Prenatal Iron and Malaria (PIMAL) Study

SAFETY AND EFFICACY OF IRON INTERVENTIONS IN AFRICAN PREGNANT WOMEN

VERSION: 01 JANUARY 2011

TRIAL REGISTRATION IDENTIFIER: ClinicalTrials.gov NCT01308112

Principal Investigator: Dr. Hans Verhoef, London School of Hygiene and Tropical Medicine, UK (hans.verhoef@wur.nl)

Collaborating institutions:
1. University of Nairobi, College of Agriculture and Veterinary Sciences, Department of Food Technology and Nutrition, Unit of Applied Nutrition, Uthiru, Nairobi, Kenya
2. Maseno University, School of Public Health and Community Development, Department of Human Nutrition, Maseno, Kenya
3. London School of Hygiene and Tropical Medicine (LSHTM), Nutrition and Public Health Intervention Research Unit, England
4. Wageningen University, Cell Biology and Immunology Group/Division of Human Nutrition, Wageningen, The Netherlands
5. Swiss Federal Institute of Technology (ETH), Laboratory for Human Nutrition, Zürich, Switzerland

Funding: EU KP7 Project no. 211484: Novel staple food-based strategies to improve micronutrient status for better health and development in sub-Saharan Africa (INSTAPA) - Work package 5: Safety of food-based interventions

Sponsor: London School of Hygiene and Tropical Medicine, Keppel Street 281, London WC1E 7HT, UK (contact: Clinical Trials QA Manager, Tel: +44 207 927 2626; e-mail: patricia.henley@lshtm.ac.uk; LSHTM sponsor ref no: QA268)

Ethical clearance:
- Ethics Review Committee, London School of Hygiene and Tropical Medicine, Keppel Street 281, London WC1E 7HT, UK (review no: 5664)
- Ethics and Research Committee, Kenyatta National Hospital-University of Nairobi
Indemnity: LSHTM holds Public Liability ("negligent harm") and Clinical Trial ("non-negligent harm") insurance policies which apply to this trial.

Trial coordination: For general queries, supply of trial documentation, and collection of data, please contact the Trial Manager: Mr. Martin Ndegwa Mwangi (contact details: see below).

Clinical queries:
Clinical queries should be directed to Dr. Hans Verhoef (contact details: see below).

Protocol authorized by:

Name: Hans Verhoef
Role: Principal Investigator
Signature: HANS VERHOEF
Date: 01 January 2011

This protocol describes the PIMAL study and provides information about procedures for each participant. It should not be used as a guide for the treatment of other participants; every caution has been taken in its drafting, but corrections or amendments may be necessary. These will be circulated to all investigators in the study, but centres entering participants for the first time are advised to contact the trials centre to confirm they have the most recent version.

Problems relating to this trial should be referred, in the first instance, to the study coordinator.

This trial will adhere to the principles outlined in the International Conference on Harmonisation Good Clinical Practice (ICH GCP) guidelines, protocol and all applicable local regulations.
RESEARCH TEAM

Dr. Hans Verhoef, PhD  
(Principal Investigator)  
Wageningen University/London School of Hygiene and Tropical Medicine, Nutrition and Public Health Intervention Research Unit, Keppel Street 281, London WC1E 7HT, England. Mobile +255 787 882596; Tel +44 20 79588140; Fax + 44 20 79588111; e-mail: hans.verhoef@wur.nl

Mr. Martin Ndegwa Mwangi, MSc  
(Trial Manager/ PhD fellow)  
Maseno University, Kenya/Wageningen University, Cell Biology and Immunology Group, P.O. Box 338, 6700 AH Wageningen, The Netherlands  
Mobile +31 61 2058997  
Fax +31 317 482718  
E-mail: martinndegwa.mwangi@wur.nl

Dr. Alice Mboganie Mwangi, PhD  
University of Nairobi, College of Agriculture and Veterinary Sciences, Department of Food Technology and Nutrition, Unit of Applied Nutrition  
P.O. Box 442, Uthiru, Nairobi, Kenya  
Mobile +254 728 458066  
E-mail: amwangi@uonbi.ac.ke

Dr. Pauline E.A. Andang’o, PhD  
Maseno University, School of Public Health and Community Development  
P.O. Box 333, Maseno, Kenya  
Mobile + 254 728 485729  
E-mail: paulango@hotmail.com

Alida Melse-Boonstra, PhD  
Wageningen University, Division of Human Nutrition, P.O. Box 8129, 6700 EV Wageningen, The Netherlands  
Tel +31 317 484317; Fax +31 317 483342  
E-mail: alida.melse@wur.nl

Professor Michael B. Zimmermann, PhD MD  
Wageningen University/Swiss Federal Institute of Technology (ETH), Laboratory for Human Nutrition, ETH Zentrum, Schmelzbergstrasse 7, LFV E19, CH-8092 Zürich, Switzerland  
Tel +41 44 6328657; fax +41 44 6321470  
email: michael.zimmermann@ilw.agrl.ethz.ch

Hala Ghattas, PhD  
London School of Hygiene and Tropical Medicine, Nutrition and Public Health Intervention Research Unit, Keppel Street 281, London WC1E 7HT, England. Mobile + 44 20 79588139; Tel +44 20 79588140; Fax + 44 20 79588111; e-mail: hala.ghattas@lshtm.ac.uk

Sharon E. Cox, PhD  
London School of Hygiene and Tropical Medicine, Nutrition and Public Health Intervention Research Unit, Keppel Street 281, London WC1E 7HT, England  
Tel + 44 20 79588140; Fax + 44 20 79588111  
Sickle Cell Disease Research Programme  
Muhimbili University of Health & Allied Sciences, Dar es Salaam, Tanzania.  
Mobile +255 765 406115  
E-mail: sharon.cox@lshtm.ac.uk

Professor Huub F.J. Savelkoul, PhD  
Wageningen University, Cell Biology and Immunology Group, P.O. Box 338, 6700 AH Wageningen, The Netherlands. Tel +31 317 483922; Fax +31 317 482718; e-mail: huub.savelkoul@wur.nl
1

SUMMARY

Background: 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. Based on our previous work, the Kenyan government is currently drafting legislation for mandatory iron fortification of industrially milled flour.

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. Implementation of the new fortification policy means that pregnant women will receive iron through a combination of fortified foods and supplementation. We are concerned about the safety of the high iron intake resulting from such a policy.

Objectives: Our objectives are: 1) 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 (primary objective); 2) to identify baseline factors that are prognostic for the NTBI response to consumption of a single iron supplement; 3) to assess intervention effects on neonatal iron stores at 1 month of age; 4) to assess intervention effects on maternal haemoglobin concentrations, prevalence of iron deficiency anaemia and iron stores at 1 month after delivery; 5) to assess intervention effects on maternal intestinal pathogens at 1 month after delivery; and 6) to develop methods for community-based flour fortification with iron.

Methods: The main study concerns a double-blind, community-based efficacy trial in a rural area within Nyanza Province, Kenya. We will deploy a community-based surveillance system to detect women at an early stage of pregnancy. Women with singleton pregnancies at a gestational age between 13 and 20 weeks, with haemoglobin concentrations \( \geq 90 \text{ g/L} \), and who are willing to deliver in a predesignated health facility, will receive chemotherapy for helminth infections (albendazole and praziquantel) and will be randomised to receive daily supplements with either placebo or iron (60 mg iron as ferrous sulphate). At randomisation we will collect venous blood and measure the serum NTBI response to consumption of the first supplement. Local millers will be trained and supervised to fortify flour from households of participating women by adding fortificant to grain while it is being milled. For this purpose, we will develop and apply protocols to fortify small quantities of flour homogenously with iron at WHO-recommended target levels (20 mg iron as NaFeEDTA per kg flour). We expect that this fortification level will supply 27% of the Recommended Nutrient Intake for pregnant Kenyan women. At delivery, we will collect maternal blood from the placenta and the median cubital vein, and neonatal blood from the umbilical cord. The intervention will continue until 1 month after delivery, when venous blood will be collected from mothers, and peripheral blood from their infants. We plan to include 150 pregnant women per intervention group; resources allowing, this number will be increased to 225/group. A sample size of 150 women/group will yield 80% probability of excluding the null value from the 95% CI of the effect estimate (assumptions: prevalence of infection, control group: 50%, intervention-associated increase in prevalence: 35%). In the primary analysis, we will assess group...
differences in the prevalence of *Plasmodium* infection in parturient women as indicated by the presence of malarial antigens or malarial DNA in placental, venous or cord blood.
TABLE OF CONTENTS

1 SUMMARY ........................................................................................................................................... 5

2 INTRODUCTION ...................................................................................................................................... 10

2.1 BACKGROUND AND RATIONALE ................................................................................................. 10

2.1.1 Background .................................................................................................................................. 10

2.1.2 Literature review ............................................................................................................................ 10

2.1.3 Rationale ....................................................................................................................................... 14

3 OBJECTIVES ........................................................................................................................................ 15

3.1 HYPOTHESIS ...................................................................................................................................... 15

3.2 GENERAL AND SPECIFIC OBJECTIVES ....................................................................................... 15

3.2.1 General objective ............................................................................................................................ 15

3.2.2 Specific objectives ............................................................................................................................ 15

1. TO COMPARE THE PRESENCE OF *Plasmodium* INFECTION IN PARTURIENT WOMEN WHO
   ANTENATALLY RECEIVED A COMBINATION OF IRON-FORTIFIED FOODS WITH IRON SUPPLEMENTS VERSUS
   IRON-FORTIFIED FOODS ONLY (PRIMARY OBJECTIVE) ........................................................................ 15

2. TO IDENTIFY BASELINE FACTORS THAT ARE PROGNOSTIC FOR THE NTBI RESPONSE TO CONSUMPTION
   OF A SINGLE IRON SUPPLEMENT; .................................................................................................................. 15

3. TO ASSESS INTERVENTION EFFECTS ON NEONATAL IRON STORES AT 1 MONTH OF AGE; .............. 15

4. TO ASSESS INTERVENTION EFFECTS ON MATERNAL HAEMOGLOBIN CONCENTRATIONS AND
   PREVALENCE OF IRON DEFICIENCY ANAEMIA AT 1 MONTH AFTER DELIVERY; .............................. 15

5. TO ASSESS INTERVENTION EFFECTS ON MATERNAL INTESTINAL PATHOGENS AT 1 MONTH AFTER
   DELIVERY; ............................................................................................................................................. 15

6. TO DEVELOP METHODS FOR COMMUNITY-BASED FLOUR FORTIFICATION WITH IRON. ............. 15

6.2.3 Justification ..................................................................................................................................... 15

4 TYPE OF STUDY ...................................................................................................................................... 16

4.1 PILOT SURVEY .................................................................................................................................... 16

4.2 PREPARATORY ACTIVITIES CARRIED OUT SO FAR ......................................................................... 16

5 STUDY METHODS: PARTICIPANTS AND INTERVENTIONS ...................................................................... 17

5.1 STUDY SETTING ................................................................................................................................... 17

5.2 STUDY POPULATION ............................................................................................................................ 17

5.3 INTERVENTIONS ................................................................................................................................... 18

5.3.1 Premedication .................................................................................................................................. 18

5.3.2 Pre-randomisation/pre-registration evaluations .............................................................................. 18

5.4 ELIGIBILITY CRITERIA ........................................................................................................................ 19

5.5 PARTICIPANT TIMELINE ..................................................................................................................... 19

5.6 TARGET SAMPLE SIZE ......................................................................................................................... 19

5.7 RECRUITMENT AND RETENTION STRATEGIES .............................................................................. 20

6 STUDY METHODS: PARTICIPANT ALLOCATION .................................................................................... 24

6.1 RANDOMISATION ............................................................................................................................... 24

6.1.1 Sequence generation and allocation concealment ............................................................................ 24

6.1.2 Implementation .................................................................................................................................. 24

6.2 BLINDING ............................................................................................................................................ 26

7 STUDY METHODS: DATA COLLECTION, MANAGEMENT AND ANALYSIS ........................................... 26

7.1 OUTCOMES .......................................................................................................................................... 26

7.2 DATA COLLECTION METHODS .......................................................................................................... 27

7.3 DATA MANAGEMENT ............................................................................................................................. 29
8 STUDY METHODS: TRIAL MONITORING ................................................................. 30
8.1 MONITORING AND REPORTING OF Harms ......................................................... 30
8.1.1 Safety reporting for non-drug trials ............................................................... 31

REPORTING PROCEDURES: .............................................................................. 31
8.1.2 Non serious AEs ......................................................................................... 31
8.1.3 Serious AEs ............................................................................................... 31
8.2 OVERSIGHT..................................................................................................... 32
8.2.1 Data Safety and Monitor ........................................................................... 32
8.2.2 Withdrawal criteria .................................................................................... 32
8.2.3 Interim analyses .......................................................................................... 33
8.3 EMERGENCY CODE-BREAKING PROCEDURE ............................................ 33

