) gravidity: the number of times a woman reports to have been pregnant, regardless of the outcome of these pregnancies, with twins and other multiple births counted as 1, and including the current pregnancy; *Plasmodium* infection was defined as any infection: one or more positive results for the presence of pLDH or HRP2 in plasma (dipstick tests), *Plasmodium* pigment upon histological examination of placental biopsies), or *P. falciparum* DNA (PCR test); current or recent *P. falciparum* infection (similarly, but restricted to results from dipstick tests and PCR tests); or current infection (similarly, but restricted to results from PCR tests).

Statistical analysis: Data were analyzed using SPSS version 22 (IBM, Armonk, NY). Data

were described as means (SDs), medians (25th- and 75th-percentiles) or prevalence values in the overall population, or in women without inflammation (plasma concentrations CRP ≥10 mg/L or AGP ≥1.0 g/L), HIV infection or *Plasmodium* infection.

Univariate linear analysis was used to explore associations between ZPP (log-transformed) and iron markers (including anemia), factors known or suspected to be associated with iron status (age, gestational age, gravidity, plasma concentrations of folate and vitamin B12), inflammation markers, infections (*Plasmodium* infection, HIV infection) and other disorders suspected to be associated with ZPP (α+-thalassemia, plasma concentrations of bilirubin and LDH). Multivariate linear analysis with a backward elimination procedure was used to derive a parsimonious model of factors that were independently associated with ZPP.

We assessed the diagnostic performance of ZPP (both in whole blood and erythrocytes) in detecting iron deficiency (plasma ferritin concentration <15 μg/L). Because plasma ferritin concentration reacts as an acute phase protein, we restricted these analyses to women without inflammation, *Plasmodium* infection or HIV infection.

Combinations of ZPP and haemoglobin concentration may have better ability than single markers to distinguish between children with and without iron deficiency. Thus we used scatter plots and logistic discriminant analysis to assess the diagnostic performance of ZPP combined with haemoglobin concentration. Receiver Operating Characteristics (ROC) curves were produce using the probability of iron deficiency as a function of ZPP and haemoglobin concentration as a quantitative test outcome. Diagnostic performance was assessed by visual inspection of these curves and by assessing differences in the Area-Under-the-Curve (AUC) with corresponding p-values. Similar analyses were performed for EP concentration.

We subsequently assessed the diagnostic performance of ZPP as a dichotomized variable, with various thresholds. First, we used threshold values for ZPP of 70 μmol/mol and 40 μmol/mol heme (NCCLS, 1996), (WHO/CDC, 2007), depending on whether the assay is conducted in whole blood or washed erythrocytes. The whole blood ZPP value of 70 μmol/mol heme (NCCLS, 1996), (WHO/CDC, 2007), (WHO, UNICEF, & UNU, 2001) (2.7 μg/g Hb) was derived from the 95% upper limit of the reference values for women and children participating in the US National Health and Nutrition Examination Survey (NHANES) II, after excluding individuals with anemia, low transferrin saturation and elevated blood lead concentrations. The cut-point for erythrocyte ZPP of 40 μmol/mol heme is based on several small studies comparing iron-deficient and iron-replete individuals. (Hastka et al., 1992), (Hastka, Lasserre, Schwarzbeck, & Hehlmann, 1994) Lastly, we calibrated ZPP cutpoints to produce unbiased estimates of the prevalence of iron deficiency using methods described elsewhere (Talsma, Verhoef, Brouwer, Mburu-de Wagt, Hulshof and Melse-Boonstra: submitted).

Results

Plasmodium infection was highly prevalent but contained, as judged by low plasma concentrations of inflammation markers, LDH and bilirubin **(Table 1)**. One-fifth of women had HIV infection, and one-third had inflammation. They had poor iron status, with 37% being anemic, 53% being iron deficient and 27% being iron replete. Iron status was uncertain in 20% of women because they had inflammation with plasma ferritin concentrations in the normal range, which indicates either iron repletion or iron deficiency with elevated ferritin concentrations due to inflammation.

The prevalence of iron deficiency as defined by whole blood ZPP >70 μmol/mol heme, erythrocyte ZPP >70 μmol/mol heme and erythrocyte ZPP >40 μmol/mol heme was 73%, 23.4% and 46.4%, respectively.

α+-thalassemia was common, with 41% and 8% of women being heterozygous and homozygous, respectively.

Both in univariate and multivariate analysis, whole blood and erythrocyte ZPP were associated with iron deficiency, plasma concentrations of soluble transferrin receptor and folate **(Table 2)**. For example, in univariate analysis, each increment in plasma concentrations of soluble transferrin receptor by 1 mg/L was associated with a 32% increase in whole blood ZPP. Although whole blood ZPP seemed associated with gestational age in multivariate analysis, such an association was not found for erythrocyte ZPP. Being multigravid was independently associated with ZPP. Both univariate and multivariate analysis suggested that bilirubin concentration was associated with reduced erythrocyte ZPP. Neither *Plasmodium* infection nor inflammation were associate with ZPP, regardless of the case definition for *Plasmodium* infection, whether assessed in whole blood or erythrocytes, or whether examined by univariate or multivariate analysis. We also found no evidence that ZPP was associated with α+-thalassemia genotype. In univariate analysis, ZPP was associated with plasma vitamin B12 concentration, but this association disappeared in multivariate analysis.

In the restricted population (i.e. women without inflammation, *Plasmodium* infection or HIV infection), whole blood ZPP, erythrocyte ZPP and EP concentration had only modest ability to discriminate between women with and without iron deficiency (Figure 1, panels A and B). Erythrocyte ZPP scored best of these three markers, with an AUC of 0.73 **(Figure 1**, footnote). Haemoglobin concentration also performed poorly when used individually, and had no added diagnostic value when used in combination with whole blood ZPP, erythrocyte ZPP or EP concentration (Figure 1, panels C-H).

Table 1: Characteristics of the populations studied

Characteristic	All women		Women without either inflammation, any *Plasmodium* infection or HIV infection	
n	470		175	
Age				
< 20 years	20.6%	(97)	17.1%	(30)
≥ 20 years	79.4%	(373)	82.9%	(145)
Gestational age				
13-14 weeks	9.1%	(43)	6.3%	(11)
15–16 weeks	25.7%	(121)	25.1%	(44)
17–18 weeks	29.6%	(139)	26.3%	(46)
19–21 weeks	24.5%	(115)	30.3%	(53)
22–25 weeks	11.1%	(52)	12.0%	(21)
Gravidity				
Primigravidae	18.1%	(85)	17.7%	(31)
Secundigravidae	19.6%	(92)	16.0%	(28)
Multigravidae	62.3%	(293)	66.3%	(116)
Plasma CRP concentration, mg/L	4.3	[2.1–10.4]	0	
Plasma AGP concentration, g/L	0.72	[0.60–0.93]	0	
Inflammation				
Plasma CRP concentration ≥ 10 mg/L	26.8%	(126)	0	
Plasma AGP concentration ≥ 1 g/L	18.1%	(85)	0	
Plasma concentrations of CRP ≥10 mg/L or AGP ≥ 1.0 g/L	32.3%	(152)	0	
HIV infection	21.1%	(99) [2]	0	
Plasmodium infection				
Any *Plasmodium* infection, [3] by dipstick or PCR	37.2%	(175)	0	

Table 1: Characteristics of the populations studied

Current or recent *P. falciparum* infection, by either HRP2- or LDH-based dipstick	19.5%	(91) [3]	0	
P. falciparum, by PCR	34.7%	(163)	0	
Hb concentration, g/L	113.2	(11.4)	115.7	(10.8)
Anemia (haemoglobin concentration <110 g/L)	37.2%	(175)	25.7%	(45)
Plasma ferritin concentration, µg/L	13.9	[8.2–29.2]	10.6	[7.0–18.5]
Iron status				
Iron deficient (plasma ferritin concentration <12 µg/L)	52.8%	(248)	64.6%	(113)
Iron replete (plasma ferritin concentration ≥12 µg/L, without inflammation)	27.2%	(128)	35.4%	(62)
Uncertain (plasma ferritin concentration ≥12 µg/L, with inflammation)	20.0%	(94)	0	
Whole blood ZPP, µmol/mol heme	90	[68–121]	87	[63–121]
Whole blood ZPP > 70 µmol/mol heme	73.4%	(345)	69.1%	(121)
Erythrocyte ZPP, µmol/mol heme	36	[20–66]	42	[20–74]
Erythrocyte ZPP > 70 µmol/mol heme	23.4%	(110)	28.6%	(50)
Erythrocyte ZPP > 40 µmol/mol heme	46.4%	(218)	52.6%	(92)
EP concentration, µg/L	203	[117–428] [2]	224	[130–476] [4]
Plasma sTfR concentration, mg/L	1.94	[1.48–2.63]	1.87	[1.36–2.62]
Plasma transferrin concentration, g/L	3.12	(0.56)	3.21	(0.54)
Plasma folate concentration, µg/L	6.91	[5.45–9.39] [3]	6.55	[5.21–8.87] [4]
Plasma vitamin B_{12} concentration, pmol/L	425	[311–651] [3]	413	[307–638] [4]
Plasma bilirubin concentration, µmol/L	6.9	[4.9–9.4] [3]	7.1	[4.8–9.4] [4]
α^+-thalassemia genotype				
Normal	51.2%	[109/213] [5]	48.4%	[44] [6]
Heterozygote	41.3%	[88/213] [5]	42.9%	[39] [6]
Homozygote	7.5%	[16/213] [5]	8.8%	[8] [6]

Table 1: Characteristics of the populations studied

Values indicate mean (SD), median [25th- and 75th-percentile] or % (n)
AGP: α_1-acid glycoprotein protein; CRP: C-reactive protein; EP: erythrocyte protoporphyrin; HRP2: *P. falciparum*-specific histidine-rich protein-2; LDH: *P. falciparum*-specific lactate dehydrogenase; sTfR: soluble transferrin receptor; ZPP:H: zinc protoporphyrin:haem
[1] Only one participant had infection by a *Plasmodium* species other than *P. falciparum*; missing values resulted in n=468, [2] n=466, [3] n=174, [4] 213 [5] and n=91 [6]

TABLE 2: Factors associated with ZPP measured in whole blood or erythrocytes

	Univariate analysis		Multivariate analysis	
	Difference (95% CI)	**p**	**Difference (95% CI)**	**p**
Whole blood ZPP				
Gravidity		0.68		0.04
Primigravidae	[Reference]		[Reference]	
Secundigravidae	–2.3% (–15.6% to 13.1%)		0.5% (–9.6% to 11.8%)	
Multigravidae	2.7% (–8.9% to 15.7%)		9.4% (0.1% to 19.6%)	
Gestational age		0.87		0.008
13–14 weeks	[Reference]		[Reference]	
15–16 weeks	5.4% (–11.3% to 25.3%)		6.7% (–5.6% to 20.7%)	
16–18 weeks	6.1% (–10.4% to 25.7%)		–4.0% (–14.9% to 8.3%)	
19–21 weeks	2.0% (–14.3% to 21.3%)		–7.3% (–18.2% to 5.1%)	
22–25 weeks	–1.0% (–19.0% to 20.9%)		–11.0% (–23.0% to –2.8%)	
Anaemia	64% (51.2% to 77.8%)	<0.001	33.5% (23.9% to 43.9%)	<0.001
Iron deficiency (plasma ferritin concentration <15 μg/L)	26.3% (15.8% to 37.8%)	<0.001	16.6% (9.1% to 24.6%)	<0.001
Plasma sTfR concentration, mg/L	31.9% (28.1% to 35.9%)	<0.001	24.5% (20.6% to 28.5%)	<0.001
Plasma transferrin concentration, g/L	30.9% (21.4% to 41.2%)	<0.001	–	
Plasma folate concentration, 10 μg/L	6.1% (–7.2% to 21.4%)	0.39	13.5% (3.0% to 25.2%)	0.01
Plasma vitamin B_{12} concentration, 100 pmol/L	–1.8% (–3.4% to –0.2%)	0.03	–	
Plasma total bilirubin concentration, μmol/L	0.7% (–0.4% to 1.8%)	0.20	–	

TABLE 2: Factors associated with ZPP measured in whole blood or erythrocytes

α^+-thalassemia genotype		0.67		
Normal	[Reference]			
Heterozygote	–3.8% (–16.1% to 10.2%)		–	
Homozygote	–10.0% (–30.2% to 16.1%)		–	
Plasmodium infection				
Any *Plasmodium* spp., by any dipstick or PCR	8.0% (–1.6% to 18.4%)	0.10	–	
Current or recent *P. falciparum* infection [1]	5.1% (–6.2% to 17.8%)	0.39	–	
P. falciparum, by dipstick [1] or PCR	8.1% (–1.4% to 18.6%)	0.10	–	
P. falciparum, by PCR	9.9% (0.1% to 20.7%)	0.05	–	
HIV infection	5.2% (–14.9% to 6.2%)	0.37	–	
Plasma CRP concentration, mg/L	0.2% (–0.1% to 0.5%)	0.27	–	
Plasma AGP concentration, g/L	21.5% (3.2% to 43.0%)	0.02	–	
Inflammation [3]				
Plasma CRP concentration ≥10 mg/L	5.0% (–5.1% to 16.1%)	0.34	–	
Plasma AGP *concentration* ≥1.0 g/L	14.0% (–1.5% to 27.9%)	0.03	–	
Plasma CRP concentration ≥10 mg/L or AGP ≥1.0 g/L	6.6% (–3.1% to 17.2%)	0.19	–	
Erythrocyte ZPP [1]				
Gravidity		0.13		0.007
Primigravidae	[Reference]		[Reference]	
Secundigravidae	23.4% (–4.3% to 59.2%)		21.7% (–0.5% to 49.0%)	
Multigravidae	23.2% (0.0% to 51.7%)		31.8% (–11.0% to 56.6%)	
Gestational age		0.48		
13–14 weeks	[Reference]			
15–16 weeks	–4.6% (–29.4% to 29.0%)		–	
16–18 weeks	13.9% (–15.3% to 53.2%)		–	
19–21 weeks	6.1% (–21.6% to 43.8%)		–	
22–25 weeks	–5.0% (–33.1% to 34.8%)		–	
Anaemia	94.6% (–67.5% to 126.2%)	<0.001	47.1% (27.5% to 69.7%)	<0.001

TABLE 2: Factors associated with ZPP measured in whole blood or erythrocytes

Iron deficiency (plasma ferritin concentration <15 μg/L)	87.6% (–62.1% to 117.1%)	<0.001	53.4% (34.8% to 74.6%)	<0.001
Plasma sTfR concentration, mg/L	48.3% (–40.0% to 57.1%)	<0.001	35.1% (27.2% to 43.6%)	<0.001
Plasma transferrin concentration, g/L	85.3% (–63.2% to 110.4%)	<0.001		
Plasma folate concentration, 10 μg/L	0.6% (–1.8% to 2.9%)	0.64	24.0% (3.0% to 49.3%)	0.02
Plasma vitamin B12 concentration, 100 pmol/L	–4.4% (–9.0% to 0.5%)	0.08	–	
Plasma total bilirubin concentration, μmol/L	–3.0% (–4.8% to –1.1%)	0.002	–3.2% (–4.7% to –1.7%)	<0.001
α^+-thalassemia genotype		0.87		
Normal	[Reference]			
Heterozygote	–5.9% (–25.2% to 18.3%)		–	
Homozygote	–4.7% (–37.9% to 46.2%)		–	
Plasmodium infection				
Any *Plasmodium* spp., by any dipstick or PCR	–3.0% (–17.5% to 14.1%)	0.71	–	
Current or recent *P. falciparum* infection [1]	–10.2% (–26.3% to 9.5%)	0.29	–	
P. falciparum, by dipstick, [1] or PCR	–3.0% (–17.5% to 14.1%)	0.71	–	
P. falciparum, by PCR	–1.4% (–16.3% to 16.3%)	0.87	–	
HIV infection	–8.5% (–24.6% to 11.0%)	0.37	–17.9% (–29.9% to –4.0%)	0.01
Plasma CRP concentration, mg/L	–0.1% (–0.6% to 0.5%)	0.82	–	
Plasma AGP concentration, g/L	1.4% (–24.0% to 35.2%)	0.92	–	
Inflammation [3]				
Plasma CRP concentration ≥10 mg/L	–2.1% (–18.0% to 16.8%)	0.81	–	
Plasma AGP *concentration* ≥1.0 g/L	3.2% (–15.8% to 26.5%)	0.76	–	
Plasma CRP concentration ≥10 mg/L or AGP ≥1.0 g/L	–4.2% (–19.0% to 13.2%)	0.61	–	

AGP: α_1-acid glycoprotein; CRP: C-reactive protein; HRP2: *P. falciparum*-specific histidine-rich protein-2; LDH: *P. falciparum*-specific lactate dehydrogenase; sTfR: soluble transferrin receptor; ZPP: zinc protoporphyrin

[1] Either HRP2- or LDH-based dipstick

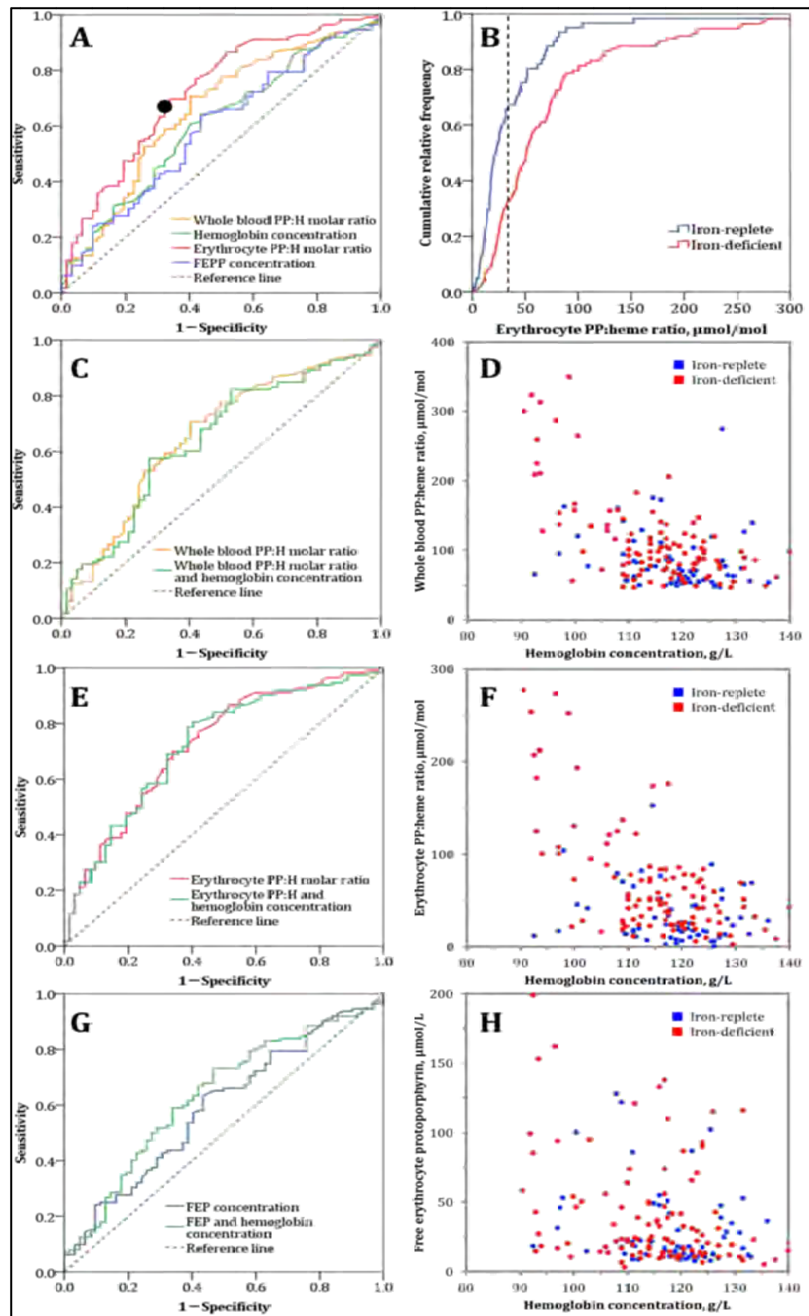

Figure 1: Ability of erythrocyte protoporphyrin, either alone or combined with haemoglobin concentration, to discriminate between pregnant women with and without iron deficiency (see next page for details of panels A to H).

Panel A: Receiver operating characteristics (ROC) curve for various blood markers, used alone, to discriminate between iron-deficient and iron-replete women. Area-under-the-curve, AUC (95% CI): whole blood ZPP: 0.66 (0.57–0.74); erythrocyte ZPP: 0.73 (0.65–0.80); EP: 0.59 (0.50–0.68); Haemoglobin concentration: 0.61 (0.52–0.70).

Panel B: Cumulative relative frequency distribution of erythrocyte ZPP, the best indicator when used as a single test (Panel A), to discriminate between iron-deficient and iron-replete women.

The black circle in Panel A and the dotted black line in Panel B indicate the erythrocyte PP:heme ratio of 34 μmol/mol whereby the total diagnostic error is minimized at a prevalence of iron deficiency of 50%.

Panels C, E and G: ROC curves for whole blood ZPP, erythrocyte ZPP and EP, either alone or each in combination with haemoglobin concentration. AUC (95%CI): combined whole blood ZPP with haemoglobin concentration: 0.64 (0.56–0.73); combined erythrocyte ZPP with haemoglobin concentration: 0.72 (0.64–0.80); combined EP with haemoglobin concentration: 0.64 (0.55–0.73).

Panel D: bivariate scatterplot for whole blood protoporphyrin and haemoglobin concentration, by iron status;

Panel F: bivariate scatterplot for erythrocyte protoporphyrin and haemoglobin concentration, by iron status;

Panel H: bivariate scatterplot for free erythrocyte protoporphyrin and haemoglobin concentration, by iron status.

Grey dashed lines in ROC curves indicate a ‘worst’ possible test, which has no discriminatory value and an area-under-the-curve (AUC) of 0.5. An ideal marker would have a curve that runs from the lower-left via the upper-left to the upper-right corner, yielding an AUC of 1.0.

At a cut-point of 70 μmol/mol heme, whole blood ZPP had sensitivity and specificity of 78% and 47%, respectively, of detecting iron deficiency **(Table 3)**. This low specificity results in low positive predictive values (i.e. the probability of a test result correctly indicating iron deficiency) and gross overestimates of the prevalence of iron deficiency, particularly when the true prevalence is low. For example, at a hypothetical prevalence of 10%, the positive predictive value would be 14%, and the estimated prevalence would be 56% (Table 3).

Table 3: Diagnostic performance of ZPP, measured in whole blood or erythrocytes, in detecting iron deficiency [1] at hypothetical prevalence values (50%, 30% and 10%) for iron deficiency [2]

Cut-point	Justification	Sensitivity	Specificity	True prevalence	PPV	NPV	Estimated prevalence
Whole blood ZPP, µmol/mol heme							
>70	Cutpoint corresponding to 2.7 µg/g, which has been selected to define the presence of iron-deficient erythropoiesis(E. L. Senga, Koshy, & Brabin, 2012)	78%	47%	50%	59%	68%	66%
				30%	39%	83%	61%
				10%	14%	95%	56%
>49	Cutpoint selected for screening, with a sensitivity of 95%	95%	3.2%	50%	50%	39%	96%
				30%	30%	60%	96%
				10%	10%	85%	97%
>85	Cutpoints selected to yield unbiased estimates of the prevalence of iron deficiency (Figure 2; top panels)	63%	63%	50%	63%	63%	50%
>102		43%	76%	30%	43%	76%	30%
>160		13%	90%	10%	13%	90%	10%
Erythrocyte ZPP, µmol/mol heme							
>70	Recommended range to indicate iron deficiency in the absence of infection(WHO, 2001)	38%	87%	50%	75%	58%	26%
				30%	56%	77%	21%
				10%	25%	93%	16%
>40	Cutpoint recommended to distinguish between iron deficient erythropoiesis and iron sufficient erythropoiesis(WHO/CDC, 2007)	64%	68%	50%	66%	65%	48%
				30%	46%	81%	42%
				10%	18%	94%	35%
>11	Cutpoint selected for screening, with a sensitivity of 95%	95%	19%	50%	54%	80%	88%
				30%	34%	90%	85%
				10%	12%	97%	82%
>34	Cutpoints selected to yield unbiased estimates of the prevalence of iron deficiency (Figure 2; top panels)	67%	67%	50%	67%	67%	50%
>52		48%	77%	30%	48%	77%	30%
>81		27%	92%	10%	27%	92%	10%

Table 3: Diagnostic performance of ZPP, measured in whole blood or erythrocytes, in detecting iron deficiency [1] at hypothetical prevalence values (50%, 30% and 10%) for iron deficiency [2]

PPV: positive predictive value; NA: not applicable; NPV: negative predictive value. Cutpoint values for ZPP (column 1) expressed as µmol/mol heme.
[1] Defined as serum ferritin concentration <15 µg/L; [2] analysis restricted to women without inflammation, *Plasmodium* infection or HIV infection

Erythrocyte ZPP >70 µmol/mol heme had much better specificity (87%) but a low sensitivity (38%), whilst values >40 µmol/mol heme yielded intermediate values for sensitivity and specificity (64% and 68%, respectively; values obtained from ROC curve analysis, Figure 1). With our sensitivity and specificity values, unbiased estimates for hypothetical prevalence values of 50%, 30% or 10% would be produced at whole blood ZPP cutpoints of 85 µmol/mol heme, 102 µmol/mol heme and 160 µmol/mol heme, respectively. Corresponding cutpoints for erythrocyte ZPP would be 34 µmol/mol heme, 52 µmol/mol heme and 81 µmol/mol heme.

Even at a sensitivity of 95%, as may be applied for screening purposes, a negative test result obtained as whole blood ZPP ≤ 49 µmol/mol heme would be insufficient to rule out iron deficiency, because negative predictive values (i.e. the probability of a test result correctly indicating absence of iron deficiency) would only be 63%, 76% and 85% at prevalence values of 50%, 30% or 10%, respectively (Table 3).

At a prevalence of 19%, erythrocyte ZPP ≤ 11 µmol/mol heme (corresponding to a sensitivity of 95%) would yield 97% probability of ruling out iron deficiency, resulting in iron deficiency being excluded in 18% (=100%–82%; Table 3) of women. At higher prevalence values, this sensitivity is insufficient to rule out iron deficiency as judged by negative predictive values.

Discussion

In the population studied, both whole blood ZPP and erythrocyte ZPP were mostly determined by iron markers including anemia, whilst inflammation, *Plasmodium* infection and HIV infection played only minor roles. When used individually, whole blood ZPP, erythrocyte ZPP and EP had limited ability to discriminate between women with and without iron deficiency, whilst combining each of these markers with haemoglobin concentration had no additional diagnostic value. This limited diagnostic value was also apparent when using dichotomized variables for whole blood ZPP and erythrocyte ZPP. Conventional cut off points for whole blood ZPP (>70 μmol/mol heme) can result in gross estimates of the prevalence of iron deficiency, particularly when the true prevalence is low. Erythrocyte ZPP may have limited value to rule out iron deficiency when used for screening in conditions with a low prevalence (e.g. 10%).

Our study was designed to investigate the diagnostic utility of ZPP in a malaria endemic, resource-poor setting that has a high prevalence of α+-thalassemia and other haemoglobin disorders. This is particularly relevant because the World Health Organization (WHO) no longer recommends that children in malaria-endemic areas should receive universal iron supplementation; instead, children should be screened and supplementation should be restricted to those with iron deficiency. (WHO, 2007) Unfortunately, however, there are no simple, rapid tests available to implement this recommendation under field conditions. The WHO has pointed to the need to validate ZPP in malarious areas. (WHO, 2007)

Iron status is commonly monitored by haemoglobin concentration, hematocrit, and plasma ferritin concentration. (Romslo et al., 1983) Whereas cutpoints for these markers have been established in non-pregnant individuals, they are probably unreliable in pregnancy because these markers are affected by plasma expansion. By contrast, ZPP content can be expressed as a molar ratio to heme, which should theoretically be independent of hemodilution. Thus ZPP has been proposed as a preferred marker for iron status in pregnancy. (Ron B. Schifman et al., 1987) Furthermore, erythrocyte ZPP was reported to be a sensitive and specific indicator in the detection of iron deficiency in non-pregnant women and young children aged 1-5 years in the United States and in areas where the prevalence of elevated blood lead concentration is not high. (Mei, Parvanta, Cogswell, Gunter, & Grummer-Strawn, 2003)

A strong point in our study was ZPP measured both in whole blood and in washed erythrocytes. We strictly adhered to protocol, conducted measurements in duplicate, and ran control samples as per instructions by the manufacturer. Lead exposure in our study population was probably very low. We ensured comprehensive assessment of iron status in all the participants using different markers as recommended by various guidelines. (WHO/CDC, 2007), (British Committee for Standards in Haematology, 2011) The iron markers, inflammation markers, and hemolysis markers reported in this study, as well as EP

concentrations, were assessed independently by laboratories that were not involved in the fieldwork. Further, were probably detected most asymptomatic *Plasmodium* infections using most of the known methods of assessing *Plasmodium* infection during pregnancy (dipsticks, PCR, and histopathology of placental biopsies). The high acceptance rate for HIV testing (98%) enabled us to study the diagnostic performance of ZPP in the presence of HIV as a chronic disease. We also performed statistical analyses restricted to women without inflammation, *Plasmodium* infection or HIV infection because plasma ferritin concentration reacts as an acute phase protein.

Our study had several limitations. First, we could not assess α+-thalassemia status of all the participants due to practical reasons. However, we found no associations between ZPP and α+-thalassemia in the women in whom α+-thalassemia genotype was established. Secondly, we studied pregnant women. Although we do not expect that the diagnostic performance of ZPP is better in children, we cannot exclude this possibility. In addition, other conditions and genetic disorders such as sickle cell anemia and G6PD that may influence the diagnostic utility of ZPP were not studied.

Several other studies also found that detection of iron deficiency by ZPP leads to marked overestimates of the prevalence of iron deficiency. (Grant et al., 2012), (Rebecca J. Stoltzfus et al., 1997), (Asobayire, Adou, Davidsson, Cook, & Hurrell, 2001), (Crowell, Ferris, Wood, Joyce, & Slivka, 2006) This may have been due at least in part to inflammation or infection. (WHO/CDC, 2007), (WHO, 2007) Several studies have shown that ZPP content in whole blood can be markedly higher than values measured in washed erythrocytes. Various reasons including interference by bilirubin have been cited. (Hastka et al., 1992), (Hastka et al., 1994), (R B Schifman & Finley, 1981), (Janousek, Rosa, Jirova, & Kejlova, 2010), (Buhrmann, Mentzer, & Lubin, 1978) Our findings show, however, that this overestimation is also in large part due to low specificity to ZPP at conventionally used cutpoints, whether measured in whole blood or in erythrocytes. Selection of cut-points for dichotomized diagnostic tests should depend on the diagnostic aims. When used as an initial screening marker to manage iron deficiency, ZPP should be highly sensitive, with a view to rule out iron deficiency (i.e. a high negative predictive value, no longer needing work-up) or to identify individuals who are iron deficient or who need further diagnostic work-up. Our findings show, however, that a high sensitivity will inevitably be accompanied by a low specificity, and thus an unacceptably low negative predictive value. For example, in Table 3, we have shown that for whole blood ZPP, a sensitivity of 95% can be obtained with a cutpoint of 49 μmol/mol heme. However, the corresponding specificity is 3.5%, resulting in negative predictive values that will be unacceptably low with true prevalence values for iron deficiency in most conditions. As an exception, erythrocyte ZPP may have limited value to rule out iron deficiency in populations with low prevalence of iron deficiency (Table 3). These results were obtained in a restricted dataset, with exclusion of women with inflammation, *Plasmodium* infection or HIV infection. The diagnostic performance of ZPP in an unrestricted dataset would presumably have been even worse.

When applied to estimate the prevalence of iron deficiency, ZPP cutpoints can be calibrated to produce unbiased estimates.

In this population, both whole blood ZPP and erythrocyte ZPP have little diagnostic utility as a screening marker to manage iron deficiency, whether used as single tests or combined with haemoglobin concentration. When used to estimate the prevalence of iron deficiency, conventional cutpoints for whole blood ZPP can result in marked overestimates.

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Chapter 4

Antenatal iron supplementation and serum concentrations of non-transferrin bound iron in pregnant women in a malaria-endemic region of Kenya: a randomised controlled trial

Abstract

Background: Iron supplementation can lead to increased incidence of malaria and other infectious diseases in children, possibly because of non-transferrin bound iron (NTBI). We aimed to measure the influence of ingestion of a supplement with iron (60 mg, as ferrous fumarate) on serum NTBI concentrations in pregnant Kenyan women. We also assessed the influence of iron status, gravidity, maternal age, *Plasmodium* infection, HIV infection and α+-thalassemia genotype on the serum NTBI response to oral iron supplementation.

Design and methods: Double-blind, randomised intervention trial comparing supplementation with iron versus placebo. Pregnant women (gestation age 13-23 weeks, haemoglobin >90 g/L) ingested the iron supplement. They were offered an optional non-standardized lunch meal approximately 1.5 hours after ingestion. At 3 hours after ingesting the iron supplement, blood was collected in trace elements free tubes. Serum NTBI, iron, and inflammation markers were then assessed. Statistical analysis was done according to a pre-defined plan.

Results: 379 women participated in the study. Compliance was 100%. Intervention groups were similar at baseline except for mild imbalances. NTBI concentrations were similar between groups (mean for both: 0.18 µmol/L; difference, 95%CI: 0.01 µmol/L, –0.03 µmol/L to 0.05 µmol/L). NTBI was present (values ≥0.18µmol/L) in 40.9% (76/186) and 46.1% (89/193) of women in high-dose iron versus low-dose iron groups (difference: 5.3%, –15.0% to 4.7%).

Conclusion: There was no effect of oral ingestion of 60 mg ferrous fumarate on serum NTBI concentration three hours after ingestion; the NTBI response to iron was also not influenced by *Plasmodium* infection, iron deficiency, iron deficiency anaemia or α-thalassaemia. In addition, the effect was not substantially changed by other covariates such as gravidity, maternal age, and HIV infection status.

Submitted for publication

Introduction

Supplementation with ferrous iron salts can lead to an increased incidence of malaria and other infectious diseases in children, (Sazawal et al., 2006) which is possibly mediated by non-transferrin bound iron (NTBI). (WHO, 2007) NTBI does not normally occur in healthy individuals. (Espósito, Epsztejn, Breuer, & Cabantchik, 2002) Supplementation with ferrous salts can transiently produce NTBI **(Table 1)**, although the supportive evidence was obtained from small, mostly non-randomised studies using volunteers with adequate iron status. Although transferrin-mediated uptake is the major route of iron delivery to erythroblasts, reticulocytes and mature erythrocytes can take up NTBI via transferrin-independent mechanisms. (Morgan, 1988) (Egyed, 1988) (Kovar et al., 2006) (Valis et al., 2008) (Sanchez-Lopez & Haldar, 1992) Thus, it would seem likely that *Plasmodium* parasites can access NTBI as a nutritional source of iron for their development and propagation (R J Stoltzfus, 2008; WHO, 2007). Elimination of NTBI is also a therapeutic goal (Brissot, Ropert, Le Lan, Loréal, & Loreal, 2012) because it is thought to play a role in various pathological conditions and also in the production of reactive oxygen species which may contribute to tissue damage.

Little is known about factors that influence the magnitude of the serum NTBI response to iron ingestion. It is plausible that these factors include determinants of iron absorption, since NTBI production probably occurs when the absorption rate of iron and its release into circulation exceeds the rate of iron binding to transferrin. Thus, women may be particularly vulnerable in the second trimester of pregnancy, when iron deficiency is common and non-haem iron absorption may be as high as 25%. (Barrett, Whittaker, Williams, & Lind, 1994) In pregnancy, iron status is also known to be related to gestational age, (Leif Hallberg, 2001) gravidity, and maternal age. *Plasmodium* infection (de Mast, Nadjm, et al., 2009; Andrew M Prentice et al., 2012) and HIV infection can lead to decreased iron absorption, and thus perhaps to a reduced NTBI response. Thalassaemia is characterised by ineffective erythropoiesis and increased plasma iron turnover. (Gafter-Gvili, Prokocimer, Breuer, Cabantchik, & Hershko, 2004) When extreme, this can lead to excessively low hepcidin production, resulting in iron being absorbed at a faster rate than being used for erythropoiesis. Although this is the case in severe forms of β-thalassemia, even without transfusions, (Gardenghi et al., 2007; Musallam, Taher, & Rachmilewitz, 2012) it remains to be established for α+-thalassaemia, which does not result in manifestations other than mild anaemia in homozygotes.