9 ETHICAL CONSIDERATION AND DISSEMINATION ........................................... 33
9.1 RESEARCH ETHICS APPROVAL ...................................................................... 33
9.2 PROTOCOL AMENDMENTS ............................................................................ 33
9.3 CONSENT/ ASSENT ....................................................................................... 34
9.4 CONFIDENTIALITY .......................................................................................... 34
9.5 COMPETING INTERESTS ................................................................................ 34
9.6 ROLE OF INVOLVED PARTIES: PARTNERSHIPS AND KEY PUBLICATIONS BY THE RESEARCH GROUP ................................................... 34
9.7 POST-TRIAL CARE .......................................................................................... 36
9.8 CONTRIBUTION TO LOCAL CAPACITY BUILDING ....................................... 36
9.9 AUDITS AND INSPECTIONS .......................................................................... 37
9.10 DISSEMINATION OF RESULTS AND PUBLICATION POLICY ....................... 37

10 APPENDICES .................................................................................................... 43
10.1 DATA COLLECTION FORMS .......................................................................... 43
10.1.1 Form 1 - Venous blood processing – field .................................................. 43
10.1.2 Form 2: Cord blood processing – field ....................................................... 43
10.1.3 Form 3 - Placental blood processing – field ................................................ 43
10.1.4 Form 4 - Screening - medical examination ................................................ 43
10.2 INFORMED CONSENT MATERIALS .............................................................. 43
10.2.1 Information about the study ..................................................................... 43
10.2.2 Informed consent form (first screening meeting) ....................................... 45
10.2.3 Informed consent form for HIV testing ...................................................... 46
10.3 OTHER DOCUMENTS .................................................................................... 47
10.3.1 Flow chart for safety reporting .................................................................. 47
10.3.2 Maize consumption and processing in sub-Saharan Africa ....................... 47
10.3.3 Quality control procedures to assess levels of iron as NaFeEDTA in flour .. 48
10.3.4 Planned study area (Kisumu District, Nyanza Province) ......................... 49
10.3.5 Estimated intake of iron and EDTA by population group, assuming fortification levels of 20 mg iron as EDTA per kg flour ......................... 50
10.3.6 Curriculum vitae, Principal investigator .................................................... 51
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBI</td>
<td>Wageningen University, Cell Biology and Immunology Group</td>
</tr>
<tr>
<td>CBS</td>
<td>Kenya Central Bureau of Statistics</td>
</tr>
<tr>
<td>CDC</td>
<td>US Centres for Disease Control and Prevention</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>ETH</td>
<td>Swiss Federal Institute of Technology</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization</td>
</tr>
<tr>
<td>GMP</td>
<td>Good Manufacturing Practice</td>
</tr>
<tr>
<td>HRP-2</td>
<td>Histidine-rich protein-2</td>
</tr>
<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LSHTM</td>
<td>London School of Hygiene and Tropical Medicine</td>
</tr>
<tr>
<td>MMC</td>
<td>Meander Medical Centre, Amersfoort, The Netherlands</td>
</tr>
<tr>
<td>MOH</td>
<td>Ministry of Health</td>
</tr>
<tr>
<td>NTBI</td>
<td>Non-transferrin bound iron</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>SAMs</td>
<td>Soluble adhesion markers</td>
</tr>
<tr>
<td>sTfR</td>
<td>Soluble transferrin receptor</td>
</tr>
<tr>
<td>UNK</td>
<td>University of Nairobi – Kenya</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WU</td>
<td>Wageningen University, The Netherlands</td>
</tr>
</tbody>
</table>

### KEYWORDS
Iron supplementation, iron fortification, malaria, pregnancy
2 INTRODUCTION

2.1 Background and rationale

2.1.1 Background
In a randomised trial among children aged 1-35 months in Pemba, Tanzania, daily supplementation with iron (12.5 mg as ferrous sulphate) and folic acid increased rates of hospitalisation and all-cause mortality (Sazawal et al. 2006). This reinforced earlier concerns that iron interventions can increase the incidence of malaria and infectious disease, even in individuals without iron overload (Oppenheimer 2001). An expert group convened following the Pemba trial by the World Health Organization (WHO) recommended that iron supplementation should be restricted in areas where malaria transmission is intense and infectious disease highly prevalent, but that these restrictions are not to be applied to fortification or food-based approaches for delivering iron (WHO 2006a). This advice was issued despite a lack of evidence that consuming iron-fortified foods in malaria-endemic areas is safe, but it was based on the assumption that iron fortification is likely to avoid the potential adverse effects of a large bolus of iron taken in a single dose, because the iron would be consumed in smaller amounts throughout the day and therefore absorbed more evenly (WHO 2007).

Despite the findings from the Pemba study, universal iron supplementation continues to be recommended by WHO for women during pregnancy and 3 months postpartum (Dr F. Branca, Director, Department of Nutrition for Health and Development, WHO, Geneva; personal communication, September 2009). Potential effects of iron on infection are likely to be most pronounced in pregnancy, when iron absorption is very high. In observational studies among pregnant women, iron deficiency was recently found to be associated with a reduction of the prevalence and density of Plasmodium parasites in placental blood, whether or not adjusted for gravidity status (Kabyemela et al. 2008, Danqua et al. 2008). Thus in Tanzania, the prevalence of placental infection as assessed by microscopy was 7.5% in women with iron deficiency (serum ferritin concentration <30 µg/L and <70 µg/L if C-reactive protein concentration ≤ 8.2 mg/L or >8.2 mg/L, respectively), as compared with 30.2% in iron-replete women (Kabyemela et al. 2008). In Ghana, the corresponding prevalence values were 21.5% and 38.8% when infection in peripheral blood was assessed by microscopy, and 56.5% and 67.3% when assessed by PCR (Danqua et al. 2008). However, malaria-induced inflammation can produce increased serum ferritin concentrations independent of iron status, so that malaria may have been a cause rather than an effect in the associations reported by Kabyemela et al. (2008) and Danqua et al. (2008). Nonetheless the question arises whether combined iron fortification and supplementation is safe, and whether supplementation policies should be revisited in pregnant women (Daily and Wylie 2008).

2.1.2 Literature review
Malaria in pregnancy: Although good estimates on the effects on maternal mortality are scarce, malaria in pregnancy should be avoided because of its devastating effects on the foetus and neonate: it is associated with an increased risk of low birth weight (which itself is strongly associated with infant mortality), intrauterine growth retardation, preterm delivery, spontaneous abortion, stillbirth and neonatal mortality (Desai et al. 2007). Because they can selectively bind to chondroitin sulphate A expressed on placental villi (Fried and Duffy 1996), malaria parasites can sequester and concentrate in vast numbers in placentas of women with scant peripheral parasitaemia (Brabin and Rogerson 2001) and may persist in the placenta after the start of treatment (Sartelet et al. 1997). Placental malaria is associated with an increased risk of stillbirth, reduced transfer of maternal antibodies and cellular immune responses in infants to several other infectious diseases, reduced development in
infants of cellular and antibody responses to *P. falciparum* epitopes, and an increased risk in infants of infection (reviewed in Brabin and Rogerson 2001, Desai et al. 2007).

The risk of malarial infection is higher in pregnant than in non-pregnant women, higher in the second trimester of pregnancy than in the third trimester, and, particularly in highly endemic areas, higher in primiparous women than in multiparous women (Desai et al. 2007). In highly endemic areas, malaria-associated anaemia is most pronounced in primigravidae because women develop specific protective responses to malaria over successive pregnancies (Desai et al. 2007). By contrast, the protection afforded by iron deficiency against placental infection seems particularly elevated in primiparous women, and appears more pronounced in the second and third trimesters of pregnancy (Kabyemela et al. 2008, Danqua et al. 2008). In a non-randomised study in Papua New Guinea, intravenous infusion of iron dextran increased the risk of malaria among primigravidae but not in multigravidae (Oppenheimer et al. 1986). However, in this study, the intervention was not randomised and – considering that iron infusion was preferentially given to primiparae and was strongly associated with decreased haemoglobin concentration at baseline – the findings may easily have been confounded. In multigravidae in The Gambia, iron supplementation did not increase the prevalence of infection in peripheral blood or placental biopsies (Menendez et al. 1994). However, the women in this study were examined 4-6 weeks before delivery and received chemotherapy when infected, so that possible effects of iron on infection may have been rescinded by the time of delivery. Nonetheless, it may be possible that differences between gravidity groups in iron-deficiency associated placental infection (Kabyemela et al. 2008, Danqua et al. 2008) are related to acquired immunity, which may limit the risk of malaria due to iron supplementation in multigravidae.

**Mechanisms:** It has been speculated that malaria parasites can access extra-erythrocytic labile serum ferric iron that is bound to ligands other than transferrin (WHO 2007, Stoltzfus 2008). Although this non-transferrin bound iron (NTBI) does not normally occur in healthy individuals (Esposito et al. 2002), oral ferrous salts as contained in supplements can transiently produce NTBI in anaemic, adult volunteers (Hutchinson et al. 2004, Dresow et al. 2008) and pregnant women (Baron et al. 2008); in addition, findings from a small study suggest that consumption of a meal containing 13.1 mg iron (of which 10 mg supplemental iron as ferric chloride) leads to postprandial NTBI production in patients with hereditary haemochromatosis but not in healthy, iron-replete controls (Hutchinson et al. 2008). A high dose of supplemental iron may overwhelm the hepcidin-mediated regulation of iron release from enterocytes across the basolateral membrane into circulation (Andrews 2000, Frazer and Anderson 2005), resulting in the fast release of large quantities of NTBI into circulation. Although transferrin-mediated uptake is the major route of delivery of iron to developing erythrocytes, reticulocytes and mature erythrocytes can take up NTBI via transferrin-independent mechanisms (Morgan 1988, Egyed A 1988, Kovar et al. 2006, Valis 2008, Sanchez-Lopez and Haldar 1992).

If iron intake affects NTBI production, this is likely to be most pronounced in the second trimester of pregnancy, when the absorption of non-haem iron may be as high as 25% (Barrett et al. 1994). Although little is known about the factors that determine the magnitude of the NTBI response to iron, the dose and type of ingested iron are probably important factors, as well as pregnancy status, gestational age and the amount of body iron stores, all of which are known to be important determinants of iron absorption (Hallberg 2001). In addition, NTBI production is possibly caused by vitamin B<sub>12</sub> deficiency (Gafter-Gvili et al. 2004), suggesting that catabolic iron derived from ineffective erythropoiesis is a possible source of NTBI (Gafter-Gvili et al. 2004). This would imply that NTBI may also be produced in deficiencies of vitamin A and folate, and genetic disorders of haemoglobin synthesis (thalassemia and sickle cell) that are common in Africa.
Another mechanism that may explain the findings of the Pemba study is that oral intake of iron may cause a change in composition of gastrointestinal macro- and microbiota, resulting in the proliferation and evasion of some pathogens. This corroborates findings that bacteraemia caused by non-typhoid *Salmonella* and other species is a complicating factor associated with death in African children with severe malarial anaemia (Verhoef and Veenemans 2009).

**Causes of iron deficiency:** Poor people in developing countries have a high burden of iron deficiency, primarily because their diets are monotonous, low in animal food sources, and heavily based on unrefined cereals grains and legume seeds. Although these foods have reasonable iron content, they also contain high concentrations of phytate. This phosphate-storage molecule occurs naturally in seeds and binds with iron to form insoluble complexes that are not absorbed in the intestine. As a result, only 2.5%-3.7% of iron from meals containing high-phytate maize is absorbed (Galan et al. 1990, Hurrell et al. 2002). These estimates are indicative because the cereal contribution and composition of meals vary widely (Bothwell et al. 1982), and because they were obtained from apparently healthy volunteers, presumably with adequate iron stores. Absorption may be higher in iron deficient individuals, because the fractional absorption of non-haem iron is known to be greatly increased in individuals with low iron stores (Hallberg 2001). In agreement with these findings, an expert group crudely estimated that approximately 5% of iron is absorbed from a simple, monotonous diet as customary in rural, poor African populations (FAO/WHO 1988). As a consequence, the dietary intake level that is required to meet the iron requirements of the poor in developing countries is much higher than those with diets that are typical in Western countries. Iron demand is highest during periods of growth and in pregnancy. Among pregnant women, haemoglobin concentrations are lowest in multigravidae. Iron demands are further increased by losses due to chronic bleeding from intestinal wounds caused by helminth infections (hookworm, *Trichuris trichuria* and *Schistosoma* spp.).

In Africa, anaemia is also caused by deficiencies of vitamin A and vitamin B$_{12}$, α$^+$-thalassaemia, and infections, notably malaria and HIV, but possibly also *Helicobacter pylori*. There is no strong evidence that folate deficiency anaemia is a public health problem in Africa (Fishman et al. 2000, WHO/FAO 2006).

**Benefits of iron interventions in pregnancy:** Iron deficiency anaemia in pregnancy is associated with preterm delivery, low birth weight (Allen 2000) and maternal mortality. Although it is generally assumed that iron status of the foetus, and subsequently the infant, is relatively independent of maternal iron status during pregnancy, there is mounting evidence that maternal iron deficiency in pregnancy reduces foetal iron stores, perhaps well into the first year of life (Allen 2000). There is little doubt that iron requirements increase dramatically during pregnancy (IOM 2001), and that iron supplementation in pregnancy increases maternal iron stores and haemoglobin concentrations.

Despite this evidence, the benefits of iron interventions in pregnancy are contentious (Rush 2000, Beaton 2000, Rioux and LeBlanc 2007). The controversy is rooted at least in part in uncertainty about the markers of adequate iron status in pregnancy. Reference values for haemoglobin concentration in pregnancy are not defined by functional criteria, but are based on studies in iron-supplemented women. It is not known whether high haemoglobin concentrations as achieved by supplementation are needed to avert adverse maternal or pregnancy outcomes (Beaton 2000). This is corroborated in a recent Cochrane review of 49 trials, which concluded that universal iron supplementation leads to increased haemoglobin concentrations before and after birth, and a reduced risk of anaemia at term, but there is no evidence of a marked reduction in substantive maternal and neonatal adverse clinical outcomes (low birthweight, delayed development, preterm birth, infection, postpartum haemorrhage) (Peña-Rosas and Viteri 2009). Also an effect on maternal mortality has not been demonstrated. In addition, adherence to iron supplementation by pregnant women is
notably low, and the effectiveness of community-wide or population-based iron supplementation programmes is further impaired by difficulties in delivery of iron supplements during antenatal services (Galloway and McGuire 1994, Mason et al. 2001). There is no convincing evidence that decades of implementation of such programmes in many countries have had much, if any, demonstrable effect on either iron stores or haemoglobin concentrations (Rush 2000, Mason et al. 2001).

There is also evidence that the effect of iron supplementation may be influenced by sickle cell genotype: in Gambian pregnant women with normal (AA) haemoglobin genotype, iron supplementation resulted in increased maternal haemoglobin concentrations after delivery, and an increased birth weight of the neonate; by contrast, it resulted in reduced haemoglobin concentrations and reduced birth weight when given to pregnant women with sickle cell trait (AS) (Menendez et al. 1995). This genetic haemoglobin disorder is very common in areas where malaria is highly endemic (e.g. prevalence of sickle cell trait is approximately 25% in western Kenya).

**Progress in iron fortification:** National and international efforts to establish iron fortification programmes are now beginning to bear fruit. As of 2008, 77 countries with 3.85 billion people were routinely adding iron to wheat flour (FFI 2008a), as compared with two countries in 1990. Mass fortification with iron of maize flour occurs only in three African countries (Uganda, South Africa and Zambia) (FFI 2008b), but several countries are in the process of developing national programmes. The World Health Organization (WHO 2009a) recently endorsed recommendations by the Flour Fortification Initiative, which proposed NaFeEDTA as the preferred iron fortificant in high-phytate flour (FFI 2008c). Based partly on our previous work (Andang’o et al. 2007), the Kenyan government is now drafting legislation for mandatory fortification of industrially processed maize and wheat flour with iron as NaFeEDTA (Dr. S. Shafir, Director, Ministry of Public Health and Sanitation, personal communication, November 2008). At an average per capita consumption level of 200-300g per flour/day, as occurs in Kenya (225 g/d; FAO Food Balance Sheets 2003; [http://faostat.fao.org/site/368/DesktopDefault.aspx?PageID=368](http://faostat.fao.org/site/368/DesktopDefault.aspx?PageID=368)), the recommended fortification level is 20 mg iron as NaFeEDTA (FFI 2008c).

Maize is the main staple food in sub-Saharan Africa ([Annex 8](#)), Mexico and Central America. In eastern and southern Africa, maize flour products are consumed throughout the year in large quantities and every day, often at every meal, and by all age groups including infants. Subsistence farming families grow maize on small plots of land, which they grind in posho mills and further process into food at home. Maize is simply ground to a whole-grain flour that contains all or part of the germ, which gives it a high fat content and a short shelf-life (because of the development of rancidity due to the formation of soluble iron and zinc salts). The germ also contains high levels of phytic acid. In eastern Africa, maize is usually consumed as uji, a gruel of maize flour cooked in water, or ugali, a stiff porridge that is produced similarly, but with a higher flour content. Leavening or fermentation can reduce phytate levels to some extent but, unlike wheat, this is not generally practised with maize.

Technologies for small-scale fortification have been developed in pilot studies in Zambia, Malawi, Zimbabwe and Nepal (Philar and Johnson 2005); however, we have been unable to identify any published information on the validity of fortification protocols, i.e. the extent to which fortification protocols yield flour that is uniformly and consistently blended with fortificant iron at the indicated target level.

**Measuring the burden of malaria in pregnancy:** Peripheral blood parasitaemia is widely used to assess the burden of infection and disease severity in malaria. It is only a weak predictor of mortality in falciparum malaria, because the circulating stages do not necessarily reflect the total body load of parasites: the latter also comprises the more pathogenic mature
stages in parasitized erythrocytes that are usually sequestered by cytoadherence to endothelial cells in the microvasculature of organs (Dondorp et al. 2005).

Brightlight microscopy, the conventional standard for the detection and quantification of malaria parasites in blood, is in many aspects deficient for this purpose. The method suffers from high inter- and intra-observer variability and, even under ideal circumstances, it yields very imprecise estimates of parasite density when parasitaemia is low (Shapiro and Perlmutter 2008).

Several new methods are available to measure the burden of malarial infection. **PCR-based methods** can detect parasites at lower densities and are more specific than microscopical detection of parasitaemia in peripheral blood. Sub-microscopic infections detected by a PCR test in pregnant women have been associated with anaemia (Mockenhaupt et al. 2006).

At schizont rupture, sequestered falciparum parasites secrete histidine-rich protein-2 (HRP-2) into the plasma. This protein is found in the parasite cytoplasm or parasite food vacuole, but it is also found in the host erythrocyte cytoplasm and red cell membrane. **Plasma HRP-2 concentrations** accurate indicate the total body parasite biomass in acute falciparum malaria, and can be measured with a commercially available enzyme-linked immunosorbent assay that is based on monoclonal-antibodies specific for *P. falciparum* (Cellabs, Sydney, NSW, Australia). In pregnant women, even when parasites are sequestered in the placenta and not detectable in peripheral blood, HRP-2 is still detectable in peripheral blood samples. Dipsticks based on HRP-2 detection show a higher sensitivity for this population than does microscopy (Murray et al. 2008). Sub-microscopic infections detected by a HRP-2 test in pregnant women have been associated with both anaemia and premature delivery (Mockenhaupt et al. 2006).


Histological examination of placental biopsy seems more sensitive in detecting placental infection than microscopical examination of placental blood films, but there was no evidence that the former was associated with birth weight or maternal haemoglobin concentrations (Anchang-Kimbi et al. 2009).

**2.1.3 Rationale**

Implementation of the new flour fortification policy means that pregnant women will receive iron through a combination of fortified foods and universal iron supplementation. In view of the findings from the Pemba trial, our current study aims to assess whether the iron intake resulting from such a policy is safe.
3 OBJECTIVES

3.1 Hypothesis
We hypothesize that flour fortification alone will reduce the risk of *Plasmodium* infection in parturient women as compared to combined supplementation and flour fortification.

3.2 General and specific objectives

3.2.1 General objective
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.

3.2.2 Specific objectives
1. To compare the presence of *Plasmodium* infection in parturient women who antenatally received a combination of iron-fortified foods with iron supplements versus iron-fortified foods only (primary objective).
2. To identify baseline factors that are prognostic for the NTBI response to consumption of a single iron supplement;
3. To assess intervention effects on neonatal iron stores at 1 month of age;
4. To assess intervention effects on maternal haemoglobin concentrations, prevalence of iron deficiency anaemia and iron stores at 1 month after delivery;
5. To assess intervention effects on maternal intestinal pathogens at 1 month after delivery;
6. To develop methods for community-based flour fortification with iron.

3.2.3 Justification
Alternatively, as the comparator, we could have chosen to select women who receive iron supplements only. However, this would have had several disadvantages. First, this comparison would result in a relatively small contrast in iron intake, so that intervention effects may be 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 we would find that a combination of fortification and supplementation results in increased malarial infection among pregnant women, then African governments should reconsider universal iron supplementation programmes rather than iron fortification: as reviewed in the preceding paragraphs, universal iron supplementation in pregnancy is 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.
4 TYPE OF STUDY
The main study is a double-blind, randomised controlled trial with parallel groups. The study is primarily designed as an explanatory trial (i.e. it aims at measuring potential effects when iron is administered under optimal, controlled conditions), the study population is relatively homogeneous, and the primary analysis is chiefly per protocol. To increase the relevance of trial findings for policy decisions, the intervention will be provided under conditions of normal health care.

4.1 Pilot survey
Within the study area, the prevalence of malarial infection in parturient women has been extensively measured for other purposes (e.g. Ayisi et al. 2003, 2004; Parise et al. 2003; Van Eijk et al. 2002, 2003, 2004, 2007). Because the results from these studies were sufficient to guide our sample size calculations, we restricted the aims of our pilot survey to:

1. Development of skills in collecting maternal placental blood;
2. Testing of key laboratory protocols and procedures;
3. Preparation for the trial by fostering participation with local health facilities and research institutions.

On pre-specified days, we selected consecutive parturient women with uncomplicated pregnancies delivering in Siaya District hospital, Kenya. 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. Compared to other methods (perfusion, incision, biopsy, and tissue grinding), this method results in less contamination with foetal blood (Othero et al. 2006). In addition, we collected placental biopsies as described by Bulmer et al. (1993a,b) from near to the centre of each placenta, adjacent to the basal plate. 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 we do not foresee a formal analysis of data.

4.2 Preparatory activities carried out so far
The following preparatory activities have been carried out:

1. Detailed research proposal developed;
2. Study area indentified where malaria is highly endemic;
3. Workshop organised on flour fortification with local millers and AkzoNobel (Nairobi: 21 November 2008);
4. Companies (AkzoNobel, Fortitech) consulted that will produce nutrient premix for the trial;
5. Stakeholders identified and strategy developed for dissemination of findings.
6. Protocol developed for blood collection: we have checked and ensured the feasibility of conducting multiple laboratory tests with the small volumes of venous blood that can be collected in the trial; a protocol is currently being written up.
7. A population census conducted of the targeted area in Kanyawegi, Ojolla, and Osiri sublocations, Kisumu North District, Nyanza province, Kenya.
8. Pilot study conducted at Siaya District hospital (in partnership with KEMRI, Kisumu).
5 STUDY METHODS: Participants and interventions

5.1 Study setting
The trial will be conducted in the target area in Kanyawegi, Ojolla, and Osiri Sub-locations. These sub-locations are in Winam Division, Kisumu District, Nyanza Province, Kenya (Annex 9). Based on a census that we conducted in April 2010, we estimated the total population in this area as 12,202 people. Out of these, 2806 were women of child bearing age (14–45 years). If needed, there will be possible expansion of the targeted study area into Maseno and Kombewa Divisions.

5.2 Study population
Local families are mostly poor subsistence farmers from the Luo tribe. Their diet is monotonous and predominantly based on maize, with a low content of animal products. Posho mills are very common in the area. Customers typically bring 1-10 kg grain per visit for milling. Maize is occasionally pre-blended with sorghum or – less frequently – with millet or cassava. Portions are measured in standard tins that have a capacity of 2 kg, and the miller is paid accordingly (Verhoef, unpublished observations, 2009).

In this area, malaria is highly endemic and transmission is perennial and intense, with seasonal peaks occurring during the long and short rains. In accordance with guidelines from the Ministry of Health (MOH 2002), pregnant women routinely receive intermittent preventive treatment for malaria with two single doses of sulfadoxine-pyrimethamine: the first dose between 16 and 27 weeks of pregnancy, and the second dose between 28 and 36 weeks, at least at a 4-week interval after the first dose.

Intestinal schistosomiasis is also common, but the prevalence declines rapidly within a few kilometres away from Lake Victoria, whilst urinary schistosomiasis is rare (Dr. Diana Karanja, KEMRI/CDC, personal communication, August 2009). In South-West Seme Location, Kombewa Division, Kisumu District, close to the shores of Lake Victoria, the following prevalence values were found in adult women: serum ferritin concentrations <12 µg/L: 62%; haemoglobin concentration <100 g/L (pregnant) or <110 (non-pregnant): 20%; hookworm: 71%; S. mansoni: 20%; T. trichuria: 19%; malaria: 26% (Olsen et al. 1998). In neighbouring Bondo District, on the shores of Lake Victoria, the following prevalence values were found among pregnant women attending clinics for routine antenatal care and with a gestational age between 14 and 24 weeks: serum ferritin concentrations <12 µg/L: 32%; haemoglobin concentration <105 g/L: 38%; hookworm: 49%; S. mansoni: 62%; T. trichuria: 23% (Alusala et al. 2008).