Our main aim was to measure the influence of ingestion of a supplement with iron (60 mg, as ferrous fumarate) on serum NTBI concentrations in pregnant Kenyan women. Additionally, we assessed the influence of iron status, gravidity, maternal age, *Plasmodium* infection, HIV infection and α+-thalassemia genotype on the serum NTBI response to oral iron supplementation.

Table 1: Summary of studies into effect of iron interventions on non-transferrin bound iron (NTBI) concentrations

Reference	Population (sample size)	Intervention	Follow-up/measurements	Key findings	Comments
Hutchinson et al. 2004	UK adult women with anaemia (n=7)	65mg iron as ferrous sulphate given with or without test meal low in inhibitors of iron absorption (white bread, margarine, honey and dilute orange cordial)	Serial collection of serum samples at 30min-intervals for 4hrs NTBI measurement: Gosriwatana et al. with detection by HPLC	NTBI increased over time relative to baseline, whether taken with or without food (both $p<0.001$) No evidence that food had an effect	Absence of detectable effect of food may be because the meal was low in compounds that inhibit iron absorption, or due to small sample size
Hutchinson et al. 2008	UK males with HH (HFE C282Y+/+; n=5) *versus* iron-replete male controls (n=6)	Test meal with 10mg extrinsically added ferric iron as enriched $^{58}FeCl3$	Serum samples collected at baseline and at 0.5h and 3h thereafter NTBI measurement: Gosriwatana et al. (Anal Biochem 1999;273:212e20) with detection by ICP-MS	HH: NTBI seemed increased at 3h after baseline, p=0.06 Controls: no postprandial increase in NTBI	None
Baron et al. 2008	Pregnant Israeli women (n=57)	100mg oral iron as ferrous calcium citrate (n=35) *versus* iv 100mg iron as ferric hydroxide sucrose (n=22)	None of the patients had fasted overnight Blood samples collected at baseline and at 1hr thereafter NTBI measurement: Breuer, Cabntchik (Anal Biochem 2001;299:194–202)	No NTBI detectable at baseline NTBI concentration at 1hr: 0.8091 µM versus 0.0743 µM, $p<0.01$	Non-randomised study (iv iron given to those with severe anaemia unresponsive to oral iron treatment or because they could not tolerate oral iron treatment due to severe side effects)

Table 1: Summary of studies into effect of iron interventions on non-transferrin bound iron (NTBI) concentrations

Reference	Population (sample size)	Intervention	Follow-up/measurements	Key findings	Comments
Dresow et al. 2008	German healthy adult volunteers [1] or patients with IDA (n=2)	Healthy volunteers: 10–150 mg iron in various formulations [1] Patients with IDA: 100mg ferrous iron as ascorbate	Plasma samples collected at baseline and at 1–8h thereafter NTBI measurement: Singh et al. (Anal Biochem 186:320–323) with modification of using atomic absorption spectroscopy	Peak NTBI concentration at 1–8h: 6–12 µM, $p<0.01$	
Schümann et al. 2012a	Fasted non-anaemic iron-replete Guatemalan adult men (n=8)	0, 15mg, 30mg, 60mg, 120mg and 240mg iron as ferrous sulphate	Serum samples collected at baseline and at 1h, 2h and 3h thereafter NTBI measurement: Breuer, Cabntchik (Anal Biochem 2001;299:194–202)	Iron resulted in a dose-dependent increase in NTBI	Cross-over trial with incremental doses and a 7-day or 14-day washout period between testing, depending on dose
Schümann et al. 2012b	Fasted non-anaemic healthy adult Guatemalan men without inflammation (n=10)	100mg ferrous iron as sulphate *versus* 100mg ferric iron as NaFeEDTA *versus* 100mg ferric iron in polymaltose complex *versus* placebo, with a light snack (pancakes/fruit) after 180min	Plasma samples collected at baseline and at 90min, 180min and 270min thereafter NTBI measurement: Breuer, Cabntchik (Anal Biochem 2001;299:194–202)	Increase in NTBI with FeSO4 was significantly greater than that with water, NaFeEDTA, or IPM, with no differences among the latter three	Randomised cross-over trial with a 7-day washout period between testing
Schümann et al. 2013	Apparently healthy, fasted non-pregnant adult Guatemalan women	100mg iron as ferrous sulphate *versus* 100mg iron as NaFeEDTA	Serum samples collected at baseline and at 90min, 180min and 270min	Increase in NTBI with FeSO4 was significantly greater	Trial with parallel groups; however, report that intervention was

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Reference	Population (sample size)	Intervention	Follow-up/measurements	Key findings	Comments
	without inflammation, with ferritin concentration <30µg/L and Hb concentration ≥112g/L (n=40)	*versus* 100mg iron in polymaltose complex *versus* placebo, with a light snack (pancakes/fruit) after 180min	thereafter NTBI measurement: Breuer, Cabntchik (Anal Biochem 2001;299:194–202)	than that with water, NaFeEDTA, or IPM, with no differences among the latter three	randomly allocation seems contradicted by equal sample size per group

HH: hereditary haemochromatosis; IDA: iron deficiency anaemia

[1] Iron dosage: 10mg as ferrous ascorbate (n=6), 100mg as ferrous ascorbate (n=8); 100mg as ferrous sulphate (n=6); 50mg as ferrous glycine sulphate (n=4); 100mg as ferrous glycine sulphate (n=7); 80.5mg as ferrous gluconate (n=5); 150mg ferric polysaccharide complex (n=5)

Subjects and Methods

This study was part of a randomised controlled trial to assess the effect of iron supplementation in Kenyan women. Details about study design and main results will be reported elsewhere. The trial received ethical clearance from ethical review committees in Kenya (Kenyatta National Hospital/University of Nairobi) and England (London School of Hygiene and Tropical Medicine), and was registered at www.ClinicalTrials.gov (NCT01308112). Written informed consent was obtained from all study participants.

Study area and population

The study was conducted (October 2011–April 2013) in the administrative areas of Kanyawegi, Osiri and Ojolla, Nyanza Province, Kenya, where malaria is highly endemic and transmission is perennial. Anaemia is highly prevalent (69%)(Ouma et al., 2007) and intestinal schistosomiasis is common(Samuels et al., 2012). The population consists mainly of poor families from the Luo tribe, and has a diet mostly based on maize and with a low content of animal products, with sorghum, millet, and cassava being consumed in smaller quantities.

Study design

The study was a double blind, randomised intervention trial comparing supplementation with iron versus placebo. To prevent severe anemia, all women were dewormed, and all received a small daily dose of iron through flour fortification in the 2–3-week period before randomisation.

Recruitment and eligibility criteria

Local women aged 15–45 years who were married or cohabitating were invited for pregnancy testing when having missed their menstrual period for 10 weeks. Those who were not in stable relationships were invited for pregnancy testing every 12 weeks. In consenting women, we administered a urine pregnancy test, a medical examination including obstetric ultrasonography, and preventive antihelminth chemotherapy with praziquantel and albendazole.

Local mill operators added fortificant iron (target dose: 20mg/kg as NaFeEDTA) (WHO et al., 2009) to grain routinely brought by a member of a participating homestead from screening until women left the study. Based on weighed intake studies, we estimate that fortification supplied on average 5.7mg fortificant iron daily in pregnant women (unpublished data).

At the second visit to the research clinic (14 to 21 days after initial visit), we collected venous blood in K2EDTA tubes, and measured haemoglobin concentration (HemoCue301, Ängelholm, Sweden) and zinc protoporphyrin (Aviv 206d, Lakewood, NJ). Buffy coat and erythrocyte sediment were stored separately in DNA-stabilizing buffer (AS1, Qiagen, Valencia, CA) at 2–8˚C in the field and subsequently at–80˚C until polymerase chain reaction (PCR) assays. Plasma was stored at –196˚C in the field and subsequently at–80˚C until analysis.

Women were included when aged 15-45 years; consent had been obtained; likely to be available for study until 1 month after delivery and planning to deliver in the pre-designated health facility. They were excluded when having obvious mental retardation or a metabolic disorder, a medical history of sickle cell anemia, epilepsy, diabetes, an obstetric history suggestive of eclampsia or pre-eclampsia; carrying multiples; gestational age at the second visit was <13weeks or >23weeks; no venous blood was collected, haemoglobin concentration was <90g/L.

Randomisation, interventions and blinding

Supplements comprised capsules with starch as filler that were identical in appearance except that the shell for each type was into two colors (blue and dark green for iron, white and buff for placebo). Iron supplements contained 60 mg iron as ferrous fumarate (Dr. Paul Lohmann®, CAT No.500005025200). Ferrous fumarate was used for practical reasons,

knowing that it has similar iron bioavailability as ferrous sulfate. (Harrington et al., 2011) The code linking each color to the type of supplement was kept in sealed envelopes. One of us (HV) not involved in the fieldwork used tables with random numbers to produce sequentially numbered sealed, opaque envelopes containing the code. Eligible women were allocated in order of enrolment to the color indicated in the next available envelope. Participants and field staff were blinded to intervention until data analysis. To ensure adherence, women were observed when swallowing the capsule.

Follow-up of study participants

Approximately 1.5h after ingesting the first supplement, the participant was offered a non-standardised lunch that could include fish, omena (Rastrineobola argentea), beef, beans, green grams (Vigna radiata), bread, ugali (stiff maize porridge), rice, and chapati (unleavened flat-bread made from wheat flour), vegetables (mostly kale) and drinking water. We did not keep records of what was consumed by each participant.

At 3h after ingesting the first supplement, blood was collected in trace elements free tubes (Becton-Dickinson, Franklin Lakes, NJ, USA; catalogue 368380). Serum was stored at –196˚C in the field and subsequently at –80˚C during transport and storage until assessment of NTBI concentration in the UK. Although women were retained for further study, this is outside the scope of this paper, and will not be described here.

Laboratory analysis

Serum NTBI concentration was measured at King's College, London, UK, using a flow cytometry-based assay. This assay allows for accurate detection of NTBI concentrations down to 0.1µM. (Hider, Silva, Podinovskaia, & Ma, 2010) The detailed procedure can also be found in a patent application which was published in 2010, UK Patent Application number 1007209.8. For practical reasons, NTBI concentration was determined in the first 379 consecutively randomised women.

Haemoglobin concentrations were measured using a photometer (HemoCue model 301, Ängelholm, Sweden). The molar ratio of zinc protoporphyrin to haemoglobin (ZPP:H) in whole blood and washed erythrocytes was measured in duplicate for each sample using a haematofluorometer (Model ZPP 206D, AVIV Biomedical, Lakewood, NJ, USA). Control samples at low, medium, and high levels (AVIV) were run after every 30 readings while two level (low, high) calibration samples (AVIV) were run twice per year.

Iron markers (plasma concentrations of ferritin, soluble transferrin receptor, transferrin), and inflammation markers (plasma concentrations of C-reactive protein [CRP] and α1-acid glycoprotein) were measured on a Beckman Coulter UniCel DxC 880i analyser as per manufacturer's instructions.

HIV infection was determined using rapid antibody tests (Alere Determine™, Alere™,

Waltham, Massachusetts, USA); a positive result was confirmed using other antibody tests (Unigold™, Trinity Biotech PLC™, Co Wicklow, Ireland, and/or Bioline™, Pantech, Umhlanga, Kwazulu-Natal, South Africa).

Plasmodium antigenaemia was assayed by rapid dipstick tests (Access Bio Inc, Somerset, NJ, USA; CareStart, catalogue G0151 and G0171) that can detect *P. falciparum*-specific histidine-rich protein-2 (HRP2), *Plasmodium* lactate dehydrogenase (pLDH) specific to either *P. falciparum* or to non-*falciparum* species, i.e. *P. ovale*, *P. malariae* or *P. vivax*. Whereas HRP2-based tests detects current or recent *P. falciparum* infection, pLDH-based tests only indicate current infection.(Makler et al., 1998; Moody, 2002; Piper et al., 1999)

DNA was extracted from buffy coats and erythrocytes with a QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany). The –α3.7 deletion type of α+-thalassemia and *P. falciparum* infection were detected by separate PCRs.(Liu et al., 2000)(Hermsen et al., 2001) Due to practical constraints, α+-thalassemia genotype was assessed for the first 216 consecutively randomised women only.

Definitions

We used the following definitions: gravidity: the number of times a woman reported to have been pregnant, regardless of the outcome of those pregnancies, with twins and other multiple births counted as 1, and including the current pregnancy; compliance: percentage of participants who consumed the iron supplement or placebo and for whom a venous blood sample was obtained at 3h thereafter; *Plasmodium* infection: positive result for a pLDH-based dipstick test, a HRP2-based dipstick test or PCR test; anaemia: haemoglobin concentration <110 g/L;(WHO, 2011a) iron deficiency: plasma ferritin concentration <15 µg/L; (WHO, 2011b) iron deficiency anaemia: concurrent anaemia and iron deficiency; iron-deficient erythropoiesis: erythrocyte ZPP:haem molar ratio >40 µmol/mol; (Zimmermann, 2008) (Hastka et al., 1992) (Hastka et al., 1994) inflammation: either C-reactive protein (CRP) >10 mg/L (Nielsen et al., 1990) or α1-acid glycoprotein (AGP) >1 g/L (Filteau et al., 1993).

Statistical methods

Haemoglobin concentration and ZPP:haem molar ration were calculated as the mean of duplicate measurements, or as median values if more than two recordings were available.

Data analysis was done using IBM-SPSS Version 21 (SPSS, Chicago, USA) and CIA 2.2.0 (http://www.som.soton.ac.uk/research/sites/cia/). The primary analysis concerned the relative difference in serum NTBI concentrations at 3h after ingestion of the first supplement between groups that received iron or placebo. Serum NTBI concentrations were normalised by log-transformation. We considered NTBI to be present at values exceeding 0.18 µmol/L (Robert Hider, unpublished results). All analyses were by intention-to-treat.

Using linear regression analysis, we assessed confounding by comparing effects with and without adjustment for baseline factors suspected to be prognostic for the primary outcome (iron status, indicated by haemoglobin concentration, plasma ferritin concentration [<12 μg/L or ≥ 12 μg/L], gestational age, gravidity [primigravidae, secundigravidae, multigravidae] and maternal age [< 20 years and ≥ 20 years]); HIV infection; *Plasmodium* infection status; and α+-thalassemia genotype. We used stratified analysis and multivariate analysis to explore effect modification by baseline factors.

Results

Of 2,015 women invited for screening, we randomised 470 (23%) to intervention **(Figure 1)**, and included 379 women in this paper. Compliance was 100%.

Regarding baseline characteristics, intervention groups were similar except for mild imbalances in marital status, gravidity, anaemia and the prevalence of iron deficiency anaemia **(Table 2)**. 99% of participants who tested positive for *Plasmodium* infection had *P. falciparum* while only one participant (1%) was infected with other *Plasmodium* species.

At 3h after ingesting the supplement, NTBI concentrations were similar between groups (mean for both: 0.18 µmol/L; difference, 95%CI: 0.01µmol/L, –0.03 µmol/L to 0.05 µmol/L). NTBI was present (values ≥0.18µmol/L) in 40.9% (76/186) and 46.1% (89/193) of women in high-dose iron versus low-dose iron groups (difference: 5.3%, –15.0% to 4.7%).

Adjustment for possible confounders did not substantially change the effect estimate; neither was any of these factors independently associated with NTBI concentrations (not shown).

There was no evidence that NTBI response was dependent on *Plasmodium* infection status at baseline (difference, 0.01 µmol/L in groups with and without infection; 95% CI: 0.01 µmol/L, -0.06 to 0.04) **(Figure 2)**. There was also no evidence that NTBI response to iron depended on initial haemoglobin concentrations. Similarly, given placebo or iron, a 10-fold increase in the zinc protoporphyrin:haem ratio was associated with a 0.04 and 0.07 µmol/L decrease in NTBI concentrations respectively (difference, 95% CI: 0.03 µmol/L, -0.124 to 0.064, p=0.53). NTBI values seemed slightly reduced in participants with iron deficiency anaemia as compared to iron-replete individuals (95% CI: -0.04 to 0.06, p=0.11), irrespective of intervention group.

We found no evidence for an effect of normal (mean, 95% CI: -0.02 µmol/L, -0.09 to 0.04), heterozygous (0.04 µmol/L, -0.01 to 0.09) or homozygous (0.04 µmol/L, -0.07 to 0.16) α-thalassaemia genotypes in the serum NTBI response to ferrous fumarate. The effect of iron on NTBI concentrations is found to be -0.02 µmol/L in the normal genotype and equal in both the heterozygous and homozygous genotypes (0.04 µmol/L, difference 0.06 µmol/L)

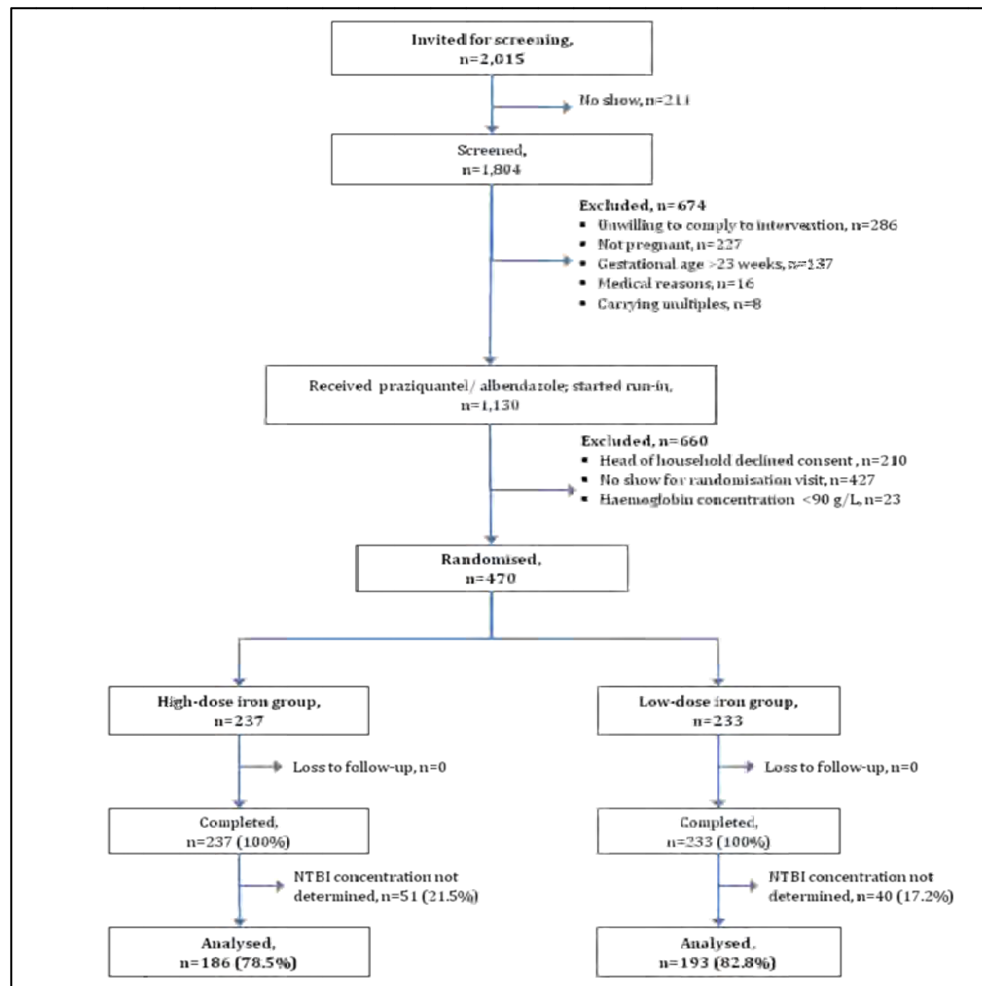

Figure 1: Participant flow

Table 2: Characteristics of study participants, by intervention group

Characteristics	High-dose iron	Low-dose iron
n	186	193
Height, cm	162.5 (6.0)	162.3 (6.8)
Weight, kg	58.3 (8.0)	57.5 (7.8)
Body mass index, kg/m^2	22.1 (2.8)	21.8 (2.6)
Marital status		
Married or living together	162 [87.1%]	157 [81.3%]
Divorced or separated	5 [2.7%]	7 [3.6%]
Never married	19 [10.2%]	29 [15.0%]
Age, years	25.3 (6.2)	24.5 (5.9)

Table 2: Characteristics of study participants, by intervention group

Characteristics	High-dose iron	Low-dose iron
Gestational age, weeks [1]	17.8 (2.5)	17.8 (2.5)
Gravidity		
Primigravidae	26 [14.0%]	38 [19.7%]
Secundigravidae	42 [22.6%]	33 [17.1%]
Multigravidae	118 [63.4%]	122 [63.2%]
Plasmodium infection [2]	60 [32.3%]	67 [34.7%]
HIV infection[4]	33 [17.9%]	42 [21.8%]
Plasma CRP concentration, mg/L	4.32 [7.3]	4.39 [9.2]
Plasma AGP concentration, g/L	0.76 (0.25)	0.78 (0.26)
Inflammation [3]		
Plasma CRP concentration ≥10 mg/L	45 [24.2%]	55 [28.5%]
Plasma AGP concentration ≥1.0 g/L	28 [15.1%]	33 [17.1%]
Plasma concentrations of CRP ≥10 mg/L or AGP ≥1.0 g/L	56 [30.1%]	62 [32.1%]
Haemoglobin concentration, g/L	113.9 (11.2)	112.3 (11.6)
Anaemia, haemoglobin concentration <110g/L	63 [33.9%]	79 [40.9%]
Iron deficiency, plasma ferritin concentration <15µg/L		
All women	103 [55.4%]	102 [52.8%]
Those with CRP concentration <10 mg/L	82 [44.1%]	79 [40.9%]
Those with AGP concentration <1.0g/L	93 [50.0%]	88 [45.6%]
Those with concentrations of CRP <10mg/L or AGP <1.0 g/L	97 [52.2%]	91 [47.2%]
Iron deficiency anaemia	31 [16.7%]	43 [22.3%]
Plasma ferritin concentration, µg/L	15.4 [20.3]	15.9 [19.9]
Plasma sTfR concentration, mg/L	1.90 [1.09]	2.09 [1.16]
Plasma transferrin concentration, g/L	3.1 (0.6)	3.1 (0.5)
Whole blood ZPP:haem molar ratio, µmol/mol	94.7 [53.1]	98.4 [57.1]
Erythrocyte ZPP:haem molar ratio, µmol/mol	38.3 [48.3]	41.1 [55.8]
Plasma folate concentration, nmol/L	16.85 (6.88)	17.27 (7.33)
Plasma vitamin B_{12} concentration, pmol/L	497.9 (266.2)	499.3 (274.8)
α^+-thalassaemia genotype [5]		
Normal	54/ 105 51.4%	57/ 111 51.4%
Heterozygote	45/ 105 42.9%	44/ 111 39.6%
Homozygote	6/ 105 5.7%	10/ 111 9.0%

Values indicate mean (SD), geometric mean [IQR], n [%] or n/n [%]. AGP: α_1-acid glycoprotein; CRP: C-reactive protein; FEP: free erythrocyte protoporphyrin; HRP2: *P. falciparum*-specific histidine-rich protein-2; LDH: *P. falciparum*-specific lactate dehydrogenase; sTfR: soluble transferrin receptor; ZPP: zinc protoporphyrin.

[1] **All participants were in the second trimester of pregnancy except for one who was in the first trimester;** [2] Any species, by any dipstick or PCR; **only one participant (high-dose iron group) had infection by a *Plasmodium* species other than *P. falciparum*;** [3] **Only one participant (high-dose iron group) had current fever defined as axillary temperature ≥ 37.5 °C;** [4] **HIV infection status was not determined for two participants**; [5] Determined in 216 women only.

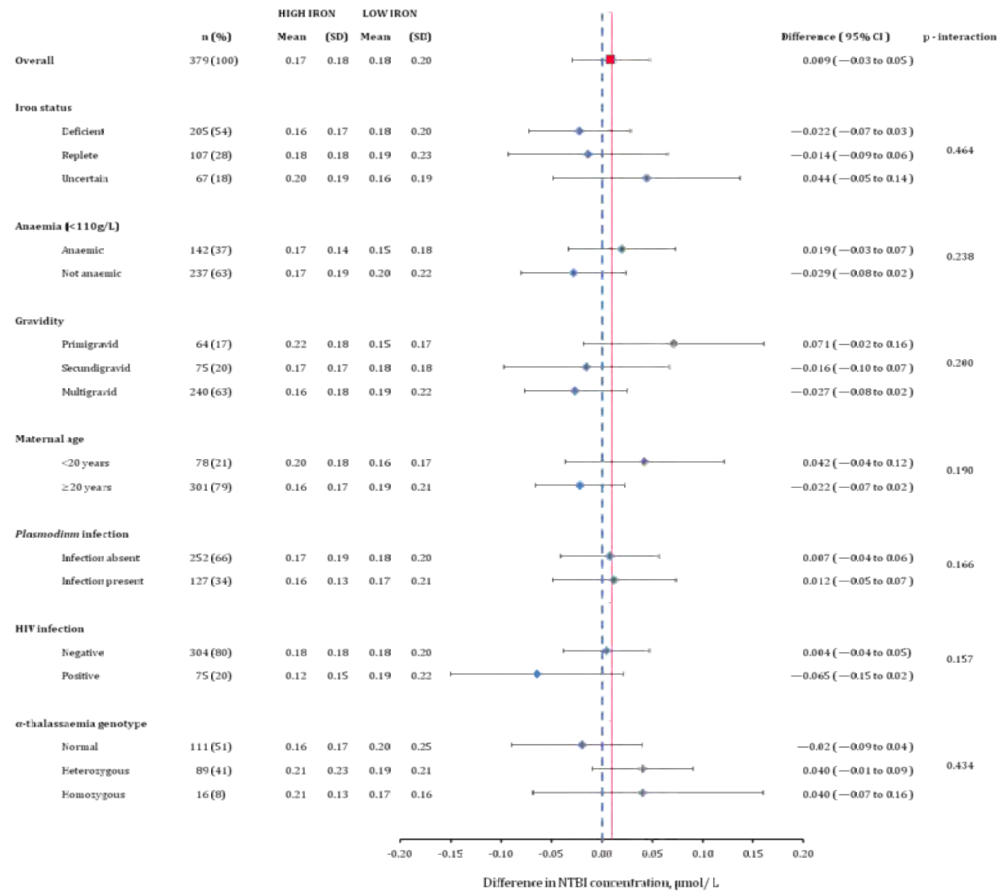

Figure 2: Subgroup analyses. Horizontal lines indicate 95% CIs.

Discussion

We found no effect of oral ingestion of 60 mg ferrous fumarate on serum NTBI concentration three hours after ingestion; the NTBI response to iron was also not influenced by *Plasmodium* infection, iron deficiency, iron deficiency anaemia or α-thalassaemia. In addition, the effect was not substantially changed by other covariates such as gravidity, maternal age, and HIV infection status.

This was despite the fact that iron deficiency anaemia leads to increased iron absorption (Leif Hallberg & Hulthén, 2002) which would lead to higher serum NTBI concentrations. (Chung & Wessling-Resnick, 2003) Thalassaemias are associated with increased iron absorption as a result of ineffective erythropoiesis, and increased plasma iron turnover

(Gafter-Gvili et al., 2004), thus NTBI concentration is expected to be higher in individuals with thalassaemia disorders. However, *Plasmodium*-induced inflammation has been shown to lead to a decreased iron absorption (de Mast, van Dongen-Lases, et al., 2009) and is therefore likely to result in a reduced NTBI concentration.

To our knowledge, this is the first study on the effect of iron supplementation on serum NTBI involving pregnant African women living in a region of high malaria endemicity. Compliance was 100%, and there was no attrition during follow-up. Blinding was highly effective because the taste of the iron and placebo supplements was masked by the capsules. Malaria rates in Kenya have been declining, (A. Noor et al., 2009) (Ministry of Public Health and Sanitation (MOPHS) & Ministry of Medical Services, 2010) thus in order to quantify the total malaria burden on the participants, we assessed all asymptomatic *Plasmodium* infection using combinations of dipstick tests and *Plasmodium falciparum* specific PCR. PCR tests for other *Plasmodium* species were not done because results of dipstick tests showed that all infections except one were due to *P. falciparum*. Assessment of inflammation by combinations of C-reactive protein and α1-acid glycoprotein allowed us to determine iron status in the study population. All analyses were done according to a pre-specified plan.

Our study had some limitations. A meal was consumed by most of the study participants during the three-hour waiting period, especially if the intervention period occurred during lunchtime. Ideally, participants should not have ingested anything else apart from the study supplement during this period. For ethical reasons and in the hope to boost compliance, however, we decided to offer an optional meal during the intervention. We did not control or record amounts or types of food eaten after ingestion of the supplement. Generally, meals contained relatively high concentrations of iron inhibitors, such as phytates from maize flour, which may have reduced iron absorption and thus NTBI production. This is strengthened by the recommendation of the WHO to take iron supplements with food, to avoid a rapid increase of serum iron concentrations and transferrin saturation, exceeding the binding capacity of transferrin and leading to the production of NTBI. (Hurrell, 2011) (WHO, 2007) A study that compared the NTBI response in participants with and without taking a meal (Hutchinson et al., 2004), found no difference between NTBI concentrations. The meal served in that study was however low in iron inhibitors, and the sample size (n=7) was small, which made it unlikely to detect a difference if it existed.

The nature of NTBI is still largely unknown, resulting in little consensus on its true levels and how it should be measured. Research groups throughout the world use a variety of test principles to measure NTBI. A round robin compared NTBI measurements from twelve serum samples in six laboratories; five laboratories (including ours) used chelation methods and one a bleomycin assay. This round robin showed considerable inter- and intra-method variation between serum NTBI levels (Jacobs et al., 2005). None of the methods thus far has however shown to be clearly superior. There is need of additional information on the

nature and relevance of NTBI and the development of more robust quantification methods before introducing it into clinical practice.

Increased concentrations of transferrin receptor indicate an increased erythropoiesis or tissue iron deficiency, and thus possibly with an enhanced response in NTBI concentration to oral ferrous iron. NTBI samples were obtained in the second trimester, between 13 and 23 weeks of pregnancy. Transferrin receptor levels are lowest around 13-20 weeks of pregnancy and far lower than in non-pregnant women (4.5 and 7.0 mg/L respectively). (Beguin, Lipscei, Thoumsin, & Fillet, 1991) This implies that iron uptake for erythropoiesis was likely to be down regulated at the moment of sample collection for NTBI analysis, which may be a contributing factor for not finding a difference in NTBI response between the intervention and placebo group.

Little is known about factors that determine the magnitude of serum NTBI concentration. It is assumed that factors leading to increased iron absorption from gut enterocytes positively affect the amount of NTBI released into the circulation. (Chung & Wessling-Resnick, 2003) The relatively high levels of NTBI in iron replete subjects as compared to iron deficient women are possibly caused by higher levels of apotransferrin circulating in people with iron deficiency anaemia, whereby the binding capacity of transferrin is increased and less NTBI is formed (Dresow, Petersen, Fischer, & Nielsen, 2008).

Ineffective erythropoiesis in severe forms of β-thalassaemia can lead to excessively low hepcidin production, resulting in iron being absorbed at a faster rate than being used for erythropoiesis, which likely leads to NTBI production (Musallam, Cappellini, Wood, & Taher, 2012). Unlike severe β-thalassaemia, α+-thalassaemia causes only minor symptoms and has not shown to be associated with iron loading. (Veenemans et al., 2008) (Veenemans, Milligan, et al., 2011) This can possibly explain the absence of an evident difference in NTBI production between normal and α+-thalassaemia genotypes (Figure 4), although a slightly positive effect on the NTBI concentration of heterozygous and homozygous genotypes was observed as compared to the normal genotype (Figure 4 and 5).

Our findings are in contrast with other studies, which found NTBI production upon ingestion of iron supplements. (Schuemann et al., 2012) (Hutchinson et al., 2004) (Dresow et al., 2008) These studies issued 10-240 mg ferrous iron to participants and all observed NTBI after three hours, even when unsaturated transferrin was still present. This probably occurs when the iron influx exceeds the rate of iron uptake by transferrin, a process that may be enhanced by the relatively slow binding between iron and transferrin (Aisen, 1974). Healthy Guatemalan males (n=8), anaemic British women (n=7) and a combination of healthy and anaemic German participants (n=43) were included in the respective studies, indicating that NTBI after the ingestion of ferrous iron occurs in iron replete as well as anaemic subjects.

Conclusions

We found no effect of oral iron supplementation on NTBI concentrations when the supplement was given with food to women in their second trimester of pregnancy. This study does not support changing existing policies for daily supplementation with 60 mg iron as ferrous salts. Further studies are needed to determine the effect of iron supplementation on NTBI in pregnant women in a fasting state in various stages of pregnancy, especially the third trimester of pregnancy when transferrin receptor concentrations are highest.

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Chapter 5

Factors that predict *Plasmodium* infection in pregnancy

Introduction

Malaria prevalence among pregnant women and their offspring has declined in various malaria endemic regions. (Kalayjian, Malhotra, Mungai, Holding, & King, 2013) Emphasis on asymptomatic malaria is increasingly common especially because of the observed detrimental effects during pregnancy. Several studies have found that sub-patent infections (i.e. low parasitaemias that are detected by PCR but not by microscopy or dipstick tests) are associated with reduced birth, intrauterine growth retardation, preterm delivery, and increased neonatal mortality. (Malhotra, Dent, Mungai, Muchiri, & King, 2005), (Adegnika et al., 2006), (Arango, Maestre, & Carmona-Fonseca, 2010), (Mohammed et al., 2013) The pathological consequences of maternal infection are possibly dependent on population-specific factors such as immunity, transmission intensity, parity, maternal age, access to treatment, coverage and quality of antenatal services.

Prompt diagnosis of malaria is vital for reduction of malaria morbidity and mortality. Laboratories in most resource-poor malaria regions are not equipped to effectively detect asymptomatic *Plasmodium* infection. Doctors and other clinical care specialists often make critical diagnostic decisions on clinical grounds alone using very limited information. Commonly used symptoms to diagnose malaria, e.g. fever, can be a result of a myriad other causes. Over-prescription of malaria treatment drugs is therefore common. As a result, resistance to existing drugs is on the rise and global efforts to contain the resistance have been initiated. (WHO Global Malaria Programme, 2010, 2011)

Malaria rarely occurs in isolation. Usually, other co-morbidities co-exist. In addition to malaria, other diseases such as anaemia, HIV infection, tuberculosis, bilharzia, and helminthic infections are also common. (Stevens, 2004) Drug supply to counter these diseases in resource-poor countries is not always adequate. (Thiam, Kimotho, & Gatonga, 2013) The social cultural environment also plays a role in hindering the elimination of these

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diseases. A poor social economic status has been attributed to disease burden in sub-Saharan Africa. (Stevens, 2004) Currently, scenarios of low coverage of micronutrient supplementation and inadequate health care access and coverage manifests in many countries. (Anna Maria van Eijk et al., 2011) Problems of coverage of known interventions such as intermittent preventive treatment (IPT), (Thiam et al., 2013) and bed nets have been reported. (Malhotra et al., 2009)

Global and national maps of malaria risk have been important tools in targeting of malaria control interventions (A. M. Noor et al., 2008). Spatial prediction of malaria prevalence has enabled researchers and program implementers to map out high-risk zones in most geographical areas. (Haque et al., 2010; A. M. Noor et al., 2008) At patient level, the World Health Organisation has guidelines for the identification of severe malaria. (WHO, 2010) Decision-making tools such as the malaria severity score (MSA) and the Glasgow coma score (GCS) exist and are a great aid to medical practitioners. (Mishra, Panigrahi, Mishra, & Mohanty, 2007) Predictive scores are now widely used in the management of various clinical conditions in resource-rich countries. (Antman et al., 2000; Fine et al., 1997) However, there is no guideline for the prediction of asymptomatic *Plasmodium* infection especially in pregnancy, in resource-poor settings.

The new challenge in resource-poor countries involve targeting interventions to the most at risk persons in groups that are already living in malaria hot-spots. Medical practitioners are increasingly faced with the dilemma of how to diagnose asymptomatic *Plasmodium* infection. In the future, resource-poor malaria endemic countries will probably result to dipstick tests to diagnose asymptomatic infection. These tests will most likely be selected based on cost, ease of use, storage stability under field conditions, sensitivity and/or specificity of the selected test. In our previous work, we combined two types of dipstick tests that detected current and recent *Plasmodium* infection. They were only able to detect 50% of the asymptomatic infection when compared to quantitative PCR. Doctors need to correctly predict the remaining 50% as adverse outcomes are also existent in these persons.