The crude birth rate in Nyanza Province is 45.8 per 1,000 population (Kenya Population and Housing Census 1999, Ministry of Planning and National Development, Central Bureau of Statistics). Antenatal clinic attendance in the area is high, with more than 90% of pregnant women visiting antenatal care clinics at least twice during pregnancy (M. Parise and others, unpublished data; referenced in Ter Kuile et al. 2003). However, fewer than 5% of women in these districts make their first visit to an antenatal clinic in the first trimester of pregnancy (Parise et al. 2003). In a recent study conducted among parturient women in Nyanza Provincial General Hospital, Kisumu and Siaya District Hospital, it was found that the prevalence of Plasmodium infection in placental blood was 35.7% (real-time PCR) and 17.2% (microscopy); the corresponding figures in peripheral blood were 33.1% and 15.9% (Perrault et al. 2009). Similar values have been reported in the same area by Van Eijk et al. (2003).
5.3 Interventions
All participating women will have their flour fortified and will be randomly allocated to daily iron supplements, or supplements containing placebo. Flour will be fortified to a level of 20 mg iron as NaFeEDTA per kg flour, in accordance with recent guidelines from the Flour Fortification Initiative (2008d) that have also been adopted by the World Health Organization (WHO 2009a). For pregnant women, we estimate that this will provide 9 mg/day, or 27% of the recommended iron intake (calculations available upon request). This will keep the intake of EDTA for each population group below the maximum level for the Acceptable Daily Intake as defined by JECFA (1983, 2008). This dose is relatively small compared to 60 mg iron (approximately 1 mg/kg body weight) provided through preventive supplementation, and 120 mg iron (approximately 2 mg/kg body weight) for therapeutic supplementation.

Iron supplements will contain 60 mg iron as ferrous sulphate, as recommended by the World Health Organization (WHO 2001). Participating women will be advised to refrain from taking iron supplements from other sources for the duration of the intervention period, and this will also be discussed in advance with personnel from local health facilities. The intervention will continue until 1 month after delivery.

5.3.1 Premedication
To prevent severe anaemia, all women will receive preventive chemotherapy for infections by *Schistosoma* spp. (praziquantel; 3×20 mg/kg body weight as a 1-day treatment) and geohelminths (albendazole; 400 mg, single dose) at a gestational age between 13 and 20 weeks, depending on the gestational age at the screening visit. This therapy will be provided in or at the start of the second trimester of pregnancy, but before randomisation. Women in their first trimester of pregnancy will be asked to return at the start of the second trimester, and the run-in period will be extended accordingly. Preventive therapy is in accordance with recommendations by the World Health Organization to provide mass treatment with these drugs of entire communities, including women in the second trimester of pregnancy and lactating women, in populations with a high prevalence of schistosomal or geohelminth infection (*Ascaris, Trichuris* and hookworms) (WHO 2002, 2006). In addition, praziquantel and albendazole can be safely co-administered (WHO 2006b).

Interaction with other drugs
To avoid interaction, praziquantel will not be administered to those already receiving treatment with rifampicin.

Dispensing and accountability
A physician employed by the project will stock and dispense praziquantel and albendazole, and keep records of the doses received and dispensed.

5.3.2 Pre-randomisation/pre-registration evaluations
The following tests will be done and results will be available before randomisation: pregnancy tests; haemogram (including haemoglobin concentration, haematocrit, differential leucocyte count); zinc protoporphyrin:haem ratio in whole blood and washed erythrocytes; plasma C-reactive protein concentration; HIV tests (voluntary).

Samples will be collected at randomisation for the following tests, but results will be available afterwards: genotyping for haemoglobin disorders (incl. α-thalassaemia; sickle cell; glucose-6-phosphate dehydrogenase deficiency; haptoglobin polymorphisms); malarial infection by dipstick tests and PCR; plasma concentrations of histidine-rich protein 2, ferritin, soluble transferrin receptor, α1-acid glycoprotein, retinol, cobalamin and folate; presence and density in stool of *Trichuris trichuria*, hookworm, *Schistosoma* spp., *Giardia intestinalis*, *Entamoeba histolytica* and *Helicobacter pylori*.
5.4 Eligibility criteria
Resident women aged 15-45 years will be excluded when having a reported medical history suggestive of sickle cell anaemia, epilepsy, diabetes; an personal obstetric history suggestive of eclampsia or pre-eclampsia; obvious mental retardation or metabolic disorder; or when not having provided written consent; when not pregnant (i.e. a negative result for the dipstick test or ultrasonography); when carrying multiples; when the gestational period exceeds 20 weeks; when planning to leave the homestead or to be absent for prolonged periods in the course of the pregnancy or within a 1-month period thereafter; or when planning to deliver outside the research clinic.

Pregnant women will be eligible for randomisation when a) all homestead members consent and agree to obtain their flour exclusively from one or several mills participating in the study; b) the head of the homestead or his/her representative has provided written consent on behalf of all homestead members; c) the pregnant woman has provided a blood sample; and d) the haemoglobin concentration is ≥90 g/L.

5.5 Participant timeline

![Figure 1. Participant timeline](image)

5.6 Target sample size
Within the study area, the prevalence of malarial infection in parturient women has been extensively measured for other purposes (e.g. Ayisi et al. 2003, 2004; Parise et al. 2003; Van Eijk et al. 2002, 2003, 2004, 2007). These studies guided sample size calculations of our trial. We plan to include 150 pregnant women per intervention group (total: 300 women). Assuming that 5% of women will drop out of the iron group, no ‘drop-in’ will occur of women crossing over from the placebo group to the iron group, a prevalence in the control group of the primary outcome of 50%, and an anticipated prevalence ratio of 1.35, this sample size will yield 80% probability of excluding the null value from the 95% CI of the effect estimate (Figure 2).

Resources allowing, we will increase the sample size to 225 women per group, which increase the probability of excluding the null value from the 95% CI of the effect estimate to 92%. At a prevalence ratio of 1.30, the corresponding probability is 83%. Assuming a crude birth rate of 43 per 1,000 population (Kisumu District, 1999 census data; Kenya Central Bureau of Statistics, Ministry of Planning and National Development), and expecting recruitment to take place over a 1.5-year period, we need to conduct the study in a population of approximately 7,000 people.
Figure 2. Effect of sample size on the probability of excluding the null value from the 95% CI, under various assumptions of the true group difference in prevalence of infection. Red arrow: intended sample size. Assumptions: α=0.05; prevalence of infection in control group without supplements: 50%; with correction factor to account for 5 drop-out in iron group (formulae according to Wittes 2000).

5.7 Recruitment and retention strategies
Study participants will be recruited from communities in the study area. To collect data and samples at baseline and during the intervention period, we will use an existing public health facility that is centrally located in the study area. Medical staff at this facility (i.e. not employed by the project) will provide antenatal care to all women as usual, without supervision by project staff. The heads of homesteads and women of child-bearing age living in those homesteads will be invited for group meetings, where field staff will inform them of the study goals and procedures. Eligible women (see Section 5.4) will be registered so that they can be followed to detect pregnancy at an early stage, using procedures that are summarised in Figure 3.

For women who are married or living together, community volunteers will solicit the menstrual history every 4 weeks, and report the results to field staff. These women will be invited to a centrally located research dispensary, and will be offered pregnancy testing by urine test when the start of the last menstrual period is reported to have occurred 10 weeks earlier. Women who never have been married, or who are divorced, separated, or widowed will be asked to visit the research clinic every 12 weeks for pregnancy testing by urine test. After delivery, women will be excluded from pregnancy monitoring for the first 3 months of the neonatal period. For those who delivered more than 3 months ago, urine tests will be done at 12-weekly intervals until menstrual periods have recurred; thereafter, pregnancy detection procedures will be as described above.

Women with pregnancy confirmed by urine test will receive a screening number; vital information and a medical/obstetrical history will be recorded using a standardised questionnaire, and a medical examination performed.
For consenting women, pregnancy will be confirmed and gestational age will be determined by ultrasonography (SonoSite Titan, SonoSite, Bothell WA, USA). Those carrying multiples, with gestational age >20 weeks, who are unlikely to comply with follow-up procedures or who plan to deliver outside a clinic where samples can be obtained will be excluded. Immediately after the screening visit, but at least after 13 weeks of gestational age (counted from first day of last menstrual period), participating women will enter a run-in period to sterilise infections by *Schistosoma* spp. and geohelminths, to obtain consent from the members from her homestead, and to avoid drop-outs due to early miscarriage. To prevent severe anaemia, all women will receive preventive chemotherapy for infections by *Schistosoma* spp. (praziquantel; 3×20 mg/kg body weight as a 1-day treatment) and geohelminths (albendazole; 400 mg, single dose) at a gestational age between 13 and 20 weeks, depending on the gestational age at the screening visit, but in any case at least 14 days before randomisation (Figure 4). Women in their first trimester of pregnancy will be asked to return at the start of the second trimester, and the run-in period will be extended accordingly. Preventive therapy is in accordance with recommendations by the World Health Organization.

*All others: see text

**Figure 3. Decision tree for early detection of pregnancy**
Organization to provide mass treatment with these drugs of entire communities, including women in the second trimester of pregnancy and lactating women, in populations with a high prevalence of schistosomal or geohelminth infection (Ascaris, Trichuris and hookworms) (WHO 2002, 2006). In addition, praziquantel and albendazole can be safely co-administered (WHO 2006). Praziquantel will not be administered to those already receiving treatment with rifampicin. At around the same time, women should also receive the first dose of intermittent preventive therapy with sulfadoxine-pyrimethamine as part of their routine antenatal care. Thus, to avoid frequent dosing, we will not administer such preventive treatment.

Within 48 h following the detection of eligible women, field staff will visit the homestead to ask fellow homestead members for consent for their flour to be fortified. The head of the household will be asked to sign a consent form on behalf of the entire homestead.

Pregnant women will be medically examined at randomisation, and a venous blood sample (6 mL) will be collected. Women will receive voluntary counselling and testing for HIV as per national guidelines. An overview of the measurements is provided in Table 1. We will detect malarial infection by rapid dipstick tests (see section Laboratory methods below) and by microscopical examination of blood films; haemoglobin concentration, haematocrit and cellular indicators by haematology analyser (Cell Dyn 3200, Abbott, Abbott Park, IL, USA), the ratio of zinc protoporphyrin:haem in whole blood and washed erythrocytes (Aviv Biomedical, Lakewood, NJ, USA), and plasma C-reactive protein concentration by rapid field test (QuikRead, Orion Diagnostics, Espoo, Finland). Blood sediment, including buffy coats, will be preserved at 2-8 °C in microcentrifuge tubes containing a DNA-stabilizing buffer (AS1, Qiagen, Valencia, CA, USA) for subsequent DNA extraction and genotyping for haemoglobin disorders. In addition, we will store plasma specimens for subsequent measurement of concentrations of histidine-rich protein 2, ferritin, soluble transferrin receptor and α₁-acid glycoprotein, and retinol, cobalamin and folate. A stool sample will also be collected for microscopical detection and counting of eggs of *T. trichuria* by direct smears and concentration methods (Fleck and Moody 1988, WHO 1991). Aliquots of fresh stool without preservative will be frozen and stored at -20 °C for subsequent detection of gastrointestinal pathogens.

Depending on the gestational age at the time of the screening visit, the procedure outlined above will ensure that eligibility has been established at a gestational age between 16 and 23 weeks.

On the day of randomisation, exactly 3 h after women have ingested their first supplement, when we expect the NTBI response to iron to be close to its peak (Hutchinson et al. 2004), we will collect a venous peripheral blood sample in a tube suitable for trace element analysis (Becton Dickinson). Serum will be stored for subsequent measurement of concentrations of NTBI and soluble adhesion molecules (sICAM).