There has been few assessments of the combinations of prognostic factors that best predict asymptomatic *Plasmodium* infection during pregnancy. The effectiveness of interventions given by program implementers and clinicians could be boosted by tools that enable them predict the most at-risk individuals within population groups. We therefore sought to identify factors that predict *Plasmodium* infection during pregnancy. We also aimed to develop a prediction model that can reliably predict asymptomatic *Plasmodium* infection during pregnancy, in women who are *Plasmodium*-negative by dipstick tests but *Plasmodium*-positive by PCR. To do this, we examined the sensitivity and specificity of the predictive variables in order to derive simple models to identify pregnant women at risk of asymptomatic *Plasmodium* infection.

Methods

Study background

The data for this study was obtained from the main study (Chapter 2 of this thesis). The fieldwork was conducted from October 2011 to April 2013 in the administrative area of South-West Kisumu county, formerly Kisumu district, Kenya. This area is along the shores of Lake Victoria, at around 1234m above sea level. Malaria is endemic and transmission is perennial. People living in the area are exposed to 30─100 infectious mosquito bites per year, (A. Noor et al., 2009) although this rate is reducing. (Ministry of Public Health and Sanitation (MOPHS) & Ministry of Medical Services, 2010) Anaemia (69%) (Ouma et al., 2007) is highly prevalent among pregnant women while intestinal schistosomiasis is highly prevalent in school-aged children.(Samuels et al., 2012) Coverage of intermittent preventive treatment is low: in 2008-2009, 17% of women in Nyanza Province received at least two doses of sulfadoxine-pyrimethamine during pregnancy, and 35% received at least one dose. (Kenya National Bureau of Statistics (KNBS) & ICF Macro, 2010)

An obstetric ultrasound instrument to enable accurate determination of gestational age was installed in one of the two health facilities in the study area, Ober Kamoth health centre. The study received clearance from ethical review committees in Kenya (Kenyatta National Hospital/University of Nairobi) and England (London School of Hygiene and Tropical Medicine), and was registered at ClinicalTrials.gov (NCT01308112).

Study design and data collection

The parent study was a double blind, randomised, placebo-controlled intervention trial with two parallel groups. Pregnant women received daily supplementation with iron or placebo. To prevent severe anaemia, all women were dewormed before the start of the intervention, and received a small dose of iron through flour fortification during the intervention. However, a cross-sectional design was used for the analysis of the results presented in this chapter. Baseline data from the two arms of the randomised trial was pooled and used for the cross-sectional analysis. All *Plasmodium* positive cases were identified by various methods (see table 2) and used in this analysis.

The data analysed according to pre-defined statistical plan. We validated the obtained results with data obtained at delivery from all the clients who provided *Plasmodium* infection data at delivery.

Detailed recruitment procedures are reported elsewhere (chapter 2 of this thesis). We re-describe some of the procedures that were relevant to this study. At baseline, women were counselled and asked for consent for HIV testing. Following medical examination, a venous blood sample was collected in a tube with K2EDTA (Becton Dickinson, Nairobi, Kenya; catalogue 367863). Haemoglobin concentration was measured by photometer (HemoCue

301, Ängelholm, Sweden). The ratio of zinc protoporphyrin:haem (ZPP:H) was measured in whole blood and washed erythrocytes by haematofluorometer (see section Laboratory analyses). Washed erythrocytes were stored in a DNA-stabilizing buffer (AS1, Qiagen, Valencia, CA, USA) at 2 – 8 °C for subsequent DNA extraction and detection of *Plasmodium* infection by PCR test. Plasma samples were stored for subsequent detection of *Plasmodium* antigens and HIV antibodies by rapid dipstick tests. Additional plasma samples were stored in liquid nitrogen (-196 °C) in the field and at –80 °C during transport and storage for subsequent assessment of iron markers (concentrations of ferritin, soluble transferrin receptor, transferrin), C-reactive protein, α1-acid glycoprotein, bilirubin, vitamin B12, lactate dehydrogenase, haptoglobin, folate, pre-albumin, and albumin.

Women were included when aged 15–45 years; consent had been obtained; and likely to be available for larger study until 1 month after delivery. They were excluded when having obvious mental retardation or a metabolic disorder, a medical history of sickle cell anaemia, epilepsy, diabetes, an obstetric history suggestive of eclampsia or pre-eclampsia; carrying multiples; gestational age at the second visit was below 13 weeks or exceeded 23 weeks; homestead members had not provided consent; no venous blood sample was collected, haemoglobin concentration was <90 g/L. Recruitment continued until the target sample size (470) had been attained.

As per procedures of the main study, maternal blood (6 mL) was collected by venipuncture in tubes containing K2EDTA tubes (Becton Dickinson) within 1 hour after delivery. Each woman was requested for her clinic attendance booklet, and information regarding the number of doses received for intermittent preventive treatment against malaria was recorded.

Laboratory analysis

ZPP:H ratio was measured for each sample in duplicate using a haematofluorometer (Model ZPP 206D, Aviv Biomedical, Lakewood NJ, USA). Control samples at low, medium, and high levels (AVIV, catalogue 9999-40839) were run after every 30 readings while two level (low, high) calibration samples were run twice per year (AVIV, catalogue 9999-112562). *Plasmodium* antigenaemia was assayed by rapid dipstick tests (Access Bio Inc, Somerset, NJ, USA; CareStart, catalogue G0151 and G0171) that can detect *P. falciparum*-specific histidine-rich protein-2 (HRP2), *Plasmodium* lactate dehydrogenase (pLDH) specific to either *P. falciparum* or to non-*falciparum* species, i.e. *P. ovale*, *P. malariae* or *P. vivax*. Whereas HRP2-based tests detect current or recent *P. falciparum* infection, pLDH-based tests only indicate current infection. (Makler et al., 1998; Moody, 2002; Piper et al., 1999)

In order to detect of *Plasmodium* infection by PCR, blood samples were centrifuged (8min @ 600g), and 120 µl red blood cells were transferred to 0.5 mL tubes prefilled with 120 µl PBS and 140µl DNA-stabilizing buffer AS1. They were then mixed, and stored at -196°C

for subsequent shipment to the Netherlands for further laboratory analysis. Nucleic acid isolation was performed and DNA was extracted using Qiagen whole blood DNA isolation protocol according to the manufacturer's instructions. However, DNA was eluted in 50 µl nuclease free water. The extracted DNA was stored in -20°C until PCR analysis. Analysis of samples for the presence of *P. falciparum* DNA was done according to the protocol of Hermsen et al(Hermsen et al., 2001) with the following modifications: An internal amplification control detecting the human household gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was added to the protocol by using a Texas Red labeled probe and Gapdh specific primers: Fwd: 5` GAA-GGT-GAA-GGT-CGG-AGT-C `3; Rev: 5` GAA-GAT-GGT-GAT-GGG-ATT-TC `3; Probe: 5` Texas Red CAA-GCT-TCC-CGT-TCT-CAG-CC HBQ1 -`3. Amplification reactions were performed in a volume of 25 µl, a total of 2.5 µl 1x Qiagen buffer, 6.5 mM MgCl2, 250 µM dNTP's, 400 nM of each *P. falciparum* 18S primer, 100 nM *P. falciparum* 18S probe, 40 nM of each GAPDH primer, 100 nM GAPDH probe, 0.625 U HotstarTaq polymerase Qiagen, and 2.5µl DNA sample. The primers and probes were all provided by Biolegio. Amplification comprised of 10 minutes at 95°C, followed by 45 cycles of 20 seconds at 95°C and 1 minute at 60°C. In each PCR run, water controls, no-template controls, high and low positive controls were all included in duplicate. The high and low positive controls contain a known amount of *P. falciparum*; thus, the Ct values of these controls were used to determine the PCR amplification efficacy. The Ct values of these controls have a range between 2x the standard deviation (which was calculated by measuring the controls 12 times over several days) and the Ct values of the controls were required to fall within this range.

HIV infection was assessed by rapid antibody tests (Alere™, Waltham, Massachusetts, USA); a positive result was confirmed using other antibody tests (Unigold; Trinity Biotech, Co Wicklow, Ireland and/or Bioline; Pantech, Umhlanga, Kwazulu-Natal, South Africa).

Plasma concentrations of ferritin, soluble transferrin receptor (sTfR), transferrin, C-reactive protein, and α1-acid glycoprotein were measured on a Beckman Coulter UniCel DxC 880i analyser according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were done using SPSS version 21.0 (SPSS, Chicago, USA). We described *Plasmodium* infection as assessed by HRP-2, LDH, HRP-2 and LDH, and PCR. The primary outcome, which was adopted as the gold standard in this analyses, was defined as the presence of maternal *Plasmodium* infection in samples collected at baseline, regardless of species, as indicated by one or more positive results for the presence of parasite-derived LDH or HRP2 in plasma (dipstick tests), or *Plasmodium* DNA (any species, by PCR test). Results of microscopy were excluded from this case definition as they would be considered incorrect if discordant with results from PCR tests.

The following definitions were used: anaemia: haemoglobin concentration <110 g/L

(WHO, 2011a); iron deficiency: plasma ferritin concentration <15 µg/L (WHO, 2011b); iron-replete: plasma ferritin concentration ≥15 g/L in the absence of inflammation; iron status uncertain: plasma ferritin concentration ≥15 g/L in the presence of inflammation; iron deficiency anaemia: anaemia co-existing with iron deficiency; inflammation: plasma concentrations of C-reactive protein (CRP) >10 mg/L (Nielsen et al., 1990) and/or α1-acid glycoprotein >1 g/L (Filteau et al., 1993); gravidity: the number of times a woman reported to have been pregnant, regardless of the outcome of these pregnancies, with twins and other multiple births counted as 1, and including the current pregnancy.

Since the outcome was dichotomous (yes/no *Plasmodium* infection), a logistic regression was used. Logistic regression does not make assumptions of linearity, normality and homogeneity of variance for the independent variable. We assessed the contribution of individual determinants to the overall effect estimate. A stepwise forward multivariate analysis was then fitted and the weight of the determinants was estimated using estimations of the maximum likelihood ratio. We checked for multi-colinearity and estimated accuracy of the effect estimate by visual assessment of the calibration plots.

Receiver operating characteristics (ROC) curves and area under the curve (AUC) estimates were generated. We used the following criteria to judge the quality of the AUC estimates:

0.90 to 1.0 = excellent (A); 0.80 to 0.90 = Good (B); 0.70 to 0.80 = Fair (C); 0.60 to 0.70 = Poor (D); 0.50 to 0.60 = Fail (F)

Finally, we checked for internal validation of the selected model(s) using data collected at parturition from women who completed the follow-up period.

Results

Out of the 2,015 women invited for screening, 470 (23%) were randomised to intervention and included in the main analysis for this study. **Table 1** and **Figure 1** contains the characteristics of study subjects.

Table 1: Characteristics of study participants

Characteristics	
n	**470**
Height, cm	162.4 (6.2)
Weight, kg	57.9 (7.6)
Body mass index, kg/m^2	21.9 (2.7)
Marital status	
Married or living together	388 [82.6%]
Divorced or separated	18 [3.8%]
Never married	64 [13.6%]
Age, years	24.8 (6.1)

Table 1: Characteristics of study participants

Gestational age, weeks [1]	17.8 (2.6)
Gravidity	
Primigravidae	85 [18.1%]
Secundigravidae	92 [19.6%]
Multigravidae	293 [62.3%]
Plasmodium infection	
Current *Plasmodium* infection (pLDH)	31 [6.6%]
Current or recent *Plasmodium* infection (pLDH + HRP2)	90 [19.1%]
Current *Plasmodium* infection (q-PCR)	163 [34.7%]
Current or recent *Plasmodium* infection (pLDH + HRP2 + Pf-PCR)[2]	175 [37.2%]
HIV infection	99 [21.1%]
Plasma CRP concentration, mg/L	4.3 [8.3]
Plasma AGP concentration, g/L	0.78 (0.27)
Inflammation [3]	
Plasma CRP concentration ≥10 mg/L	126 [26.8%]
Plasma AGP *concentration* ≥1.0 g/L	85 [18.1%]
Plasma CRP concentration ≥10 mg/L or AGP ≥1.0 g/L	152 [32.3%]
Haemoglobin concentration, g/L	113.2 (11.4)
Anaemia, haemoglobin concentration <110g/L	175 [37.2%]
Plasma ferritin concentration, µg/L	13.9 [20.9]
Plasma sTfR concentration, mg/L	1.94 [1.15]
Plasma transferrin concentration, g/L	3.1 (0.6)
Iron deficiency, plasma ferritin concentration <15µg/L	
All women	248 [52.8%]
Those with CRP concentration <10 mg/L	197 [41.9%]
Those with AGP concentration <1.0g/L	221 [47.0%]
Those with concentrations of CRP <10mg/L or AGP <1.0 g/L	228 [48.5%]
Whole blood ZPP:haem ratio, µmol/mol	89.5 [52.8]
Erythrocyte ZPP:haem ratio, µmol/mol	36.5 [46.9]

Mean (SD), Median [IQR], n [%]

AGP: α1-acid glycoprotein; CRP: C-reactive protein;HRP2: *P. falciparum*-specific histidine-rich protein-2); LDH: *P. falciparum*-specific lactate dehydrogenase; sTfR: soluble transferrin receptor; ZPP: zinc protoporphyrin

1. All women except one were in the 2nd trimester of pregnancy; 2. Only one participant (high-dose iron group) had infection by a *Plasmodium* species other than *P. falciparum*; 3. Only one participant (high-dose iron group) had current fever defined as axillary temperature ≥ 37.5 °C; 4. One missing value in the per protocol analysis dataset; 5. HIV status of two participants was not determined

Table 2 describes the different type of tests used to assess *Plasmodium* infection and the number of positive cases. Majority of the *Plasmodium* infected women were detected by PCR (93.1%), followed by a combination of HRP2 and pLDH dipsticks (51.4%) . pLDH, which detects only current infection, detected only 17.7% of positive cases when used independently.

Table 2: Prevalence of asymptomatic *Plasmodium* infection at baseline, by various test methods

Type of *Plasmodium* infection	Type of test	Number infected, n/n (%)
Current infection	pLDH	31/ 175 (17.7)
Current or recent infection	pLDH + HRP2	90/ 175 (51.4)
Current infection	*P. falciparum* PCR	163/ 175 (93.1)
Current and recent infection [AND]	All (pLDH & HRP2 & PCR)	27/ 175 (15.4)
Current and recent infection [OR]	All (pLDH or HRP2 or PCR)	175/ 175 (100)

We developed seven models (A to G) to predict asymptomatic *Plasmodium* infection during pregnancy in women who are *Plasmodium*-negative by dipstick tests but *Plasmodium*-positive by PCR. **Table 3** is a list of the various determinants used to obtain models A to G.

Table 3: Determinants in the various models*

Model A:	Plasma α1-acid glycoprotein + Number of live children + HIV infection + Plasma pre-albumin + Plasma haptoglobin + Plasma lactate dehydrogenase + Plasma bilirubin + Plasma ferritin + Hb concentration +Maternal age + Blood group + Systolic blood pressure + parity
Model D:	Number of live children + HIV infection + Hb concentration + Blood group + Plasma α1-acid glycoprotein + Plasma haptoglobin + Plasma pre-albumin + Plasma lactate dehydrogenase + Plasma bilirubin
Model B:	Plasma α1-acid glycoprotein + Plasma pre-albumin + Plasma haptoglobin + Plasma lactate dehydrogenase + Plasma bilirubin + Plasma ferritin concentration
Model C:	Number of live children + HIV infection + Hb concentration + Blood group
Model E:	N Number of live children + HIV infection + Hb concentration + Blood group + Plasma α1-acid glycoprotein + Plasma haptoglobin + Plasma pre-albumin
Model F:	Number of live children + HIV infection + Hb concentration + Blood group + Plasma α1-acid glycoprotein + Plasma lactate dehydrogenase + Plasma bilirubin
Model G:	Number of live children + HIV infection + Hb concentration + Blood group + Plasma α1-acid glycoprotein

***Models arranged in order of appearance in the ROC curves**

We assessed the potential of various baseline determinants to predict asymptomatic *Plasmodium* infection as assessed by q-PCR. **Figure 2** shows the obtained ROC curves and the criteria used to build the final models. Model A and D had the best area under the curve (AUC) (0.82 and 0.80). Model C which was composed of easy to assess determinants, had an AUC of 0.68.

Based on our pre-specified criteria, the models were judged as follows: Model A = Good; Model B = Fair; Model C = Poor; Model D = Good; Model E = Fair; Model F = Fair; Model G = Fair

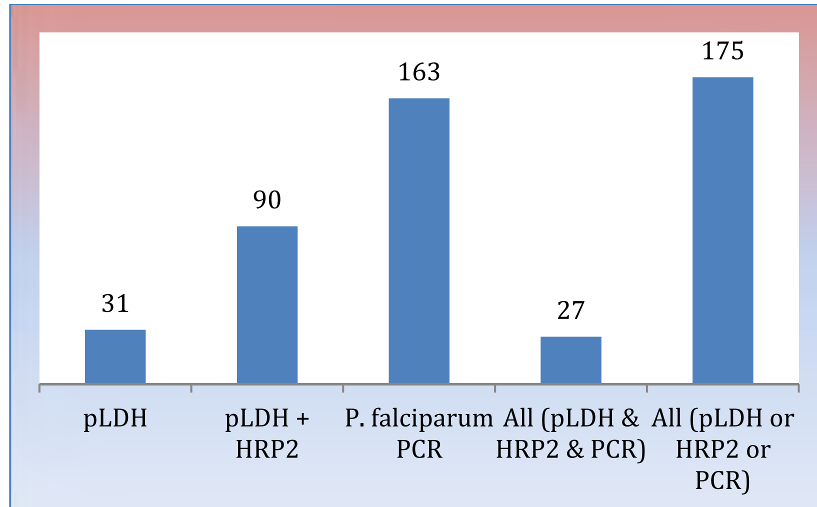

Figure 1: Prevalence of asymptomatic *Plasmodium* infection at baseline, by various test methods

Two best models (A and D) were judged as "good" as they had an area under the curve above 0.80. All the remaining models except one were judged as "fair". We made a model (model C) composed of easy to measure determinants in resource-poor settings (number of live children + HIV infection + Haemoglobin + Blood group). Addition of an extra determinant to this model (α1-acid glycoprotein) improved its AUC from 0.68 to 0.72 thus it was judged as fair (model G).

The best predictors of asymptomatic *Plasmodium* infection were: α1-acid glycoprotein, number of live children, HIV infection, pre-albumin, haptoglobin, lactate dehydrogenase, bilirubin, ferritin, haemoglobin, age, blood group, systolic blood pressure, and parity.

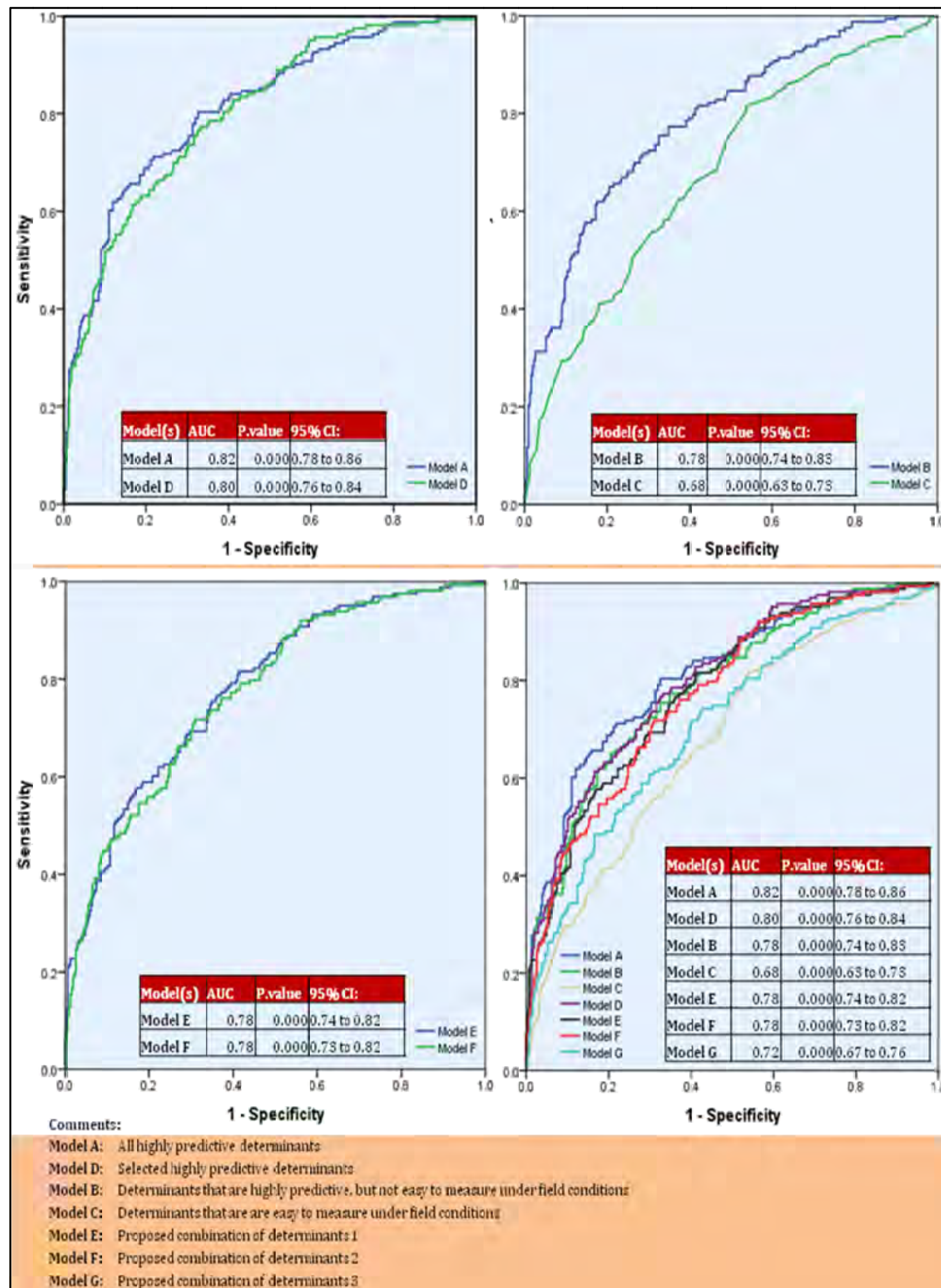

Figure 2: ROC curves of various models to determine asymptomatic *Plasmodium* infection

Discussion

The factors that were best in predicting asymptomatic *Plasmodium* infection were HIV infection, plasma haemoglobin concentration, number of live children, blood group, plasma α1-acid glycoprotein concentration, plasma haptoglobin concentration, plasma pre-albumin concentration, plasma lactate dehydrogenase concentration, and plasma bilirubin concentration. Out of these, four (HIV infection, plasma haemoglobin concentration, number of live children, blood group) are routinely assessed in most resource-poor settings. The other predictive determinants are assessed in more specialised laboratories in larger medical and research institutions.

Out of the seven models developed to predict asymptomatic *Plasmodium* infection during pregnancy in women who are *Plasmodium*-negative by dipstick tests but *Plasmodium*-positive by PCR, Model A was best with an AUC of 0.82 ("good"). However, it was composed of many determinants (12) and was thus not easy to apply it under conditions of usual healthcare. Model C, which was composed of determinants that are easy to assess in resource poor settings, was judged as "poor". Although addition of α1-acid glycoprotein to this model improved its AUC to 0.72 ("fair" – model G), a predictive curve of above 0.9 ("excellent") would have been the best predictor.

Our study had various strengths. First, as a gold standard, we assessed *Plasmodium* infection by PCR. Compared to the two dipstick tests used in this study (pLDH and HRP2), PCR detected many more *Plasmodium*-infected subjects (34.7% versus 19.1%). Secondly, because all the PCR analysis was performed by the same lab, there was no verification bias of the gold standard as PCR analysis for all subjects was done in the same laboratory using the same protocol. Lastly, the number of responders (*Plasmodium* positive cases) was large enough to allow us to assess the predictive ability of the determinants. According to Harrell and Peduzzi et al, 1996, for each determinant studied in multivariate prediction research, at least 10 individuals with the disease are needed in the smallest category of the outcome variable to allow proper statistical modelling. [Harrell et al., 1996; Peduzzi et al., 1996].

Our participants were part of a randomised controlled trial which was conducted in a malaria endemic area. The data used in this study was obtained after the participants were randomised into the trial thus no selection bias could be associated with the subjects selection criteria. Furthermore, we were able to study the discriminatory ability of many determinants to correctly pick up *Plasmodium*-infected and non-infected subjects in one population. We were also able to compare the predictive reliability of different determinants whether independently or in combination with others (interaction). The assumptions of binary logistic regression made it possible to study multiple determinants and derive their probabilities which could be used to plot the ROC curves.

Our study had some limitations. In the studied population, multiple morbidities exist thus it is reasonable to expect some co-morbidity bias because known or unknown diseases may

affect the positivity or negativity of test. Secondly, some determinants e.g. past miscarriages, past still births, use of insecticide treated nets, and use of insecticide sprays although possibly of high predictive value, were not assessed because of unavailability of data. Lastly, other area-specific demographic and geographic determinants such as house-type and distance from known water masses were not used despite the fact that spatial prediction of malaria risk zones has been done elsewhere. (Haque et al., 2010; A. M. Noor et al., 2008)

Various studies have explained the association between *Plasmodium* and most of the determinants outlined in this study. Silamut et al found that plasma α1-acid glycoprotein concentrations were consistently raised in acute malaria.(Silamut, Molunto, Ho, Davis, & White, 1991) The association between *Plasmodium falciparum* and HIV infection has been shown by others. (Kuile et al., 2004) We expected that due to compromised malarial immunity, women that were HIV infected would be more susceptible to *Plasmodium* infection. Steketee et al found that HIV infection diminishes a pregnant woman's capacity to control *P. falciparum* parasitaemia. (Steketee et al., 1996)

Cserti et al studied the association between blood group and *Plasmodium falciparum* infection and found that the worldwide prevalence of blood group O parallels malarial regions suggesting that survival from malaria is associated with group O, and mortality is associated with group A. (Cserti & Dzik, 2007). Gravidity, (M. Desai et al., 2007) and maternal age, (Tako et al., 2005) are also associated with *Plasmodium* infection. In highly endemic areas, primigravidae are at greater risk of malarial infection (Nosten et al., 1991) (A. M. Greenwood et al., 1992), probably because they have a reduced immunity. Young primigravidae and multigravidae are at greater risk of malaria and its adverse effects than older primigravidae or multigravidae, respectively (Espinoza et al., 2005; Leenstra et al., 2003; Marques et al., 2005) (S J Rogerson et al., 2000) (Walker-abbey et al., 2005). Furthermore, it is conceivable that a subject with many live children in a resource-poor setting may be immune-compromised due to excessive workload, poor dietary intake, and cumulative poor health.

To the best of our knowledge, no other studies have investigated the reliability of multiple determinants to predict asymptomatic *Plasmodium* infection even though scoring methods exist for prediction of fatality from other clinical conditions such as burn injuries, (Ryan et al., 1998) stroke, (Poungvarin, Viriyavejakul, & Komontri, 1991) acute pancreatitis (Wilson, Heath, & Imrie, 1990) etc. In a study in Lagos, Nigeria, young maternal age, and use of insecticide spray was associated with increased risk of malaria infection in pregnant women. (Agomo & Oyibo, 2013) However, the authors did not study more determinants or combinations of determinants. According to Cottrell et al, although an infection during late pregnancy (after 28 weeks of pregnancy) was found to be strongly related to the presence of a placental infection, the best predictive model was given by taking into account parasitological information during whole pregnancy. (Cottrell et al., 2006) Such

information is seldom available in resource-poor settings. Although investigators frequently use ROC curves to assess diagnostic tests, this method is also appropriate for prognostic prediction using probabilistic estimates from logistic regression. (Levi, 1985) Medical decision making is then made easier by enabling the physicians' probabilistic judgments using predictive models.

Conclusions

This study identified the best predictive factors for asymptomatic *Plasmodium* infection. The study also confirmed that the number of live children that a subject has, coupled with HIV infection, low haemoglobin concentration levels, and elevated inflammation are fair predictors of asymptomatic *Plasmodium* infection. However, despite the potential benefits of combining different determinants to arrive at a set of predictive criteria that can be used to judge whether a subject may or may not be having asymptomatic *Plasmodium* infection, a highly predictive field-friendly predictive model was not plausible. For future research, plasma α1-acid glycoprotein concentration deserves further investigation as it was highly predictive of *Plasmodium* infection. Similarly, there is need to investigate the predictive reliability of plasma haptoglobin concentration, plasma pre-albumin concentration, plasma lactate dehydrogenase concentration, and plasma bilirubin concentration whether independently or in combinations with each other.

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Chapter 6

Early identification of pregnant women at high risk of giving birth to a neonate with low birth weight

Introduction

Approximately 16.5% of infants in developing countries, or 20 million infants, are born annually with a birth weight <2,500g. Epidemiological evidence indicates that such neonates die at a 20 times faster rate than heavier babies (UNICEF/WHO 2004), but low birth weight has also been associated, among others, with increased morbidity in the neonatal and post-neonatal period. Mortality rates can vary 100-fold across the range of birth weight, and rises continuously with decreasing weight (UNICEF/WHO 2004).

A better understanding of the etiology of low birth weight may provide clues for its prevention. In a randomised controlled trial among Kenyan women, we have recently shown that daily iron supplementation in the second and third pregnancy trimesters can reduce the risk of low birth weight by 65%, whilst correction of iron deficiency resulted in an increase in birth weight by 250g (Mwangi et al., submitted). Data that were collected as part of this trial at baseline and at delivery may allow for a further analysis of factors associated with birth weight.

In the period 2005–2012, 43% of pregnant African women attended the minimum four times antenatal care as recommended by the World Health Organization (WHO). Yet, many continue to deliver outside health facilities. For example, in western Kenya, in an area adjacent to where we conducted our trial, 80% of women delivered outside a health facility; among these, traditional birth attendants assisted 42%, laypersons assisted 36%, while 22% received no assistance (Van Eijk et al. 2006). Thus it would be important to develop methods that allow for identification of women at an early stage of pregnancy who need specific care to prevent low birth weight or who need urgent referral for safe delivery in a health care facility.

Submitted for publication

Thus we aimed a) to identify factors associated with birth weight; b) to develop a methodology to predict cases of low birth weight, using a single risk score that is based on prognostic variables collected at the second trimester of pregnancy.

Subjects and Methods

Population

This study used samples collected for a double blind, randomized controlled trial to investigate the safety and efficacy of iron supplementation in Kenyan pregnant women (Mwangi et al. submitted). The study (www.ClinicalTrials.gov: NCT01308112) was conducted from October 2011 to April 2013 in western Kenya (South-West Kisumu). Ethical clearance was received in Kenya and England. All participants gave written informed consent. Pregnant women in the late stage of the first trimester to early stage of second trimester were screened and assessed for eligibility into the study.

The study area was rural and highly endemic for malaria. The participants were recruited from married (or cohabiting) local women aged 15 – 45 years. If a woman missed her menstrual period for 10 weeks, she was invited for a pregnancy test. Women who were not in stable relationships were invited for a free pregnancy test every 12 weeks. Pregnant women received preventive chemotherapy with albendazole and praziquantel against helminth infections and intestinal schistosomiasis to prevent severe anemia. Gestation age was determined by ultrasound examination.

The population consists mainly of poor families from the Luo tribe. Their diet is mostly based on maize with a low content of animal products. Sorghum, millet, and cassava are consumed in smaller quantities. In the study area, anaemia is highly prevalent (69%) (Ouma et al., 2007) and intestinal schistosomiasis is common (Samuels et al., 2012).

We included pregnant women who: gave informed consent; were aged 15─45 years; likely to be available for study until 1 month after delivery and planning to deliver in the pre-designated health facility. We excluded women who had: obvious mental retardation; a metabolic disorder; a medical history of sickle cell anemia, epilepsy, diabetes; an obstetric history suggestive of eclampsia or pre-eclampsia; carrying multiples; gestational age at the second visit was <13weeks or >23weeks; no venous blood was collected, or haemoglobin concentration was <90g/L.

As part of the main trial, the flour from the homesteads of all participants was fortified with NaFeEDTA at the local mill level from screening until women left the study. The mill operators added fortificant iron (target dose: 20mg/kg as NaFeEDTA) (WHO et al., 2009) to grain routinely brought by a member of a participating homestead. Based on weighed intake studies, we estimate that fortification supplied on average 5.7mg fortificant iron daily in pregnant women (unpublished data).

The baseline data and blood samples were collected 14─21 days after the initial screening visit. Blood pressure was assessed during the first and second visits to the research clinic. We did not assess smoking in the population studied as very few women smoke. Consumption of alcohol and coffee is also probably very low. We collected venous blood in K2EDTA tubes, and measured haemoglobin concentration (HemoCue301, Ängelholm, Sweden) and zinc protoporphyrin (Aviv 206d, Lakewood, NJ). Buffy coat and erythrocyte sediment were stored separately in DNA-stabilizing buffer (AS1, Qiagen, Valencia, CA) at 2–8˚C in the field and subsequently at–80˚C until PCR assays. Plasma was stored at –196˚C in the field and subsequently at–80˚C until analysis.

Randomization and blinding

Pregnant women were randomly allocated to 60 mg iron as ferrous fumarate (Dr. Paul Lohmann®, CAT No.500005025200) or to placebo group. The supplements comprised capsules with starch as filler that were identical in appearance except that the shell for each type was into two colors (blue and dark green for iron, white and buff for placebo). The code linking each color to the type of supplement was kept in sealed, opaque envelopes. One of us (HV) not involved in the fieldwork used tables with random numbers to produce sequentially numbered envelopes containing the code. Eligible women were allocated in order of enrolment to the color indicated in the next available envelope. Participants and field staff were blinded to intervention until data analysis.

Laboratory analysis

Haemoglobin concentrations were measured using a photometer (HemoCue model 301, Ängelholm, Sweden). Iron markers (plasma concentrations of ferritin, soluble transferrin receptor, transferrin), vitamins (plasma vitamin B12 and folate concentration), and inflammation markers (plasma concentrations of C-reactive protein [CRP] and α1-acid glycoprotein) were measured on a Beckman Coulter UniCel DxC 880i analyser as per manufacturer's instructions. ZPP content in whole blood was measured by hematofluorometer (Aviv 206D, Lakewood Township, NJ, USA). Control samples at low, medium, and high levels (AVIV) were run after every 30 readings while two level calibration (Aviv; low, high) samples were run twice per year.

ZPP was also measured in the washed erythrocytes. To wash erythrocytes, blood samples were centrifuged (8min, 600xg), plasma was removed and replaced with an equal volume of phosphate buffered saline (Medicago, Uppsala, Sweden; catalogue no. 09-2051-100) in 100 mL deionized water. Following renewed centrifugation (8min, 600×g), the supernatant and buffy coat were discarded.

HIV infection was determined using rapid antibody tests (Alere Determine™, Alere™, Waltham, Massachusetts, USA); a positive result was confirmed using other antibody tests (Unigold™, Trinity Biotech PLC™, Co Wicklow, Ireland, and/or Bioline™, Pantech,

Umhlanga, Kwazulu-Natal, South Africa).

Plasmodium antigenaemia was assayed by dipstick tests (Access Bio Inc., Somerset, NJ, USA; CareStart, catalogue G0151 and G0171) that can detect *P. falciparum*-specific histidine-rich protein-2 (HRP2), *Plasmodium* lactate dehydrogenase (pLDH) specific to either *P. falciparum* or to non-*falciparum* species, i.e. *P. ovale*, *P. malariae* or *P. vivax*. Whereas HRP2-based tests detect current or recent *P. falciparum* infection, pLDH-based tests only indicate current infection. (Makler, Piper, & Milhous, 1998) (Piper et al., 1999) (Moody, 2002) HIV infection was assayed using antibody tests (Alere, Waltham, MA; confirmed by Unigold; Trinity Biotech, Bray, Ireland and/or Bioline; Pantech, Umhlanga, South Africa).

We determined α+-thalassaemia genotype by polymerase chain reaction; (Veenemans et al., 2008) (Veenemans et al., 2011) because of practical reasons, we could perform this analysis only in the first 213 successively recruited women.

Supervised daily administration of the study supplement was ensured for all the randomized participants. At delivery, we recorded the date of birth and the neonatal data (gender, anthropometric data). Maternal blood (6 mL) was collected by venipuncture within 1 hour after delivery. All blood samples were collected in tubes with K_2EDTA (Becton Dickinson). In addition, placental biopsies were collected and stored in 40% formaldehyde solution with PBS at pH 7.2 for subsequent histological examination.

Definitions

Hypertension is usually defined as diastolic blood pressure ≥ 90 mmHg or systolic blood pressure ≥ 140 mmHg. Because blood pressure is reduced in the first half of pregnancy by 25 mmHg,(WHO 1987) however, were defined hypertension as diastolic blood pressure ≥ 75 mmHg or systolic blood pressure ≥ 125 mmHg, each determined as the average of two measurements taken on different days.

Plasmodium infection status in placental biopsies was classified as non-infected, active infection, active-chronic infection and past-chronic infection.(Bulmer et al. 1993)

Low vitamin B12 status was defined by plasma cobalamin concentrations <200 ng/L (148 pmol/L). (Carmel et al. 2003)

Statistical analysis

Objective 1: First, we used one-way ANOVA and univariate linear regression analysis to identify factors possibly associated with birth weight. In this analysis, we used variables collected at recruitment into the trial, as well as data collected at delivery. We used dummy-coded categorised explanatory variables because several factors have been reported to be associated with birth weight of fetal growth in a non-linear fashion (e.g. maternal age,

gravidity, haemoglobin concentration). We subsequently developed a multivariate linear regression model with backward elimination to identify factors that were independently associated with birth weight. Gestational age at delivery was not included in this model, because its inclusion would adjust and thus eliminate effects on birth weight by factors that affect gestational duration. For iron deficiency and iron supplementation, we assumed interaction because pre-planned subgroup analysis of the randomisation trial had confirmed our expectation that women with initial iron deficiency had a greater response in birth weight to iron supplementation than their peers who were initially iron-replete (Mwangi et al., submitted). We found no support for effect modification between iron supplementation and other baseline factors assessed in the present paper (not shown).

Objective 2: We modelled low birth weight using multivariate logistic discriminant analysis with a stepped forward selection procedure to examine the added prognostic value of variables collected at baseline (second trimester of pregnancy). We started the model with the variable iron supplementation, then also entered variables that could easily be obtained from an interview and a routine medical examination, subsequently added laboratory data that can be collected using point-of-care tests that are commercially available (haemoglobin concentration, *Plasmodium* antigenaemia, HIV antibodies, C-reactive protein concentration), and lastly data that would require measurement in a tertiary care facility or reference laboratory. Thus variables were categorised into four types, depending on the practical ease of performing each test and the inputs required for their assessment **(Table 1)**.

Table 1: Categorisation of prognostic variables assessed at 2 trimester of pregnancy

Variable	Measure [1]
Type 1 (routinely assessed at most rural care facilities, or could be added at no cost)	
Iron supplementation	Binary
Maternal age	Nominal
Marital status	Nominal
Gravidity	Ordinal
Height	Nominal
Body mass index	Nominal
Hypertension	Binary
Category 2 (not assessed at all rural care facilities, but can be added at with relatively small inputs)	
Haemoglobin concentration	Nominal
Plasmodium dipstick test (any test positive)	Nominal
Type 3 (can be assessed at rural care facilities with relatively large added inputs)	
Inflammation (plasma C-reactive protein concentration >10 mg/L)	Binary

Table 1: Categorisation of prognostic variables assessed at 2 trimester of pregnancy

Erythrocyte ZPP content	Ordinal
Type 4 (requires assaying in reference laboratory) [3]	
Inflammation (plasma α_1-acid glycoprotein concentration >10 mg/L)	Binary
Iron status (plasma ferritin concentration and inflammation [2])	Nominal
Folate status	Binary

[1]As analysed in the present paper; [2]Elevated plasma concentrations of both C-reactive protein concentration and α_1-acid glycoprotein concentration; [3]α^+-thalassaemia genotype was not considered because of its relatively high cost, practical problems in its measurement (e.g. DNA isolation in a DNA-free environment), and because it probably will never be used because it is not informative about other health conditions.

Type 1 and Type 2 variables include variables that are routinely assessed in most rural care facilities in developing countries, or that can be assessed with relatively small inputs; Type 3 variables concern point-of-care tests that can also be performed in such facilities, but this would require substantial inputs in the form of capital investments, recurrent costs, training, supervision or a reliable supply of consumables. Type 4 variables need to be assessed in a reference laboratory. The prognostic value of added variables was evaluated using likelihood ratio tests, and variables were rejected if p>0.15.

The coefficients obtained by multiple logistic regression analysis were used to produce a linear predictor (LP) that was converted into a predicted probability (P) of low birth weight for each mother, based on the logistic regression equation. By allowing the variable P to vary in the range [0□1], we produced Receiver Operating Characteristics (ROC) curves, which were used to assess the ability of various models to discriminate between women who gave birth to a neonate with or without low birth weight. This discriminating ability can be expressed by the area-under-the-curve (AUC). We predefined AUC values in the ranges 0.50□0.60, 0.60□0.70, 0.70□0.80, 0.80□0.90 and 0.90□1 to indicate failed, poor, fair, good and excellent performance.

Results

Table 2 show results of the analyses to identify factors associated with birth weight. Teenage pregnancy, short stature (<155cm), being not married hypertension, inflammation and active-chronic placental infection with malarial parasites were associated reductions in birth weight. On the other hand, birth weight increased with gravidity, iron supplementation, body mass index and gestational age. There was weak evidence that birth weight was associated with haemoglobin concentration class at baseline, and there was no consistent monotonous trend in this association.

Associations with low vitamin B12 status are not reported because we found only 3 women (0.6%) to have low vitamin B12 status. There was no evidence of an associated between plasma cobalamin concentrations and birth weight (not shown).

Table 3 shows associations with birth weight obtained by multivariate regression analysis. Being never married, presence of inflammation, being a girl and presence of iron deficiency were independently associated with reductions in birth weight, whereas being overweight (body mass index ≥ 25 kg/m2) was associated with increased birth weight.

Evidence that markers of *Plasmodium* infection were associated with birth weight was weak. Results of dipstick tests from blood collected from the antecubital vein were not associated with birth weight, and these were rejected in the multivariate analysis.

We nonetheless retained placental markers of infection in the multivariate model because there is strong evidence from randomised trials that malaria causes a reduced birth weight that can be reversed by control measures, (Garner and Gulmezoglu 2006, Eisele et al. 2012, Kayentao et al. 2013) and our data indicated that active-chronic placental infection is independently associated with a reduction in birth weight by 213g (Table 3). Our reduced multivariate logistic regression model (‘Model 1’) of low birth weight only contained iron supplementation, maternal height and HIV infection from the Type 1 and Type 2 variables (**Table 4)**.

Because the other Type 1 and Type 2 variables can be relatively easily assessed, we also added the full model (‘Model 2’); however, there was no statistical evidence that this model performed better (p=0.72). For Type 3 and Type 4 variables, only inflammation as indicated by plasma α1-acid glycoprotein concentration ≥ 1 mg/L (‘Model 3’) had added prognostic value (p=0.02).

The results from the logistic regression analyses shown in Table 4 were used to calculate the predicted probability of low birth weight. For example, for Model 1, P was computed as follows: $P=1/((1+e^{(-LP)}))$, where LP = 1.059 [Iron supplementation] + 2.108 [Height1] + 0.490 [Height2] + 1.096 [Height3] + 1.783 [Height4] – 0.870[HIV infection] – 4.092 (variable names indicated between straight brackets; [Height1–4] indicate binary dummy variables indicating categories 155–159.9cm, 160–164.9cm, 165–169.9cm and ≥ 170 cm, respectively).

Table 2: Factors associated with birth weight (g)

Factor	n	Birth weight, g	(SD)	p-value	Effect (95% CI)
Determined at baseline or in samples collected at baseline					
Maternal age				0.05	
<20 years	67	3,008	(459)		–141 (–255 to –27)
20–34.99 years	290	3,149	(423)		Reference
≥35 years	36	3,085	(416)		–64 (–213 to 85)
Maternal height				0.11	
< 155 cm	45	2,999	(481)		–169 (–313 to –26)
155–159.9 cm	75	3,071	(390)		–97 (–217 to 23)
160–164.9 cm	146	3,169	(449)		Reference
165–169.9 cm	75	3,099	(436)		–69 (–189 to 51)
≥ 170 cm	52	3,183	(361)		14 (–122 to 150)
Marital status				0.004	
Married or living together	341	3,146	(431)		Reference
Never married	39	2,908	(391)		–238 (–379 to –96)
Divorced or separated	13	3,059	(386)		–87 (–23 to –150)
Body mass index				0.001	
≤ 18.5 kg/m^2	30	3,072	(436)		–20 (–179 to 139)
18.5–25 kg/m^2	321	3,093	(405)		Reference
≥ 25.0 kg/m^2	42	3,357	(542)		264 (–128 to 401)
Hypertension				0.56	
Absent	358	3,123	(432)		Reference
Present [1]	35	3,078	(424)		–45 (–195 to 105)
Gravidity				0.003	
Primigravida	54	2,932	(437)		Reference
Secundigravida	76	3,126	(413)		194 (45 to 343)
Multigravida	263	3,156	(426)		224 (99 to 349)
Haemoglobin concentration				0.06	
< 100 g/L	60	2,996	(443)		–99 (–230 to 32)

Table 2: Factors associated with birth weight (g)

100–100.9 g/L	83	3,149 (437)		54 (–64 to 171)
110–119.9 g/L	134	3,095 (401)		Reference
120–129.9 g/L	98	3,180 (447)		85 (–27 to 196)
≥ 130 g/L	18	3,238 (425)		142 (–69 to 354)
Inflammation			0.001	
Absent	275	3,165 (418)		Reference
Plasma CRP concentration ≥10mg/L and AGP concentration <1.0 g/L	53	3,105 (395)		–59 (–184 to 66)
Plasma CRP concentration <10mg/L and AGP concentration ≥1.0 g/L	21	3,012 (433)		–153 (–341 to 36)
Plasma CRP concentration ≥10mg/L and AGP concentration ≥1.0 g/L	44	2,905 (483)		–260 (–395 to –125)
Iron status			0.02	
Deficient (plasma ferritin concentration <15µg/L)	211	3,145 (433)		7 (–91 to 105)
Replete (ferritin concentration ≥15µg/L; inflammation absent)	113	3,152 (417)		Reference
Uncertain (ferritin concentration ≥15µg/L; inflammation present)	69	2,988 (428)		–163 (–290 to –36)
Iron supplementation			0.001	
No	199	3,050 (410)		Reference
Yes	194	3,191 (441)		141 (56 to 225)
Erythrocyte ZPP content			0.34	
≤40 µmol/mol haem	203	3,107 (411)		
40–70 µmol/mol haem	94	3,175 (481)		67 (–38 to 173)
> 70 µmol/mol haem	96	3,090 (419)		–17 (–122 to 88)
Folate status			0.66	
Plasma folate concentration <5 µg/L	322	3,115 (436)		25 (–87 to 138)
Plasma folate concentration ≥5 µg/L	69	3,140 (404)		Reference
Plasmodium infection [3]			0.19	
Absent	247	3,136 (443)		Reference
Sub-patent	68	3,150 (421)		14 (–102 to 130)
Patent	78	3,040 (396)		–95 (–205 to 14)

Table 2: Factors associated with birth weight (g)

HIV infection			0.12	
Absent	304	3,137 (431)		Reference
Present	87	3,055 (425)		–82 (–185 to 21)
α^+-thalassaemia genotype			1.00	
Normal (*αα*/*αα*)	94	3,147 (471)		Reference
Heterozygote (*α*–/*αα*)	79	3,143 (396)		–4 (–133 to 125)
Homozygote(*α*–/*α*–)	14	3,154 (256)		7 (–235 to 248)
Determined at delivery or in samples collected at delivery				
Infant sex			0.03	
Boys	196	3,167 (427)		Reference
Girls	197	3,072 (430)		–96 (–181 to –11)
Gestational age			<0.001	
≤ 37.99 weeks	131	2,952 (409)		–233 (–321 to –144)
38–41 weeks	234	3,185 (401)		Reference
≥ 41.00 weeks	25	3,392 (517)		207 (37 to 377)
Haemoglobin concentration			0.18	
< 100 g/L	70	3,050 (417)		–124 (–260 to 13)
100–100.9 g/L	64	3,079 (410)		–95 (–235 to 45)
110–119.9 g/L	71	3,074 (402)		–100 (–236 to 36)
120–129.9 g/L	84	3,174 (424)		Reference
≥ 130 g/L	101	3,177 (471)		4 (–121 to 129)
Plasmodium infection [3]				
Assessed in circulating venous blood			0.43	
Absent	278	3,137 (451)		Reference
Sub-patent	45	3,077 (333)		–60 (–196 to 76)
Patent	68	3,073 (400)		–64 (–179 to 50)
Assessed in placental biopsies			0.05	
Absent	192	3,188 (440)		Reference
Active	8	3,138 (429)		–50 (–353 to 252)
Active-chronic	28	2,975 (398)		–213 (–383 to –44)

Table 2: Factors associated with birth weight (g)

Past-chronic	88	3,089 (403)	–98 (–206 to 10)

CRP: C-reactive protein; AGP: α_1-acid glycoprotein

[1] Diastolic blood pressure ≥ 75 mmHg or systolic blood pressure ≥ 125 mmHg, each determined as the average of two measurements taken on different days; [2] p-value for interaction between initial iron status and iron supplementation; [2] Categories defined as follows: deficient: plasma ferritin concentration <12 µg/L, regardless of the presence or absence of inflammation; replete: plasma ferritin concentration ≥12 µg/L in the absence of inflammation (plasma concentrations of C-reactive protein <10 mg/L or α1-acid glycoprotein <1.0 g/L); uncertain: plasma ferritin concentration ≥12 µg/L in the presence of inflammation; [3] Mutually exclusive categories defined as follows: absent: negative results for any dipstick test or PCR test; sub-patent: positive result for *P. falciparum*-specific PCR test but negative result for any dipstick test; patent: at least one positive dipstick test result (i.e. presence of either HRP2, *P. falciparum*-specific pLDH, or pLDH specific human *Plasmodium* species other than *P. falciparum*), regardless of PCR test result.

Table 3: Factors independently associated with birth weight (g), multivariate linear regression analysis

	Effect on birth weight, g	(95% CI)	p
Marital status			**0.02**
Married or living together	Reference		
Never married	–207	(–358 to –55)	
Divorced or separated	–90	(–344 to 165)	
Body mass index			**0.001**
≤ 18.5 kg/m^2	–22	(–210 to 167)	
18.5–25 kg/m^2	Reference		
≥ 25.0 kg/m^2	264	(124 to 404)	
Inflammation at baseline			**0.06**
Absent	Reference		
Plasma CRP concentration ≥10mg/L and AGP concentration <1.0 g/L	–129	(–289 to 32)	
Plasma CRP concentration <10mg/L and AGP concentration ≥1.0 g/L	–150	(–385 to 85)	
Plasma CRP concentration ≥10mg/L and AGP	–235	(–406 to –63)	

Table 3: Factors independently associated with birth weight (g), multivariate linear regression analysis

concentration ≥1.0 g/L			
Infant sex			**0.02**
Boy	Reference		
Girl	–110	(–199 to –20)	
Iron status at baseline [1]			**0.001 [2]**
Iron-deficient	–158	(–309 to –6)	
Iron-replete	Reference		
Uncertain	–11	(–248 to 227)	
Placental infection status by *Plasmodium* spp.			
No infection	Reference		**0.283**
Active	–12	(–298 to 275)	
Active-chronic	–158	(–324 to 9)	
Past	–55	(–162 to 51)	

AGP: α_1-acid glycoprotein; CRP: C-reactive protein

[1] Iron status categories: iron-deficient: plasma ferritin <15 µg/L regardless of inflammation status; iron replete: plasma ferritin ≥15 µg/L without inflammation (both plasma CRP concentration ≥10mg/L and AGP concentration <1.0 g/L); iron status uncertain (either plasma CRP concentration ≥10mg/L or AGP concentration <1.0 g/L). The associations reported for iron-deficiency and uncertain iron status apply to women who did not receive supplementation. The model also included effects for iron supplementation and its interaction terms with iron status categories, but these effects will be reported elsewhere. [2] P-interaction

Table 4: Prognostic factors for low birth weight (g), based on multivariate logistic regression analysis

	Model 1 (Type 1□2 variables; reduced model)	Model 2 (Type 1□2 variables; full model)	Model 3 (Type 3□4 variables; Reduced model)
Type 1 variables			
Constant	4.092	–3.895	–3.572
Iron supplementation	1.059	0.961	0.942
Maternal height			
155–159.9 cm	2.108	2.276	2.162
160–164.9 cm	0.490	0.512	0.53
165–169.9 cm	1.096	1.142	1.036
≥ 170 cm	1.783	1.734	1.721
HIV	–0.870	–1.209	–1.016
Maternal age			
20–34.99 years	–	–0.351	–0.503
≥35 years	–	–0.640	–0.769
Marital status			
Never married	–	0.061	0.119
Divorced or separated	–	–0.530	–0.268
Gravidity			
Secundipara	–	1.043	0.871
Multipara	–	0.609	0.716
Body mass index			
18.5–25 kg/m^2	–	0.100	0.128
≥ 25.0 kg/m^2	–	–0.583	–0.41
Hypertension	–	1.068	1.211
Type 2 variables			
Hb concentration			
100–100.9 g/L	–	0.411	0.38
110–119.9 g/L	–	–0.521	–0.509
120–129.9 g/L	–	–0.177	0.025
≥ 130 g/L	–	–0.915	–0.812
Plasmodium infection [3]			
Sub-patent	–	–0.015	0.431
Patent	–	–0.352	–0.26
Type 3-4 variables			
Inflammation (plasma α1-acid glycoprotein ≥ 1 mg/L)			–1.288

Values indicate coefficient estimates associated with each factor.

Figure 1 shows the ROC curves for Models 1□3. The ability to discriminate between women who would deliver a neonate with low or normal birth weight was fair for Model 1, and good for Model 3. Under Model 1, the decision rule of P > 0.0360 would have 90% sensitivity in predicting low birth weight, and the corresponding specificity would be 46%. Under Models 2 and 3, this 90% sensitivity is attained with P > 0.0375 and P > 0.0348, respectively, yielding specificity values of 58% and 63%.

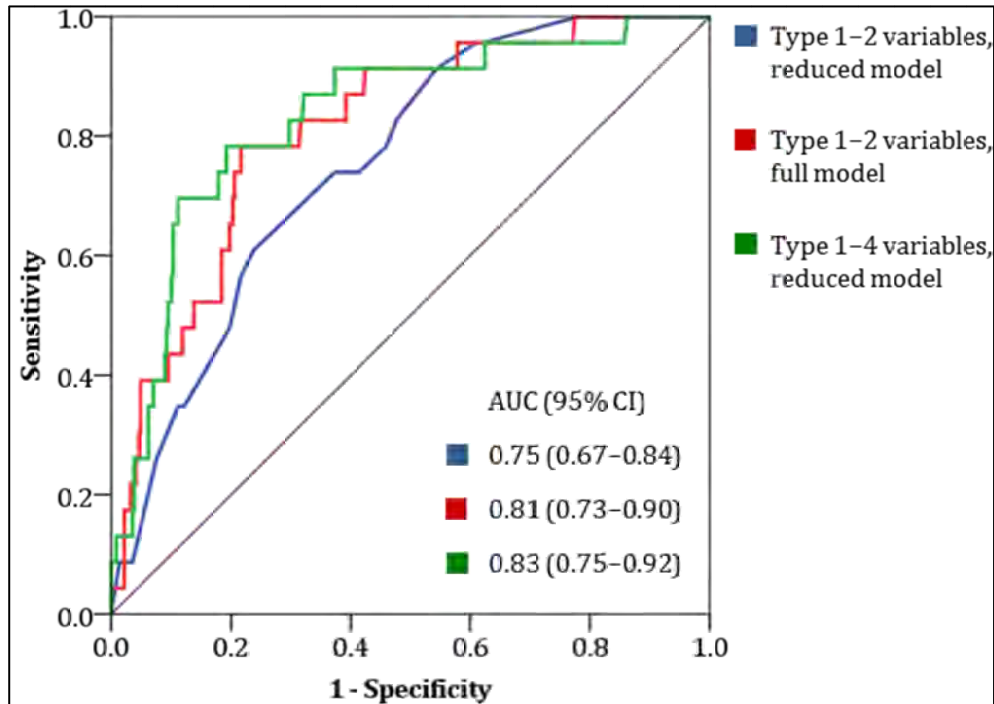

Figure 1: ROC curve showing the ability of various combinations of variables collected at second trimester of pregnancy to discriminate between women who will or will not given birth to a neonate with low birth weight (<2,500g)

AUC: Area-under-the-curve. Blue, red and green curves are based on Models 1, 2 and 3 (Table 4), respectively). For explanation of variables included in each model, see Table 4 and text. The diagonal line represents the theoretically worst test with no discrimination between groups. Diagonal segments in curves are produced by ties.

When the decision rule of P > 0.0360 under Model 1 (see preceding paragraph) were applied to Kenya, where 11% of neonates are born with low birth weight, (UNICEF/WHO 2004) a predicted outcome of normal birth weight would be correct with a probability of 97%, whilst a predicted outcome of low birth weight would be correct with a probability of 17% **(Table 5)**. The decision rules P > 0.0375 and P > 0.0348 under Models 2 and 3,

respectively, would give virtually identical probabilities of correctly predicting normal birth weight, and yield only small improvements in the probability of correctly predicting low birth weight (21% and 23%, respectively; table 5).

Table 5: Performance of decision rules obtained with Models 1, 2 and 3 in predicting low birth weight with 90% sensitivity in pregnant women, at a prevalence of low birth weight reported for Kenya (11%)

Model 1. Decision rule to predict low birth weight: $P > 0.0360$ [1]					
Predicted	**Birth weight**				
	<2,500g	**≥2,500g**			
Low birth weight	0.10	0.48	0.58	Specificity	46%
Normal birth weight	0.01	0.41	0.42	PPV	17%
	0.11	0.89	1.00	NPV	97%

Model 2. Decision rule to predict low birth weight: $P > 0.0375$ [1]					
Predicted	**Birth weight**				
	<2,500g	**≥2,500g**			
Low birth weight	0.10	0.37	0.47	Specificity	58%
Normal birth weight	0.01	0.52	0.53	PPV	21%
	0.11	0.89	1.00	NPV	98%

Model 3. Decision rule to predict low birth weight: $P > 0.0348$ [1]					
Predicted	**Birth weight**				
	<2,500g	**≥2,500g**			
Low birth weight	0.10	0.33	0.43	Specificity	63%
Normal birth weight	0.01	0.56	0.57	PPV	23%
	0.11	0.89	1.00	NPV	98%

PPV: positive predictive value, i.e. the probability of correctly predicting low birth weight; NPV: negative predictive value, i.e. the probability of correctly predicting the absence of low birth weight.

[1] $P = 1/(1 + e^{-LP})$**; model-specific** ***LP*****s can be derived from Table 4 (see text).**

Discussion

Our data showed that being never married, inflammation, being a girl and iron deficiency were independently associated with reduced birth weight, whereas being overweight was

associated with increased birth weight. We have also shown that methods can be developed with fair accuracy in discriminating between women due to give birth to neonates with low birth weight versus normal birth weight, using a single risk score that is based on a combination of prognostic variables collected at the second trimester of pregnancy.

Our two research questions have driven separate approaches to data analysis, with different results. First, we considered an etiological question about the causal factors that determine birth weight. In this part of the research, we used birth weight as a continuous outcome variable, because dichotomisation into a derived variable 'low/normal birth weight' inevitably results in a loss of information and statistical precision, (e.g. MacCallum et al. 2002) and mortality rates are believed to rise continuously with decreasing birth weight (UNICEF/WHO 2004). In addition, etiological research requires the exclusion of confounders, i.e. external factors that form an alternative explanation for an apparent association between a potential determinant of birth weight) and consideration of effect modification, i.e. the measure of association between a potential determinant and birth weight depends on an external factor. A backward elimination procedure, as we applied, is commonly used and recommended to eliminate confounding between factors, and to identify factors that are independently associated with outcome. In this analysis, we also did not include gestational age because it is likely in the causal pathway between other factors and birth weight. A separate analysis would be required to identify factors that are independent determinants of gestational duration but this was outside the scope of the present paper.

Second, we considered a prognostic question about factors that predict the risk of low birth weight. In this analysis, we dichotomised birth weight, mimicking the decision eventually to be made by care providers as to whether or not to take special precautions to prevent low birth weight or to ensure safe delivery. The objective of this part of our analysis is not to explain the occurrence of low birth weight, so confounding and effect modification are irrelevant, and the factors included in the statistical model do not necessarily have to be causally related to low birth weight. A stepped forward approach allows the assessment of the predictive value of each factor when sequentially added to those factors already included. From a practical point of view, it is logical to start with those factors that can be easily measured under field conditions. For these reasons, the factors included the models thus generated (Table 4) are different from those included in the first part of this paper (Table 3).

A problem that is often encountered in prognostic (and diagnostic) research is that associations are reported between single factors and outcome. In medical care, however, one is usually interested in the added value of a factor over and above the prognostic information that is already contained in other variables. One strength of our paper is the multivariate approach that allowed us to overcome this problem, and that produced a single risk score (P) to predict the risk of low birth weight based on the predictive information contained in a combination of variables. A limitation of the present paper, however, is that

it used a randomised trial as the vehicle for research. As a consequence, we may have failed to collect key variables that could be helpful in the prediction of low birth weight, and generalizability of our results may be limited by the eligibility criteria used in the trial. We have nonetheless developed a promising methodology that can be further advanced in future studies. Another issue that may be raised concerns the question whether the prognostic factors were influenced by our experimental intervention, i.e. iron supplementation. We believe that we have accounted for this by including iron supplementation as a separate variable in the analysis. A last limitation is that our decision rules should be considered with caution, because they need to be validated in other settings.

Several of the risk factors identified in univariate analysis (Table 3) were similarly associated with birth weight as reported elsewhere. For example, it has been described that girls weigh less than boys, neonates from single mothers and divorced mothers have an increased risk of low birth weight, and second and third children are heavier than first children (Kramer 1987, Valero De Bernabé et al. 2004). We did not find an inverted U-shaped association between haemoglobin concentration at delivery and birth weight, (Valero De Bernabé et al. 2004) but this may be in part because we collected blood immediately after birth, which may not properly reflect haemoglobin status and iron stores immediately before birth. We also found limited evidence for an association between markers of *Plasmodium* infection and birth weight, despite strong evidence from randomised trials that malaria can cause a reduced birth weight that is reversed by control measures. (Garner and Gulmezoglu 2006, Kayentao et al. 2013) However, blood markers of *Plasmodium* infection measured in our samples collected at baseline and at delivery may not have captured the entire history of exposure to infection during pregnancy, particularly in this population of women who, due to frequent exposure to infection, presumably have developed considerable protective immunity to suppress parasitaemia.

A notable finding in the univariate and multivariate analyses presented in Tables 3 and 4, as well as the results presented in Table 5, is the marked reduction in birth weight associated with inflammation, particularly as indicated by elevated plasma concentrations of α1-acid glycoprotein. Contrary to C-reactive protein concentration, which increases rapidly after an inflammatory stimulus and drops rapidly after the cessation of such a stimulus, α1-acid glycoprotein may indicate more chronic inflammation because it rises slowly and also drops slowly. (e.g.Thurnham et al. 2010) Pregnancy is characterised by a low-grade systemic inflammatory response, but infection-induced inflammation is also a common cause of preterm birth. (Romero et al. 2007, Genc and Ford 2010, Mor et al. 2011) Further work would be needed to assess the cause of inflammation in our study population; considering that the absence of an associations between birth weight with either *Plasmodium* infection or HIV infection, it seems likely that the cause of inflammation was due to other causes.

Lastly, our prediction models (Table 4, Figure 1) show that measurement of plasma α1-acid glycoprotein concentration overall has added value in predicting low birth weight.

However, this added benefit leads to only small gains in predictive performance when applied to screen women with a view to better management (table 5). In such conditions, one wishes to have a high specificity to ensure that the majority of women at high risk of giving birth to a neonate with low birth weight are detected, and to rule out cases of low birth weight on the basis of the prediction. When applied with a sensitivity of 90% and an 11% risk of low birth weight as extant in Kenya (table 5), the addition of α1 acid-glycoprotein concentration leads to only a small reduction in the proportion of women who are falsely indicated as being at high risk. On this basis, it may be better to base decision rules on Models 1 or 2, which were equally effective in correctly ruling out cases of low birth weight.

Conclusion

We conclude that it is technically feasible to predict with fair accuracy women at high risk of giving birth to a neonate with low birth weight, based on variables that can be collected rapidly at relatively low cost and ease in the second trimester of pregnancy.

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Chapter 7

General discussion

The aim of this thesis was to assess the effect of iron supplementation on safety indicators, and on iron status, in pregnant women and their neonates. The main study was implemented against the backdrop of new fortification regulations in Kenya that required all industrially milled flour to be fortified with micronutrients including iron in the form of NaFeEDTA. (Government of Kenya, 2012) We hypothesized that consumption of food products fortified with iron combined with intake of iron supplements especially in pregnancy, could increase malaria rates, with adverse health consequences for pregnant women and their neonates. The specific aims of the thesis were as follows: 1) To compare the presence of malarial infection in parturient women who received a combination of iron-fortified foods with iron supplements versus iron-fortified foods only [**chapter 2**]; 2) to assess intervention effects on the maternal prevalence of iron deficiency anaemia at 1 month after delivery [**chapter 2**]; 3) to assess intervention effects on neonatal iron stores at 1 month of age [**chapter 2**]; 4) to assess the diagnostic utility of Zinc protoporphyrin in diagnosing iron deficiency in malaria endemic regions [**chapter 3**]; 5) to identify baseline factors that are prognostic for the NTBI response to consumption of a single iron supplement [**chapter 4**]; 6) to determine the factors that predict *Plasmodium* infection in pregnancy [**chapter 5**]; 7) to identify factors associated with birth weight [**chapter 6**]; 8) to develop a methodology to predict cases of low birth weight, using a single prognostic score that is based on prognostic variables collected at the second trimester of pregnancy [**chapter 6**]; 9) to develop methods for community-based flour fortification with iron; 10) to assess intervention effects on maternal intestinal pathogens at 1 month after delivery. At the time of completion of this thesis, data analysis on maternal intestinal pathogens was ongoing; these findings will be reported elsewhere.

Main findings of this thesis

Figure 1 is a summary of the main findings, outlined sequentially from safety issues regarding iron supplementation, beneficial aspects of iron supplementation, main conclusions of all chapters, and finally, policy implications of the thesis findings. Contrary to our hypothesis, iron supplementation did not result in an increased risk of *Plasmodium*

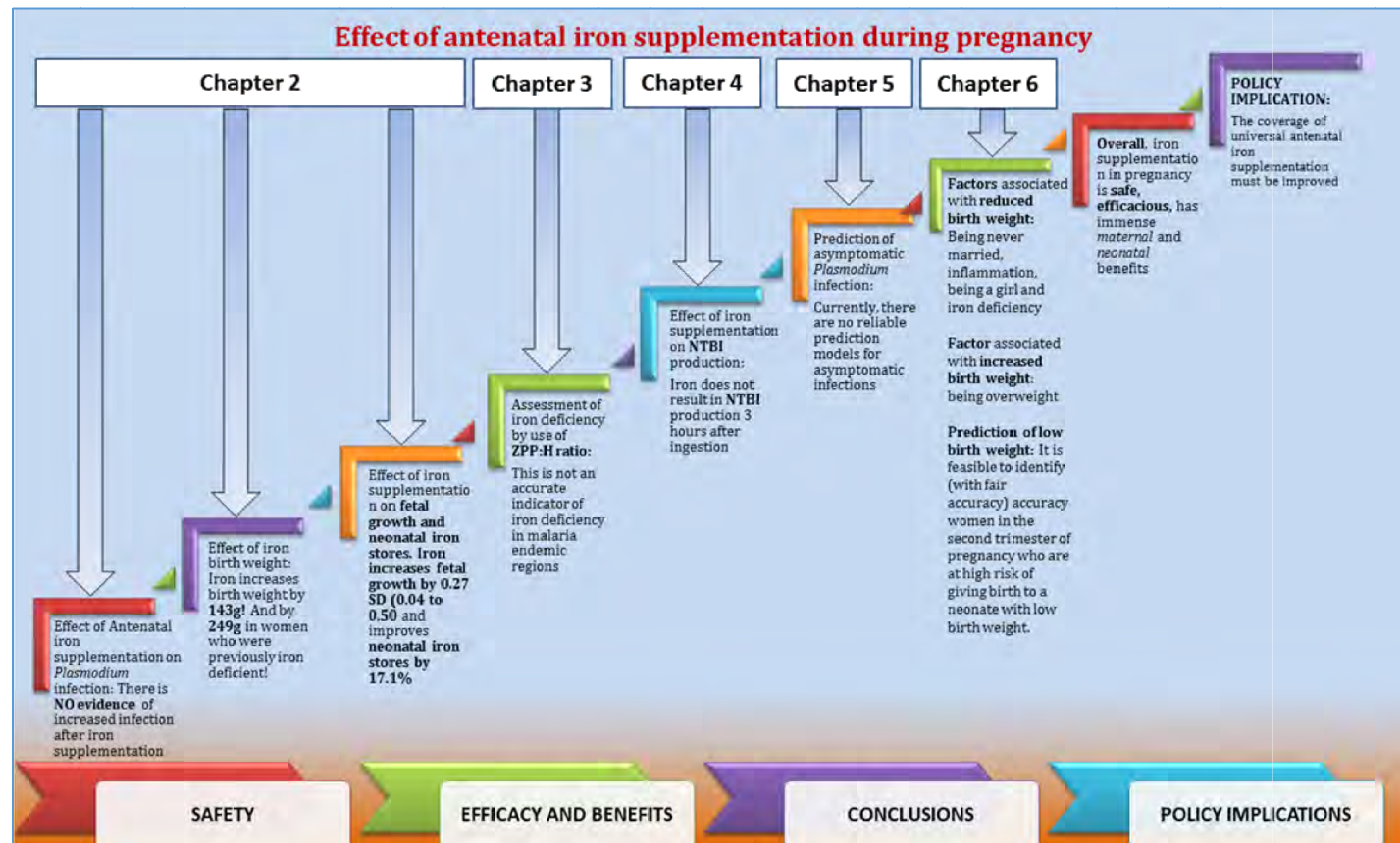


Figure 1: Overview of main findings

infection. We did not find evidence that the effect of iron on *Plasmodium* infection depended on the use of Intermittent Preventive Treatment (IPTp). We nonetheless agree with the current World Health Organisation recommendation that "in malaria-endemic areas, provision of iron and folic acid supplements should be implemented in conjunction with measures to prevent, diagnose and treat malaria". (WHO, 2012a)

Although this recommendation has obvious benefits for the target population, programme implementers and governments in resource-poor settings are held-back by the increased cost of malaria control, iron deficiency, and anaemia control programmes. Usually, the end result is a poorly implemented programme with low coverage. This exacerbates the problem of malaria and iron deficiency anaemia control in malaria-endemic countries. Considering the upper limit of the 95% CI of the main effect of iron supplementation (─9.3% to 9.3%), we cannot exclude the possibility that iron supplementation may have led to a 9% increase in *Plasmodium* infection, which may have reduced birth weight. (Desai et al. 2007) Overall, however, iron supplementation led to large increase in birth weight (143g). Particularly in women who were initially iron deficient, iron supplementation increased birth weight by 249 g. Although it is theoretically possible to screen and treat only those who are iron deficient, this is difficult to achieve in developing countries because of practical and financial constraints. For these reasons, we recommend universal iron supplementation in pregnant women, even in areas with poor coverage of IPTp.

The results reported in this thesis have tremendous implications for neonatal health. We showed that iron supplementation reduced the risk of low birth weight, which is the primary determinant of neonatal and post-neonatal mortality. Low birth weight is also an important cause of neonatal morbidity, inhibited infant growth, and cognitive development (chapter 2). Our calculations indicate that if our results are applied to all women in developing countries in order to eliminate iron deficiency, we could avoid 3 million births with low birth weight annually and save the lives of more than half a million neonates. These figures should be treated with caution, however, because they are based on many assumptions.