Local health workers will be advised to counsel women to take the supplement with food in case these women report mild adverse events that may be associated with oral iron supplements (e.g. black stools, nausea, diarrhoea or constipation). Participating women reporting adverse events or sickness will be referred to normal health facilities where they will receive standard care. The Field Coordinator will arrange for transport to a local health facility for participating pregnant women who are severely ill.
START OF SCREENING:
Women with pregnancy confirmed by urine test

Informed consent procedure; questionnaire; medical and ultrasound examination

Women excluded when:
- Non-pregnant
- Carrying multiples
- Gestational age >20 weeks
- Unlikely to complete follow-up period;
- Planning to deliver outside the research clinic

Start run-in period

Within 48 h after report of pregnancy: Project staff visits home to obtain consent from homestead members; start of fortification

Gestational age

<13 weeks

Ask to return at the start of 2nd trimester of pregnancy

At 14-21 days after start of preventive chemotherapy:
informed consent procedure for HIV testing; collect blood specimen

Women excluded when:
- Blood sample not obtained
- Haemoglobin concentration <90 g/L
- Homestead ineligible

Homestead excluded when:
- Pregnant woman ineligible
- No written consent received from Head of homestead

END OF SCREENING:
- Eligible pregnant women will be randomised and included in intention-to-treat analysis

FIGURE 4. Overview of recruitment procedures
Fortification: Preblend containing the fortificant will be given to millers, who will keep a limited stock in closed plastic containers. Grain will be brought to the mill by the customer (a registered member of a participating homestead), and the miller will fortify the flour by continuous addition by hand sprinkling of preblend with the grain (whole or dehulled) in the mill feed hopper while it is being milled. For this purpose, the miller will use a calibrated scoop or spoon to measure an amount proportional to the weight of grain. In pilot projects in Malawi and other African countries, this procedure has resulted in adequate mixing (Quentin Johnson, personal communication, November 2008); nonetheless, we will conduct preparatory studies to ensure that the procedures used will result in adequate levels of fortification, and in homogenous distribution of the fortificant iron in flour.

Supplementation: Participating women will receive either fortified flour with daily oral iron supplements, or fortified flour with daily oral supplements containing placebo. The production of supplements are currently being produced by Lab & Allied, Nairobi, with ferrous sulphate (dried, BP 2010) obtained from Dr. Paul Lohmann GmbH, Emmenthal, Germany (catalogue no: 501022005500). As foreseen, the supplements will be in the form of tablets in blister packs. Participating women will be advised to refrain from taking iron supplements from other sources for the duration of the intervention period, and this will also be discussed in advance with personnel from local health facilities. The intervention will continue until 1 month after delivery.

A Field Investigator and a driver, who will be based in the study area, and continuously available to coordinate the study and to ensure that all procedures are followed will be appointed.

6 Study methods: Participant allocation

6.1 Randomisation

6.1.1 Sequence generation and allocation concealment
The randomisation list will be generated by a person not involved in the allocation of treatment, using tables of random numbers. No restriction will be applied. Treatment arms will be colour coded, and the colour allocated to each woman will be kept in sealed, opaque, and sequentially numbered envelopes. The field worker(s) allocating treatment will not be allowed to open these envelopes until it has been formally established that the woman is eligible and will enter the trial, and she has been registered in a central list.

6.1.2 Implementation
At randomisation, each participating woman will be sequentially allocated to the colour indicated in the next available envelope, and this colour will be marked on a registration card that will be kept by the woman. In addition, the homestead to which she belongs will receive a limited number of registration cards on which the colour code is not indicated. These cards will allow homestead members to identify themselves with local posho millers, so that their flour will be fortified during the milling process. The study team will also ensure that participating millers will each regularly receive a updated list of participating homesteads.
<table>
<thead>
<tr>
<th>Measure</th>
<th>At baseline</th>
<th>At delivery</th>
<th>At delivery</th>
<th>At 1 month after delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV infection</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin concentration</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Differential blood cell count</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Malaria dipstick tests (pLDH/HRP2)</td>
<td>X(^3)</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Malaria PCR tests</td>
<td>D</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Microscopical examination of blood films</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>ZPP:haem ratio(^4)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α+ -thalassaemia genotype</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sickle cell genotype</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haptoglobin gene polymorphisms</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma HRP2 concentration</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Plasma pLDH concentration</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Plasma ferritin concentration</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Plasma sTfR concentration</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Plasma CRP concentration</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Plasma α1-acid glycoprotein</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma retinol concentration</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Plasma cobalamin concentration</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Plasma folate concentration</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Plasma concentrations of cytokines/SAMs</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

Abbreviations: X: to be measured; D: actual measurement may depend on the availability of sufficient resources

HRP2: histidine-rich protein-2; pLDH: parasite-specific lactate dehydrogenase; sTfR: soluble transferrin receptor; SAMs: and soluble adhesion markers. In addition, we will measure \(^1\) concentration of non-transferrin bound iron in serum collected from venous blood at 4 hours after ingestion of the first supplement, and \(^2\) the presence of gastrointestinal infections (hookworm, *Schistosoma* spp., *Giardia intestinalis*, *Entamoeba histolytica* and *Helicobacter pylori*, *Trichuris trichuria*) in stool collected from women at baseline and at 1 month after delivery. \(^3\) Will be done retrospectively after randomisation; \(^4\) Both in whole blood and washed erythrocytes
6.2 Blinding
The supplements will be contained in opaque capsules with four colours (tentatively: red, blue, yellow and black) that encode for two types of supplements (iron versus placebo). The colour code will be prepared by a person who is not involved in the field work. Copies of the colour code will be kept in three separate places in sealed envelopes. The colour code will not be available to the field team, and will not be broken until after the field work is finished, the database is locked, preliminary statistical analyses have been done to describe baseline and end-of-intervention characteristics (aggregated by all treatment groups), and a statistical analysis plan is sent to the Data Safety and Monitoring Committee. Clinical officers and laboratory personnel will be blinded to treatment when medically examining trial participants.

7 Data collection, management and analysis

7.1 Outcomes
The primary outcome concerns the presence of infection by any *Plasmodium* spp. in parturient women, as indicated by a positive result of a pLDH-based or HRP2-based dipstick test, or a *Plasmodium*-specific PCR test, each conducted in maternal venous blood, placental blood or cord blood.

Iron stores will be measured by plasma concentrations of ferritin and soluble transferrin receptor. Iron deficiency anaemia in post-partum women will be defined as coexisting anaemia (haemoglobin concentration <120 g/L) and depleted iron stores (plasma concentration <15 μg/L; De Benoist et al. 2008). Plasma ferritin concentration will be used as an indicator of iron stores in individuals without malaria or inflammation. Plasma concentration of soluble transferrin receptor and the ratio of zinc protoporphyrin:haem in washed erythrocytes will be used as indicators of the supply of iron for erythropoiesis; iron-deficient erythropoiesis will be defined as a ratio of zinc protoporphyrin:haem >40 μmol/mol (Zimmermann 2008). In addition, depending on the availability of new assays and funds, we may use novel iron indicators such as urinary hepcidin concentration, which can detect iron deficiency in patients with multiple medical problems (Nemeth and Ganz 2006). Serum NTBI concentration will be measured using described by Breuer and Chabanchik (2001).

Collection of end-point data and samples: At delivery, birth weight will be recorded and samples will be collected of maternal placental blood, maternal venous blood (6 mL), and cord blood (6 mL). Maternal blood will be collected from the placenta by puncturing the intervillous space, which results in less contamination with foetal blood than other collection methods (perfusion, incision, biopsy, and tissue grinding) (Othero et al. 2006). Cord blood will be obtained after careful cleaning by direct sampling of the umbilical vein. Maternal venous blood will be collected within 1 hour after delivery, and at 1 month after delivery. All blood samples will be collected in tubes with EDTA as an anticoagulant. In addition, placental biopsies will be collected and stored in paraformaldehyde for future detection of infection by histological examination of tissue sections.

At the end of intervention, i.e. 1 month after delivery, we will collect another venous blood sample to determine iron status, and a stool specimen to assess the presence of infection with *Schistosoma* spp. and geohelminths. These infections can also influence iron indicators independently of iron status, with the exception of plasma soluble transferrin receptor, which is influenced by malaria-induced haemolysis (Verhoef et al. 2002) but not by worm infections. All women will subsequently receive a therapeutic dose of praziquantel and albendazole. At 1 month after delivery, we will collect peripheral blood by heel puncture from neonates to determine iron stores.
7.2 Data collection methods

To collect data and samples at baseline and during the intervention period, we will use an existing public health facility that is centrally located in the study area. Medical staff at this facility (i.e. not employed by the project) will provide antenatal care to all women as usual, without supervision by project staff. We will appoint a Field Investigator and a driver, who will be based in the study area, and continuously available to coordinate the study and to ensure that procedures are followed.

Sample collection and handling: Venous blood collection will be done by experienced laboratory technicians or nurses under medical supervision, using sterile equipment and techniques. The small risk of bleeding, infection and/or phlebitis after venipuncture will be explained to participating women. We will comply with Kenyan regulations for handling of biological specimens collected for research purposes.

Fortification premix will be produced in Denmark and shipped to Kenya by a specialised manufacturer (Fortitech Inc., Gadstrup, Denmark), with NaFeEDTA as a source of iron (Ferrazone®, AkzoNobel, Amsterdam, The Netherlands). Dilution of premix to a preblend is essential because the quantity of concentrated premix required to fortify a typical 1-20 kg batch of maize meal is too minute to measure out and achieve even distribution in flour at the correct levels. The premix will be diluted at a central location by project staff, using maize flour that will be provided by the project.

We will use a haematology analyser (Cell Dyn 3200, Abbott, Abbott Park, IL, USA), a fluorometer to measure the ratio of zinc protoporphyrin:haem in whole blood and washed erythrocytes (Aviv Biomedical, Lakewood, NJ, USA), and a battery-operated machine to measure plasma C-reactive protein concentration by rapid field test (QuikRead, Orion Diagnostics, Espoo, Finland). Medical and laboratory supplies will be obtained through regular suppliers (VWR) in Kenya or abroad. We are currently evaluating whether the supplements with active ingredients and matching placebo can be produced in Kenya; if needed, we will obtain them from a manufacturer abroad that operates under EU Good Manufacturing Procedures (GMP), and that we have used in a previous micronutrient supplementation trial in Tanzania (Laboratory Medisan, Heerenveen, The Netherlands; http://www.lab-medisan.com/).

Procedures – field, laboratory, quality control: At around week 35 of gestational age, a meeting will be held with participating women. In that meeting, they will be counselled to deliver at a hospital (tentatively: Kisumu District Hospital and/or Bondo District Hospital). A mobile phone will be provided until delivery, so that the woman, her relatives or a volunteer can warn field staff of the start of the delivery. Field staff will provide or arrange for transport to the hospital, or cover the cost of public or private transport if and as required; a field coordinator and/or driver will be available continuously for this purpose in the study area. Medical staff will be supervised by project staff to collect data and samples, and will receive a financial incentive for work that has been satisfactorily performed. Cord clamping will be done as usual, in accordance with the aim of the trial to assess intervention effects under conditions of usual care.

Tables 1 and 2 provide overviews of the indicators to be measured, the site of analysis, and the institution responsible for the analysis.

Two types of dipstick tests will be stored at 2°C–8°C and deployed to detect malaria infection (AccessBio, USA; www.acsbio.com; CareStart G0151 for detection of *P. falciparum*-specific lactate hydrogenase (pLDH), and CareStart G0171 for detection of *P. falciparum*-specific histidine-rich protein-2 (HRP2) and *P. ovale*/P. *malariae*/P. *vivax*-pLDH). The pLDH-based
tests detect current infection (Makler et al. 1998, Piper et al. 1999, Moody 2002) and can distinguish between current falciparum and non-falciparum malarial infections. By contrast, the HRP2-based test detects current or recent infection (because the protein can remain present in the blood for up to several weeks after parasitaemia clearance; Moody 2002), and is specific for *P. falciparum* only. These tests are among the best of 41 brands and types of dipstick tests that were recently evaluated under auspices of the World Health Organization, and have excellent performance (>95%) in detecting low and higher parasite density (200 parasites/μL or 2000 parasites/μL, respectively) (WHO 2009b).

*Plasmodium* infection will also be detected by conventional microscopical examination microscopical examination of thick and thin blood films, and by detecting the presence in blood of haemozoin-containing leucocytes (Abbott, Cell Dyn 3200; raw data will be downloaded for subsequent quantitative analysis of haemozoin-containing leucocytes using standard flow cytometry software). Funds permitting, we will also measure plasma concentrations of pLDH and HRP2 by ELISA assays as an indicator of the total body biomass of *P. falciparum* parasites (Dondorp et al. 2005; Kifude et al. 2008, Martin et al. 2009). For HRP2, we will use a commercially available assay (Malaria Ag CELISA, Cellabs, Sydney, NSW, Australia).

Haemoglobin concentrations will be measured and whole and differential leucocyte counts will be performed using a haematology analyzer (Abbott, Cell Dyn 3200). In the field, plasma C-reactive protein concentrations will be measured by a portable machine (QuikRead, Orion Diagnostica, Espoo, Finland).