The effect of iron on birth weight was achieved at least in part through increased fetal growth (effect: 0.27 SD, 95%CI: 0.04 to 0.50). In this analysis, we assessed fetal growth by z-scores. Compared to dichotomised indicators of fetal growth such as small-for-gestational age, z-scores have the advantage that dichotomising of continuous outcomes leads to loss of information and reduced statistical precision when comparing groups. (MacCallum, Zhang, Preacher, & Rucker, 2002)

Our study also showed improved neonatal iron stores one month post-partum as indicated by an increase in plasma ferritin concentration by 17.1% (95% CI: 2.0% to 34.3%) in neonates of mothers who received high-dose iron compared to those who received low-dose iron. It is a common belief that the fetus obtains and stores normal amounts of iron even when its mother is mildly to moderately iron deficient. (Allen, 1997).

Most studies that failed to show an association between maternal and infant iron status examined the relationships between iron status in late pregnancy and iron markers in cord blood in women who were not iron deficient. (Allen, 1997) (Ajayi, 1988; Doyle, Crawford, Wynn, & Wynn, 2009; Okuyama, Tawada, Furuya, & Villee, 1985; Rios, Lipschitz, Cook, & Smith, 1975). In areas where anaemia is prevalent, however, cord blood iron markers were closely associated with maternal concentrations. (Agrawal, Tripathi, & Agarwal, 1983; Gaspar, Ortega, & Moreiras, 1993) The findings of this thesis provide more impetus to the need to offer iron supplements to pregnant women with an aim of boosting infant iron stores. Although infants born at term are not usually iron deficient, neonatal stores are important because they probably delay the time until infants develop iron deficiency.

We also assessed whether zinc protoporphyrin is accurate when used to assess iron deficiency in malaria endemic regions (Chapter 3). After comparisons with other iron indicators such as plasma ferritin concentrations it was found that zinc protoporphyrin is of unreliable diagnostic utility when discriminating between pregnant women with and without iron deficiency. Whole blood ZPP content overestimated the proportion of iron deficient population. Erythrocyte ZPP content performed slightly better but still over-estimated the proportion of iron deficient population. Further research is required to explain the factors that influence ZPP synthesis in patients with chronic diseases. In addition, there is need for a review of the guidelines on the diagnosis and management of iron deficiency as assessed by ZPP in pregnancy in regions where chronic diseases are prevalent. Our results indicate that whole blood ZPP should have no role as a screening marker to manage iron deficiency in pregnant women. Erythrocyte ZPP may have limited value to rule out iron deficiency in populations with low prevalence of iron deficiency, but a positive test result should be followed by other, more specific diagnostic tests such as plasma ferritin concentration. Haemoglobin concentration is not suitable for this purpose.

Non-transferrin bound iron has been the subject of many scientific discussions especially after the publication of the Pemba trial results. (Sazawal et al., 2006) The appearance of NTBI in circulation after oral ingestion of iron supplements has been thought to aid the growth and multiplication of *Plasmodium* parasites thereby increasing malaria induced morbidity and mortality. We conclude that oral ingestion of 60mg of ferrous fumarate will not lead to a detectable increase in NTBI concentrations provided that it is taken with food(Chapter 4). The absence of an effect is consistent with the lack of an effect of iron on *Plasmodium* infection (Chapter 2).

In chapter 5 of this thesis, we aimed to develop a field friendly tool that can be used to predict asymptomatic *Plasmodium* infection. This was motivated by the fact that most point-of-care dipstick tests used to detect *Plasmodium* infection are not able to detect 100% of all the infection present yet asymptomatic infections are increasingly associated with adverse maternal and neonatal outcomes. In addition, with the observed reduction in malaria prevalence rates, focus is probably going to shift towards methods to detect

asymptomatic *Plasmodium* infection. Although many likely predictive factors were assessed individually or in combination with others, we did not get a reliable tool that is easy to apply in resource-poor malaria endemic settings.

Low birth weight neonates die at a 20 times faster rate than heavier babies (UNICEF/WHO 2004). We postulate that a better understanding of the aetiology of low birth weight can provide clues for its prevention. In chapter 6, we aimed to identify factors associated with birth weight and then to develop a methodology to predict cases of low birth weight, using a single prognostic score that is based on variables collected at the second trimester of pregnancy. We used data collected as part of the trial presented in Chapter 2 and undertook further analyses. We show that being never married, inflammation, being a girl and iron deficiency were independently associated with reduced birth weight, whereas being overweight was associated with increased birth weight. Our findings indicate that methods can be developed with fair accuracy in discriminating between women due to give birth to neonates with low birth weight versus normal birth weight, using a single risk score that is based on a combination of prognostic variables collected at the second trimester of pregnancy.

Methodological issues: study design, implementation, and analysis

Selection of study area and participants

A prudent selection of study area and participants is key to measuring effects in randomised controlled trials. The population under study must be “at-risk” of the outcome studied. In our case, we selected an area that is highly malaria endemic. We confirmed this by analysing not only the available literature about the area, but also by visiting research institutions in the area and discussing their raw data, surveys and observations. Key institutions that were visited included Kenya Medical Research Institute/US Centres for Disease Control and Prevention (KEMRI/CDC Kisumu), and the Walter Reed Project in Kombewa. We also held meetings with the Director of the Kenya national malaria control programme who confirmed that the selected area was a “malaria hot spot”. In addition, there was also a high prevalence of anaemia, iron deficiency, helminth infections, and schistosomiasis.

Participants were recruited over a long time period stretching over a year. Because of the envisaged long duration of follow-up, and to avoid attrition, we carefully selected participants who were most likely to cooperate until the end of the intervention. We therefore strictly observed our inclusion and exclusion criteria. We engaged community volunteers to help in identification and recruitment of study participants, and recruited research assistants who would help in supervised administration of the iron supplements. These were respected members of the study population who had participated in health interventions in the study area in the past. We coupled every participant with a community volunteer and a research assistant, and also ensured that participants could call the study

personnel any time of day or night. In the end, we learnt that building a trust system with participants was key to realising our study objectives.

We achieved very high compliance rates in our study. Only two women failed to meet our compliance criteria which was measured as the proportion of women who consumed >90% of the scheduled supplements. We attributed the success rates in compliance to supervised administration of iron supplements. To our knowledge, there are no other studies that have ensured supervised administration of iron supplements during pregnancy in a malaria-endemic region. A recent Cochrane review highlighted compliance and attrition as major problems that affected studies that investigated the relationship between iron supplementation and maternal and neonatal outcomes. (Peña-Rosas et al., 2012)

Justification of inclusion of fortification plus supplementation group

In the study design, we compared supplementation with iron versus placebo in women who all received fortification. We could instead have chosen to compare fortification and supplementation versus supplementation only. However, this would have several disadvantages. First, this comparison would result in a relatively small contrast in iron intake, so that intervention effects could have been too small to measure with sufficient precision. Iron supplementation, on the other hand, leads to a larger contrast in iron intake, so that in the absence of evidence of effects on infection, we can be more confident that lower iron intakes of iron as supplied by fortification is safe.

Second, if a combination of fortification and supplementation increased malarial infection among pregnant women, then governments would be asked to reconsider universal iron supplementation programmes rather than iron fortification. Universal iron supplementation in pregnancy has been controversial because of the uncertainty about the functional improvements that can be achieved, poor adherence by women, and the poor performance of supplementation programmes. In addition, fortification benefits not only pregnant women but also other population groups.

Assessment of malarial parasitaemia in pregnancy

Various studies have used different methods to assess malarial parasitaemia in pregnancy (**Table 1**). This not only makes it difficult to compare results from different studies but also poses a dilemma regarding the most appropriate method to use. To accurately detect asymptomatic parasitaemia, we used combinations of methods of assessment, namely dipstick tests, PCR, and histopathology of placental biopsies. The last two methods are not feasible in remote field conditions.

Table 1: Studies that assessed malarial parasitaemia in pregnancy in Kenyan women using different methods

Reference	Year; place	Study population	Prevalence (outcome)
Perrault et al. 2009	Year not reported; New Nyanza Provincial General Hospital, Kisumu (urban; n=92) Siaya District Hospital (rural; n=65)	Parturient women aged 15-40 y, apparently healthy, with singleton pregnancy	35.7% (placental blood; rt-PCR) 17.2% (placental blood; microscopy) 33.1% (peripheral blood; rt-PCR) 15.9% (peripheral blood; microscopy) 10.8% (cord blood; rt-PCR) Siaya: 52.3% (peripheral blood; by rt-PCR)
Van Eijk, Ayisi et al. 2004	June 1999 – June 2000; NNPGH	All women who delivered at NNPGH with uncomplicated pregnancy of a gestational age ≥ 32 weeks, residing in Kisumu municipality, willing to be tested for HIV and who attended ANCs	13.9% (peripheral blood; microscopy) SP lead to 46% reduction in prevalence of placental malaria, but was only taken by 38% of women. 19% of women reported having used iron/folic acid supplements.
Ter Kuile et al. 2003	1996-99; Rarieda Division, Siaya District	Pregnant women in the cohort area	19.6% in ITN villages vs 26.1% in control villages (maternal placental or peripheral blood; microscopy) Cord blood parasitaemia: 2.8% vs 4.1%
Van Eijk et al. 2003	1996-99; NNPGH, Kisumu	Pregnant women, no known underlying disease, attending ANC with uncomplicated singleton pregnancy ≥ 32 weeks' gestation, residing in the Kisumu area	21.9% (15.2% in peripheral blood and 19.0% in placental blood; microscopy)
Schulman et al. 2001	January 1996-July 1997; Kilifi District Hospital	Parturient women with singleton pregnancies	Primigravidae: 24%, 23%, 34% Secundigravidae: 18%, 13%, 24% 3,4-gravidae: 8%, 7%, 13% >4: 10%, 7%, 12% (peripheral blood, placental blood and placental biopsies; microscopy)
Parise et al.	5 clinics in Kisumu District and 4	Parturient women	Kisumu: 27.1% and 23.8%

Table 1: Studies that assessed malarial parasitaemia in pregnancy in Kenyan women using different methods

Reference	Year; place	Study population	Prevalence (outcome)
2003	clinics in Mombasa District		Kilifi: 24.5% and 19.5% (peripheral and placental blood; microscopy)
Parise et al. 1998	1994-96; NNPGH and Kisumu District Hospital, Kisumu	Women in their 1st and 2nd pregnancies attending antenatal clinics with 16-26 weeks gestation	Case management: 27.0% and 27.1% : 2-dose SP: 9.8% and 11.5% Monthly SP: 6.7% and 8.9% (peripheral and placental blood; microscopy)
Shulman et al. 1998	September 1994-November 1995 Women attending ANC at Kilifi District Hospital	Primigravidae or women with a previous history of a pregnancy <12 weeks, with a singleton pregnancy	Control: 5.8% and 35.8% ITNs: 12.7% vs 41.3 (peripheral and placental blood; microscopy)

NNPGH, New Nyanza Provincial General Hospital-Kisumu; ANC, Antenatal clinic; ITN, Insecticide treated net

Table 2: Case definitions of *Plasmodium* infection

	HRP2-based dipstick test	LDH-based dipstick test		Microscopy of peripheral blood [1]	Placental biopsy		PCR, peripheral blood		PCR, maternal placental blood	
		P. falciparum	Other spp.		Pigment	Parasites [1]	*P. falciparum*	Other spp.	*P. falciparum*	Other spp.
Case 1 (primary outcome) [2]	●	●	●	○	●	○	●	●	●	●
Case 2 (patent)	●	●	●	○	●	○	○	○	○	○
Case 3 (sub-patent)	○	○	○	○	○	○	●	●	●	●
Case 4 (current or recent)	●	●	●	○	○	○	●	●	●	●

Diagnostic tests are included (●) or excluded (○) in each case definition, and are linked through 'OR' Boolean operators.
[1] Regardless of parasite stage (i.e. asexual or sexual stages).

Histological examination of placental biopsies is often considered the 'gold standard' of malaria diagnosis in pregnancy, and results in infection status being classified into four categories depending on the presence and distribution of *Plasmodium* parasites and haemozoin (Bulmer, Rasheed, Francis, Morrison, & Greenwoods, 1993):

1. Active infection: parasites are present in maternal erythrocytes the intervillous spaces with or without pigment in intervillous erythrocytes or monocytes;
2. Active-chronic infection: parasites are present in the intervillous spaces along with pigment as deposits in fibrin or in macrophages within fibrin;
3. Past infection: pigment is present in fibrin or in macrophages within fibrin but not in parasites; and
4. Not infected.

Although placental histopathology is expensive, laborious and requires highly skilled manpower, it provides a unique opportunity for researchers to study past *Plasmodium* infections retrospectively. For ethical reasons, it is not feasible to assess *Plasmodium* infection during the intervention period without providing chemotherapy to those found to be infected. Investigators would like to quantify the presence of *Plasmodium* infections during pregnancy in a way that does not compromise ethical standards. Placental histopathology provided an opportunity to study the occurrence of past infections during pregnancy without compromising ethics.

Any form of *Plasmodium* infection that is associated with adverse maternal or pregnancy outcomes is bad for the pregnant mother and her new-born baby. We formulated the primary outcome as the presence of maternal *Plasmodium* infection in samples collected at delivery, regardless of species, as indicated by one or more positive results for the presence of *Plasmodium*-derived LDH or HRP2 in plasma (dipstick tests), past placental infections, or *Plasmodium* DNA (any species, by PCR test). Although we assessed the presence and density of *Plasmodium* parasites in microscopical examination of blood films, we excluded these results from our case definition because results from microscopy would be considered incorrect if discordant with results from PCR tests. Table 1 shows the case definitions used in this study.

Pigment deposition has generally been found to be associated with low birth weight, particularly in the presence of parasites in the intervillous spaces (Menendez et al., 2000). There is uncertainty, however, about the effect of past infections (i.e. with pigment deposition but without parasitaemia), on neonatal outcomes: such infections have been found to be associated with reduced birth weight in several studies Walter, Garin, Blot, & Philippe, 1981, Watkinson & Rushton, 1983, Rogerson et al., 2003, but not in others (Matteelli et al., 1996, Menendez et al., 2000, Muehlenbachs, Mutabingwa, Fried, & Duffy, 2007, Muehlenbachs et al., 2010).

PCR is considerably more sensitive in detecting maternal infection than either microscopy

or rapid dipstick tests that assay the presence of *Plasmodium*-specific proteins (e.g. HRP2 or LDH). (Kattenberg et al., 2011) Several studies have found that sub-patent infections (i.e. low parasitaemias that are detected by PCR but not by microscopy or dipstick tests) are associated with reduced birth weight ((Malhotra, Dent, Mungai, Muchiri, & King, 2005), (Adegnika et al., 2006), (Arango, Maestre, & Carmona-Fonseca, 2010), (Mohammed et al., 2013)) but others have not (Mankhambo, Kanjala, Rudman, Lema, & Rogerson, 2002, Mockenhaupt et al., 2006,Rantala et al., 2010)).

We are not aware of studies on the maternal or pregnancy outcome of *P. malariae* or *P. ovale* infection during pregnancy in Africa. *P. vivax* is associated with low birth weight ((Luxemburger et al., 2001), (Nosten, ter Kuile, Maelankirri, Decludt, & White, 1991), (Nosten et al., 1999)) but this species rarely occurs in Africa.

Various studies targeting placental malaria have been done in Kenya. Table 3 shows the prevalence of placental malaria as reported by some studies that used combinations of the methods discussed above.

Selection bias

Selection bias is unlikely to occur in a trial if participants are individually and properly randomised and sample size is large. In all the chapters of this thesis, we present results after comparing the full population sample, thus ensuring that group similarity at baseline is maintained. We also assessed for possible confounding by comparing crude and adjusted prevalence ratios. The latter was obtained by including baseline covariates that could be prognostic for outcome. These covariates were either likely to be prognostic for the primary outcome, as predefined on the basis of the literature review or simply possibly prognostic for the primary outcome. They included: gravidity (M. Desai et al., 2007), maternal age (M. Desai et al., 2007) (Tako et al., 2005), HIV infection status (Kuile et al., 2004), *Plasmodium* infection status, iron status, and gestational age at delivery. When the potential value of the adjustment was in doubt, we considered the unadjusted analysis as the one of primary attention, and the adjusted analysis as supportive.

Throughout this thesis, various sub-group analyses have been reported. It is possible that subgroup analysis could distort the balance achieved by randomisation of participants at baseline. We interpreted the results of subgroup analysis cautiously. (Assmann, Pocock, Enos, & Kasten, 2000), (Wang, Lagakos, Ware, Hunter, & Drazen, 2007), (Fletcher, 2007; Oxman, 1992; Sun, Briel, Walter, & Guyatt, 2010; Yusuf, 1991)

Effect modification

We expected the relationship between iron supplementation and malaria parasitaemia at parturition to be modified by various factors as discussed below.

a. *Gravidity:* In highly endemic areas, primigravidae are at greater risk of malarial

infection (Nosten et al., 1991) (A. M. Greenwood et al., 1992), probably because they have reduced immunity. Thus, we expected that their ability to suppress a possible increase in parasitaemia resulting from iron would be reduced.

b. *Age: Plasmodium* infection was expected to be higher in women ≤20 years old. Young primigravidae and multigravidae are at greater risk of malaria and its adverse effects than older primigravidae or multigravidae, respectively (Espinoza et al., 2005; Leenstra et al., 2003; Marques et al., 2005) (S J Rogerson et al., 2000) (Walker-abbey et al., 2005). This is probably because age-associated immunity plays a role in controlling malaria infection during pregnancy in highly endemic areas. We expected that women ≤20 years old had a reduced ability to suppress a possible increase in parasitaemia resulting from iron.
c. *HIV infection:* HIV infection compromises malarial immunity such that HIV infected multigravidae have at least as high a risk of placental infection as non-HIV-infected primigravidae (M. Desai et al., 2007). HIV exacerbates the burden of malaria and increases the degree to which malaria is associated with maternal severe anaemia. We expected that due to compromised malarial immunity, women that were HIV infected would be more susceptible to iron-induced infection.
d. *Maternal iron status at baseline:* Low iron status at baseline would be protective against malaria infection but would also result in increased absorption of the supplemented iron. The vice versa was also true thus we expected the effect of iron supplementation on malaria parasitaemia to be influenced by iron status at baseline.

Adverse outcomes

We defined adverse outcomes as "any untoward medical occurrence in a study participant". The most serious adverse events (unrelated to study treatment) that occurred were deaths of two mothers and eight neonates (see participant flowchart in Chapter 2). One of the mothers died soon after delivery due to post-partum haemorrhage while the other mother died three weeks after delivery due to pneumonia and cardiac complications. The cause of death of most neonates as reported by attending nurses was neonatal sepsis. All adverse events were reported to the principal investigator within 24 hours and then to the data safety monitoring committee. The data safety committee was independent, blinded, and periodically reviewed cumulative trial data and advised on safety issues.

There were two cases of neural tube defects which were referred to Bethany kids – Kijabe mission hospital, a specialised hospital dealing with spina bifida and neural tube defects (http://kijabehospital.org/). Both babies had classic wounds on their spine and hydrocephalus. After extensive surgery and close follow-up, both babies survived.

Intermittent preventive treatment (IPT)

Intermittent preventive treatment data were obtained retrospectively from the participants

antenatal clinic attendance booklets. In Kenya, nurses fill in the information in these booklets during the antenatal clinic visits. This is standard practice countrywide and it has major benefits. In situations where there are virtually no existent hospital records regarding a pregnant mother, the antenatal booklets serve as a key source of information for medical practitioners. All clinical and non-clinical interventions received by the pregnant mother are recorded in these booklets.

We examined whether intermittent preventive treatment had an effect on malaria parasitaemia at birth. Because IPT data was recorded after randomisation, theoretically, it could have been influenced by the intervention and thus it was considered separately in the analysis. Contrary to our expectations, mothers who had two recorded IPT interventions had the highest rates of *Plasmodium* infection at parturition compared to mothers who had one or three IPT interventions (preliminary data, not shown). Most mothers received two IPT doses. It is possible that mothers came for a follow-up antenatal visit because they were experiencing malaria-related symptoms. Although the clinic attendance booklets provide a wealth of information, interpretation of data there-in deserves critical and logical thought.

Important pre-trial procedures

This section of the discussion highlights important issues related to implementation of studies of similar nature in resource poor settings. This is arguably a “mine-field” for most researchers and successful navigation of critical aspects of trial implementation determines to a great extent, the success of the research. Reference is made to experiences gathered during fieldwork in Kenya.

Legal requirements and issues regarding ethical clearance

Implementation of a study in humans in developing countries is not as straight-forward as often perceived. There are complex networks of government regulations, institutional collaborations, finance obligations, and often, conflicting bureaucracies. Planning for a study must take into account time spent on the vital but necessary pre-trial registrations and legal issues.

The first step in launching the study in Kenya involved applying for research permits for the main researchers. This application is submitted to the Kenya National Council for Science and Technology. The procedure involves submitting the study proposal and detailed field wok implementation plans. These are legal requirements without which no research can be undertaken in Kenya. Once research permits are granted, the process of obtaining ethical review permits can begin. It is prudent to apply for these documents as early as possible to avoid delays. In addition, with research permits, certain activities are permitted e.g. holding meetings with community leaders etc. This can be useful as the researcher awaits final ethical clearance. Though the national council for science and technology does not require annual reports of trial implementation progress, it does require

the researcher to deposit two copies of the final report of findings at its depository. If a researcher applies for a new research license and yet he/ she did not submit reports of past research, a denial of license could be imposed.

Ethical clearances were obtained from two Ethical Review Committees (ERCs): Kenyatta National Hospital/University of Nairobi ERC (Kenya), and the London School of Hygiene and Tropical Medicine ERC (United Kingdom). Obtaining ethical clearance in the United Kingdom was vital because London School of Hygiene and Tropical Medicine was the legal trial sponsor and was thus responsible for insurance of all trial participants etc. To conduct the research in Kenya, local ethical clearance was mandatory hence the reason for obtaining the second ethical clearance in Kenya.

Unlike the research license requirements, most ethical committees in Kenya require submission of an annual progress report. Adverse events and serious adverse events are also required to be reported "as they occur – based on a pre-specified criteria". The process of obtaining ethical clearance is usually slow and proper planning of field activities is vital so as to maximise utility of available resources. **Figure 2** shows the administrative process prior to, and during trial implementation. Since there are several institutions from which ethical clearance can be obtained, it is important to carefully select the institution that has the quickest processing times etc. This information can be obtained by gathering information from fellow researchers in the target study area.

The success of many research projects hinges on goodwill not only from study participants but also from the governance framework in the target study area. Before start of the trial, we approached key government departments and briefed the lead persons verbally and in writing, about the impending research work. It was hoped that this would provide a platform upon which dissemination of the trial findings would be done i.e. after trial completion and publication of findings. Because of the envisaged policy implications upon completion of the study, we expanded the list of "contacted" key authorities at national level to include the Division of Malaria Control, Division of Reproductive Health, and the Ministry of Public Health and Sanitation. Officials at Provincial and District levels responsible for health in the target area, especially those from the Ministry of Medical Services and the Ministry of Public Health and Sanitation, were visited to explain study aims and procedures, and to obtain their approval. We were lucky that the available trial funding enabled us to do all these preparatory activities. While we recognise that funding limitations curtail the extent to which researchers can do preparatory work prior to trial commencement, we emphasize the benefits and good will derived from these efforts. A notable example was when we were experiencing clinical staff shortages. A visit to the provincial medical officer of health solved the problem as he was already aware and in support of our work, thus he immediately approved the appointment of 3 extra nurses at our research station.

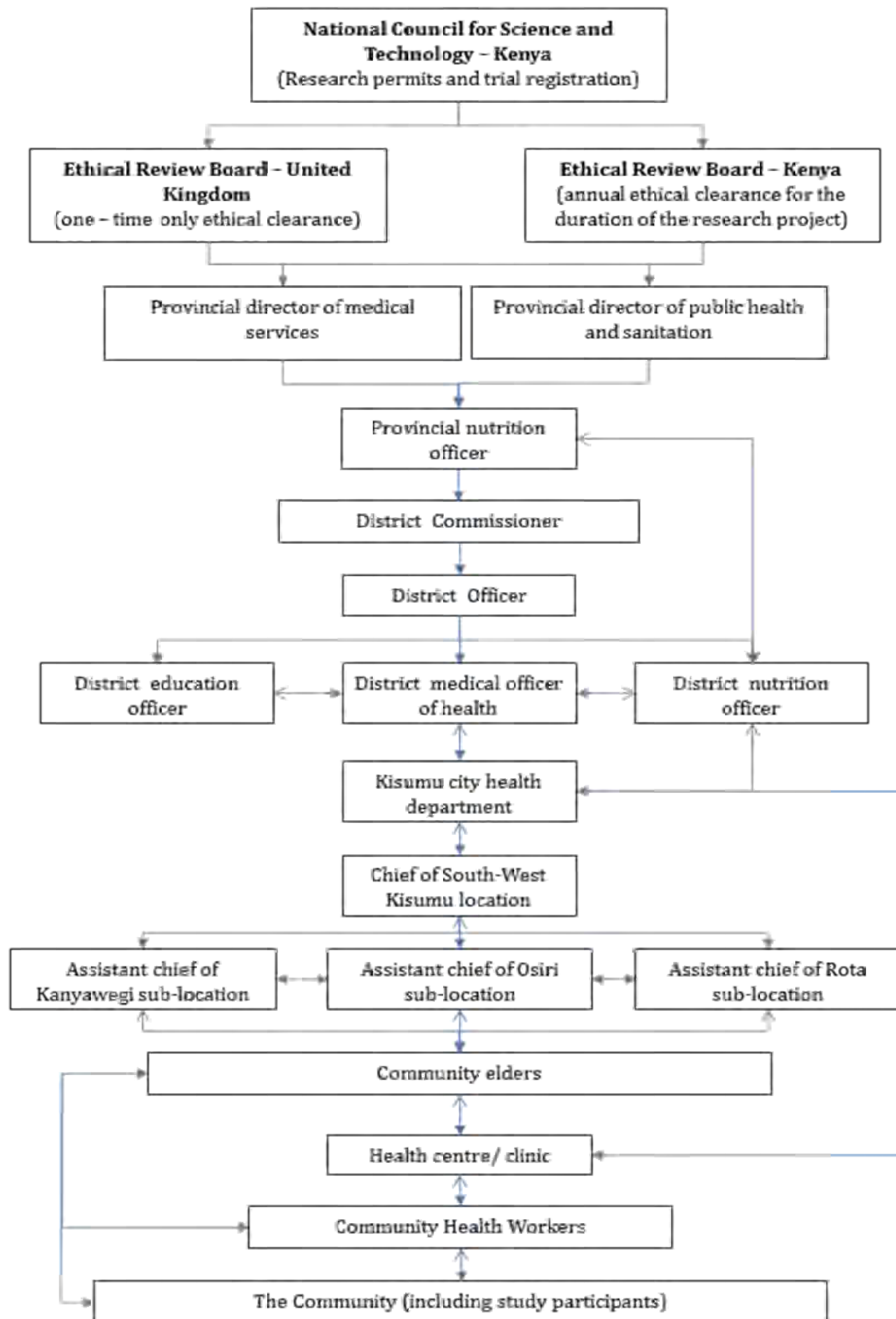


Figure 2: Decision tree prior to, and during trial implementation

Numerous meetings were held with community members, community health workers, provincial administrators and local leaders to sensitise and inform them about study aims and procedures. Local health facilities (including local dispensaries, District, and Provincial Hospitals) were visited to learn about standard clinical procedures (including HIV testing) and to coordinate our study with on-going health activities in the area. Although virtually all women in the study area currently accept HIV testing, we still took time to demystify the HIV testing procedures that we were going to use and to assure all the interested persons that the results of the tests would be completely confidential. This paid off in the implementation phase of our study as all our study participants accepted HIV testing despite the cultural myths surrounding this procedure.

The census

In order to implement the trial effectively, it was necessary that we know approximately how many people were resident in the study area. This was especially because we intended to fortify with NaFeEDTA, all the flour in all the participating households and their homesteads, for the entire duration of follow-up of the index subject. The only estimates of population size available to us were from the previous Kenya Demographic and Health Survey 2008-2009.(Kenya National Bureau of Statistics (KNBS) & ICF Macro, 2010) Unfortunately, detailed data regarding the target area were not available and thus the solution was to conduct a census.

The area selected for study consisted of three administrative units (‘sub-locations’) in South-West Kisumu location. Enumeration of all persons, and households in this area involved careful planning, recruitment of personnel, training, and closely monitored data collection. The first step was obtaining the necessary legal permissions from the provincial administration. We then developed a five-page census questionnaire that was aimed at collecting vital information necessary for implementation of our research work.

For efficient census data collection, we coupled each census enumerator with a village elder. The elders were key to obtaining community support to our efforts. Enumerators were much more likely to be accepted by the community when accompanied by the elders. In addition, the data was more correct and trustworthy because the elders usually had very good insight of the family situations of the visited households. By equipping the elders and enumerators with key information regarding our intended study, community mobilisation and support was obtained.

Global Positioning System (GPS) data of each household was collected in addition to the regular census data. This aided follow-up of study participants as there are no person-specific location/ home addresses in this area. In addition, we enquired about grain milling procedures and patterns. This information proved very useful when planning for the community based flour fortification programme.

Vital fieldwork collaborations before trial launch

The term "independent researchers" in this section refers to researchers who are formally not staff of local research institutions such as Kenya Medical Research Institute or US Centers for Disease Control and Prevention, Kisumu. Some PhD fellows venture out as independent researchers without support by local institutions. Institutions ensure that the research infrastructure (offices, laboratories, vehicles etc.) is ready for use when needed. Usually, they also map out areas where they implement their trials. The studies presented in this thesis were done within the framework of an "independent researcher".

Unlike researchers who are part of local institutions, independent researchers have to set up the research infrastructure from scratch. We identified our "partner" health clinic after an extensive tour of the proposed study area. This clinic (Ober Kamoth health centre) was favoured because it was centrally located in the research area. Furthermore, there were referral hospitals within a radius of 10-15 km from the health centre. Although it had facilities that could be converted to research offices, they were in dilapidated conditions and needed extensive renovations before research work could commence. The health centre was also severely under-staffed. The community had very little confidence in its ability to deliver quality health services. We decided to transform this health centre into a beacon of health services delivery in the area as part of our contribution to better health services in the area.

The first step was undertaking extensive discussions with the Department of Health Services, Kisumu municipality, as it was the owner and manager of the health centre. A Memorandum of Understanding (MoU) was signed between all parties. The MoU recognised our presence in the study area and gave permission to not only work from the health centre, but to also under-take extensive repairs of the health facility. Periodic meetings would be held to discuss and approve plans before major work was done to the existing buildings. As part of the MoU, we requested for additional nurses from the Ministry of Health and from the Health Department, Kisumu. Luckily, following recommendations from leaders we had met in the initial planning meetings, we received additional staff.

It is important for independent researchers to secure support by other research institutions in their target area. This is necessary for many reasons e.g. emergency laboratory support or when demarcating geographical research areas to avoid "crossing-over" of study participants from one study to another. In our study area, three other research institutions were identified. These were:

1. Kenya medical Research Institute/ US Centers for Disease Control (KEMRI/ CDC) – Kisumu
2. Walter Reed Programme – Kisumu and Kombewa field sites
3. Maseno University – Maseno

We held meetings with the institutions above and explained our research aims and objectives. We then mapped out our proposed study area and sought to find out whether there were any active studies involving human subjects ongoing in the area. In return, the representatives of the research organisations explained to us their research interests, focus, capabilities, and geographical area of operation. They also gave us their commitment to support us during the implementation of the research project. Some of them even visited our field research offices and offered valuable advice on how to transform the site for research purposes.

Conflict of interests with humanitarian aid organisations based in the study area

In the preparatory period before trial commencement, a new humanitarian aid project – the Output-Based Approach (OBA) project begun in parts of our target area (www.output-based-aid.net). This project aimed at promoting hospital delivery of newborn children (as opposed to the practice of unsafe home deliveries in rural villages). The project had collaborated with World Food Programme, which would provide iron-fortified flour (7 kg/month) to the recruited women. The OBA project thus indirectly led to delays in our procedures as we evaluated the probability of participants in our study receiving additional fortified flour from the Output-Based Approach project.

We carried out a survey to determine how many women in the study area were in the OBA project. We interviewed 40 pregnant women. Out of these, only 17% were in the OBA project. As such, in order to avoid iron overload, we decided to avoid recruiting women who were already in the OBA project. Women in our study were given instructions about the repercussions of obtaining fortified flour from other sources. The antenatal clinic books of all our participants were clearly stamped on the front-page with information pertaining to the fact that the subject was part of an ongoing study and thus giving additional iron as either fortification or supplementation would possibly be injurious to the health of the mother.

Community mobilization and incorporation of traditional birth attendants into research activities

The success of a research project based in a remote place depends largely on the level of support given by the local community. African communities are organised in complex ways. There are multiple factors that determine if a community will support a research study. In Western societies, an individuals' decision to participate is based on written information, mass media, and probably ones assessment of the perceived benefits. In Kenya, the decision to participate depends also on other additional factors such as advice from community elders, opinion leaders, religious leaders, and health advocates such as community health workers.

We embarked on a community mobilisation campaign in order to garner support by all parties involved. Initially, we gave key information to census enumerators and their

accompanying elders. We also held meetings with the local administration (chief, sub-chiefs, and elders of the community). We explained in detail what the study was about, the stages that the study would go through, how long it would take, who would participate, participants' benefits, and compensation mechanisms in-case of harm to participants. It was very important that the administrative team gives their support to the project otherwise the study would probably not be allowed to start. In addition, we made sure that we did not over-state the benefits that individual participants or the community would get so as to avoid future misunderstandings. Written meeting minutes proved useful during periodic evaluation meetings.

Following advice by the administrative team, we held more meetings with opinion leaders, religious leaders, millers, and community health workers. The objective was to provide a platform for them to ask as many questions as possible and for us to provide answers to their questions and clarifications whenever necessary. This was vital in view of the fact that our participants were a highly vulnerable group. The millers were also interested in knowing whether the fortification of locally-sourced flour would change the taste of the flour product and whether they would be required to charge more money to customers. We assured everyone that all our activities would not require a financial investment from their side and that participants would not be required to pay extra for the fortificant.

One of the outcomes of the community mobilisation activities was the realisation that we needed to also target the traditional birth attendants (popularly known as TBAs). TBAs are highly respected amongst the women folk in rural Africa not only because they provide a service that the government sometimes cannot, but also because their work is deeply rooted in the local culture and customs. Although the government health officials had made a point that they were not in support of the work of TBAs due to health risks to the pregnant women, it turned out that a lot of women still sought the services of traditional birth attendants. Modern hospitals are not many in the area considering the geographical expanse of the region. It was not a surprise to us that women in labour went to the nearest help (TBA) available. The TBAs brought up an issue that we had not anticipated. Our project was going to cut-off their revenue source. In addition, government was not supporting them so they could not officially be seconded to the project. Following extensive discussions with the TBAs, it was agreed that we train and then engage them as community health workers in the project. This way, when women went to them to give birth, they would immediately call the project ambulance and the woman would be moved to the hospital where she would deliver under skilled attendance. This was a win-win solution for all and it proved to be one of the key success pillars of the project.

Global position system (GPS)-aided research project administration

Modern technology is increasingly becoming the tool that resource-poor countries use to circumvent problems. Global position system (GPS) has been recognised as a unique research tool in remote research locations. Unlike in developed countries, most research areas in developing countries have no formal address codes. Usually, the population relies

on post office boxes that are centrally located in the nearest mid-size town for their mail. GPS has enabled easier follow-up of study participants in such areas.
As part of the census, we collected GPS data of 2,564 households. The GPS data was entered into an electronic database, and linked to the census data. With a view to assist in locating homes of the women in the study area, a high-resolution photographic satellite map was produced of the study area, and integrated with GPS data so that individual households were visible and indicated.

Figure 3 is a map generated from Google maps and overlaid with the GPS coordinates of the study participants who were randomised into the study. It also shows the location of the research health centre relative to the whole study area. From the map, we could easily locate the home of a participant especially in times of emergencies such as during delivery. Women would call for the project ambulance as soon as they went into labour. Using the GPS coordinates of the home of the woman (or sometimes just relying on local knowledge of the area), we would rush to the home of the woman and bring her to the research hospital where skilled staff would ensure a smooth delivery of her baby. In addition, GPS data enabled us to know if a woman came from within the boundaries of our research project. Furthermore, as randomisation of trial participants progressed, we were able to see from the map whether we were randomising from only one side of the area or from the whole area.

The success of the community based flour fortification programme

a) Estimating flour intake and milling practices in the area:

In 2010-11, we conducted studies in the study area to assess flour intake and grain milling practices of the target population. Dietary assessment was done to quantify flour intake and to identify sources of flour. Methods used to collect this data included food frequency questionnaires (n=50; conveniently selected households), weighed food records (n=18 households; in duplicate), and field observations of all posho mills in the area. The results indicated that 93% of households in the target population obtained their flour by milling grain at local hammer (posho) mills, whilst only 2% of households reported to obtain their flour exclusively from the shops (centrally processed flour). The mean daily per capita intake of flour was 252 g (SD: 41 g). Out of the 45 posho mills in the area, 24 run on diesel and 21 on electricity. Most flour was produced from maize only, or maize together with sorghum or cassava. The smallest amount of flour milled was 1 kg. Many households also used flour as animal feed.

From these data, we concluded that flour fortification at hammer mill level was plausible; maize/ corn was obviously the target food vehicle though other foods such as cassava, millet, and sorghum could also be fortified; the target client would be the pregnant mother though the whole homestead would have to benefit from the fortification. The latter point was motivated by the observation that food was shared between household members and it was not uncommon for the mother to serve everyone else and serve herself last. This is typical of most African communities.

Figure 3: Visualization of the GPS coordinates of the geo-locations of the study participants (map courtesy of Google maps. GPS coordinates visualized using GPS visualizer - www.gpsvisualizer.com)

b) Field-testing of a protocol for community-based fortification:

Fortificant iron (as NaFeEDTA; ±13% iron), in premix form, was obtained from Fortitech (Fortitech Europe ApS, Gadstrup, Denmark). The target fortification level (20mg iron as NaFeEDTA per kg of flour) was based on guidelines from the Flour Fortification Initiative that were adopted by the World Health Organization (WHO, 2009) and by the Kenya Bureau of Standards (KEBS 2010, GoK 2008), and based on an estimated per capita consumption of flour of 225g of flour per day.

The premix contained iron in highly concentrated form. The manufacturers prefer to ship it in this form in order to reduce shipping and storage costs. It is therefore not possible to achieve a homogenous mixture of the premix in flour when adding directly to the grains being milled. For this reason, an additional step was required whereby premix was diluted into a preblend, which was suitable for addition to the grains being milled.

We diluted premix into three preblend master batches at a ratio of 1:100. During trial runs in the Netherlands, we used an electric cement mixer (new) to mix the flour and the premix so as to obtain a preblend. However, contrary to expectations, it proved impossible to procure an electrical cement mixer in Kenya. Available mixers were all diesel-powered, which are unsuitable for food preparation purposes due to the risk to carcinogenic compounds from the petroleum products.

The problem was solved by using an "ODJOB" home cement mixer procured from the USA. This drum-like mixer works by being rolled on the ground. Since it has specially designed grooves on the inside, the manufacturers claim that cement is adequately mixed within 10 seconds of continuous rolling. We decided to apply this principle in the production of fortificant premix albeit with a few additional tests.

Two "ODJOB" cement mixers were obtained and thoroughly cleaned before being used exclusively for fortification work. To improve efficiency and reduce human workload, the "ODJOB" mixer was mounted on a stand that was locally designed and assembled **(Figure 4)**. The stand had a mounting section for the "ODJOB" drum and a handle used to rotate it while standing or sitting on a high chair. It was also specially designed to give a tilt to the angle of rotation so that the flour not only mixed because of the irregular form of the "ODJOB" mixer but also because the drum was tilted at various angles. This increased efficiency of the assembly. Figure 4 shows the "ODJOB" mixer, mounted on a locally-made stand.

We assessed the effect on homogeneity of preblend iron content after various mixing times. Each of the batches was mixed for 60 minutes; 6 preblend samples (25 g) were collected according to a pre-established plan from various locations in the mixer at 20, 40 and 60 minutes. We similarly assessed the effect of producing preblend in batches of various volumes (8 kg, 10 kg and 12 kg). Lastly, we diluted preblend into flour in 10 different

mills, at a ratio of 1:100. The fortificant iron content was determined for all samples in duplicate.

There was no demonstrated effect of preblend mixing volume on fortificant iron homogeneity. With a mixing time of 20 minutes, the iron concentration of preblend was 2.01 g/kg (co-efficient of variation: 2%) (Target: 2 g/kg); there was no evidence that a longer mixing time resulted in a more homogenous sample. Fortification at the mill resulted in an average level of fortification of 18.0 mg iron per kg flour, SD: 2.2 mg/kg (target: 20.0 mg/kg). The difference could be explained by losses due to the retention of flour in the mill after the first run. Thus we were confident that our fortification procedure resulted in adequate levels and adequate homogeneity of fortificant iron in flour, and that our target level (20 mg/kg) in the final fortified product was appropriate in view of the per-capita amount of flour consumed by the participating women. 45 local millers were trained in fortification procedures and all but three participated in the study. The three were excluded because their mills had porous sieves thus the iron in the fortified flour product was not homogenously mixed.

Figure 4: "ODJOB" mixer, mounted on a locally-made stand

Issues related to the manufacture of trial supplements

We had originally foreseen that the iron supplements in our trial would contain dried ferrous sulphate, the compound that is conventionally used in iron tablets. To mask the metallic taste, we decided to have this formulated into capsules. When conducting

laboratory tests for stability of the iron supplements, we became aware that ferrous sulphate is hydrophilic, withdrawing water from gelatine capsules that naturally contain 16% water. As a result, there was a serious threat that the capsules would eventually crack and its contents would leak. This would also allow the study participants to taste the contents of the iron capsules and to distinguish between capsules containing iron and placebo. Thus we decided to switch to ferrous fumarate, which is not hydrophilic but which has a similar molecular weight (thus not requiring bigger capsules, which may be hard to swallow for pregnant women) and similar fractional absorption of iron as in ferrous sulphate.

Issues of study administration

Implementation of a research project with multiple research partners can exert a lot of pressure on the implementation team. Some major decisions are so urgent that there is no time to wait for consultations to take place up and down the management ladder. It is vital that the implementation team is given a leeway in situations that demand urgent action.

Staff administration is a key component of successful project implementation. However, it rarely gets the appraisal necessary because some projects are short-term. In long-term projects such as the one described in this thesis, the ability of the project team to deliver the expected results depends largely on the interrelationships between various team members. Figure 5 is the organisational structure of the human resource in the research project.

Researchers in poor, remote communities sometimes result to borrowing from the research budget due to humanitarian demands. Pregnancy is a period full of surprises for most couples including those that participated in our project. Despite our repeated messages about the extent of our mandate, most project participants were of the idea that the project team could sort out any of the pregnancy-related issues that arose. We were very careful not to overstep our mandate while at the same time keeping within ethical boundaries.

Although we intended to hold regular technical meetings with the project management team spread out in Africa and Europe, we quickly realised that teleconferences and video conferences were the way forward. Every team member has to be up-to-date with current events and procedures in the field. It is challenging to call for meetings when field procedures such as the birth of children are not predictable. Sometimes, we had to participate in teleconferences while at the same time driving through the bush to locate a woman in labour.

Another interesting realisation was that data is not only closely guarded by researchers but also by the participating individuals and communities. The last two quickly realised that we would accumulate quite a lot of health-related information about them. We explained clearly what information would be collected, who would see it, how it would be stored, and how the results would be disseminated to them. Trial planners and implementers should

never take it for granted that the community is at ease with collection of health related information.

We ensured that there was a framework for data back-up, archiving, and protection from theft or loss of data or samples. The trial sponsor (London School of Hygiene and Tropical Medicine) ensured that all collected information was kept in encrypted memory sticks and could be regularly sent to the principal investigator for back-up. These sticks were virtually indestructible, can survive very harsh weather conditions, and most importantly, can be remotely wiped-off all data.

A minor but nevertheless important extra point was the number of staff members involved in this project. Since the principle investigator (HV) was based in the Netherlands, the PhD fellow had to manage more than 84 members of staff on a daily basis. The roles of these people are indicated in the organisational structure – figure 5. In addition, there was the usual handling of suppliers, part-time workers etc. As such, the research work inevitably went beyond the realms of research and demanded good management and leadership skills.

Community based pregnancy testing and surveillance

Pregnant women in the study area delay making their first antenatal clinic visit to the hospital sometimes till the third trimester. As such, we decided to deploy a community-based pregnancy surveillance programme aimed at identifying and encouraging pregnant women to make the visit earlier i.e. before 20 weeks of gestation. Pregnancy testing was necessary in order to identify pregnancies at an early stage. However, excessive testing is expensive, puts an unnecessary burden on field staff and women, and may lead to false positive test results. In addition, very early testing is unethical because 60% of pregnancies are lost due to natural causes before the end of the first trimester is reached. In view of these issues, we developed a Standard Operating Protocol (SOP) for surveillance and detection of early pregnancy (see annex II). This SOP reviewed outlined strategies and appropriate procedures for early detection of pregnancies in the study area.

Because unmarried women may initially 'hide' their pregnancies, we invited them for regular urine testing. Married women were questioned monthly about the occurrence of their menstrual periods. A nurse administered urine tests at the research clinic to:

a) All women who had never been married, or who were divorced, separated, or widowed: at 12-weekly intervals;
b) All women who were married or living together: when women reported not to have had a menstrual period for 10 weeks since the date of the onset of the last menstrual period.

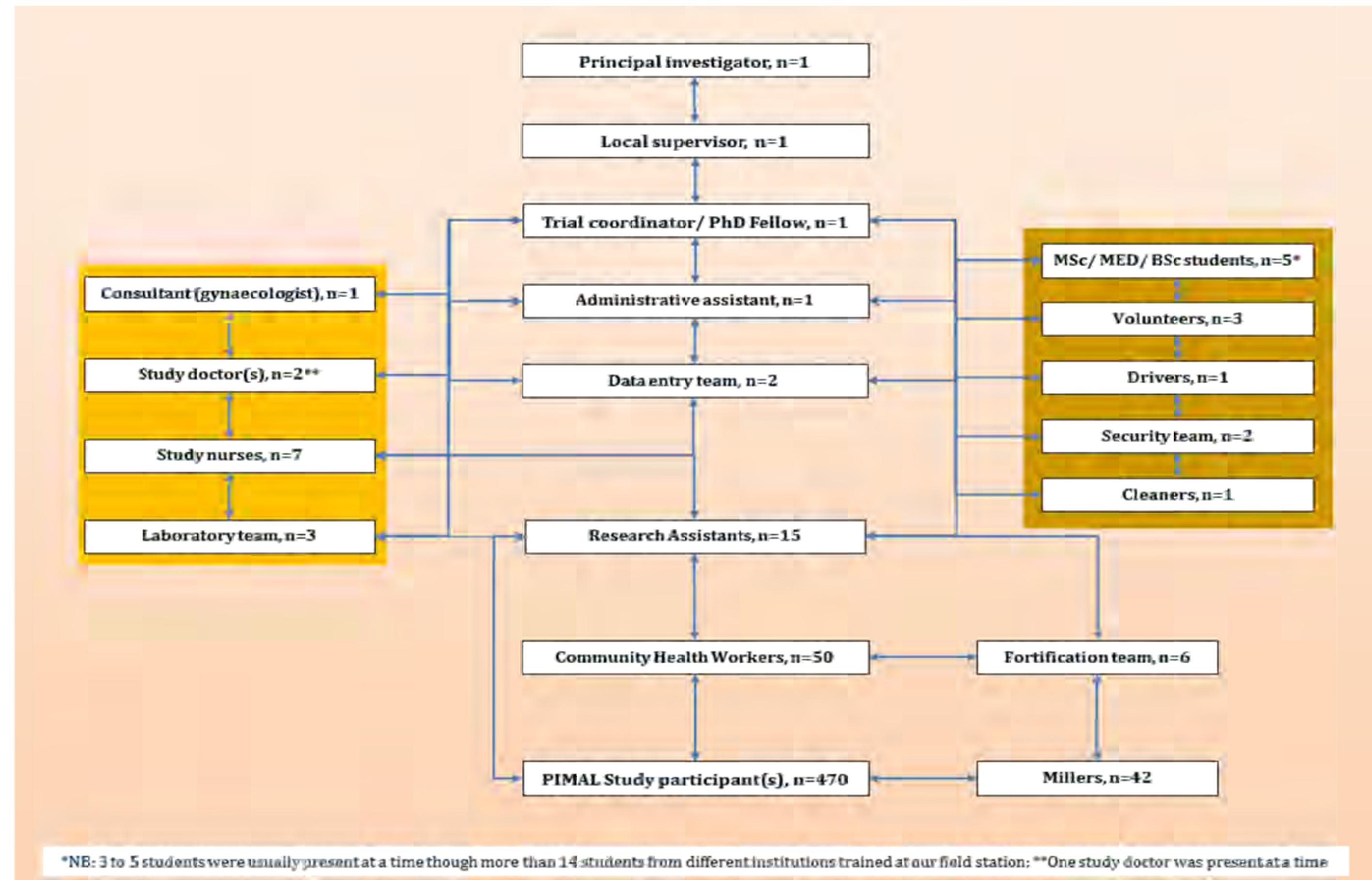


Figure 5: Organisational structure

Confirmation by obstetric ultrasound was done if the time since the onset of the last menstrual period exceeded 10 weeks. A nurse conducted an ultrasound examination immediately after pregnancy had been confirmed by urine test, or as shortly as possible thereafter.

Ultrasound techniques served to confirm pregnancy, determine if the pregnancy concerned a singleton, and determine the gestational age. Five nurses from the research clinic were trained on ultrasonography. These nurses actively participated in the screening and recruitment of pregnant women and also in conducting successful deliveries of babies from the project mothers. However, only one nurse did most of the ultrasound scans. During a period when this particular nurse was on pregnancy leave, a stand in nurse failed to identify two twin pregnancies. These mothers were followed up until the end to be included in the full analysis set, but they were excluded from the per protocol analysis.

Pregnancy surveillance: the use of common electronic tools

Pregnancy surveillance and testing in the source population (approximately 4,025 women of childbearing age (15-45 years) was a challenging task that could easily have become inefficient and chaotic. We aimed to detect all pregnancies in the source population within the first trimester. An electronic MS-Excel based pregnancy surveillance and detection database was designed and used to help detect potentially pregnant women. Its key features were:

1. Resident women in the study area within the eligible age range (15-45 years) were listed in the 'Source population', together with information about their date of birth, marital status, village of residence, GPS coordinates, and parity.
2. Information about community health workers, including their village of residence, and contact details.
3. For each woman, pregnancy surveillance was assigned to a community health worker or a study nurse, who also supervised the community health workers. If a woman was married, she was assigned to a community health worker. Unmarried women were assigned to the study nurses. This criterion for assigning women to a community health worker or study nurse was based on previous experience in an adjacent area, which indicated that married women were generally open about their pregnancies i.e. their reported history of menstrual periods was largely reliable. By contrast, unmarried women tended to initially hide their pregnancies, because these pregnancies are often unplanned or unwanted (Mrs. Joyce Nduku, formerly Kenya Medical Research Institute; personal communication). For these reasons, pregnancy monitoring in women who were married or living together was done by community health workers and traditional birth attendants (through questioning, with follow-up by a nurse in case of missing menstrual periods), whereas in women who were unmarried, divorced, separated, or widowed), it was done by a study nurse (through urine testing).

4. The database had summary sheet that automatically provided summary real-time information on the total number of women in the database, the total under surveillance by community health workers and the study nurse, etc. It also warned automatically if there were women in the database that were not accounted for, and provided a breakdown of the reasons for not being under surveillance (e.g. current pregnancy).

The surveillance database was dynamic in the sense that it automatically included registered young women when becoming of childbearing age (based on their birth dates) and automatically excluded others when exceeding our cut-off age of 45 years. We updated the database every month with data from participating households brought by community health workers. The achieved efficiency of pregnancy detection was a key milestone in the fieldwork.

Sustaining a high recruitment rate

In the study protocol, we assumed a crude birth rate of 43 per 1,000 population (Kisumu District, 1999 census data(Central Bureau of Statistics, Ministry of Finance and Planning, 2002), which allowed us to recruit and attain the minimal target sample size of 300 women within a 1.5-year period. We thus needed to conduct the study in a population of approximately 7,000 people. In our study proposal, however, we had asked for permission to include 450 women if allowed by available resources. To improve the rate of recruitment, we expanded the study area to cover a population of approximately 12,000 people.

The projected end of recruitment and end of follow-up of project participants was based on an average recruitment rate of 1.21 pregnant women per day. This rate improved as the fieldwork teams became more efficient. Mobilization and awareness meetings were held regularly with community leaders and elders to improve the recruitment rate. A progress graph of the rate of recruitment is shown below.

Between the 70th and 95th day since the start of recruitment, we observed a plateau in the recruitment progress. At the time, there was a severe drought (and food shortage) in our study area. Most pregnant women who had been scheduled to come for randomisation visits did not honour the appointment. To remedy the situation, we sourced for maize grain and gave some grain to the mothers who came for recruitment as motivation to join the study. The recruitment progress then continued according to plan.

Strategies to promote hospital delivery and to minimise attrition

In Western Kenya, 80% of rural women deliver outside a health facility; among these, traditional birth attendants assisted 42%, laypersons assisted 36%, while 22% received no assistance. (Van Eijk et al. 2006) These statistics coupled with information obtained from resource persons familiar with health related practices in our study area prompted us to

develop a comprehensive strategy aimed at retaining recruited participants in the study and hence minimising attrition.

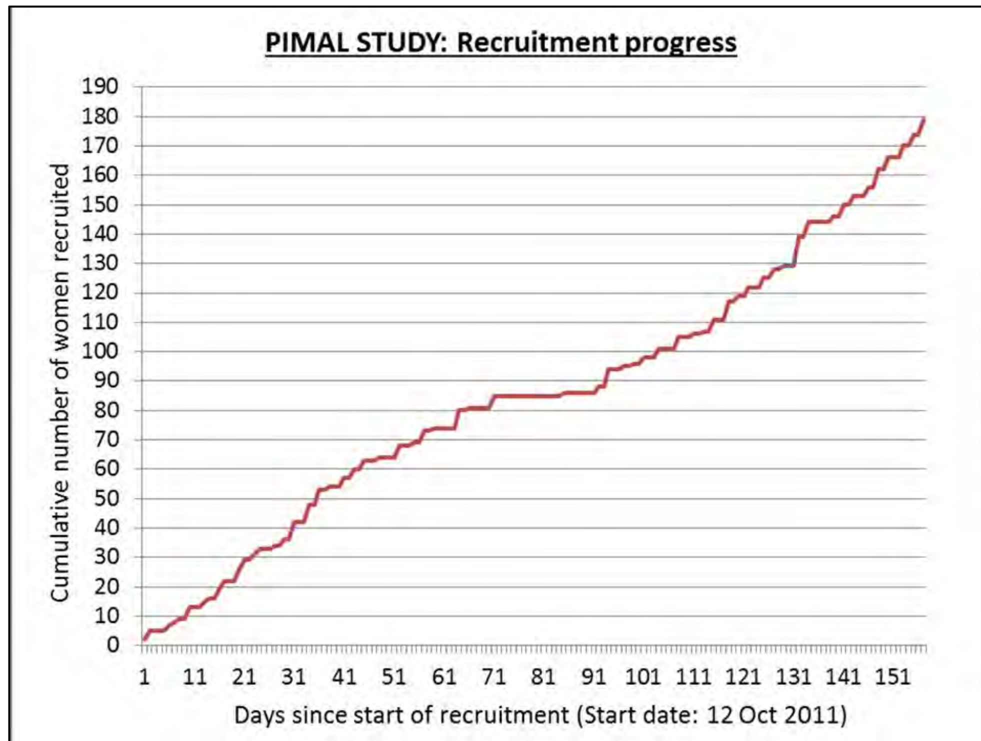


Figure 6: Recruitment progress based on actual rate of recruitment

We offered various incentives to participants and devised strategies aimed at promoting the overall good will among our participants. These strategies included the following:

1. Building a trust system with the local community: we quickly realised that the local community had very little trust in the health care system around them. We intensified efforts aimed at boosting the trust and confidence of the local community (especially pregnant women) thus we ensured that when they approached us for help, we responded promptly and helped in whatever way we could. We also developed a working philosophy that "the (pregnant) mother is the queen". This made all our members of staff treat the patients with care and respect thereby promoting the confidence of the locals with our service delivery.
2. Transportation of pregnant women to and from the hospital: we sent the project ambulance to transport our participants to and from the hospital for all their pregnancy

related appointments. Bearing in mind that the homes of some participants were over 15km away, the transportation service was highly appreciated by the participants.

3. Ambulance services (24hr) during labour: when women went into labour, they called the project ambulance and were then transported free of charge to the health centre where the study staff were waiting to help in delivery of the baby. We ensured that for all participating women, we were available continuously on all days of the week including public holidays.
4. Hospital delivery at no cost: no fees were charged for delivery of babies of our study participants. If a mother was referred to a bigger hospital, the project paid all fees related to the delivery.
5. General assistance in referrals to specialised health institutions: we acted as intermediaries for our clients whose babies had medical conditions that were outside the scope of our research. For example, when two babies were born with spina bifida, we contacted a specialised hospital near Nairobi (340km away) and assisted in transporting the babies to the hospital for specialised care. Further, we managed to negotiate with the hospital administration so that the babies were treated free of charge. This level of assistance was deeply appreciated by the surrounding community.
6. Recruitment of research assistants and community volunteers from the study area: in order to secure support and good will from members of the community, we recruited local people with health related training as staff in our project.
7. Regular meetings with spouses of pregnant women: we recognised that the spouses of the pregnant women played a big role especially when the women went into labour. Usually, the spouses are the ones who would call us and ask us to send the ambulance. We therefore held regular meetings with the spouses to make sure that they not only supported our efforts but also that they got an opportunity to clear any doubts that they had about the project.
8. Visiting participants (and their new born babies): we made it a practice to visit as many already current and already graduated participants as possible. This was not only to ensure that we maintained a good rapport with our present and past participants but also to check on the welfare of the new born babies and their mothers. We later realised that these women referred a lot of their friends to our study.
9. Training of traditional birth attendants (TBAs) and engaging them in our project as community health workers: TBAs are respected members of the community. When women realised that the TBAs supported our project, they accepted to participate.
10. Other motivations:
    a. Maize grain – during times of food scarcity and drought, we gave some maize grain to the participating mothers. This grain was obtained locally and was distributed “as is” i.e. without milling or value addition. Women would then take it to the mill and have it fortified as they would have done with their own grain. We did not supply a lot of grain. Further, we calculated the amount of grain given to a woman based on

her stage of pregnancy. For example, a woman who was eight months pregnant received slightly more grain than a woman who was 4 months pregnant. It is worth mentioning that this grain was consumed by all members of the household thus we did not measure exactly what the woman consumed.

b. Delivery gift-pack: when women came to give birth, our staff gave them a standard gift pack that comprised of antiseptic, soap, cotton wool, glycerine, baby oil, a nappy and baby powder. The idea to prepare a small gift pack was arrived at after we realised that most mothers would need a hygiene pack soon after delivery of their baby. Unlike in Western countries, mothers in our study area did not attend formal antenatal lessons where they were instructed about preparing for the delivery of their babies. They also got little or no support from their husbands.
c. Fortification of flour at the mills: most mothers liked the idea that micronutrients were added to their flour products.

A lot of wisdom is required in deciding what sort of incentives to offer participants in a study. Usually, a financial compensation is worked out and offered to all participants. This is normally calculated based on established formulas which vary from institution to institution. However, researchers working in resource poor settings must ensure that the motivational incentives should not influence the decision of the potential participant i.e. in making their decision to participate. We devised direct and indirect motivational strategies that would not unnecessarily impair the independent decision of potential participants. Judging from the fact that our study had very high compliance rates and very low attrition, we can conclude that our strategies worked.

Future research

Researchers may not be able to repeat the study described in this thesis due to ethical dilemmas especially in light of the described benefits of iron supplementation during pregnancy. However, there are questions that still need answers. Below are potential research areas that need further understanding:

1. Although we did not observe an effect of oral ingestion of 60mg ferrous fumarate on non-transferrin bound iron (NTBI) three hours after ingestion, it is possible that the meal consumed by the participants hindered NTBI expression. Further research would be needed to assess the NTBI response to consumption of different types and amounts of oral iron supplements.
2. As per national and international guidelines, the daily supplementation dose for pregnant women should be doubled to 120 mg iron if they are anaemic or if 6 months duration cannot be achieved in pregnancy (Chapter 2). Further studies would be needed to establish whether such high iron doses are safe.

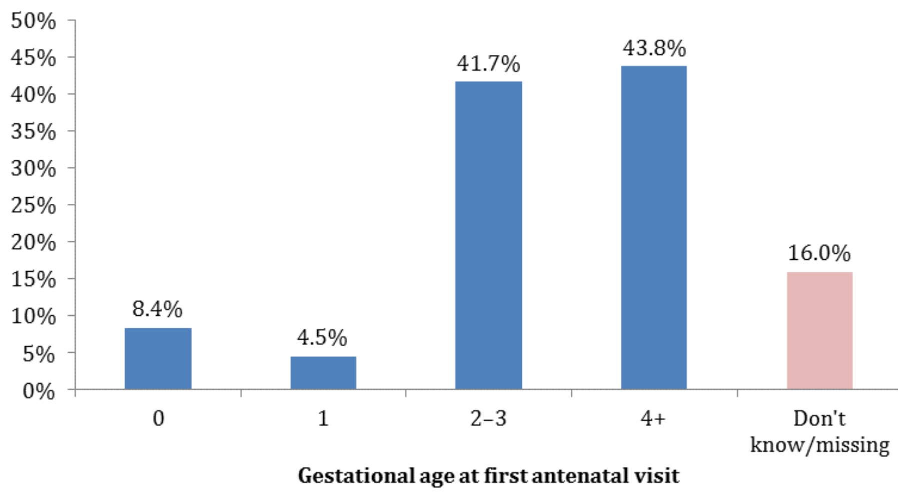
3. Further studies are needed to assess the diagnostic performance of zinc protoporphyrin in children.
4. Although the rates of malaria are declining, and despite the implementation of IPTp in the study area, we still found the risk of asymptomatic infections to be unacceptably high (45%) in our population. The detrimental effects of asymptomatic infections are already well documented as outlined in this thesis. There is urgent need for diagnostic tools that can be used in resource-poor settings to diagnose asymptomatic infections.
5. In developed countries, it may be possible to screen women at an early stage of pregnancy, with the aim to identify women who need iron supplementation to prevent anaemia and low birth weight. Our studies suggest that screening based on haemoglobin concentration, as now practiced in many countries, is inaccurate in discriminating between women at high and low risk of delivering neonates with low birth weight. Further studies are needed to identify appropriate markers and cutpoints that are suited to this purpose.

Policy implications

The findings presented in this thesis should eliminate doubts about the safety of daily antenatal supplementation with 60 mg iron as ferrous salts in malaria endemic areas. Our findings show that universal iron supplementation should be strongly encouraged, also in malaria-endemic regions.

Most developing countries already have iron supplementation policies that are well aligned to the policies by the World Health Organization. (WHO, 2012a, 2012b) Information about coverage of antenatal supplementation is scarce and difficult to interpret, because it does not routinely include estimates of adherence to supplementation throughout pregnancy. What is available suggests that it can be drastically scaled up. In Kenya, pregnant women should receive iron supplements during antenatal visits, as per national guidelines (Chapter 2). The vast majority of rural women who obtain antenatal care go to government care facilities (85%; (KNBS/ICF Macro 2010), at least in part because these facilities provide antenatal care free of charge. Only 60% of rural pregnant women receive their first antenatal care before the third trimester (Figure 7). Thus women do not benefit fully from iron supplementation because they start attending antenatal services late in pregnancy. A survey conducted in 2002 among rural women in western Kenya found that only 53% women who had recently delivered had received any iron supplements during pregnancy. (Anna M Van Eijk et al., 2006) Because care facilities are of out of stock or low in stock, the supply of supplements provided per visit, if any, will provide cover for only small periods. Only 44% of rural pregnant Kenyan women make four or more antenatal visits (Figure 7), and this number seems to be declining. (Kenya National Bureau of Statistics (KNBS) & ICF Macro, 2010) Although supply problems have been a major constraint, adherence to supplementation is also problematic. (Galloway & McGuire, 1994) Thus even

women who report to have received iron supplements are likely to have been covered for only a small proportion of their pregnancy.



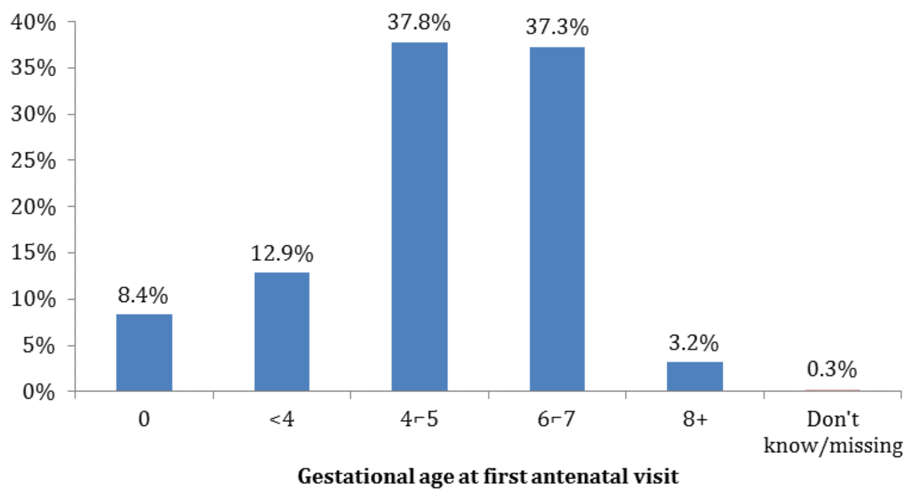


Figure 7: Frequency distribution of number of antenatal care visits during the most recent live birth (top) and the timing of the first visit (bottom).

The survey was representative for rural Kenyan women aged 15-49 years with a live birth in the five years preceding the survey. (KNBS/ICF Macro 2010)

The pattern of visits of antenatal care shown in Figure 7 varies widely between countries, but is worse in many other countries in sub-Saharan Africa and South Asia. (WHO, 2003) A review of implementation and progress of national programmes to control micronutrient deficiencies concluded that the supply of iron tablets procured from the UN Children's Fund and external sources is very low relative to the need, calculated on the basis of the birth rate and the number of iron tablets to fully cover a 40-week pregnancy. (Mason et al., 2001) Although the distribution framework for iron supplementation is in place in most resource-poor countries, the pharmaceutical supply chain could be improved by elimination of key systemic barriers. (Thiam, Kimotho, & Gatonga, 2013)

The evidence provided by this thesis is only applicable to pregnant women and cannot be extrapolated to children, for whom there is substantial evidence that iron supplementation can increase malaria rates. (Sazawal et al., 2006) As per current WHO policy, children should be screened for iron status, and iron supplementation should be restricted to those with iron deficiency. (WHO/CDC, 2007; WHO, 2004)

Conclusions

We found no evidence that antenatal iron supplementation increases the risk of *Plasmodium* infection, but it leads to substantial improvements in birth weight, fetal growth and infant iron stores, with potentially immense benefits for infant survival and health that should outweigh any possible concerns about risks of malaria. Scaling up universal iron supplementation in pregnancy in developing countries is likely to generate major public health gains.

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Annex I
Statistical analysis plan

TRIAL FULL TITLE	PRENATAL IRON AND MALARIA (PIMAL) STUDY: Safety and efficacy of iron interventions in African pregnant women
ClinicalTrials.gov NUMBER	NCT01308112
SAP VERSION	1.0
LSHTM ERB NUMBER	5664
SAP VERSION DATE	08 October 2013
TRIAL STATISTICIAN	Hans Verhoef (PhD)
TRIAL CHIEF INVESTIGATOR	Hans Verhoef (PhD)
SAP AUTHORS	Martin Ndegwa Mwangi and Hans Verhoef

Abbreviations and Definitions

A list of the abbreviations and acronyms used in the Statistical Analysis Plan (SAP) and their definitions are shown here below in alphabetical order.

AE	Adverse Event
AGP	Alpha-1 acid glycoprotein
BMI	Body Mass Index
CRP	C-reactive protein
Hb	Haemoglobin
HIV	Human Immunodeficiency virus
IPT	Intermittent preventive treatment
IUGR	Intra uterine growth restriction
NaFeEDTA	sodium ethylenediaminetetraacetate
NTBI	Non Transferrin Bound Iron
PCR	Polymerase Chain Reaction
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
sTfR	Serum transferrin
WHO	World Health Organization
ZPP:Heme ratio	Zinc protoporphyrin to haemoglobin ratio

Introduction

A recent trial in children reinforced earlier concerns that supplemental iron can lead to an increased burden of malaria, perhaps through the transient production in plasma of non-transferrin bound iron (NTBI). An expert group convened by the World Health Organization (WHO) recently recommended that iron supplementation in children should be restricted in malaria-endemic areas, but that these restrictions are not to be applied to food fortification with iron. The Kenyan government has recently made mandatory iron fortification of industrially milled flour a legal requirement. Universal iron supplementation continues to be recommended for women during pregnancy and 3 months postpartum. Potential effects of iron on infection are likely to be most pronounced in pregnancy, when iron absorption is very high. This seems to be confirmed by observational studies, which indicate that iron deficiency in parturient women is associated with a marked reduction in the prevalence and density of malarial parasites in the placenta. Such infections have devastating effects on the foetus and neonate, causing low birth weight, intrauterine growth retardation, preterm delivery, spontaneous abortion, stillbirth and neonatal mortality. This study was designed to compare daily high-dose iron (i.e. iron-fortified foods plus iron supplements) versus low-dose iron (i.e. iron-fortified foods only) during pregnancy regarding the presence of *Plasmodium* infection at delivery.

Definitions

1. *Plasmodium* infection: see Table 4.
2. Patent infection: an infection that results in detectable parasites or parasite antigens based on HRP2 and/ or pLDH dipstick tests.
3. Sub-patent infection: an infection with a negative result upon blood examination by microscopy or *Plasmodium* antigen dipstick test (HRP2 and/ or pLDH) but with a positive result for a PCR test.
4. Gravidity: the number of times a woman reports to have been pregnant, regardless of the outcome of these pregnancies, with twins and other multiple births counted as 1, and including the current pregnancy.
5. Presence of intrauterine growth restriction(IUGR): a fetus whose weight is below the 10th percentile based on a reference population with similar gestational age, or whose birth weight at term is less than 2500g (Galan, Ferrazzi, & Hobbins, 2002);
6. Fever: axillary temperature ≥ 37.5 °C;
7. Anaemia: haemoglobin concentration <110 g/L (WHO, 2011a);
8. Iron deficiency: plasma ferritin concentration <15 μg/L (WHO, 2011b);
9. Iron deficiency anaemia: presence of both anaemia and iron deficiency;
10. Inflammation: either C-reactive protein (CRP) >10 mg/L(Nielsen et al., 1990) or alpha-1-acid glycoprotein (AGP) >1 g/L(Filteau et al., 1993);

Parasite density was calculated as the median of four counts by microscopic examination of thick blood films (duplicate counts by each of two laboratory technicians).

Haemoglobin concentration was calculated as the mean of duplicate measurements or median values if more than two recordings were available.

Purpose of the analyses

This plan is restricted to the statistical analysis of the primary study objective as formulated in the original study proposal, i.e. to compare the presence of *Plasmodium* infection in parturient women who received a combination of iron-fortified foods with iron supplements versus iron-fortified foods only. The plan is based on the original proposal, and has been finalised before the treatment code was known/revealed. No interim analyses were planned or done.

Analyses populations

1. **Full Analysis Population**

   All subjects who were randomised and who a) received at least one dose of the experimental supplements (iron or placebo); b) *Plasmodium* infection status could be ascertained from blood or placental samples collected at birth or within 48 h after birth. In case of maternal or (unborn) child death, a study subject contributes only to the full analysis population when meeting both criteria (a) and (b) above.

2. **Per Protocol Population**

   The per-protocol population comprises the full analysis set but restricted to participants who a) consumed >90% of scheduled supplements; and b) had singleton pregnancies.