Plasma concentrations of ferritin and soluble transferrin receptor will be measured on an enzyme-linked immunosorbent assays (BN-prospec, Dade-Behring, Marburg, Germany) and (BeckmanCoulter; Access Ferritin) on a Beckman Coulter UniCel Dx880i analyser, respectively. Plasma concentrations of folate and cobalamin will be measured by competitive electrochemiluminescence assays on a Cobas E610 analyser (Roche Diagnostics, Almere, The Netherlands), whilst plasma retinol concentration will be assayed by high-pressure liquid chromatography. Plasma s-ICAM concentrations will be measured by enzyme-linked immunosorbent assay. HIV testing will involve two concurrently conducted rapid assays (HIV-1/2, Abbott Laboratories, Dainabot, Japan, and Unigold HIV-1/2, Trinity Biotech, Bray, Ireland). Capillus HIV-1/2 (Cambridge Diagnostics, Wicklow, Ireland) will be preformed on samples with discordant results. DNA will be extracted with a QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany); the −α3.7 deletion type of α+ thalassaemia will be determined by polymerase chain reaction as described by Liu et al. (2000). *Plasmodium* infection, sickle cell genotype, haptoglobin gene polymorphisms will be determined by polymerase chain reaction and electrophoresis. Consideration will be given to use left-over blood samples for other purposes, e.g. to develop and test new diagnostic assays for iron deficiency. Hookworm, *Schistosoma* spp., *Giardia intestinalis*, *Entamoeba histolytica* and *Helicobacter pylori* will be detected quantitatively by real-time polymerase chain reaction (PCR) assay (Ten Hove et al. 2008, Verweij et al. 2007, Haque et al. 2007) or qualitatively by direct stool antigen tests (DAKO, Amplified IDEIA HpSTAR, Breda, The Netherlands; ProSpecT ELISA tests; Oxoid Ltd., Basingstoke, England). Other diagnostic strategies are described above (see section ‘Pilot study’).

Most of the immunological, biochemical and parasitological tests will be conducted in Kenya, and we will seek opportunities to train local laboratory personnel for this purpose. It should be noted, however, that some of the tests require sophisticated equipment that is not available in Kenya, or that is prohibitively expensive or requires highly specialised personnel (e.g. NTBI measurements). Additionally, few laboratories have the capacity to conduct the variety of biochemical tests that are foreseen in our project. The analyses will be conducted in laboratories with a track record in measuring these indicators, and that have internal and external quality control procedures in place. Thus, we foresee that some of the tests may...
have to be conducted abroad. In that case, we will seek opportunities for our Kenyan partners to become involved in the analyses and to be trained in the techniques employed. Export of biological specimens will be in full compliance with the regulations in Kenya.

**TABLE 2. Laboratory analysis plan**

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Location of analysis</th>
<th>Responsible ¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin concentration, haematocrit, total and differential blood cell counts; pLDH and HRP-2 dipstick tests; zinc protoporphyrin:haem ratio; rapid tests for plasma C-reactive protein concentration; density of <em>Plasmodium</em> spp. in blood smears and <em>T. trichuria</em> in stool by conventional microscopy; HIV tests; quality control tests for NaFeEDTA in flour</td>
<td>Kenya</td>
<td>LSHTM/WU-CBI</td>
</tr>
<tr>
<td>Plasma concentrations of ferritin and soluble transferrin receptor; plasma concentrations of C-reactive protein, folate and cobalamin</td>
<td>Meander Medical Centre, Amersfoort, The Netherlands</td>
<td>LSHTM/WU-CBI</td>
</tr>
<tr>
<td>Gastrointestinal infections (real-time PCRs)</td>
<td>University Medical Centre, Leyden, The Netherlands</td>
<td>LSHTM/WU-CBI</td>
</tr>
<tr>
<td>Plasma concentrations of cytokines and soluble adhesion markers; serum concentrations of NTBI; plasma concentrations HRP-2 and pLDH; DNA genotyping; gastrointestinal infections (real-time PCRs or stool antigen tests)</td>
<td>Wageningen University, The Netherlands</td>
<td>LSHTM/WU-CBI</td>
</tr>
</tbody>
</table>

¹ LSHTM: London School of Hygiene and Tropical Medicine; WU-CBI: Wageningen University, Cell Biology and Immunology Group

### 7.3 Data management

When the field work is finished, the database will be locked and preliminary statistical analyses will be done to describe baseline and end-of-intervention characteristics (aggregated by all treatment groups). An up-to-date statistical analysis plan will be sent to the Data Safety and Monitoring Committee before breaking the code.

All data will be coded and treated confidentially. After analyses have been completed, the complete and detailed results will be communicated in written and oral form to the regional health authorities. In individual cases, where laboratory results indicate a need for medical intervention, the name of the individual will be communicated to the local health service to provide appropriate medical treatment.

### 7.4 Statistical methods

When describing the flow of participants, we will account for all individuals who were invited for screening. Groups will be described regarding baseline characteristics. We will refrain from formal testing for group differences, because differences that do exist are necessarily caused by chance alone. For individuals who were randomised but who stopped their participation before the scheduled end of intervention, we will provide reasons for their dropout, if possible. Compliance will be measured as the proportion of women who have consumed >90% of scheduled supplements.
The primary measure of intervention efficacy is calculated as the percentage reduction in the prevalence of infection; in addition, we will indicate the public health importance of the intervention effect by the prevalence difference using Newcombe’s method (Altman et al. 2000). The primary analysis is per protocol, i.e. restricted to women who comply with the study protocol, but according to groups to which they were assigned; in addition, we will conduct an exploratory intention-to-treat analysis.

In the primary analysis we will adjust prevalence ratios for covariates that are prognostic for outcome, using Cox regression analysis with constant time at risk (Lee and Chia 1993, Lee 1995, Barros and Hirakata 2003). These covariates are divided into two groups: a) variables known from previous studies to be prognostic for outcome, as predefined on the basis of a literature review, and b) variables that we suspect to be associated with the outcome. In the final model, these covariates will be retained only if they substantially change the estimate of the intervention effect. Before breaking the randomisation code, we will review and if necessary revise our statistical analysis plan.

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 (Hallberg 2001). Because our primary objective is to measure intervention effects on malarial infection, regardless of iron status at baseline, we will not measure plasma ferritin concentrations before randomisation and exclude individuals on that basis. For these reasons, we will restrict the measurement of treatment efficacy to individuals with iron deficiency at baseline, as indicated by plasma ferritin concentration <15 μg/L regardless of the presence of inflammation, or by plasma concentration of soluble transferrin receptor above the cut-off value indicating iron-deficient erythropoiesis, as specified by the manufacturer, in those with inflammation (plasma C-reactive protein concentration >7 mg/L). 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 from participants without missing values in the same group. We will also examine interaction between treatment and iron stores/deficit at baseline (indicated by the ratio of transferrin receptor and log ferritin concentrations), using stratified analysis and multivariate linear regression analysis based on fractional polynomials (Royston and Sauerbrei 2004).

Data and all appropriate documentation will be stored for a minimum of 5 years after the completion of the study, including the follow-up period.

Trial participants who stop the intervention before its prescheduled end, for whatever reason, will nonetheless be asked for reasons and for agreement to collect blood samples at the scheduled end of intervention, so that they can contribute to the intention-to-treat analysis of the primary outcome. For the same reason, data collected from women who are withdrawn will not be destroyed unless this is asked specifically by the women involved.

8 Study methods: Trial monitoring

8.1 Monitoring and reporting of harm

Implementation of the new flour fortification policy means that pregnant women will receive additional iron through a combination of iron-fortified flour and universal iron supplementation. Compared to the group receiving iron-fortified flour only, we expect that this may lead to increased haemoglobin concentrations, but it is not known whether it will provide functional benefits (see preceding paragraphs). However, we hypothesize that a high intake of additional iron is associated with an increased risk of maternal infection with Plasmodium spp.
Oral ingestion of ferrous sulphate in a dose of 100 mg ferrous iron, which is higher than the level used in this study, produces few adverse effects. Gastro-intestinal manifestations are probably caused by non-absorbed iron in the stomach (nausea, pain) or in the intestine (diarrhoea or constipation). Faeces may become black. We will not monitor for these adverse events because they are well known, reversible when supplementation is stopped, and may be related to pregnancy itself. Iron loading can occur when iron is taken by persons with hereditary haemoglobin disorders that are not accompanied by iron deficiency. In eastern Africa, this only concerns persons who have undergone repeated blood transfusions as part of treatment for sickle cell anaemia; however, women with this disorder are unlikely to survive to child-bearing age, are likely to be iron deficient, and are likely to be excluded from the trial on the basis of their medical history.

8.1.1 Safety reporting for non-drug trials

Definitions:
Adverse Event (AE): any untoward medical occurrence in a patient or clinical study subject.

Serious Adverse Event (SAE): any untoward and unexpected medical occurrence or effect that:
- Results in death
- Is life-threatening – refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe
- Requires hospitalisation, or prolongation of existing inpatients’ hospitalisation
- Results in persistent or significant disability or incapacity
- Is a congenital anomaly or birth defect

Medical judgement should be exercised in deciding whether an AE is serious in other situations. Important AEs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, should also be considered serious.

Reporting Procedures: All adverse events should be reported. Depending on the nature of the event the reporting procedures below should be followed. Any questions concerning adverse event reporting should be directed to the Principal Investigator in the first instance.

8.1.2 Non serious AEs
All such events, whether expected or not, should be recorded on the appropriate form.

8.1.3 Serious AEs
Reporting of (severe) adverse events: The community volunteers who supervise daily supplementation will also be instructed to report immediately to the field staff when a woman has been hospitalised, has had a miscarriage or a blood transfusion. The Trial Manager will report Severe Adverse Events (SAEs) immediately but at least within 24 hours after being made aware of the event to both the Data and Safety Monitor and the Principal Investigator (HV); in addition, he will keep a log file and report these SAEs in tabulated form in half-yearly progress reports. A SAE is defined as any adverse event or adverse reaction that: a) results in death of participating mother or child; b) is life-threatening to participating mother or child; c) requires hospitalisation or blood transfusion; d) results in persistent or significant disability or incapacity; e) is a congenital anomaly or birth defect. However, hospitalisations for elective treatment of a pre-existing condition do not need reporting as SAEs.
All SAEs should be reported to the LSHTM ethics committee where in the opinion of the Principal Investigator, the event was:
- ‘Related’, i.e. resulted from the administration of any of the research procedures, or:
- ‘Unexpected’, i.e. an event that is not listed in the protocol as an expected occurrence.

Reports of related and unexpected SAEs should be submitted within 15 days of the Principal Investigator becoming aware of the event, using the NRES SAE form for non-IMP studies.

Local investigators should report any SAEs as required by their Local Research Ethics Committee and/or Research & Development Office.

Contact details for reporting SAEs
Fax: + 31 317 483955, attention: Dr Hans Verhoef
Please send SAE forms to: Dr Hans Verhoef
Tel: + 31 6 83252225 (Mon to Fri 09.00 – 17.00)

8.2 Oversight
Food products (including dietary supplements) are not ‘medical products’ (EU 2004), this study does not come under the scope or control of the laws, regulations and administrative provisions relating to the implementation of good clinical practice in the conduct of clinical trials (EU Directive 2001/20/EC). Nonetheless, we will appoint a Trial Monitor and an independent Data and Safety Monitor.

Trial monitor: A Trial Monitor will generally and independently oversee the progress of a clinical trial and specifically verify that the rights and well-being of the human subjects are protected; the reported trial data are accurate, complete, and verifiable from source documents; the community volunteers monitor compliance to supplementation and elicit safety concerns in an appropriate manner; and the conduct of the trial is in compliance with the currently approved protocol/amendment(s), and with the applicable regulatory requirements.

8.2.1 Data Safety and Monitoring Committee
A Data and Safety Monitoring Committee will review the unblinded data for safety purposes, monitor the progress of the trial, and the critical efficacy endpoints; recommend to the sponsor whether to continue, modify, or stop a trial; and assess whether there are any safety issues that should be brought to participants’ attention. The Data and Safety Monitoring Committee will meet in Kenya or through teleconference.

For blood transfusions, hospitalizations, maternal deaths, and miscarriage, the Data and Safety Monitoring Committee may unblind the randomisation code; however, neither the PI nor any other member of the study team will have access to the randomisation code or the unblinded data during the field work.

The Data and Safety Monitoring Committee will liaise early in the trial with the Principal Investigator to consider the protocol in detail, and to discuss how the Data and Safety Monitoring Committee might respond to hypothetical situations. The Data and Safety Monitoring Committee will assess emerging risks, such as an increase in frequency or severity of adverse events, and may recommend protocol amendments if he/she detects serious safety issues, or to terminate the study in case of compelling evidence that intervention is associated with severe adverse events.
8.2.2 Withdrawal criteria
Participating women will be withdrawn from study when having manifestations suggesting pre-eclampsia (systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg, or both, on at least two occasions four hours apart after 20 weeks' gestation but before the onset of labour, with either proteinuria or any multisystem complication; North et al. 2011), eclampsia (manifestations of pre-eclampsia with tonic-clonic seizures not caused by coincidental neurological disorders), or if the responsible clinician considers continuation no longer medically justified for whatever reason. These women will be referred for further treatment.