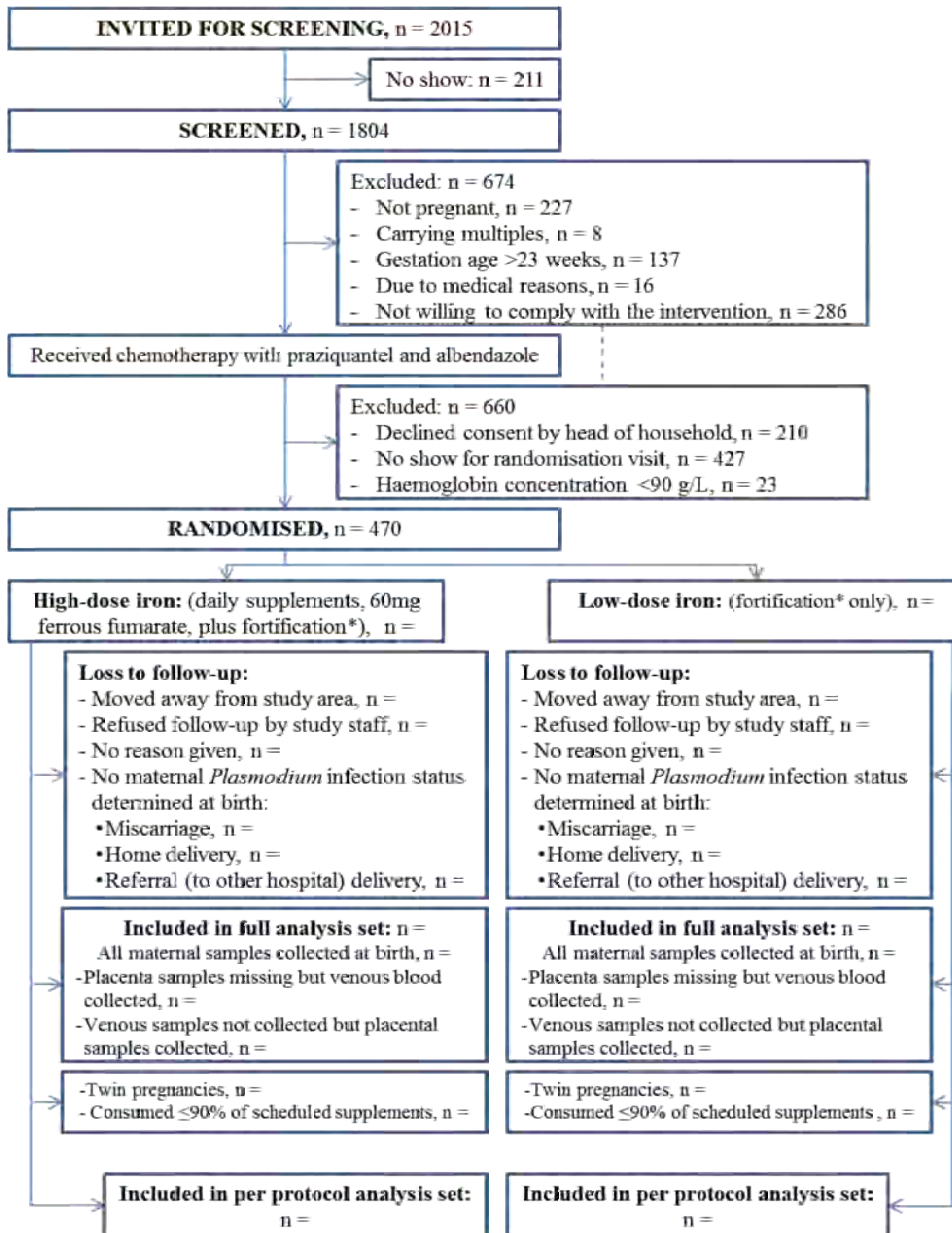
3. **Safety Population**

   All subjects who received the study treatment (including control) and are confirmed as providing complete follow-up data (including information on adverse events).

General information

In the univariate analysis, variables will be checked for distribution (normal, binomial etc.), central tendency (mean, median, mode), dispersion of the values (standard deviation, variance, range, and quartiles), and the presence of outliers/ extreme values. Similar variables will be compared by use of bar graphs, box plots and by comparing their means and distributions. In case the intervention was stopped before its prescheduled end, whether by decision of the trial participant or the field team, we will report reasons for this withdrawal.

In case data or samples were collected after stopping the intervention, we will include these data or information derived from these samples in the full analysis set. Missing data for haemoglobin concentration at the end of the intervention will be imputed blindly, before starting primary analysis, as the mean, geometric mean or median values obtained from participants without missing values in the same intervention group.



Participants' flow chart

Reporting conventions: We will report confidence intervals when possible. P-values ≥0.01 will be reported to two decimal places; p-values less than 0.001 will be reported as "<0.001". Means, standard deviations and any other statistics other than quartiles will be reported to one decimal place greater than the original data. Quartiles, such as median, or minimum and maximum will use the same number of decimal places as the original data. Estimated parameters, not on the same scale as raw observations (e.g. regression coefficients) will be reported to three significant figures.

Baseline descriptions

Data will be summarised by intervention group as n, mean, SD (symmetrically distributed continuous variables); n, median and IQR (skewed continuous variables); or numbers and percentage (categorical variables). Means for non-normally distributed variables will be reported as geometric mean.

The following tables will show the study participants' characteristics per intervention group.

Table 1: Demographic and biochemical characteristics at baseline

Characteristics
Height, cm
Weight, kg
BMI, kg/m^2
Marital status
Married or living together
Divorced or separated
Never married
Age, y[1]
Gestational age, weeks [1]
Gestational age
1st trimester
2nd trimester
Gravidity
Primigravidae
Secundigravidae
Multigravidae
Current fever (axillary temperature ≥ 37.5 °C)
Plasmodium infection, Table 4
Current or recent *P. falciparum* infection, by either HRP2- or LDH-based dipstick
P. falciparum, by PCR
P. falciparum, by HRP2- or LDH-based dipstick or PCR
Plasmodium spp. other than *P. falciparum*, by LDH-based dipstick or PCR
Any *Plasmodium* spp., by dipstick or PCR
HIV infection
Haemoglobin concentration, g/L
Anaemia, haemoglobin concentration <110g/L
Plasma transferrin concentration, g/L
Plasma sTfR concentration, µg/L

Table 1: Demographic and biochemical characteristics at baseline

Plasma ferritin concentration, μg/L
Iron deficiency, plasma ferritin concentration <15μg/L
All
Restricted to those with CRP<10mg/L
Restricted to those with alpha-1- acid glycoprotein<1.0g/L
Restricted to those with CRP<10mg/L or alpha-1- acid glycoprotein<1.0g/L
Whole blood ZPP:haem ratio, μmol/mol
Erythrocyte ZPP:haem ratio, μmol/mol
Erythrocyte FEP concentration , μg/L

Study endpoints

1. Primary outcome:

The primary outcome is defined as the presence of maternal *Plasmodium* infection in samples collected at parturition, regardless of species, as indicated by one or more positive results for the presence of parasite-derived LDH or HRP2 in plasma (dipstick tests), past infections (as indicated by *Plasmodium* pigment upon histological examination of placental biopsies), or *Plasmodium* DNA (any species, by PCR test) (Table 2, case 1). We excluded the results of microscopy from this case definition because these results would be considered incorrect if discordant with results from PCR tests.

2. Secondary outcomes

As secondary outcomes, we will consider the following indicators:

- Patent and sub-patent *Plasmodium* infection (Table 2, cases 2 and 3 respectively);
- Current or recent *Plasmodium* infection (Table 2, case 4);
- Birth weight (g);
- Gestational age at delivery (weeks);
- The presence of intrauterine growth restriction (IUGR);
- Maternal and neonatal iron status at 1 month after delivery, assessed by haemoglobin concentrations, iron deficiency anaemia and iron stores (ratio of ferritin: transferrin receptor concentrations); with and without restriction to those with inflammation.

Justification

When formulating our outcome definitions, we considered *Plasmodium* infections in pregnant women to be important in so far as they are associated with adverse maternal or pregnancy outcomes. However, the pathological consequences of maternal infection likely depends on population-specific factors such as immunity (and thus transmission intensity, parity and maternal age), access to treatment, coverage and quality of antenatal services and drug resistance. Discordant results between studies in the association between maternal *Plasmodium* infection and adverse maternal or pregnancy outcomes are also likely due to differences in the sensitivity and interpretation of diagnostic tests(Uneke, 2007).

Histological examination of placental biopsies is often considered the ‘gold standard’ of malaria diagnosis in pregnancy, and results in infection status being classified into four categories depending on the presence and distribution of *Plasmodium* parasites and haemozoin (Bulmer, Rasheed, Francis, et al., 1993):

a) Current infection: parasites are present in the intervillous spaces with or without pigment in intervillous monocytes;
b) Chronic infection: parasites are present in the intervillous spaces along with pigment as deposits or in macrophages within fibrin;
c) Past infection: pigment is present in the absence of parasites; and
d) Not infected.

Pigment deposition has generally been found to be associated with low birth weight, particularly in the presence of parasites in the intervillous spaces (C Menendez et al., 2000). There is uncertainty, however, about the effect of past infections (i.e. with pigment deposition but without parasitaemia), on neonatal outcomes: such infections have been found to be associated with reduced birth weight in several studies Walter, Garin, Blot, & Philippe, 1981, Watkinson & Rushton, 1983, Rogerson et al., 2003, but not in others (Matteelli et al., 1996, Menendez et al., 2000, Muehlenbachs, Mutabingwa, Fried, & Duffy, 2007, Muehlenbachs et al., 2010).

PCR is considerably more sensitive in detecting maternal infection than either microscopy or rapid dipstick tests that assay the presence of *Plasmodium*-specific proteins (e.g. HRP2 or LDH). Several studies have found that sub-patent infections (i.e. low parasitaemias that are detected by PCR but not by microscopy or dipstick tests) are associated with reduced birth weight ((Malhotra et al., 2005), (Adegnika et al., 2006), (Arango et al., 2010),(Mohammed et al., 2013)) but others have not ((Mankhambo, Kanjala, Rudman, Lema, & Rogerson, 2002), (Mockenhaupt et al., 2006),(Rantala et al., 2010)). With regards to *Plasmodium* species other than *P. falciparum*, we are not aware of studies on the maternal or pregnancy outcome of *P. malariae* or *P. ovale* infection during pregnancy. *P. vivax* is also associated with low birth weight but most of these infections cause symptoms ((Luxemburger et al., 2001), (Nosten et al., 1991),(Nosten et al., 1999)) and this species rarely occurs in Africa.

1. Primary analysis

The primary measure of intervention efficacy will be the difference in prevalence between intervention groups, calculated using Newcombe’s method (Altman, Machin, Bryant, & Gardner, 2000) in the per protocol population (see section 3.2.). We will also report the prevalence difference as a percentage relative to the placebo group, as well as the prevalence ratio.

2. Secondary analyses

Adjustment for baseline covariates

We will assess possible confounding by comparing crude and adjust prevalence ratios, the latter being obtained by including baseline covariates that may be prognostic for outcome in a Cox regression analysis with constant time at risk (Lee & Chia, 1993), (Lee, 1994; Osborn & Cattaruzza, 1995), (Barros & Hirakata, 2003). These covariates are divided into two groups:

a) Variables that are likely to be prognostic for the primary outcome, as predefined on the basis of the literature review:
   - Gravidity (M. Desai et al., 2007)): entered as a dummy-coded categorical variable with 3 classes: primigravidae, secundigravidae, multigravida;
   - Maternal age (M. Desai et al., 2007) (Tako et al., 2005): dummy-coded categorical variable with 2 classes: < 20 years and ≥ 20 years;
   - HIV infection status (Kuile et al., 2004) : binary variable.

b) Variables that are possibly prognostic for the primary outcome:
   - *Plasmodium* infection status (any *Plasmodium* spp., by dipstick or PCR): binary variable;
   - Iron status: haemoglobin concentration as continuous variable centred around the mean as well as plasma ferritin concentration as a binary variable (<12 µg/L or ≥ 12 µg/L);
   - Gestational age at delivery (continuous variable, centred around the mean).

We will examine the influence of these factors using a backward elimination procedure from a full model that includes all of the above variables. When judging the degree of confounding, a diversion of >15% of the crude prevalence ratio will be considered to be of public health importance. When the potential value of this adjustment is in doubt, we will consider the unadjusted analysis as the one of primary attention, and the adjusted analysis being supportive.

Full analysis set

An exploratory analysis will be conducted on the full analysis set.

Subgroup analyses

As a first step, we will use stratified analysis to assess to what extent the effect of iron on *Plasmodium* infection is influenced by gravidity, age, HIV infection and iron status, indicated by anaemia and iron deficiency (Table 3). In this analysis, we will consider variables with the following categories: gravidity: primigravidae, secundigravidae and multigravidae; age: ≤20 years, >20 years; HIV infection: infected, non-infected.

In a second step, we will assess such effect modification directly using multivariate logistic regression analysis.

Table 2: Case definitions of *Plasmodium* infection

	HRP2-based dipstick test	LDH-based dipstick test		Microscopy of peripheral blood [1]	Placental biopsy		PCR, peripheral blood		PCR, maternal placental blood	
		P. falciparum	Other spp.		Pigment	Parasites [1]	*P. falciparum*	Other spp.	*P. falciparum*	Other spp.
Case 1 (primary outcome) [2]	●	●	●	○	●	○	●	●	●	●
Case 2 (patent)	●	●	●	○	●	○	○	○	○	○
Case 3 (sub-patent)	○	○	○	○	○	○	●	●	●	●
Case 4 (current or recent)	●	●	●	○	○	○	●	●	●	●

Test with positive (●) or negative (○) results are linked through 'OR' Boolean operators.
[1] Regardless of parasite stage (i.e. asexual or sexual stages).

We will interpret the results of subgroup analysis cautiously (Assmann et al., 2000), (Wang et al., 2007),(Fletcher, 2007; Oxman, 1992; Sun et al., 2010; Yusuf, 1991).

In the analysis of haemoglobin concentrations in maternal blood at 1 month post-partum, we expect little or no treatment effect in individuals who are iron replete, because iron absorption is known to decrease with the magnitude of the iron stores (L Hallberg, 2001).

Intermittent preventive treatment (IPT)

Intermittent preventive treatment data was obtained retrospectively from the participants ante-natal clinic attendance booklets. The information in these booklets is filled in by nurses during the antenatal clinic visits. Because IPT data was recorded after randomisation, theoretically, it could be influenced by the intervention and thus it is considered separately in the analysis. We will examine whether intermittent preventive treatment has an effect on malaria parasitaemia at birth.

Intermittent preventive treatment will be a categorical variable with 5 classes: no dose (participants whose booklets were available but had no data on IPT thus no IPT dose received), single dose, two doses, three doses, unknown (participants whose booklets were not available).

Table 3: Justification of effect modifiers to be assessed

Effect modifier	Justification
a. Gravidity	In highly endemic areas, primigravidae are at greater risk of malarial infection (Nosten et al., 1991) (A. M. Greenwood et al., 1992), probably because they have a reduced immunity. Thus we expect that their ability to suppress a possible increase in parasitaemia resulting from iron to be reduced.
b. Age	*Plasmodium* infection is expected to be higher in women ≤20 years old. Young primigravidae and multigravidae are at greater risk of malaria and its adverse effects than older primigravidae or multigravidae, respectively(Espinoza et al., 2005; Leenstra et al., 2003; Marques et al., 2005)(S J Rogerson et al., 2000)(Walker-abbey et al., 2005). This is probably because age-associated immunity plays a role in controlling malaria infection during pregnancy in highly endemic areas. We expect that women ≤20 years old have a reduced ability to suppress a possible increase in parasitaemia resulting from iron.
c. HIV infection	HIV infection compromises malarial immunity such that HIV infected multigravidae have at least as high a risk of placental infection as non-HIV-infected primigravidae (M. Desai et al., 2007). HIV exacerbates the burden of malaria and increases the degree to which malaria is associated with maternal severe anaemia. We expect that due to compromised malarial immunity, women that are HIV infected will be more susceptible to iron-induced *Plasmodium* infection.
d. Iron status at baseline	Low iron status at baseline may be protective against malaria infection but will result in increased absorption of supplemented iron. The vice versa is also true thus we expect the effect of iron supplementation on malaria parasitaemia to be influenced by iron status at baseline.

Efficacy Analyses

The most important indicator for evaluating intervention benefits will be haemoglobin concentration at 1 month post-delivery. Group differences in haemoglobin concentration will measured by linear regression analysis, adjusting for possible group differences in time between delivery and blood collection (centred on 30 days post-partum). We will assess possible confounding of the intervention effect by comparing the difference in mean haemoglobin concentration with and without adjustment for baseline haemoglobin concentration (continuous variable). We will also assess the influence of haemoglobin concentration at baseline (continuous variable) on the haemoglobin response to the iron intervention, using multivariate linear regression analysis.

We will use similar procedures to assess intervention effects on plasma ferritin concentration at 1 month post-delivery.

All analyses of the continuous efficacy variables will be performed as analysis of variance with treatment group adjusting for inflammation etc. All assumptions for regression models will be assessed by viewing plots of the residual values. Dependent variables will be log-transformed as appropriate.

Safety Analyses

Summary statistics per intervention group will be produced for deaths and serious adverse events that are judged to be related to the treatment. Each subject will only be counted once and any repetitions of adverse events will be ignored; the denominator will be the total population size.

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Annex II

Surveillance for and detection of early pregnancy

Prenatal Iron and Malaria (PIMAL) Study

STANDARD OPERATING PROCEDURE

Terminology

Amenorrhoea	The absence of a menstrual period in a woman of reproductive age
Embryo	A fertilised egg in its earliest stage of development, from the time of first cell division until about eight weeks after fertilisation (thereafter called a foetus)
Fallopian tubes	Two fine tubes leading from the ovaries into the uterus
Fertilisation	Fusion of sperm cell and egg
Gestational age	The age of an embryo, foetus or new-born infant, usually calculated from the first day of the woman's last menstrual period
Implantation	An event that occurs early in pregnancy in which the embryo adheres to the wall of uterus
Menopause	The end of the fertile phase of a woman's life as a natural consequence of aging (defined in this document by the absence of a menstrual period for 6 months in women who are neither pregnant nor have postpartum amenorrhoea)
Ovary	The egg-producing reproductive organ in women
Ovulation	The process in a female's menstrual cycle by which a mature ovarian follicle ruptures and discharges an ovum (egg)
Postpartum - Amenorrhoea:	The period between childbirth and the return of menstruation following childbirth
Source- Population:	All resident women of fertile age in the study area who meet the eligibility criteria for randomisation
Stillbirth	Occurs when a foetus has died in the uterus, after 20 weeks gestation or the baby weighs more than 400 grams

Aim
This protocol describes our methods to detect pregnancies occurring in the source population at an early stage, but in any case before or at 20 weeks of gestation. Please note that gestational age is calculated as per convention from the first day of the woman's last menstrual period (see 'Terminology'), assuming that conception occurs 14 days later.

Background
Kenyan women potentially become exposed to pregnancy at a relatively young age. In 2003, 15% of girls aged 15-19 years reported to have had sexual intercourse by the age of 15 years, whilst 17% in the same age range reported to have had intercourse within the previous 4 weeks (Central Bureau of Statistics 2004). The onset of menopause increases with age from around age 30, but increases sharply after the age of 45 years. Thus in the age range 46-47 years, 32% of women reported to be menopausal; in the age range 48-49 years, this had increased to 53% (Central Bureau of Statistics 2004). Thus in our study, we consider that women may be *exposed to pregnancy in the age range 15-45 years*.

Our methods used to detect pregnancies within this age range include a) questioning women, i.e. asking them whether they have missed their menstrual period, b) urine testing and c) confirmation by obstetric ultrasound.

Questioning: Questioning has the advantage of being a relatively low-cost method to detect pregnancies. Previous experience in an adjacent area (Mrs. Joyce Nduku, KEMRI, personal communication, October 2010) indicates that women who are currently married are generally open about their pregnancies: their reported history of periods is by and large reliable. By contrast, unmarried women tend to initially hide their pregnancies. With these women, community health volunteers and traditional birth attendants may be helpful in eliciting correct responses.

When do we consider a menstrual period to be missing? Although the average cycle is 28 days, there is considerable variation in cycle length within and between women. Most menstrual cycles have a length below 40 days (e.g. McKeown et al. 1954, Jeyaseelan et al. 1992, Münster et al. 1992, Fehring et al. 2006, Das and Das 2010). Even though there are uncertainties about the validity of extrapolating these data, we define in our study *a missed menstrual period as the reported absence of menstruation within 6 weeks from the onset of the last period*.

In most postpartum women, there is for a limited time after birth no need to check for pregnancy: in women who are fully breastfeeding and amenorrhoeic but not using any contraceptive method, the probability of pregnancy in the first 6 months post-partum is below 2% (Labbok et al. 1994, Kennedy and Kotelchuck 1998, Queenan 2004, Van der Weijden et al. 2003). Postpartum amenorrhoea is largely defined, however, by the duration and intensity of breastfeeding. In 2003, only 29% of Kenyan children aged below 2 months were exclusively breastfed; at 2-3 months, almost half of all children were reported to be given complementary foods. 91% of Kenyan women reported to be amenorrhoeic or abstaining from sex in the first 3 months post-partum; the proportion reporting to be

amenorrhoeic thereafter declined rapidly (Central Bureau of Statistics 2004). For this reason, in breastfeeding women, we will consider the probability of pregnancy to be low in the first 3 months postpartum, and we will not check for pregnancy in this time period. It is also reasonably certain that the woman is not pregnant if she is within 4 weeks postpartum for non-lactating women, or within the first 7 days post-abortion or miscarriage (WHO 2004).

Urine testing: To confirm pregnancy, we will use a urine test to detect the presence of the beta subunit of human chorionic gonadotropin (hCG). This hormone is produced by the developing embryo after conception and later by the syncytiotrophoblast (part of the placenta). It can be detected in urine or blood after implantation, which occurs 6–12 days after fertilization (Wilcox et al. 1999). Home pregnancy test kits, when used by experienced technicians, are almost as accurate as professional laboratory testing (97%). When used by consumers, however, the accuracy can drop to as low as 75%, probably because many users misunderstand or fail to follow the instructions included in the kits (Bastian et al. 1998). To avoid errors, it is important to understand the reasons for their occurrence:

a. False negative readings can occur when testing is done too early. The most sensitive urine tests usually can detect hCG shortly after implantation (see **Figure 1** for an overview of the menstrual cycle); less sensitive urine tests may not detect pregnancy until 3–4 days after implantation. Menstruation occurs on average 14 days after ovulation, so *the likelihood of a false negative is low once a menstrual period is late*.

   However, ovulation may not occur at a predictable time in the menstrual cycle. A number of factors may cause an unexpectedly early or late ovulation, even for women with a history of regular menstrual cycles. The accuracy of a pregnancy test is most closely related to the day of ovulation, not of the act of intercourse or insemination that caused the pregnancy. It is normal for sperm to live up to 5 days (Weschler 2002) in the fallopian tubes, waiting for ovulation to occur. It could take up to 12 further days for implantation to occur, meaning even the most sensitive pregnancy tests may give false negatives up to 17 days after the act that caused the pregnancy. Because some home pregnancy tests have high hCG detection thresholds (up to 100 IU/L), it may take an additional 3–4 days for hCG to rise to levels detectable by these tests — meaning false negatives may occur up to 3 weeks after the intercourse that causes pregnancy.

   Note that few cycles are exactly average! Menstrual cycles are counted from the first day of menstrual bleeding. Under optimum circumstances, some sperm cells can live up to about 5 days. Once released through ovulation, the egg is capable of being fertilised for 12-48 hours before it begins to disintegrate. Thus the fertilisation period starts 5 days before ovulation, and ends 1-2 days afterwards. Fertilisation usually takes place in the ampulla, the widest section of the fallopian tubes. It usually takes 6 days for a fertilized egg to reach the uterus and to implant into the endometrium.

Early in pregnancy, more accurate results may be obtained by using the first urine of the morning when hCG levels are highest. When the urine is dilute, the hCG concentration may not be representative of the blood concentration, and the test may be falsely negative.

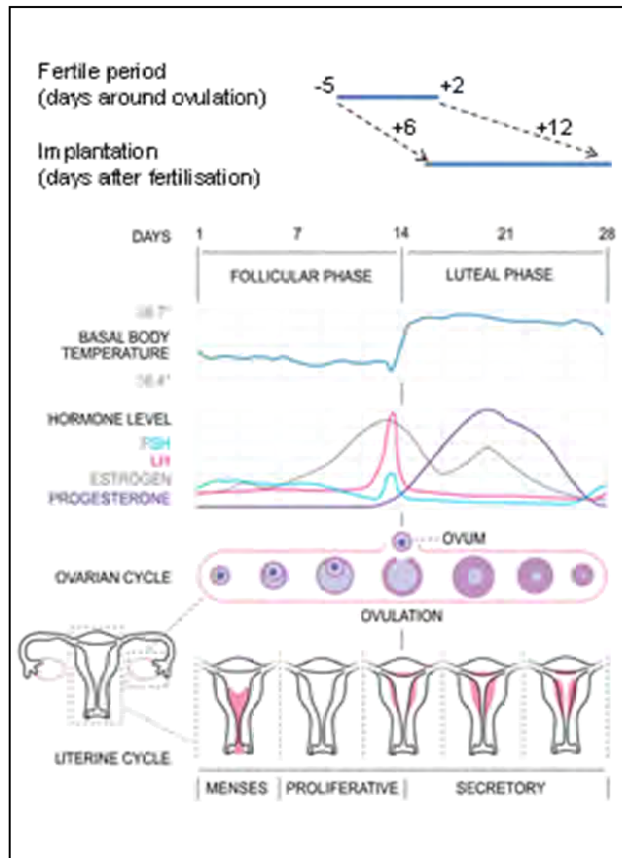

Figure 1: The average 28-day menstrual cycle

b. False positive readings may occur for several reasons. Spurious evaporation lines may appear on many home pregnancy tests if read after the suggested 3–5 minute window or reaction time, independent of an actual pregnancy. False positives may also appear on tests used past their expiration date.

Another factor that makes it undesirable to administer urine tests too frequently or too early is that many pregnancies are lost early. This is particularly important for young, unmarried women, who may wish such lost pregnancies to remain confidential. Studies using hCG tests have shown that approximately 60% of all fertilised eggs are lost due to natural causes before the end of the first trimester is reached (Goldstein 1994). Such miscarriages usually

occur without the knowledge of the once pregnant woman, and may be interpreted as a menstrual cycle of abnormal length (e.g. Edmonds et al. 1982, Kolstad et al. 1999). Even in recognised pregnancies, about 25% are lost in the first trimester. Most early pregnancy losses occur in the first month after conception, and are uncommon after the end of the embryonic period, i.e. 10 weeks after the onset of the last menstrual period (Goldstein 1994).

Obstetric ultrasound: We will use diagnostic ultrasound to confirm pregnancy and to estimate gestational age. Because gestational age probably is prognostic for many of outcome indicators considered in our trial, its estimation will allow for adjustment of intervention effects. Thus estimation of gestational age is not merely done to determine the expected date of delivery, although this is useful to plan the delivery in the predesignated health facility.

The starting day of the Last Menstrual Period, quickening, first audible foetal heart tones, uterine fundus at the umbilicus, and measurements of the fundal height are also commonly used as indicators of gestational age, but these have limited value because of inaccuracies in recall, lactation, and the subjective nature of some of these measurements.

The earlier in pregnancy verification of gestational age with ultrasound is done, the more accurate and precise that estimate will be. In the first trimester of pregnancy, foetal crown-rump length is the most important technique employed (Hohler 1984). When done properly, it allows for estimation of gestational age between 9 and 13 weeks within 5 days with 95% confidence (Hohler 1984; gestational age in days, measured from the first day of the LMP = 51.008 + 0.6 × crown-rump length in mm). Earlier than 9 weeks, the anatomical landmarks are somewhat obscure, and it is not possible to examine a single picture and be certain that the maximum longitudinal diameter of the foetus has actually been measured. Before 12 weeks gestation, one should measure the crown-rump length; after 12 weeks, one should rely on determination of the biparietal diameter (i.e. the transverse diameter of the foetal skull), ideally in combination with femur length, head circumference and abdominal circumference. The accuracy of pregnancy dating from 12 to 20 weeks is probably just as good as that done earlier in pregnancy using the crown-rump length.

Procedures

Our procedures (Figure 2) for detection of pregnancy follow a strategy that is outlined in the table below. An ultrasound examination is to be conducted immediately after pregnancy has been confirmed by urine test, or as shortly as possible thereafter.

Roles and responsibilities

Community volunteers: It is the role of community volunteers, including traditional birth attendants, to question women about their history of menstrual periods. The Field Coordinator in close consultation with local authorities and village elders will appoint volunteers. Each volunteer will do the pregnancy surveillance in a pre-allocated, fixed number of households.

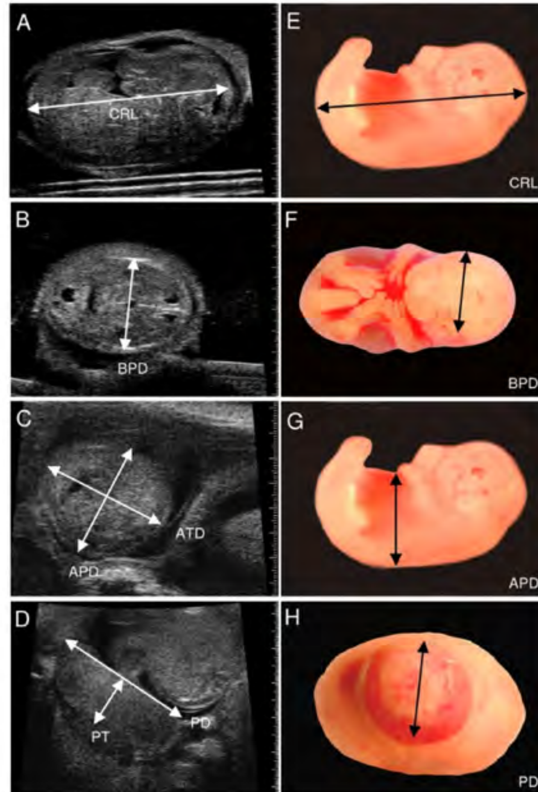

Figure 2: Illustration of measurement locations (panels A-D) for foetal growth indicators. Crown-rump length (CRL), biparietal diameter (BPD), abdominal anteroposterior diameter (APD), abdominal transverse diameter (ATD), placental diameter (PD), and placental thickness (PT); panels (E-H) obtained ex vivo by light microscopy.

Volunteers are identified and trained in surveillance procedures. The volunteers will have the following responsibilities:

- To identify all eligible women within the allocated households by listing their name, date of birth, marital status, pregnancy status;
- To ensure that all eligible women mark their menstrual periods on a calendar;
- To conduct a monthly survey by visiting women within the allocated households who are currently married or living together on a monthly basis, and by questioning them carefully about their history of menstrual periods;
- To record findings on a standard, predefined form (Annex 1), and to submit this report to the study nurse during a monthly meeting.

Target group	Action required	Comments
1. Women who *delivered a child* during the surveillance period, or within 6 months before the start of the surveillance period, regardless of their marital status	(Depending on time of delivery – see below)	
a. Delivery occurred less than 3 months ago	No questioning or urine testing required	
b. Delivery occurred more than 3 months ago	Urine tests to be done by nurse at 12-weekly intervals until menstrual periods have recurred; pregnancy detection thereafter as indicated below	
c. Unmarried women (*never* been married, *divorced, separated,* or *widowed*)	Urine testing to be done by nurse strictly every 12 weeks	At such intervals, the gestational age of pregnancies detected will likely be within the eligible range, whilst in the vast majority of pregnancies, gestational age can be established simply by obstetric ultrasound on the basis of estimated crown-rump length (accurate in the first 13 weeks of gestation)
d. Married women (*currently married,* or *living together* with a man)	Questioning to be done by nurse every month (4 weeks) by community volunteers	
	Urine testing to be done whenever women report the start of their menstrual period to have occurred 10 weeks earlier [2]	Urine tests should not be normally be administered earlier: a. In the first 7 days after menstruation, the probability of pregnancy is very low; b. In the subsequent time period until the time of the next expected menstruation, there is a high risk of false negative test results; c. In the subsequent time period until 10 weeks after the onset of the Last Menstrual Period, a woman may

Target group	Action required	Comments
		be pregnant but there is a high risk of her pregnancy being lost.

Please note:

- Volunteers must make every effort to notify a study nurse immediately when a woman reports her menstruation to be absent for 6 weeks or more from the onset of the last period (missed menstrual period), so that the nurse can set a date for a urine test at 10 weeks after the onset of the last period.
- Women who have never been married, or who are divorced, separated or widowed will be under direct surveillance by the study nurse (see preceding sections).

Study nurse

A study nurse, based at the research clinic, will have the following responsibilities:

- Ensure that all households in the study area have been assigned to a Community Volunteer;
- Keep record of all Community Volunteers, and women who have been assigned to them;
- Ensure that Community Volunteers collect data at 4-weekly intervals among women who are married or living together;
- Enter data collected by Community Volunteers daily into an electronic database, and make daily backups of this database;
- Ensure that urine tests are administered at the research clinic to:
- All women who never have been married, or who are divorced, separated, or widows: at 12-weekly intervals;
- All women who are married or living together: when women report not to have had a menstrual period for 10 weeks since the date of the onset of the Last Menstrual Period;
- Coordinate the follow-up of problem cases as and when required;

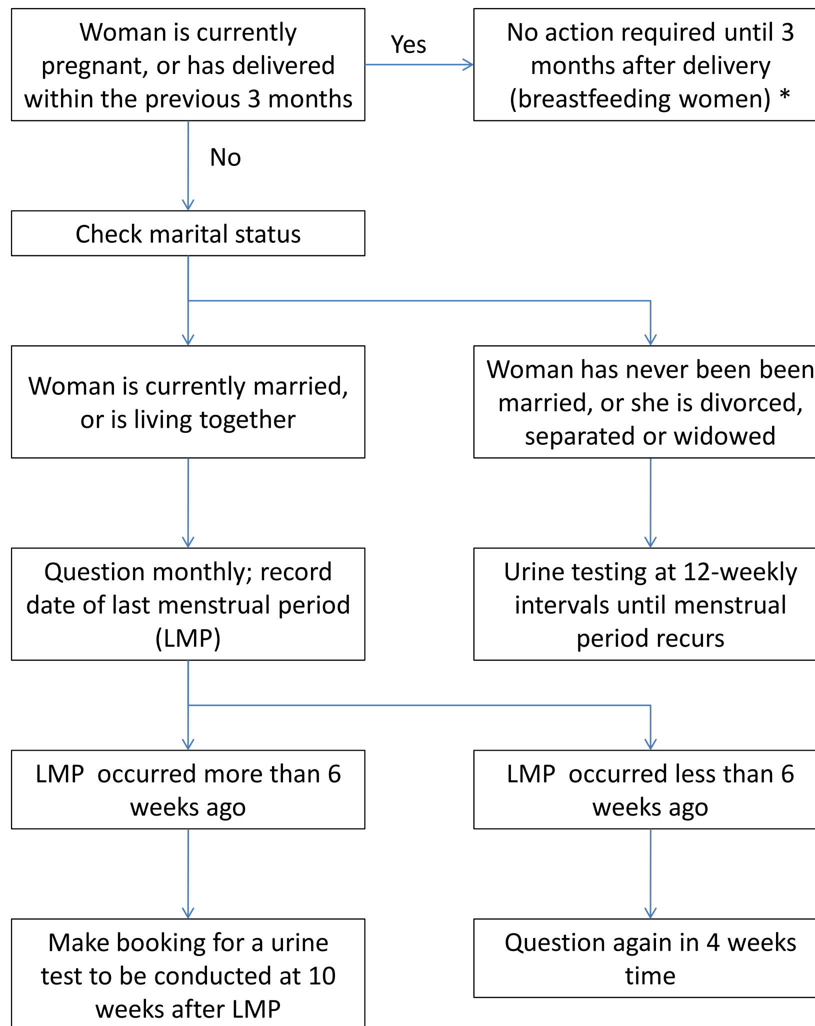

Figure 2: Decision tree for early detection of pregnancy

- Conduct an ultrasound examination immediately after pregnancy has been confirmed by urine test, or as shortly as possible thereafter;
- Organise a monthly group meeting with Community Volunteers to a) check their records; b) to identify women who missed their menstrual periods and to set dates for urine testing as needed; c) to assess and discuss the performance of the Volunteers; and d) to hand out incentives to Volunteers as appropriate;

- Keep petty cash for handing out incentives to Community Volunteers, and to keep up-to-date financial records accordingly;
- Keep an up-to-date monthly log book on surveillance activities (Annex 2);
- Prepare and submit a monthly progress report (Annex 3) to the Field Coordinator (Martin Mwangi).

Even though the nurse may delegate some duties to a data entry clerk or a student, (s)he will ultimately remain responsible.

Field Coordinator (Martin Mwangi)

- To take part in monthly meetings with Community Volunteers and the study nurse;
- To supervise and check the study nurse in all surveillance activities;
- To prepare and submit quarterly progress reports to his supervisors (Drs. Pauline Andang'o and Hans Verhoef).

All information shared by women, as well as the results of urine tests, are to remain confidential. This information may be shared with family members only after agreement has been reached with the woman concerned. Community volunteers as well as the study nurse must ensure that unauthorised persons do not have access to study records. The study nurse may disclose the pregnancy status of women with the Field Coordinator but not with other members of the Study Team, unless authorised by the Field Coordinator.