8.2.3 Interim analyses
No interim analyses are foreseen.

8.3 Emergency code-breaking procedure
For blood transfusions, hospitalizations, maternal deaths, and miscarriage, the Data and Safety Monitoring Committee may un-blind the randomisation code; the field team will remain blinded. The Data and Safety Monitoring Committee will meet early in the trial with the Principal Investigator to consider the protocol in detail, and to discuss how the Data and Safety Monitoring Committee might respond to hypothetical situations. The Data and Safety Monitoring Committee will assess emerging risks, such as an increase in frequency or severity of adverse events, and may recommend protocol amendments if he/she detects serious safety issues, or to terminate the study in case of compelling evidence that intervention is associated with severe adverse events.

9 Ethical consideration and dissemination
The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions. In preparing the current research proposal, we used new ethical guidelines as laid down in the revised Helsinki declaration (World Medical Association 2008; www.wma.net/en/30publications/10policies/b3/index.html) and the Council for International Organizations of Medical Sciences (CIOMS) in collaboration with the World Health Organization (WHO) (CIOMS 2002). In addition, we will use locally accepted procedures to obtain informed consent from communities and individuals participating in the study.

9.1 Research ethics approval
This proposal was granted ethical clearance by Ethical Review Boards both in the United Kingdom and Kenya.

Trial registration: The trial will be registered before enrolment of the first participant with www.clinicaltrials.gov.

9.2 Protocol amendments
All amendments to the protocol will first be approved by the principle investigator, who will then submit the amendments to the ethical committees in Kenya and LSHTM. Implementation will only be done after approval by the ethical committees. The principle investigator will communicate the amendments to the Trial Coordination Centre and subsequently to the field teams.
9.3 Consent/ assent
Consent to enter the study will be sought from each participant only after a full explanation has been given, an information leaflet offered and time allowed for consideration. Signed participant consent will be obtained. The right of the participant to refuse to participate without giving reasons will be respected. After the participant has entered the trial the clinician will remain free to give alternative treatment to that specified in the protocol at any stage if he/she feels it is in the participant’s best interest, but the reasons for doing so will be recorded. In these cases the participants will remain within the study for the purposes of follow-up and data analysis. All participants are free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing further treatment.

9.4 Confidentiality
Participants’ identification data will be required for the registration process. The Study Coordination Centre will preserve the confidentiality of participants taking part in the study and is registered under the Data Protection Act. Data collected will remain confidential, and study results will be reported in aggregated form so that participants will remain anonymous. Only members of the clinical team will have access to participant’s records. Field staff will sign a written statement to maintain confidentiality of any personal information from (potential) trial participants to which they may become acquainted.

9.5 Competing interests
The authors, investigators and supervisors involved in the design and implementation of this trial declare no competing interests.

9.6 Role of involved parties: Partnerships and key publications by the research group

The **Applied Nutrition Programme (ANP)** is a semi-autonomous unit in the Department of Food Science, Nutrition and Technology of the University of Nairobi. Before 2001, ANP was the only programme offering Applied Human Nutrition at Master’s degree level in Africa. It provides nutrition training at postgraduate degree level to international students, mainly from Africa, and has so far overseen the implementation of over 200 original student research projects in various countries. ANP has enormous experience in conducting capacity building in form of short courses for Government Ministries, development partners and communities. ANP has been involved with partners in other development ventures that have included development of curricula, training/teaching manuals and handbooks (e.g. with Kenyatta University, SOMANet and NECTAR Programme/Wageningen University). In addition, the ANP is involved in transfer of technology from the experimental to utilization at the household level in form of inter-departmental efforts and with Kenya Medical Research Institute (KEMRI).

**Dr. Alice Mboganie Mwangi** is Head of the ANP and has strong training, research and data analysis skills, especially relating to designing and implementing food security and consumption assessment studies, dissemination of results, designing and implementing Nutrition Programmes including community capacity building programmes as well as training (community) nutritionists. Dr. Mwangi is the Principal Investigator for the project “Improving micronutrient and nutritional status through gardening and school feeding programmes in urban and peri-urban areas of Nairobi”. She is the National Chairperson for the Kenya Coalition for Action in Nutrition.

**Dr Pauline Andang’o** is a nutritionist and lecturer at Maseno University who has been seconded to the ANP for this project because of her research expertise with iron fortification in coastal Kenya. Both Dr Mboganie Mwangi and Dr. Andang’o will provide technical and
administrative support to **Mr. Martin N Mwangi** (not related to Dr A. Mboganie Mwangi), the field coordinator, who is employed at Maseno University and registered for his PhD degree at Wageningen University, The Netherlands.

The **London School of Hygiene and Tropical Medicine** is an internationally recognized centre of excellence in public health, international health and tropical medicine with a remarkable depth and breadth of expertise. It is one of the highest-rated research institutions in the UK. The School provides a national and international focus for collaboration in teaching and research where clinical, population, laboratory and social sciences are integrated to address the broad issues of health. LSHTM currently employs over 800 staff from 37 nationalities. It is a research-led institution but also places emphasis on achieving excellence in its London-based and distance-learning Master’s programmes. The MRC International Nutrition Group is based within LSHTM’s Nutrition and Public Health Intervention Research Unit, and is led by Professor Andrew Prentice.

**Dr. Hans Verhoef** is a clinical epidemiologist with a research interest in micronutrient malnutrition and infectious diseases, particularly malaria. He will provide first-line academic supervision to Mr. Mwangi. Dr Verhoef also holds an appointment at **Wageningen University**, where he has previously conducted and coordinated studies on iron deficiency and malaria in Kenya and Tanzania.

**Drs Cox, Ghattas, Melse-Boonstra and Professors Savelkoul and Zimmermann** will advise on nutritional, medical, ethical and laboratory issues.

**Selected publications:**

9.7 Post-trial care
Immediately after delivery, participating mothers will receive a therapeutic course of artemether-lumefantrin (WHO 2002, 2006) to eliminate *Plasmodium*-associated effects on plasma indicators of iron status. All women will also receive a therapeutic dose of praziquantel and albendazole.

Remunerations: There will be no individual monetary compensation for participation, and no other compensation except financial compensation for transport of parturient women to hospital, or perhaps a small, token gift to participating women as a sign of appreciation (e.g. an impregnated mosquito net). Community volunteers will receive an allowance to compensate for time spent for the project.

9.8 Contribution to local capacity building
The proposed trial will build on a long-standing scientific research partnership between institutions in Kenya, The Netherlands and the United Kingdom. Partner-specific responsibilities are listed in Table 3. Technical staff from all the local institutions will be employed in the execution of the project and the project will thereby contribute to strengthen local expertise and build local capacity. A considerable amount of the budget will be spent in Kenya. This research project will form the basis for a PhD thesis for a Kenyan student (Mr. Martin Ndegwa Mwangi), who is appointed as a Tutorial Fellow at Maseno University and registered for his PhD degree with Wageningen University. Dr Alice Mboganie Mwangi (University of Nairobi) and Dr Pauline Andang’o (Maseno University), who are both alumni of Wageningen University, will be fully involved in all aspects of the study (design, implementation, interpretation of results, preparation and review of scientific manuscripts) and will provide locally supervision of the PhD candidate. The project will also provide opportunities for at least 2-3 MSc research projects from the University of Nairobi and/or Maseno University.

Relevance to local research and policies:
As foreseen, this study will:
- Enhance our current understanding on the extent of iron deficiency anaemia in western Kenya;
- Strengthen national fortification programme by the formulation of guidelines for small-scale flour fortification with iron;
- Guide national policies for flour fortification and the control of iron deficiency anaemia in Africa by providing evidence on the safety and efficacy of combined iron fortification and supplementation;
- Enhance partnership between the institutions involved.

**Table 3: Partner-specific responsibilities for the study**

<table>
<thead>
<tr>
<th>Partner</th>
<th>Primary responsibilities</th>
</tr>
</thead>
<tbody>
<tr>
<td>University of Nairobi, Applied Nutrition Programme, Kenya</td>
<td>Provision of personnel and local research infrastructure; field supervision and setting up local infrastructure at the research site; supervision of laboratory analyses in Kenya</td>
</tr>
<tr>
<td>Wageningen University, Division of Human Nutrition and Cell Biology and Immunology Group, The Netherlands</td>
<td>General academic and administrative supervision of the PhD fellow; support in human nutrition, particularly dietary assessment; laboratory support for measuring biochemical and immunological indicators</td>
</tr>
<tr>
<td>London School of Hygiene and Tropical Medicine, United Kingdom</td>
<td>Conception of the study; supervision of immunological and parasitological aspects; nutritional and epidemiological support; sharing medical and clinical expertise</td>
</tr>
</tbody>
</table>
9.9 Audits and inspections
The study may be subject to audit by the London School of Hygiene & Tropical Medicine under their remit as sponsor. LSHTM, the Study Coordination Centre and other regulatory bodies will assess and ensure adherence to GCP.

9.10 Dissemination of results and publication policy
Results will be reported in peer-reviewed international journals according to established guidelines about authorship (International Committee Of Medical Journal Editors 1997; Davidoff et al. 2001) and reporting of randomised controlled trials (Altman et al. 2001, Moher et al. 2001). All publications and presentations relating to the study will be authorised by the Principal Investigator. If there are named authors, these will include at least the trial's Trial Manager. The Trial Monitor and the Data Safety Monitor will be listed and contributors will be cited by name if published in a journal where this does not conflict with the journal’s policy. In addition, research results will be shared through a workshop with invited participants from international organisations and forums for advocacy and technical support (e.g. Flour Fortification Initiative, World Health Organization, Unicef, Micronutrient Initiative), national policy makers (e.g. Kenyan Ministry of Health and Ministry of Public Health and Sanitation; National Food Fortification Alliance), and local research institutions (e.g. KEMRI).

10 References
Allen LH. Anemia and iron deficiency: effects on pregnancy outcome. Am J Clin Nutr 2000;71(suppl):1280S–84S.


Annex 1. Informed consent materials

1. Information about the study

[To be translated into Dholuo; to be verbally administered to those unable to read]

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?
We want to conduct a study to test the safety of two methods to control anaemia in pregnant women. In anaemia, blood appears light red instead of a healthy dark red. A pregnant woman with anaemia is at a higher risk for delivering her baby too early, her baby may be too small, and she may die herself during childbirth. To prevent anaemia, the Kenyan government has long advised pregnant women to take iron supplements. In addition, the government is now changing the law so that all flour that is sold in shops must contain iron that is added during the milling process. If these plans go ahead, pregnant women will receive additional iron through supplementation as well as through food. Although iron is good to prevent anaemia, we are also concerned that too much iron may increase the risk of malaria. We don’t know if it is better to receive iron fortified foods only, or whether it is best to also take iron supplements. To find out, we need to compare different treatments. We put women into two groups and give each group a different treatment. The results are compared to see if one is better.

Do you have to participate?
We have asked you to take part in our study because you may be pregnant. In total, we want to study 450 pregnant women. You may decide yourself if you want to participate. We will describe the study and go through this information sheet, which we will then give to you. We will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any moment, without giving a reason. This will not affect the normal care you receive in clinics or hospital elsewhere.

What will happen to you if you take part?
We will ask you questions and carry out a medical examination to decide whether you can participate in the study. This will take several hours. We will collect a urine sample. If your pregnancy is confirmed, we will give medicine against schistosomiasis and hookworm. This will help fight anaemia, and is recommended as safe by the World Health Organization.

Two weeks later, we will collect a blood sample. We will also offer you the possibility of HIV testing. This is not compulsory, but we encourage you to do this test because knowledge of your HIV status helps to maintain your health and to reduce the risk of transmission to your baby.

You cannot participate in the study if the medical examination indicates that you are not fit, if your pregnancy is too advanced (exceeding 20 weeks), or if you indicate that you do not want to deliver in due time in a pre-designated hospital. Once we have decided that you can take part, we will start the supplementation. Also from then on, when your family members bring grain for milling, local posho millers will add iron to the flour. This will continue until one month after delivery. During this period, we ask you not to accept flour from other families or
shops, and not to share your flour with other families. We will monitor the fortification process at the posho mill. We also ask you to ensure that the miller adds iron fortificant during the milling process, and to inform us when there are problems. Your cooperation in the implementation of the study is highly appreciated.

Approximately half of all participating women will receive daily supplements with iron, and half will receive supplements that look the same, but they do not contain iron. This will be decided by chance (randomly). We will not know which supplements contain iron until after the study.

The first supplement will be given at the research dispensary; approximately 3 hours later, we will collect another blood sample. From then on, community volunteers will daily supervise the supplementation in or close to your homestead.