In our study, most ultrasound examinations will be conducted at a gestational age that is too early to (reliably) determine the sex of the foetus. Even if the sex of the foetus can be established, however, the study nurse is under no circumstance allowed to disclose this information to the parents.

Number of tests required for the study
Based on a total population in our study area of 12,202 people, a crude birth rate of 35.3 per 1000 population in rural areas in Kenya (Kenya Demographic and Health Survey 2008-09), and 10% of pregnancies being undetected in our surveillance system, we would expect to detect 388 births per year, so that the target number of 450 women would be attained in 14 months. Because a substantial proportion of women will already be pregnant at the start of our surveillance activities, however, we expect that the detection rate of pregnant women will initially exceed the rate at which they become pregnant. Thus we expect to be able to attain our target number within 1 year.

Based on a census in the study area that we conducted in 2010 (unpublished results), we expect that our source population includes 2,806 women of child-bearing age. Of these, we expect 1,686 to be married (or living together), and 1,120 to be single (never married, divorced, separated or widowed) (**Table 1**). Assuming that urine tests will be administered

monthly to 5% of women who are married (or living together), and every 12 weeks for single women, this would amount to a daily workload for the study nurse of 4 married women and 19 single women. We expect to administer 5,863 tests over a recruitment period of 1 year (**Table 2**); with an extra safety margin of 15%, this would amount to 6,750 tests.

Table 1: Distribution of women aged 15-50 years by marital status

Marital status	**Relative distribution [1]**	**Absolute number**
Currently married	54.5%	1,529
Never-married [2]	29.8%	836
Living together	5.6%	157
Divorced/separated [2]	5.9%	166
Widowed [2]	4.2%	118
Total	100%	2,806

[1] Based on data from 2003 (Anonymous 2004); [2] women in this category may not be open about their pregnancy (see text).

Table 2: Testing frequency and test requirements for the study

	Married	Single	Total
Testing frequency	5% of women/month	Every 12 weeks	
n	1,686	1,120	2,806
No. of tests to be administered per working day	4	19	23
No. of tests to be administered per month	84	404	489
No. of tests to be administered per year	1,012	4,852	5,863

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Summary

Since the British doctor Ronald Ross received the 1902 Nobel Prize in medicine for his work on malaria, more people have died from the disease than all world wars combined. This is in spite of the fact that the French chemists Pierre Joseph Pelletier and Joseph Bienaimé Caventou made quinine available from as early as 1820. According to the World Health Organisation (WHO), there were about 219 million cases of malaria in 2010 and an estimated 660,000 deaths majority of which (80% of cases and 90% of deaths) were in Africa.

Of almost two billion people who are anaemic globally, 41.8% are pregnant women. Iron supplements are used to prevent anaemia. There are concerns that iron given in high doses may increase malaria rates. Uncertainties regarding the safety of iron supplementation in malaria endemic regions were propelled by a randomized controlled trial that evaluated the effects of iron and folic acid supplementation in 32,155 children in Pemba, Tanzania. This study found that children who received iron and folic acid supplements were more likely to die or to need hospitalisation for an adverse event. At the same time, malaria is known to exacerbate anaemia; an almost inevitable consequence of malarial infection. As such, the safety of daily oral use of iron supplements by pregnant women, as a public health intervention is still not clearly established; at least not until publication of our main findings.

This thesis assessed the effects of iron supplementation on safety indicators, and on iron status, in pregnant women and their neonates. Several preparatory activities were carried out, including a census of the population in the study area and a pilot study to check the operability of the study protocols. A main study was designed with the hypothesis that consumption of food products fortified with iron combined with intake of iron supplements especially in pregnancy, would be detrimental to the health of pregnant women and their neonates.

The objectives of the study were: 1) to compare the presence of malarial infection in parturient women who received a combination of iron-fortified foods with iron supplements versus iron-fortified foods only; 2) to assess intervention effects on the maternal prevalence of iron deficiency anaemia at 1 month after delivery; 3) to assess intervention effects on neonatal iron stores at 1 month of age; 4) to assess the diagnostic utility of Zinc protoporphyrin (ZPP) in diagnosing iron deficiency in malaria endemic regions; 5) to identify baseline factors that are prognostic for the Non-Transferrin Bound Iron (NTBI) response to consumption of a single iron supplement; 6) to determine the factors that predict *Plasmodium* infection in pregnancy; 7) to identify factors associated with birth weight; 8) to develop a methodology to predict cases of low birth weight, using a single prognostic score that is based on prognostic variables collected at the second trimester of

pregnancy; and 9) to develop methods for community-based flour fortification with iron.

Most countries have enacted, or are in the process of enacting legislation for mandatory fortification of flour with iron. Thus pregnant women may receive iron from fortified foods and from universal iron supplementation programmes. This thesis provides answers to pertinent questions regarding the safety and efficacy of iron supplementation by comparing daily high-dose iron (i.e. iron-fortified foods plus iron supplements) versus low-dose iron (i.e. iron-fortified foods only) during pregnancy. The main outcome measure was the presence of maternal *Plasmodium* infection at birth, regardless of species. Chapter 1 is a detailed introduction of the background to the study and the design of the study.

This thesis presents concrete evidence that *iron supplementation to pregnant women in a highly malaria endemic region* ***does not*** *result in increased risk of malarial infection; percent difference (95%CI) = 0.0%* (−9.3% to 9.3%). Programme implementers and governments in malaria endemic regions should not be held back by previous recommendations that cautioned against issuing iron supplements to pregnant women. In light of these findings, there is no need to first screen for malaria before giving iron supplements.

Iron supplementation had major benefits for mothers and their neonates (chapter 2). The findings reported in this thesis showed a mean increase in birth weight of 143 g relative to the low-dose iron group. The effects of iron were influenced by the participants initial iron status. Correction of iron deficiency increased birth weight by 249 g, even though we cannot exclude the possibility that this may have increased malarial infection by 10%. There was no evidence that effects of iron on birth weight were influenced by intermittent preventive treatment against malaria. Iron supplementation also increased fetal growth by 0.27 SD, 95%CI: (0.04 to 0.50) probably as a result of gains in length and weight for gestation age.

We also showed improved neonatal iron stores one month post-partum as indicated by a 17.1 % (95% CI: 2.0% to 34.3%) increase in plasma ferritin concentration in neonates of mothers who received high-dose iron compared to those who received low-dose iron. This provides more impetus to the need to offer iron supplements to pregnant women with the aim of boosting infant iron stores (chapter 2).

ZPP was found to be of unreliable diagnostic utility when discriminating between pregnant women with and without iron deficiency in regions where chronic diseases are prevalent (chapter 3). The current conventional cut off points for whole blood ZPP e.g. >70 µmol/mol heme, can result in gross estimates of the prevalence of iron deficiency especially if the true prevalence is low.

The appearance of non-transferrin bound iron (NTBI) in circulation after oral ingestion of iron supplements has been thought to aid the growth and multiplication of *Plasmodium*

parasites thereby increasing malaria induced morbidity and mortality. We did not observe any increase in NTBI concentrations three hours after oral ingestion of 60 mg of ferrous fumarate (Chapter 4). We cannot exclude the possibility that iron supplementation leads to NTBI production when supplements are not consumed with food, because the lunch meal consumed by majority of our participants during the 3-hour waiting period probably contained natural compounds (phytates) that may have limited an NTBI response.

In chapter 5 of this thesis, we aimed to develop a field friendly tool that can be used to predict asymptomatic *Plasmodium* infection. This was motivated by the fact that most point-of-care dipstick tests used to detect *Plasmodium* infection are not able to detect 100% of all the infection present yet asymptomatic infections are increasingly associated with adverse maternal and neonatal outcomes. Although many likely predictive factors were assessed individually or in combination with others, we did not succeed in developing a reliable tool that is easy to apply in resource-poor malaria endemic settings.

In Chapter 6, we aimed to identify factors associated with birth weight and to develop a methodology to predict cases of low birth weight using a single prognostic score that is based on prognostic variables collected at the second trimester of pregnancy. Factors that were found to be independently associated with reduced birth weight were being never married, inflammation, being a girl, and iron deficiency. Being overweight was associated with increased birth weight. The results indicate that we can use variables collected rapidly and at relatively low cost and ease to identify with fair accuracy women in the second trimester of pregnancy who are at high risk of giving birth to a neonate with low birth weight.

The various aspects of the work presented in this thesis including the implications for policy makers are discussed in chapter 7. For policy makers, the findings of this thesis are a welcome relief. The findings therein eliminate all doubt that has hitherto been associated with antenatal iron supplementation in malaria endemic areas. Most countries already have iron supplementation policies that are well aligned to the World Health Organisation policies. Efforts to widen the coverage of antenatal iron supplementation especially in malaria endemic regions should be urgently scaled up. However, the evidence provided in this thesis is only applicable to pregnant women and cannot be extrapolated to children in malaria endemic regions. For this population, the current WHO policy must be used thus before iron supplementation, children must first be screened for malaria.

Although this thesis provides answers to key scientific questions that have hitherto baffled the scientific community, there are still research questions that can be clarified further. The effects of a high iron dose in pregnancy (as per national and international guidelines, the daily supplementation dose for pregnant women should be doubled to 120 mg iron if they are anaemic or if 6 months duration cannot be achieved in pregnancy (Chapter 2)) on maternal and neonatal outcomes need to be elucidated. Further research is needed in order to describe fully, the NTBI response to consumption different types and amounts of oral

iron supplements. There is urgent need for diagnostic tools that can be used in resource-poor settings to diagnose asymptomatic infections. Further research in children is needed to provide evidence of the safety and efficacy of iron supplementation in malaria endemic regions and to assess the diagnostic performance of zinc protoporphyrin in children. In addition, since our studies suggest that screening based on Hb concentration as now practiced in many countries, is inaccurate in discriminating between women at high and low risk of delivering neonates with low birth weight, further studies are needed to identify appropriate markers and cut-off points that are suited to this purpose.

In conclusion, this thesis has shown that there is no evidence that antenatal iron supplementation increases *Plasmodium* infection. Antenatal iron supplementation leads to large improvements in birth weight, fetal growth and infant iron stores, with potentially immense benefits for infant survival and health that should outweigh any possible concerns about risks of malaria. Epidemiological calculations indicate that if our results are applied to all women in developing countries in order to eliminate iron deficiency, we could avoid 3 million births with low birth weight annually and save the lives of more than half a million neonates. Scaling up universal iron supplementation in pregnancy in developing countries will generate major public health gains.

Samenvatting

Sinds de Britse arts Ronald Ross in 1902 de Nobelprijs voor de geneeskunde ontving voor zijn werk over malaria, zijn er meer mensen overleden aan deze ziekte dan in alle wereldoorlogen samen, ook al maakten de Franse chemici Pierre Joseph Pelletier en Joseph Bienaime Caventou in 1820 kinine beschikbaar. Volgens de Wereldgezondheidsorganisatie (WHO), waren er in 2010 ongeveer 219 miljoen gevallen van malaria en naar schatting 660.000 doden, waarvan de meerderheid (80% van de malaria-gevallen en 90% van de sterfgevallen) in Afrika.

Bijna twee miljard mensen wereldwijd, en 42% van de zwangere vrouwen, lijden aan bloedarmoede. IJzersupplementen worden gebruikt om bloedarmoede te voorkomen. Een hoge dosering ijzer verhoogt echter mogelijk ook het aantal malaria-gevallen. De twijfel over de veiligheid van ijzersuppletie in malaria-endemische gebieden kwam voort uit de resultaten van een gerandomiseerde gecontroleerde studie naar de effecten van ijzer en foliumzuur suppletie bij 32.155 kinderen in Pemba, Tanzania. Uit deze studie bleek dat kinderen die supplementen met ijzer en foliumzuur kregen, meer kans hadden om te sterven of in het ziekenhuis terecht te komen. Daarnaast is het bekend dat malaria bloedarmoede verergert; dit is een bijna onvermijdelijk gevolg van een infectie met malaria. De veiligheid van het dagelijks gebruik van ijzersupplementen door zwangere vrouwen als volksgezondheidsinterventie is nog steeds niet duidelijk vastgesteld; althans niet voor de publicatie van onze bevindingen .

Dit proefschrift onderzoekt de effecten van ijzersuppletie op veiligheids- indicatoren en op ijzerstatus bij zwangere vrouwen en hun pasgeborenen. Meerdere voorstudies zijn uitgevoerd, zoals een volkstelling in het studiegebied en een pilot-studie naar de operationalisering van de onderzoeksprotocollen. De hoofdstudie werd ontworpen met de hypothese dat de consumptie van met ijzer verrijkte voedingsmiddelen in combinatie met de inname van ijzersupplementen tijdens de zwangerschap, nadelig voor de gezondheid van zwangere vrouwen en hun pasgeborenen zou zijn.

De doelstellingen van het onderzoek waren: 1) het vergelijken van de aanwezigheid van malaria-infectie ten tijde van de bevalling bij vrouwen die een combinatie van ijzer-verrijkte voedingsmiddelen en ijzersupplementen kregen ten opzichte van vrouwen die alleen ijzer-verrijkte voedingsmiddelen kregen; 2) het bepalen van het effect van de interventie op de prevalentie van ijzergebreksanemie bij moeders, 1 maand na de geboorte; 3) het bepalen van het effect van de interventie op de ijzervoorraad van 1 maand oude babies; 4) het bepalen van de bruikbaarheid van zinkprotoporfyrine (ZPP) bij de diagnose van ijzergebrek in malaria-endemische gebieden; 5) het identificeren van factoren die prognostisch zijn voor de reactie van non transferrin-bound iron (NTBI) op inname van één enkel ijzersupplement; 6) het

vaststellen van factoren die voorspellend zijn voor Plasmodium-infectie tijdens de zwangerschap; 7) het identificeren van factoren die geassocieerd zijn met geboortegewicht; 8) het ontwikkelen van een methodologie om gevallen van laag geboortegewicht te voorspellen, met behulp van één enkele score die is gebaseerd op prognostische variabelen die verzameld zijn tijdens het tweede trimester van de zwangerschap; en 9) het ontwikkelen van methoden voor een verrijking van meel met ijzer op dorpsniveau.

De meeste landen hebben wettelijk vastgelegd dat meel moet worden verrijkt met ijzer, of zijn in het proces om dit te doen. Zwangere vrouwen kunnen hierdoor ijzer binnen krijgen via verrijkte voedingsmiddelen, maar ook via ijzersuppletieprogramma's. Dit proefschrift geeft antwoorden op dringende vragen over de veiligheid en werkzaamheid van ijzersuppletie tijdens de zwangerschap door het vergelijken van een hoge dosis ijzer (d.w.z. ijzer-verrijkte voedingsmiddelen plus ijzersupplementen) versus een lage dosis ijzer (d.w.z. alleen ijzer-verrijkte voedingsmiddelen). De belangrijkste uitkomst was de aanwezigheid van Plasmodium-infectie bij de moeder ten tijde van de bevalling, ongeacht de soort. Hoofdstuk 1 is een gedetailleerde inleiding over de achtergrond en het ontwerp van de studie .

Dit proefschrift biedt concrete aanwijzingen dat ijzersuppletie bij zwangere vrouwen in een malaria-endemisch gebied niet leidt tot een verhoogd risico op malaria infectie (risicoverschil, 95% CI: 0.0%, −9.3% tot 9.3%). Beleidsuitvoerders en overheden in malaria-endemische gebieden moeten zich niet laten tegenhouden door eerdere waarschuwingen tegen ijzersuppletie van zwangere vrouwen. Onze bevindingen in ogenschouw nemend, is het niet nodig om eerst te screenen op malaria alvorens ijzersupplementen te geven.

IJzersuppletie had belangrijke voordelen voor moeders en hun pasgeborenen (hoofdstuk 2). De in dit proefschrift beschreven bevindingen lieten een gemiddelde toename zien in geboortegewicht van 143 g ten opzichte van de groep die een lage dosis ijzer ontving. De grootte van het effect van ijzer werd beïnvloed door de ijzerstatus bij aanvang van de interventie. Bij vrouwen met ijzergebrek, leidde ijzer tot een verhoging van het geboortegewicht met 249 g, ondanks een mogelijke toename in malariainfectie met 10% in deze groep. Er waren geen aanwijzingen dat de effecten van ijzer op geboortegewicht werden beïnvloed door intermittent preventive treatment van malaria. IJzersuppletie verhoogde de groei van de foetus met 0.27 SD (95% CI: 0.04–0.50), waarschijnlijk als gevolg van een toename in zowel relatieve lengte als relatief gewicht ten opzichte van de leeftijd van de foetus.

We toonden ook aan dat ijzersuppletie leidde tot verhoogde neonatale ijzervoorraden één maand na de bevalling, zoals aangeduid door een verhoging van 17.1% (95% CI: 2.0% tot 34.3%) in de plasmaconcentratie van ferritine bij pasgeborenen van moeders die een hoge dosis ijzer kregen, vergeleken met degenen waarvan de moeders een lage dosis ijzer kregen. Dit zou een extra impuls moeten vormen om zwangere vrouwen ijzersupplementen te geven, met als doel het verhogen van de ijzervoorraden van de baby (hoofdstuk 2).

ZPP bleek onbetrouwbaar in het onderscheiden van zwangere vrouwen met en zonder ijzertekort in gebieden waar chronische ziekten voorkomen (hoofdstuk 3). Het gebruik van huidige afkapwaarden van bloedwaarden voor ZPP, namelijk > 70 µmol/mol heem, kan leiden tot grove overschatting van de prevalentie van ijzertekort, vooral als de werkelijke prevalentie laag is.

Het is aangenomen dat het voorkomen van non-transferrin bound iron (NTBI) in de bloedsomloop na orale inname van ijzersupplementen de groei en vermenigvuldiging van Plasmodium-parasieten stimuleerden, hetgeen zou leiden tot een verhoogde morbiditeit en mortaliteit door malaria. Wij vonden geen toename van NTBI-concentraties drie uur na orale inname van 60 mg ferrofumaraat (hoofdstuk 4). We kunnen niet uitsluiten dat ijzersuppletie leidt tot productie van NTBI wanneer supplementen worden ingenomen zonder voedsel, omdat de lunch, die door de meerderheid van onze deelnemers tijdens de 3 uur wachttijd werd geconsumeerd, waarschijnlijk natuurlijke verbindingen (fytaten) bevatte, die mogelijk een NTBI-respons hebben beperkt.

In hoofdstuk 5 van dit proefschrift, hebben we geprobeerd om een praktisch instrument te ontwikkelen dat in het veld kan worden gebruikt om een asymptomatische Plasmodium infectie te voorspellen. Onze motivatie hierbij was dat de meeste dipstick-testen die worden gebruikt om Plasmodium-infectie op te sporen, niet in staat zijn om 100% van alle aanwezige infecties te detecteren, terwijl asymptomatische infecties steeds vaker worden geassocieerd met nadelige gevolgen voor zowel moeder als kind. Hoewel veel mogelijk voorspellende factoren werden beoordeeld, zowel afzonderlijk als in combinatie, zijn we niet geslaagd in het ontwikkelen van een betrouwbare methode die gemakkelijk te gebruiken is in een omgeving waar malaria voorkomt en waar weinig hulpmiddelen beschikbaar zijn.

In hoofdstuk 6 hebben we ons gericht op het identificeren van factoren die geassocieerd zijn met geboortegewicht, en met het ontwikkelen van een methodologie om gevallen van laag geboortegewicht te voorspellen met behulp van een prognostische score die is gebaseerd op verschillende variabelen verzameld tijdens het tweede trimester van de zwangerschap. De volgende factoren waren onafhankelijke geassocieerd met een verlaagd geboortegewicht: nooit getrouwd geweest, ontsteking, zwangerschap, vrouwelijk geslacht van de baby, en ijzertekort. Overgewicht is geassocieerd met een verhoogd geboortegewicht. De resultaten geven aan dat we variabelen, die snel en tegen relatief lage kosten kunnen worden verzameld, bruikbaar zijn om tijdens het tweede trimester van de zwangerschap vrouwen te identificeren met een hoog risico op de geboorte van een baby met een laag geboortegewicht.

De verschillende aspecten van het werk die in dit proefschrift beschreven wordt inclusief de implicaties voor beleidsmakers worden bediscussieerd in hoofdstuk 7. De bevindingen van dit proefschrift kunnen voor beleidsmakers een opluchting zijn. Onze bevindingen nemen alle twijfel weg die tot op heden werd geassocieerd met prenatale ijzersuppletie in malaria

gebieden. De meeste landen hebben al een beleid voor ijzersuppletie dat is afgestemd op het beleid van de Wereldgezondheidsorganisatie. Inspanningen om het gebruik van prenatale ijzersuppletie te verhogen, moeten dringend worden verhoogd, vooral in malaria-endemische gebieden. Echter, de gegevens die in dit proefschrift worden gepresenteerd, zijn alleen van toepassing op zwangere vrouwen en kan niet worden geëxtrapoleerd naar kinderen in malaria-endemische gebieden. Voor deze populatie moet het huidige WHO-beleid worden gehandhaafd: alvorens tot ijzersuppletie over te gaan, moeten kinderen worden gescreend op ijzergebrek.

Hoewel dit proefschrift antwoord geeft op belangrijke vragen die wetenschappers tot nu toe bezig hielden, zijn er nog steeds vragen die om verdere opgehelderd moeten worden. Volgens nationale en internationale richtlijnen moet de dagelijkse dosis voor zwangere vrouwen worden verdubbeld tot 120 mg ijzer als ze bloedarmoede hebben, of als het niet mogelijk is om gedurende 6 maanden supplementen te slikken tijdens de zwangerschap (hoofdstuk 2). De effecten van deze hoge ijzerdosisop maternale en neonatale uitkomsten moeten worden onderzocht. Verder onderzoek is nodig om de NTBI-reactie op de consumptie van verschillende soorten en hoeveelheden van ijzersupplementen volledig te beschrijven. Er is dringend behoefte aan diagnostische instrumenten die gebruikt kunnen worden in een setting waar weinig middelen beschikbaar zijn om asymptomatische infecties vast te stellen. Bij kinderen is verder onderzoek nodig om de veiligheid en werkzaamheid van ijzersupplementen in malaria-endemische regio's te bepalen, en om de diagnostische bruikbaarheid van zinkprotoporfyrine bij kinderen te beoordelen. Omdat onze studie suggereert dat screening op basis van hemoglobine-gehalte, zoals nu wordt gedaan in veel landen, niet accuraat is voor het onderscheiden van vrouwen met een hoog en een laag risico op een baby met een laag geboortegewicht, zijn bovendien verdere studies nodig naar adequate indicatoren en afkapwaarden die geschikt zijn voor dit doel.

Samenvattend heeft dit proefschrift aangetoond dat er geen aanwijzigingen zijn dat prenatale ijzersuppletie leidt tot een verhoogd risico van Plasmodium-infectie. Prenatale ijzersuppletie leidt daarentegen tot grote verbeteringen in geboortegewicht, groei van de foetus en de ijzervoorraden van de baby, met mogelijkerwijs enorme voordelen voor de gezondheid en overlevingskansen van de baby. Deze gegevens zouden elke mogelijke bezorgdheid over de risico's van malaria moeten wegnemen. Epidemiologische berekeningen geven aan dat als onze resultaten worden toegepast op alle vrouwen in ontwikkelingslanden om zo ijzertekort te elimineren, we per jaar 3 miljoen gevallen van een laag geboortegewicht kunnen voorkomen, en mogelijk het leven kunnen redden van ruim een half miljoen pasgeborenen. Opschaling van universele ijzersupplementatie tijdens de zwangerschap in ontwikkelingslanden zal dan ook een enorme winst opleveren voor de volksgezondheid.

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Martin N. Mwangi was born on 6th July 1980 in Kiambu, Kenya. In 2000, he joined Maseno University, Kenya, to pursue a BSc degree in Nutrition and dietetics. He graduated cum laude and proceeded to work for Center for Nutrition Education and Research where he was in charge of the Xenihealth nutrition and weight management clinics in Nairobi, Kenya. Martin was then appointed as head of nutrition department at The Karen Hospital, Nairobi, Kenya. In 2007, he was granted a Unilever scholarship which enabled him to study for an MSc in Nutrition and Health in Wageningen University, The Netherlands. For his MSc thesis, Martin studied the effect of iron supplementation in young Dutch women on serine hydroxyl methyl transferase gene expression and cytokine response in peripheral blood mononuclear cells. This studies ignited his desire to pursue PhD research in micronutrients, specifically iron. He graduated in August 2009 with a specialization in Public Health Nutrition.

In September 2009, he was appointed as a PhD fellow in the INSTAPA (improved nutrition through staple foods for Africa) project. The focus of his PhD research was the safety of iron interventions during pregnancy in malaria endemic regions. Among other topics outlined in this thesis, Martin studied whether consumption of iron supplements during pregnancy increases the incidence of malaria. In December 2009, Martin was appointed as a tutorial fellow in the Nutrition and Health department of Maseno University and became involved in teaching, research and supervision of BSc and MSc students in Kenya and the Netherlands. Martin is also an alumni of the Africa Nutrition Leadership Programme and has a mission to better the nutrition and health situtation in Africa through sustainable evidence based interventions and research. He looks forward to further his career in research and/or humanitarian work.

List of publications and awards

Publications

Martin N. Mwangi, Johanna M. Roth, Menno R. Smit, Laura Trijsburg, Alice M. Mwangi, Ayşe Y. Demir, Jos P.M. Wielders, Petra F. Mens, Sharon E. Cox, Andrew M. Prentice, Inge D. Brouwer, Huub F.J. Savelkoul, Pauline E.A. Andang'o, Hans Verhoef., 2014. Antenatal iron supplementation, Plasmodium infection and birth outcomes in Kenyan women: a randomized trial. *New England Journal of Medicine. (Submitted)*

Martin N. Mwangi, Sumi Maskey, Pauline E.A. Andang'o, Johanna M. Roth, Laura Trijsburg, Alice M. Mwangi, Han Zuilhof, Barend van Lagen, Huub F.J. Savelkoul, Ayşe Y. Demir, Hans Verhoef., 2014. Diagnostic utility of zinc protoporphyrin to detect iron deficiency in Kenyan pregnant women. *Blood. (Submitted)*

Martin N. Mwangi, Johanna M. Roth, Alice M. Mwangi, Huub F.J. Savelkoul, Robert C. Hider, Petra F. Mens, Henk D.F.H. Schallig, Ayşe Y. Demir, Pauline E.A. Andang'o, Hans Verhoef., 2014. Antenatal iron supplementation and serum concentrations of non-transferrin bound iron in pregnant women in a malaria-endemic region of Kenya: a randomised controlled trial. *Clinical Chemistry. (Submitted)*

Martin N. Mwangi, Ayşe Y. Demir , Alida Melse-Boonstra, Alice M. Mwangi, Huub F.J. Savelkoul, Andrew M. Prentice, Pauline E.A. Andang'o, Hans Verhoef., 2014. Early identification of pregnant women at high risk of giving birth to a neonate with low birth weight. *American Journal of Clinical Nutrition (Submitted)*

Awards

Maelle Olive*, **Martin N. Mwangi***, Lowella Padilla*, Ana Carla Cepeda*. **Winners** of the "Too Much-Too Little" Nutrition MSc competition with the video project entitled: "The emergence of stunted obesity in developing countries". 2009. Burgerszoo Arnhem, The Netherlands.

Maelle Olive*, **Martin N. Mwangi***, Lowella Padilla*, Ana Carla Cepeda*. Invited to present the video project "The emergence of stunted obesity in developing countries" at the International Congress of Nutrition. Bangkok, Thailand. 2009.

Martin N. Mwangi 2013: Competitively selected as a participant of the Africa Nutrition Leadership Programme 2013.

Martin N. Mwangi 2014: Selected as the Africa Nutrition Leadership Programme junior staff for year 2014 together with one other candidate

Martin N. Mwangi, 2014: Winner of Sight and Life competition on the topic "Effective linkages are the key to enabling a collective and sustained approach to addressing micronutrient malnutrition", awarded travel grant to the Micronutrient Forum 2014 by Sight and Life International.

**Equal contribution*

WIAS education certificate

With the educational activities listed below, the PhD candidate has complied with the educational requirements set by the graduate school Wageningen Institute of Animal Sciences (WIAS) which comprises of a minimum total of 30 ECTS.

The basic package (3 credits)

WIAS introduction course, Wageningen	2009
Collaborative institutional training initiative (CITI Program): IRB for Health Science Research (IRB-HSR) - Human Research Curriculum	2009

Scientific exposure (15 credits)

International conferences

19th International congress of nutrition (ICN) – Bangkok, Thailand	2009
Africa Nutritional Epidemiology Conference (ANEC 4) – Nairobi, Kenya	2010
IUNS 20th International Congress of Nutrition – Granada, Spain	2013

Seminars and workshops

ECSA: Fourth Regional Workshop on Food Fortification, Nairobi, Kenya.	2009
INSTAPA annual general meeting, (www.instapa.org), Zurich, Switzerland	2010
INSTAPA WP7 Technical workshop - Cotonou, Benin	2010
3rd HADCO North-South-South Intensive Course and Meeting, Kisumu, Kenya.	2010
INSTAPA annual general meeting (www.instapa.org), Cotonou, Benin.	2011
INSTAPA annual general meeting (www.instapa.org), Durban, South Africa.	2012
Food Security and the Productivity, Health and Nutrition Nexus Workshop, Wageningen, The Netherlands.	2013

Presentations

Prenatal Iron and Malaria study: Preparatory activities and implementation strategies, Cotonou, Benin, (poster)	2011
The safety and efficacy of iron interventions in African pregnant women, Cotonou, Benin, (oral)	2011
Prenatal Iron and Malaria study: A randomized controlled trial on the safety and efficacy of iron interventions in African pregnant women, Durban, South Africa, (oral	2012
The safety and efficacy of iron interventions in African pregnant women, Granada, Spain, (poster)	2013

In-Depth Studies (7 credits)

Disciplinary and interdisciplinary courses

NIHES summer course: Principles of research in medicine and epidemiology (ESP 01)	2010
NIHES summer course: Introduction to data analysis (ESP 03)	2010
NIHES summer course: Methods of clinical research (ESP10)	2010

NIHES summer course: Markers and Prognostic research (ESP 09)	2010
NIHES summer course: Clinical trials (ESP 14)	2010
NIHES summer course: Pharmaco-epidemiology (ESP 21)	2010
*NIHES – Netherlands Institute for Health Sciences, Rotterdam, The Netherlands	
Advanced statistics courses	2010
Statistics for the Life Sciences	
PhD students' discussion groups	
Journal Club	2009-2012

Skills

Professional Skills Support Courses *(7 credits)*	
NIHES summer course: The why and how of readable articles	2010
Wageningen Graduate Schools Course: Career Orientation	2013
Wageningen Graduate Schools Course: Scientific Integrity	2013
Africa Nutrition Leadership Programme (ANLP), Potchefstrom, South Africa	2013
Research Skills *(5 credits)*	
Working with EndNote X	2009
Skills to detect placental infection, (CDC/ KEMRI), Siaya District Hospital, Kenya.	2011
Basic Obstetric Ultrasound	2011
Special research assignment: Pedestrian Safety and the Built Environment - a review of the risk Factors	2012
Didactic Skills *(28 credits)*	
Lecturing	2010-2011
Supervising 14 theses (1 BSc, 1 internship, 12 MSc)	2009-2013
Total number of credits:	**65**

Epilogue

Effective linkages are the key to enabling a collective and sustained approach to addressing micronutrient malnutrition

There is evidence that the causes of micronutrient malnutrition are multifactorial and vast in nature. The geographical spread of micronutrient malnutrition is mind-boggling. Like a spiders' web, if the synergies of different strategies to address micronutrient malnutrition are harnessed, the success of interventions will be guaranteed. Different stakeholders are equipped with different approaches and strengths to fighting micronutrient malnutrition. Independently, each stakeholder can only have a minimal impact on micronutrient malnutrition. Stakeholders must therefore seek to utilize the synergistic advantage of their approaches if great results are to be realized. Similarly, because the problem of micronutrient malnutrition is multifactorial, the approaches to combat it must have a cumulative strategy that seeks to use the documented successes of different approaches in one carefully designed strategy. For example, provision of micronutrients can be streamlined with medical care, economic empowerment, and promotion of education. Food based interventions must be implemented alongside strategies to control infectious diseases such as malaria and to promote health. Collaborative projects if well managed, will realize the greatest impact on micronutrient malnutrition. Linkages between academia, governments, Non-Governmental Organizations, civil society, and humanitarian organizations such as the World Health Organization are bound to have positive results in the fight against micronutrient malnutrition.

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Front cover (and also back cover): Land area showing study area – South West Kisumu, Kenya. The red place marks show the coordinates of the homes of study participants. GPS data visualized at www.GPSVisualizer.com. Map courtesy of Google ©2013.

Bottom left corner on the front cover: photo of Wanja Maina, a pregnant Kenyan woman. Use of photo permitted by owner.

Bottom middle picture on the front cover: ultrasound image of the fetus of one of the study participants. Use of photo permitted by owner. Photo by M.N. Mwangi.

Bottom right corner on the front cover: photo of breastfeeding baby of one of the study participants. Use of photo permitted by owner. The photo was kindly provided by Belynder Ogone.

Back cover: photo of the fourteen research assistants (and one of the MSc students) who were engaged in daily follow-up of all study participants.

Cover layout and graphics design: MN. Mwangi

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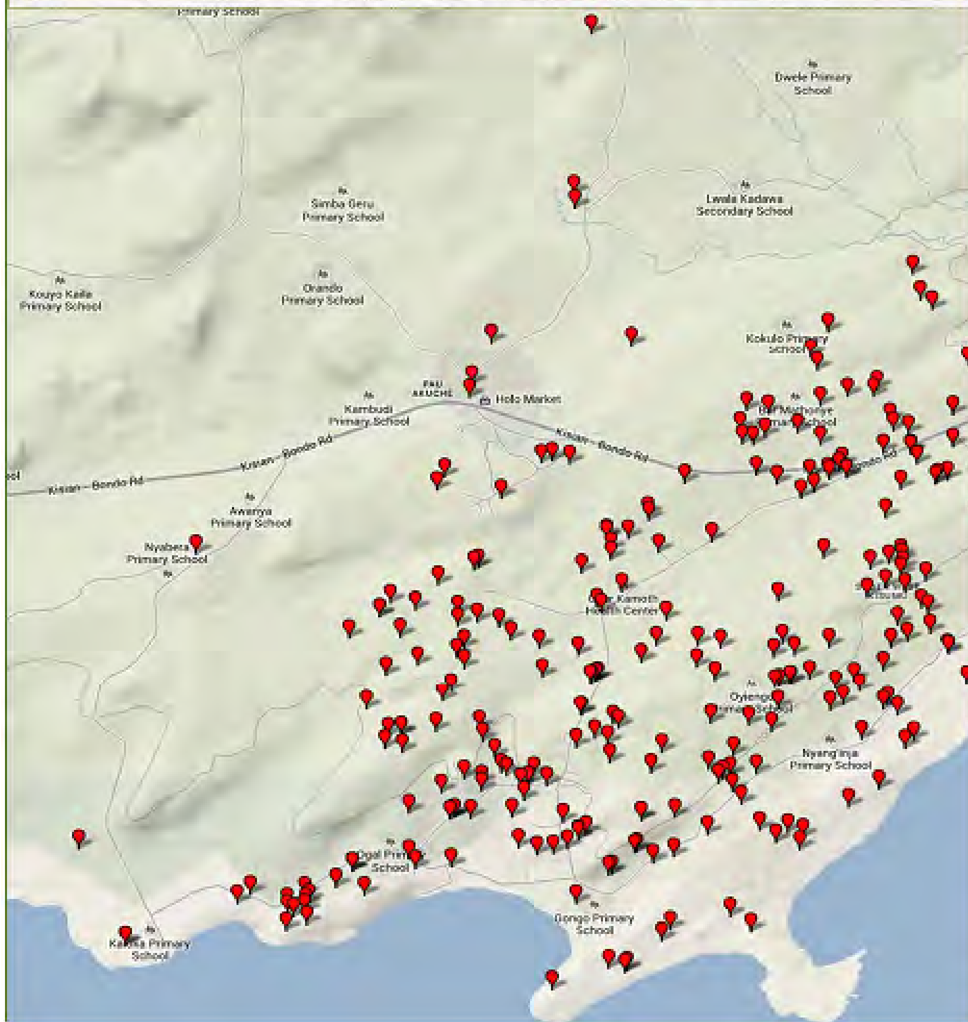

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Martin N. Mwangi, 2014