You should take the study medication regularly as directed, but we ask you to not take iron supplements from other clinics or shops. We will not provide medical care during the follow-up period: for any condition, or when you become sick, you should seek medical care from the nearest health services. You can continue to take your regular medication or other prescribed medicines. We may occasionally visit you to check if all is going well. We encourage you to visit us, however, if you have questions about the study. We will ask you to deliver in a hospital, and we will pay for your transport to the hospital where you will deliver. Shortly before delivery, we will meet with you to discuss the procedures that you should follow. The hospital staff will help you to deliver your baby free of charge. Shortly before the delivery, we will collect a blood sample from you, and immediately after delivery, we will collect additional blood samples from the placenta and the umbilical cord. At 1 month after deliver, we will collect a last blood sample. In these blood samples, we will measure anaemia, nutrient status and the presence of malaria parasites.

So in total, we will collect four blood samples from you during the study, each with a volume equal to one table spoon. At 1 month after delivery, we will also collect one small blood sample from your baby’s foot. We may take some of the blood samples abroad, where we can do special tests in specialised laboratories.

Please note that any information that you share with us will be seen only by members of the research team. All personal information will be stored securely. This means that whenever we write or talk with outsiders about anything we have been told, we never use your real name. The only information that we may have to pass on is if you or your child is at risk of serious harm.
2. Informed consent form (first screening meeting)

I have attended a meeting where I was informed about the aims and procedures of this study. I also read the information sheet about this study, or someone has read the information sheet to me. I understand why the study is being done and what I have to do to participate.

I understand that my blood and a placenta tissue sample will be used for laboratory tests. I consent to these samples being retained for unspecified use during or after the conclusion of the research project. I consent to collection of a blood sample from my newborn child at 1 month after birth. I also consent to my blood being analysed abroad.

I know that all my personal information will remain confidential.

I know that I am doing this by choice, and that I do not have to take part in this study. I understand that I can withdraw at any moment from the study, without providing reasons and without affecting the care I am usually given at local health centres.

I have asked all the questions that I wanted to ask, and they have all been answered. I know that I can ask any other questions as the study proceeds.

I agree to take part in this study.

Signature or thumb print of volunteer: __________________________

Nurse/fieldworker statement

This form has been read by / I have read the above to __________________________

(write name of volunteer)

in a language that she understands. I believe that she has understood what I have explained and she has made the free choice to participate in this study.

Signature of nurse/fieldworker: __________________________

Name of nurse/fieldworker: __________________________

Date: __ __ / __ __ / _
3. Informed consent form for HIV testing

I have asked all the questions that I wanted to ask about HIV testing, and they have all been answered.

I understand that testing is voluntary. I can decide if you want us to do such a test.

I understand that several testing options are available; whichever option I choose will not affect my chances of getting into the study. I choose the following option (tick only one of the following boxes):

☐ I agree to testing for HIV, and I want to be informed about the test result. Only the study physician and I will know the test result. The study physician will talk with me about notifying my husband or partner of possible exposure, if I test positive;

☐ I agree to testing for HIV, and I not want to be informed about the test result. I understand that no-one on the field team, not even the study physician, will be able to know my test result. However, if I change my mind later on in the study, I will be given an opportunity to either be re-tested or to have my test results provided.

☐ I do not want to be tested for HIV.

Signature or thumb print of volunteer: __________________________

Nurse/fieldworker statement

This form has been read by / I have read the above to __________________________ (write name of volunteer)

in a language that she understands. I believe that she has understood what I have explained and she has made the free choice to participate in this study.

Signature of nurse/fieldworker: __________________________

Name of nurse/fieldworker: __________________________

Date: _ _ / _ _ / _
Annex 2. Flow chart for reporting adverse events

Adverse event – is it serious?

- Yes
  - Is it expected?
    - No
      - Adverse Event (AE)
        - Does not require expedited reporting
        - Describe on Case Report Forms
    - Yes
      - Serious Adverse Event (SAE)
        - Assess severity
        - Complete SAE form and report to PI within 24 hours
        - To go on annual safety report

- No
  - Can it causally be related to the iron intervention or trial medication?
    - Yes
      - Suspected Unexpected Serious Adverse Reaction (SUSAR)
        - Report to CI within 24 hours
        - Requires expedited reporting to LSHTM and KNH-UoN Ethics Committees
    - No
      - Life-threatening or fatal?
        - Yes
          - Report to LSHTM and KNH-UoN Ethics Committees within 7 days
        - No
          - Report to LSHTM and KNH-UoN Ethics Committees within 15 days
ANNEX 3. Procedure to assess levels of iron as NaFeEDTA in flour

The Red Spot Test, which is widely used to detect elemental iron in flour, is not suitable to determine the level of iron as NaFeEDTA. The readily-applicable, colorimetric method described below has been developed by AkzoNobel, and is suitable for use in local flour mills. It is a modification of the well-established procedure to determine the level of iron in aqueous solutions using the iron(II)-complexing agent phenanthroline.

First, flour (any type) is extracted for several minutes with plain water or a mixture of water and methanol. This should dissolve 99%–100% of all FeNa-EDTA, but only 0%–1% of other formulations iron that are used for flour fortification, i.e. anhydrous FeSO₄, ferrous fumarate or electrolytically reduced iron. It is essential that all flour is subsequently separated from the aqueous solution that is used in this extraction step. This can be done by either centrifuging or filtration. To speed up the filtration process, it is recommendable to add 10% to 20% (v/v) of methanol. This addition of methanol does not affect the dissolution of NaFeEDTA, whilst the addition of methanol is not needed for centrifuging.

Then a buffer solution, a reducing agent and phenanthroline is added. A suitable buffer is one that is based on sodium or ammonium acetate, reducing agents that are suitable for this purpose can be either hydroxylamine or ascorbic acid. The former remains stable over a longer period of time as an aqueous solution, whereas the latter has the advantage of total absence of any toxicological concern. Phenanthroline is a well-known analytical reagent that is readily available all over the world, is highly stable when stored, and does not pose any specific health risks.

Maximally one hour after these compounds are added, an intensely orange colour is formed, indicating the presence of ferrous ions. The intensity of the colour is proportional to the level of NaFeEDTA in the flour. The limit of detection of was estimated to be about 2 ppm iron as NaFeEDTA. The level of iron as FeNa-EDTA can be determined unambiguously either by visual inspection through comparison of calibration samples, or by measurement in a spectrophotometer. By contrast, if fortificant iron were present in flour as anhydrous FeSO₄, ferrous fumarate or electrolytic iron, this would not generate any colour at all.

The procedure described above has been tested in several different flour types in AkzoNobel's laboratory in Arnhem, The Netherlands. The results thus obtained should be considered to be semi-quantitative, but they allow beyond any doubt to discriminate between 0, 5, 10, 15, 20 and 25 ppm iron as FeNa-EDTA added to flour.

It should be possible to apply this straightforward method under field conditions in local flour mills, so that these mills can conduct its own quality assurance programme and ensure that correct levels of iron as FeNa-EDTA have been attained in flour and premix, whether or not the iron as NaFeEDTA has been added to a premix also containing vitamins and minerals other than iron as NaFeEDTA.

Premix manufacturers determine the correct level of all micronutrients at their production facilities using relatively sophisticated equipment and methods that are not available or feasible in a local flour mill. If NaFeEDTA has been added to the premix, and the levels of the vitamins and minerals in this premix have been confirmed by the premix manufacturer to be correct, then the procedure described above will not only ensure that the levels of iron as NaFeEDTA in blended flour at the local mill are correct, but also that the levels of other micronutrients are correct (under the reasonable assumption that these other micronutrients do not separate from NaFeEDTA during the process of blending the premix with the flour).
ANNEX 5. Planned study area (Kisumu District, Nyanza Province)

Study area (may be expanded westwards if the population size in the currently foreseen area is insufficient)
### ANNEX 6. Estimated intake of iron and EDTA by population group, with fortification levels of 20 mg iron as EDTA/kg flour

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean body weight, kg</th>
<th>Dietary requirements, mg d⁻¹</th>
<th>Assumed flour intake, g</th>
<th>Fortificant iron intake</th>
<th>EDTA intake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A)</td>
<td>(B)</td>
<td>(C)</td>
<td>(D)</td>
<td>(E)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mg d⁻¹)</td>
<td></td>
<td>(mg d⁻¹)</td>
<td>% of RNI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% of UL</td>
<td></td>
<td>% of ADI</td>
<td>% of ADI</td>
</tr>
<tr>
<td>Infants 0.5-1 y</td>
<td>9</td>
<td>9.3</td>
<td>40</td>
<td>60</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Children 1-3 y</td>
<td>13</td>
<td>5.8</td>
<td>40</td>
<td>100</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Children 4-6 y</td>
<td>19</td>
<td>6.3</td>
<td>40</td>
<td>125</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Children 7-10 y</td>
<td>28</td>
<td>8.9</td>
<td>40</td>
<td>175</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Men, 11-14 y</td>
<td>45</td>
<td>14.6</td>
<td>40</td>
<td>200</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Men, 15-17 y</td>
<td>64</td>
<td>18.8</td>
<td>45</td>
<td>300</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>Men, 18-64</td>
<td>75</td>
<td>13.7</td>
<td>45</td>
<td>400</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Women, 11-14 y, premenarche</td>
<td>46</td>
<td>14.0</td>
<td>40</td>
<td>200</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Women, 11-14 y</td>
<td>46</td>
<td>32.7</td>
<td>40</td>
<td>200</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Women, 15-17 y</td>
<td>56</td>
<td>31.0</td>
<td>45</td>
<td>300</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>Women, 18- menopausal</td>
<td>62</td>
<td>29.4</td>
<td>45</td>
<td>400</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Pregnant women</td>
<td>62</td>
<td>33.8</td>
<td>45</td>
<td>450</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Lactating women</td>
<td>62</td>
<td>15.0</td>
<td>45</td>
<td>450</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Women, postmenopausal</td>
<td>62</td>
<td>11.3</td>
<td>45</td>
<td>200</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9</td>
</tr>
</tbody>
</table>

RNI: Recommended Nutrient Intake; UL: tolerable Upper intake Level; PMTDI: provisional maximum tolerable daily intake; ADI: maximum level of the Acceptable Daily Intake.  

a WHO/FAO (2004); b Assuming an iron absorption of 10%; c IOM (2001); d Calculated from absorbed iron requirements for 50- and 95-percentiles as reported by IOM (2001), but assuming an iron absorption of 10%. Computations: (E) = [(D) × 40 mg/kg]/1,000; (F) = [(E) × 100]/(B); (G) = [(E) × 100]/(C); (H) = [(E) × 5.23]/(A), with 5.23 being the ratio of the molecular weights of EDTA to iron; (I) = [(H) × 100]/1.9, with 1.9 mg kg⁻¹ d⁻¹ being the maximum level of ADI for EDTA (JECFA 2008)
ANNEX 7. Curriculum vitae, Principal Investigator

Hans Verhoef, PhD

CONTACT DETAILS

Wageningen University/London School of Hygiene and Tropical Medicine, Department of Nutrition and Public Health Intervention Research, Keppel Street 281, London WC1E 7HT, England. Mobile +255 787 882596; Tel +44 20 79588140; Fax + 44 20 79588111; e-mail: hans.verhoef@wur.nl

ACADEMIC TRAINING

- **MSc degree, Clinical Epidemiology** (course work completed), Erasmus University, Rotterdam, The Netherlands.
- **PhD degree in Nutrition, Food Technology and Biotechnology** (2001), Wageningen University, The Netherlands (Thesis: 'Iron supplementation and malaria as determinants of anaemia in African children')
- **Certificate, Human Nutrition** (2001), Graduate School VLAG, Wageningen, The Netherlands
- **MSc degree in Agricultural and Environmental Sciences** (1988), Wageningen University, The Netherlands

WORK EXPERIENCE

- **June 2008-present**: Senior Lecturer (0.7 fte), London School of Hygiene and Tropical Medicine, Nutrition and Public Health Intervention Research Unit, UK
- **Senior Researcher** (0.3 fte), Wageningen University, Cell Biology and Immunology Group, The Netherlands
- **2004-2008**: Senior Researcher, Wageningen University, Cell Biology and Immunology Group, The Netherlands, based at the Kilimanjaro Christian Medical Centre, Moshi, Tanzania
- **2001-2004**: Researcher, Wageningen University, Division of Human Nutrition
- **1996-2001**: PhD fellow, Wageningen University, Division of Human Nutrition, based in Kenya
- **1995-1996**: Researcher, Wageningen University, Division of Human Nutrition
- **1994-1996**: Consultant, vector-borne diseases (various projects, mostly western Africa)
- **1988-1991**: Medical Entomologist, World Health Organization, Honiara, Solomon Islands

SELECTED PUBLICATIONS: