Comparison of home fortification with two iron formulations: A placebo-controlled non-inferiority trial among Kenyan children protected against malaria by a single course of artemisinin-based combination therapy

By
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Declaration

I, *Emily M Teshome* wish to confirm that this thesis is my own and has not been produced elsewhere. I have acknowledged all citations made herein and any information sourced from elsewhere I have duly indicated the source. In addition, I have obtained the necessary permits from publishers for publishing my papers.

Abstract

The objectives of this study were to: 1) show non-inferiority of home-fortification with a daily dose of 3mg iron as NaFeEDTA compared with 12.5 mg iron as encapsulated ferrous fumarate. 2) Assess to what extent adherence measured by sachet count or self-reporting forms agrees with adherence measured by MEMS electronic device. 3) Assess the diagnostic performance of zinc protoporphyrin either alone or combined with haemoglobin concentration in children.

Methods: We gave chemoprevention by dihydroartemisinin-piperaquine, albendazole and praziquantel to 338 afebrile children with haemoglobin concentration ≥70 g/L. We randomly allocated them to daily home-fortification for 30 days with either placebo, 3mg iron as NaFeEDTA, or 12.5 mg iron as encapsulated ferrous fumarate. Each child received 30 sachets of micronutrient powders in a MEMS device, a self-reporting form and requested to store empty sachets. At baseline and after 30 days of intervention, haemoglobin concentration, plasma iron markers, plasma inflammation markers, Plasmodium infection in blood samples and adherence to home-fortification were assessed.

Results: Home-fortification with either of the iron interventions did not improved haemoglobin concentration, plasma ferritin concentration and plasma transferrin receptor concentration. Both self-reporting and sachet counts confirmed over-estimation in adherence measurements when compared to MEMS device. Addition of whole blood ZPP or erythrocyte ZPP to haemoglobin concentration increased the area-under-the-ROC-curve.

Conclusions: Daily home-fortification with either 3 mg iron as NaFeEDTA or 12.5 mg iron as encapsulated ferrous fumarate was not efficacious. This failure precluded further assessment of the non-inferiority of 3 mg iron as NaFeEDTA compared to 12.5 mg iron as encapsulated ferrous fumarate. Self-reporting and sachet counts are less accurate in measuring daily home-fortification with micronutrient powders compared to the MEMS device. In children, whole blood ZPP and erythrocyte ZPP combined with haemoglobin concentration have added diagnostic value in detecting iron deficiency compared to haemoglobin concentration alone.

Dedicated to

My daughter Selamawit Teshome, you are the best ever!

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Preface

This thesis has been compiled to address research gaps that are of public health concern. No study has so far compared the efficacy of daily home fortification with 12.5 mg iron as ferrous fumarate versus 3mg iron as NaFeEDTA. Information regarding accuracy in measuring adherence to home fortification powders by self-reporting and sachet counts outside a gold standard is wanting. The following chapters outline procedures, findings and conclusions;

Chapter 1: Provides background information on home-fortification with iron, issues guiding the rationale of the study, study population and study area profile, problem statement, conceptual framework, objectives and description of study endpoints and procedures for ethical approval.

Chapter 2: Details the study protocol, including the design and its rationale, sample size determination and procedures for participants' recruitment, randomization, blinding, interventions, statistical analysis and data safety monitoring. The discussion section presents arguments for and against the use of placebos and use of multiplicity in a non-inferiority study. *Published by Contemporary Clinical Trials*)

Chapter 3: This research paper covers the main aim of this study and examines the efficacy of 3mg iron as NaFeEDTA and12.5 mg iron as encapsulated ferrous fumarate in young children protected against malaria by chemoprevention. Methods and statistical analysis to achieve this objective are detailed. The paper also provides results of a meta-analysis showing the effect of iron treatment on haemoglobin concentration. Greater focus is on the discussion section that explains possible reasons for lack of efficacy. *Published by BMC-Medicine*)

Chapter 4: This research paper examines the accuracy of measuring adherence to daily home fortification of micronutrient powders using self-reporting forms and sachet count method and gives reasons why self-reporting forms and sachet count methods should not be used alone but alongside a gold standard method (MEMS electronic device) when assessing adherence to daily home-fortification in children (*Submitted for publication*)

Chapter 5: Provides information about factors associated with zinc protoporphyrin (ZPP) in children aged 1-3 years and also examines the diagnostic performance and utility of ZPP, either alone or in combination with haemoglobin, in the identification of iron deficiency (*submitted for publication*).

Chapter 6: A summary of discussions and public health implications, study limitations and recommendations for further research are presented here.

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Acronyms

ACT	Artemisinin-based Combination Therapy
ADI	Acceptable Daily Intake
AGP	Alpha$_1$-acid glycoprotein
AIDS	Acquired Immuno Deficiency Syndrome
AUC	Area Under the Curve
CDC	US Centres for Disease and Control and Prevention
CI	Confidence Interval
IOMS	Council for International Organizations of Medical Sciences
CRA	Commission on Revenue Allocation
CRP	C-reactive protein
DP	Dihydroartemisinin-Piperaquine
EDTA	Ethylene-Diamine-Tetraacetic Acid
EMA	European Medicine Agency
FDA	Food and Drug Agency
FOB	Faecal Occult Blood
HAZ	Height for Age Z-score
HF-TAG	Home Fortification Technical Advisory Group
HIV	Human Immunodeficiency Virus
HRP2	Histidine-Rich Protein-2
ICH	International Conference of Harmonisation
IPT	Intermittent Preventive Treatment
LSHTM	London School of Hygiene and Tropical Medicine
MEMS	Medication Events Monitoring System
MIC	Minimal Inhibitory Concentration
MOH	Ministry of Health
NaFeEDTA	Sodium Iron Ethylene-Diamine-Tetraacetic Acid
NTBI	Non-Transferrin Bound Iron
pLDH	*Plasmodium falciparum*-specific Lactate Hydrogenase
RDI	Recommended Daily Intake
RDT	Rapid Diagnostic Tests
ROC	Receiver Operating Characteristics curve
SEICK	Safe and Efficacious Iron in Children of Kenyan
SOP	Standard Operating Procedure
SP	Sulfadoxine-Pyrimethamine
TFR	Soluble Transferrin Receptor
WAZ	Weight for Age Z-score
WHZ	Weight for Height Z-score
WHO	World Health Organization
UNICEF	United Nations Children's Fund
USAID	United States Agency for International Development
ZPP	Zinc Protoporphyrin

Chapter 1: General introduction

1.1 Background of iron fortification powders

Iron fortification is one of the recommended iron intervention strategies aimed at providing additional iron to vulnerable target groups in an appropriate amount regardless of how much food they consume for improving their iron status. Other iron interventions may be administered as either a) iron supplementation pills, syrups, iron drops or capsules which supply an optimal amount of iron in a highly absorbable form and have been suggested to be a fast way to control iron deficiencies [1] or b) iron added to staple foods, infant formulas, sauces and other condiments during processing [2].

In 1995, the United States Agency for International Development (USAID) and the Institute of Child Health held a meeting in London having recognised that children 6-24 months old who are most at risk of iron deficiency had difficulties in swallowing prescribed iron supplemental tablets [3]. In addition, lack of low-cost generic prophylactic iron supplements suitable for them compelled UNICEF in collaboration with World Health Organisation (WHO), USAID, pharmaceutical industry and international experts to convene a consultation meeting in 1996 with the goal of identifying the most appropriate formulation and regimen suitable for this age category of children [3]. They also looked at the possibility of including other micronutrients that can easily be combined with iron in the formulation. The meeting concluded that coated or microencapsulated iron with other vitamins and minerals sprinkled on the child's semi-solid, ready prepared foods was the most appropriate solution [3]. A formulation comprising of 15 micronutrients, including iron has been evaluated and is currently in use (http://www.hftag.org/). To meet specific micronutrient needs for iron deficient children, WHO recommended universal home fortification with 12.5mg iron as ferrous fumarate for children aged 6-23 months in populations where the prevalence of anaemia in children under 5 years of age is ≥20% alongside the control of malaria in malaria-endemic areas [4]. The formulation also contains vitamin A and zinc whose deficiencies develop concurrently with iron deficiency. Follow-up trials examining therapeutic effects of the WHO formulation resulted in rapid reduction of iron deficiencies and anaemia in infants and young children [4] and were more cost-effective when compared to electrolyte iron supplementation [5].

1.2 Anaemia in pre-school children: the global perspective

Iron deficiency is a public health problem and the most common nutrition deficiency all over the world [6-8]. It also has a direct consequent effect on anaemia [9]. Other risk factors for anaemia include micronutrient deficiencies (folate, copper, zinc, vitamin A and vitamin B12), infections (malaria, intestinal parasites, schistosomiasis,

human immunodeficiency virus and inherited blood disorders (e.g., sickle cell, a-thalassemia) [10]. Globally, WHO estimates that slightly fewer than fifty per cent of pre-school children are anaemic [11] but Africa has the highest prevalence (64%) of anaemic pre-school children [12], compared to Asia, Latin America and four times higher than Europe (Figure 1). In sub-Saharan Africa, paediatric anaemia is a major public health problem especially in malaria endemic areas; hospital based statistics show that up to 75% of children are estimated to be anaemic mainly due to malaria and iron deficiency [13].

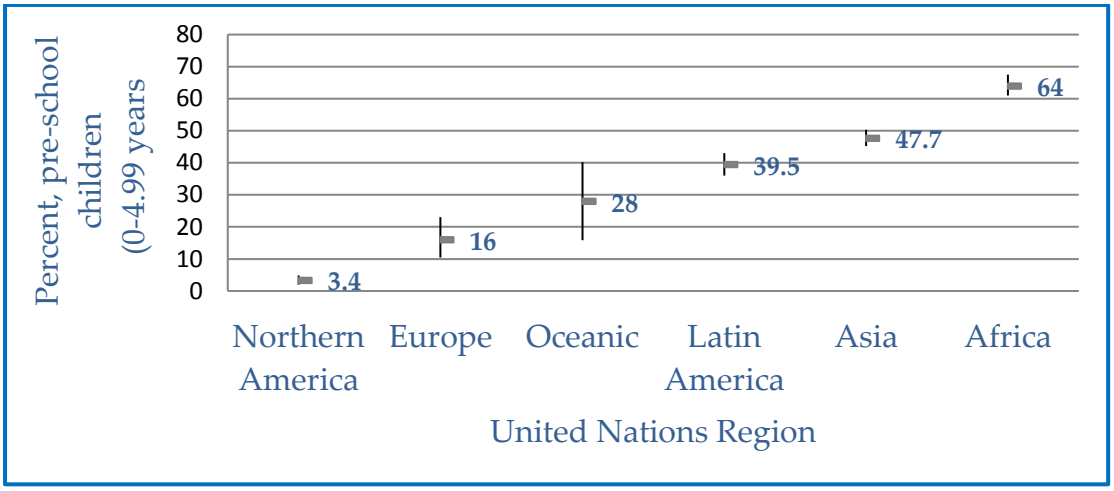

Figure 1: Global prevalence of anaemia in preschool children 1993-2005

(Adapted from: WHO/CDC, 2008).

1.3 Effect of iron interventions on infections and anaemia

Iron interventions are essential for reducing iron deficiency and enhancing growth and cognitive development in children. Trials have shown, however, that supplemental iron plays a major role in proliferation of bacteria, malaria parasites and most pathogenic micro-organisms in the body [14,15]. In sub-Saharan Africa, the most common parasitic infections among young children are malaria, schistosomiasis and soil-transmitted helminthic infections. Randomised trials in young children in Africa and Asia showed that home fortification with the recommended dose of 12.5mg iron as ferrous fumarate, although shown to reduce iron deficiency, led to an excess burden of diarrhoea, and increased numbers of potentially pathogenic enterobacteria, with a concurrent increase in gut inflammation [14-16]. Also, there is substantial evidence that iron interventions in young children can also increase proliferation of *P. falciparum* [4], which has been associated with haemolysis and dyserythropoiesis. This can be explained by: a) an increase in the destruction of the red cells; b) a high level of parasitaemia rosetting (clumping together of uninfected cells around infected cells); c) cyto-adherence; and

d) increased clearance of both infected and uninfected erythrocytes [17] which explains the fall of haemoglobin levels during and following an episode of malaria [11].. Infections can result in a) retention of intracellular iron; b) decreased blood transport of iron because of reduced transferrin; and c) elevated hepcidin production by hepatocytes, which may inhibit iron absorption needed for erythropoiesis, thus making less iron available for haemoglobin formation. This reduction of iron absorption through infection -induced inflammation has been shown in a study conducted among 18-36 months old Gambian children that showed significant inhibition of oral iron in children with malarial infection when compared with those with iron deficiency anaemia but a reverse of iron absorption was noted two weeks after treatment of malaria infection [18] .

Among WHO regions, sub-Saharan Africa carries the largest disease burden caused by co-existence of P. *falciparum* and helminth species (roundworms, *Ascaris lumbricoides*; hookworms, *Ancyclostoma duodenale* and *Necator americanus*; and whipworms, *Trichuris trichiuria*) [19]. Their growth and development is influenced by environmental factors such as favourable climate that determines survival of mosquitoes and free-living stages of helminths [20]. Resource-poor communities where the prevalence of soil-transmitted helminths exceeds 20%, such as the shores of Lake Victoria in Kenya [21], are apparently the same areas where the prevalence of *P. falciparum* infection is high, estimated at ~44% [22]. Co-infection of *P. falciparum* and soil-transmitted helminths increases the risk of low haemoglobin concentrations and a high prevalence of anaemia among pre-school and school children [23]. Also, the risk of developing falciparum malaria has been shown to be more specifically linked to increased number of helminths in the body [24].

Furthermore, co-infection of malaria parasites with bacteria can aggravate the severity of anaemia. For example, a trial conducted in Thailand among 169 patients with cerebral malaria showed that 94% of cases were anaemic (haematocrit <35%) and the severity of anaemia was worse among patients with bacterial infections [25]. In this trial and as recommended by WHO we took measures to control these infections among the study participants prior iron interventions.

1.4 Issues guiding rationale for study design

1.4.1 Pharmanutrient approaches in integrated iron intervention strategies

Iron fortification of industrially produced foods is feasible, but has limited impact in rural areas, where farmer families rely on subsistence farming. Bio-fortification entails the breeding of staple crops with high levels of micronutrients in their edible parts, but it remains to be proven if this can effectively raise the supply of bioavailable iron in African conditions. Dietary modification comprises soaking, germination and other measures to reduce the content of iron absorption inhibitors that occur naturally in cereals [26]. Whilst these interventions are useful, they have

limited efficacy, require behaviour change and are difficult to scale up in public health programmes. Thus pharmanutrient interventions remain an essential element in the control of iron deficiency anaemia of young children. Home fortification of foods with powders containing micronutrients are currently recommended as a method to increase the intake of vitamins and minerals in children, alternatively or complementary to other interventions such as oral iron supplementation.

1.4.2 Formulation and dosage of iron in home fortificants

Based on evidence that iron interventions increase the risk of malaria, bacterial and other parasitic infections [14-16,18,27], the dose of iron that is most commonly used (12.5 mg as ferrous salts) has been argued to be relatively high when administered in a single meal for children 6-23 months [28]. To solve this problem, it has been suggested to use home-fortification with reduced amounts of highly bioavailable iron (3 mg iron as NaFeEDTA) [29]. This is because in an acid condition such as in the stomach the EDTA moiety that chelates iron results in better iron absorption than iron as ferrous salts. A study conducted in Kenya showed that iron as NaFeEDTA is highly efficacious and an attractive choice even in areas with high phytate diet. Children with iron deficiency and anaemia benefitted more from iron fortification with NaFeEDTA when compared to placebo or fortification with electrolytic iron [30]. EDTA causes minimal organoleptic problems [31] and has the ability to deactivate food spoilage enzymes that would otherwise cause food products to become rancid, lose colour or flavour [32]. EDTA is less affected by inhibitors of iron absorption [33] and can be added to a variety of iron vehicles such as wheat cereal, maize flour and fish sauce **i**n children, however, the daily dose of iron as NaFeEDTA should not exceed 3 mg to remain within the acceptable daily intake of EDTA (0-1.9 mg kg-1 day-1) [34].

Reducing the iron dose may decrease the amount of luminal iron and thus prevent a shift towards increased risk of iron-induced malaria and a more pathogenic profile in intestinal biota composition as reported with oral iron supplementation interventions [35-36]. Also, it may result in fewer adverse effects, improved adherence and improved effectiveness. Thus, home fortification with a high dose of iron (12.5 mg) as ferrous fumarate iron or a low dose (3 mg) as NaFeEDTA may result in similar or even higher doses of absorbed iron, but the risks of acquiring the adverse events associated with such systemic diseases are likely to depend on the absorbed amount of iron hence the safety of this approach can potentially be maintained if adequate parasitic and bacterial control measures are put in place during iron intervention [4, 37].

1.4.3 Home fortification with iron and selected micronutrients

About 15 micronutrients have been recommended by the Home Fortification Technical Advisory Group, (http://www.gainhealth.org/hftag/products/micronutrient-powders-mnp). By contrast, and based on a systematic review, WHO recently recommended that multiple micronutrient powders for home fortification for younger children 6-23 months should include iron, vitamin A and zinc, and may contain other vitamins and minerals as need arises. For iron, WHO recommended a dose of 12.5 mg, preferably as encapsulated ferrous fumarate [4]. Since there is limited evidence that additional micronutrients to those recommended by WHO for anaemia treatment may bring health benefits to children in developing countries [38], it is important that home fortificants contain vitamins and minerals with demonstrated health benefits and low likelihood of harmful effects. Inclusion of vitamin A and zinc is well justified in view of strong evidence that supplementation with these micronutrients results in marked reductions in child mortality or morbidity [39, 40] although recent evidence is questioning whether vitamin A remains efficacious against altered levels of vitamin A nutritive in recent years. In addition, there is no evidence that zinc adversely affects malaria rates in African children [41], whereas vitamin A, given alone or in combination with zinc, may reduce malaria rates [42,43]

The inclusion of folic acid is controversial. Folate deficiency is not a public health problem among children in developing countries [44] and convincing evidence that folate deficiency is common in children in malaria-endemic areas is lacking. Also, folic acid supplementation has not been shown to have a beneficial effect on anaemia [45]. Furthermore, Kenyan children are no longer treated with antifolate antimalarial drugs (sulfadoxine-pyrimethamine. Thus in the studies described in this thesis, we excluded folic acid in the home fortification powder because of concerns that it may cause failure of other anti-folate drugs (e.g. contrimoxazole) [46] . **Table 1** presents the content of the 13 micronutrients contained in 1g sachet selected for this study compared to that which is recommended by Ministry of Health (Kenya) and Home Fortification Technical Advisory Group (HF-TAG). In our study we aligned the content of vitamin A, iron and zinc to suit the WHO recommendation but omitted folic acid for reasons mentioned above. But the rest of the micronutrients are the same with Ministry of Health and HF-TAG. We included 3mg iron as NaFeEDTA because these were our experimental iron and a placebo group that had all other micronutrients except iron. The placebo group was necessary to prove efficacy of 3mg iron as NaFeEDTA and to prove assay sensitivity of 12.5mg iron as encapsulated ferrous fumarate (details in Chapter 2).

Micronutrient	Study content	MOH content	HF-TAG
Vitamin A µg RE*	300	400	400
Vitamin D µg	5	5	5
Vitamin E mg	5	5	5
Vitamin C mg	30	30	30
Thiamin (vitamin B1) mg	0.5	0.5	0.5
Riboflavin (vitamin B2) mg	0.5	0.5	0.5
Niacin (vitamin B3)	6.0	6.0	6.0
Vitamin B6 (pyridoxine) mg	0.5	0.5	0.5
Vitamin B12 (cobalamine) µg	0.9	0.9	0.9
Folate µg*	0	150	Folic acid 90mcg
Iron			
EITHER iron as encapsulated ferrous fumarate, mg*	12.5	11.0	10.0
OR iron as NaFeEDTA, mg*	3	0	0
OR no iron (placebo)	0	0	0
Zinc mg*	5	4.1	4.1
Copper mg	0.56	0.56	0.56
Selenium µg	17	17	17
Iodine µg	90	90	90

Table 1: Comparisons of formulations for micronutrient powders

*Nutrient content in this study varied from Ministry of Health (MOH) and Home-Fortification Technical Advisory Group (HF-TAG)

1.4.4 Measuring adherence for home fortification powders

Better health outcomes are seen in patients who adhere to treatment than poorly adherent patients, even when that treatment is a placebo [47]. Treatment outcomes may depend on drugs and other nonspecific therapeutic effects [48]. Whereas researchers are interested in better outcomes, study participants are more interested in the impact of treatment on quality of life [49,50] because acceptance of any treatment strategy is a combination of true characteristics of a drug (efficacy, half-life and side effects) [51] and the consumer's perceived characteristics such as product presentation including taste, colour, odour and texture. Good adherence to treatment has been associated with better health outcome and so patients' non-adherence may impact negatively on the society by aggravating disease and increasing health care costs borne by the community, thus the participants' overall best interest and primary goal must be considered during adherence measurement [52]. However, adherence reporting for home fortification with micronutrients has been poor [53] because data obtained is either over-estimated masking the

suboptimal adherence level of treatment or under-reported causing it to be misinterpreted as low adherence to treatment. Most commonly used measurement tools for adherence in most trials are self-reporting or empty sachet counts (synonymous with pill counts) [54]. In this trial we considered the following factors to enhance adherence to treatment: a) participant's right to determine whether or not to accept treatment (adherence autonomy); b) researcher's responsibility to do no harm (non-maleficence); c) researchers not to divulge participant's personal information (privacy)[52]; d) use of a reliable tool for measuring adherence, the Medication Events Monitoring Systems (MEMS) which is considered a gold standard [55,56] was compared to self-reporting and sachet count to determine their accuracy.

1.4.5 Pre-medication for the control of malaria and helminths in children

Effective malaria control can be achieved using chemoprophylaxis [57] but this is not recommended for endemic areas, mostly because it can lead to loss of acquired immunity or delay its acquisition, resulting in increased malaria rates when the intervention is stopped [58]. The intermittent administration of full treatment courses of antimalarial medicines regardless of the presence or absence of infection aims to prevent malarial illness and it has been shown to substantially reduce malaria rates both in infants and older preschool children, without an evident rebound effect [59]. The protection offered by intermittent preventive treatment is mainly through a post-treatment prophylactic effect [60]. Among the four anti-malarial drugs recommended by WHO artemisinin-based combination therapy, dihydroartemisinin-piperaquine has been shown to be safe, well tolerated, have a long half-life (~30 days) and be highly effective [61-64] and it was the preferred choice of antimalarial drug for this trial.

In resource-poor communities where the prevalence of soil-transmitted helminths is greater than 20%, WHO recommends mass treatment for soil transmitted helminths for all persons including school-age children [65]. The inclusion of pre-school children in mass treatment has recently been considered because of increasing evidence that they are equally susceptible to helminthic infections in areas of high soil-transmitted helminth endemicity. Recently, these preventive treatments have been included in routine health programs such as the expanded program for immunizations [66]. Administering safe, efficacious and low-cost anti-helminthics, like praziquantel for schistosomiasis and benzimidazole derivates (albendazole and mebendazole) against soil-transmitted helminthiasis has been recommended by WHO [67]. Of the two benzimidazole antihelmintics, albendazole has been shown to be effective and to have the fewest side effects in children [68]; it reduces worm burden by >80% [65]. A trial conducted in the Philippines among children and adults showed that in areas of co-endemicity, praziquantel treatment had a benefit of reducing hookworm infection and subsequently anaemia [69]. Also, another study

showed that infected children administered with anti-helminthic drugs before anti-malarial prophylaxis had improved immunological response [70]. In this study we prioritised iron deficiency and anaemia related to malaria infection, taking into consideration other possible causes like schistosomiasis and soil transmitted helminths that will have to be addressed through mass treatment prior the iron fortification intervention.

1.4.6 Selection of study site

This study was conducted in Kisumu County located in western region of Kenya. In 2009, UNICEF's situation analysis report for Kenya indicated that, among pre-schoolers, parasitaemia is common: nearly 50% and 30% of children in western region and coastal regions respectively have *Plasmodium* infections. In addition, infections such as diarrhoea, family size and a mother's iron deficiency status were associated with a two-fold increase in childhood anaemia [71]. The situation analysis report further showed that spleen enlargement, mainly attributed to malaria, and was associated with a five-fold increase in the risk of childhood anaemia, while hookworm and *Schistosoma* infections were associated with a two-fold increase in risk of anaemia among mothers and men respectively.

Kisumu County profile

Kisumu county is located in Western Kenya and borders Lake Victoria, the largest lake in Africa and third largest in the world. The County has three districts: Kisumu East, Kisumu West and Nyando. The county records a population of 968,909 with a population density of 465 persons per sq km [72].

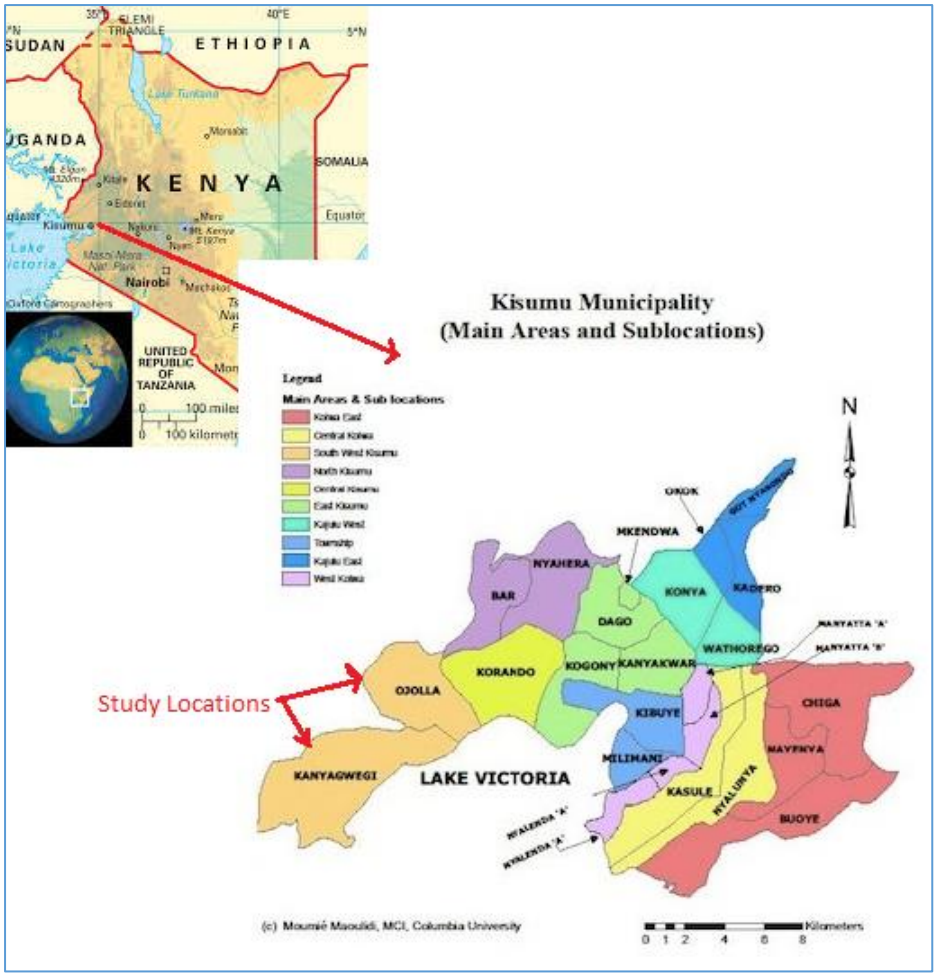

Figure 2: Map of study location

The population is predominately of a young generation with a larger proportion of the population at 30 years and below. This youthful population has led to high dependency ratio of ~1:1.2 and subsequently puts pressure on the available education, health and other social amenities [66]. The annual growth rate of the county is estimated at 2.8% [73] and 48% of the urban population live within the absolute poverty bracket nearly double the national average estimated at 29% [74]. The main causes of poverty include poor environmental conditions, low economy mostly due to unskilled workforce as most skilled workforce migrate to work in other major cities, low utilization of natural resources and socio-cultural factors. A high poverty rate in the district has contributed towards high school drop outs among the primary school population where more than 50% of the children cannot continue with their secondary school education [67]. Low education level has been associated with poor maternal and child health care practices among the *Luo* community living in the slum areas [75].

Malaria prevalence is high at 44.7% (as percent of all hospital patients) [22] compared to the average national prevalence of 27.7% [76]. Malaria is particularly rampant amongst children during the rainy season when breeding grounds for

malaria mosquitoes are ubiquitous [76]. The prevalence of *Plasmodium falciparum* infection in children aged 1-4 years has been reported to range between 39% and 63% [77]. Infant mortality stands at 75 deaths per 1,000 live births while under 5 mortality is at 105 per 1000 live births [78]. Other diseases affecting infants and young children include upper respiratory tract infections and waterborne diseases (e.g. typhoid, diarrhoea). HIV prevalence (18.7%) among the adults is the second highest in the country [79] and among infants is estimated at 10% [10]. Surveys have also shown that the area is endemic for *Schistosoma mansoni*, with a prevalence of 14% in infants [80] and infections by hookworm and *Trichuris trichiura* have contributed towards anaemia prevalence in young children [81].

1.4.7 Selection of study participants (12-36 months old children)

Children of the age category 12-36 months were selected for this trial for the following reasons. First, in full term healthy children (less than 6 months old), iron stores obtained from the mother can sustain the infant's iron requirements; later in infancy, the iron demand increases due to rapid growth and subsequent increase in blood volume and as such the need for iron increases because the iron stores are marginal [82]. Second, most infants are protected against bacterial and parasitic infections by antibodies acquired from their mothers during pregnancy and transferred by breast-milk but as they grow their immunity develops over time but their immune response to eliminate infections depends on the type of bacteria and strain that sometimes modify their pathogenicity mechanism to adapt to the host defence mechanism [83]. Consequently, severe anaemia (haemoglobin concentration <70g/L) is common in children under five years due to infections related to malaria, HIV and other parasitic infections. As such, WHO recommends praziquantel and benzimidazole antihelmintics (albendazole or mebendazole) to be administered to children aged one year and above [83]. Third, breast milk contains low levels of para-amine benzoic acid, thus proliferation of malaria parasite is reduced in exclusive breastfeeding infants (less than 6 months old) [84]. Consequently, in high malaria-endemic areas, the prevalence and density of parasitaemia reaches a peak at the age of 6–36 months, and thereafter declines with age [85]. Within this vulnerable age range, the incidence peak for severe malaria anaemia precedes that for cerebral malaria [86,87]. These children may develop the most severe response to infections because they have fewer red blood cells and it is possible that during clearance of the uninfected cells clumped to the infected cells the number of red blood cells is further reduced [88,89]. These factors either alone or combined underscore the reasons for selecting study children who are of the age category 12-36 months.

1.5 Problem statement

Generally, infants and young children's need for micronutrients increases because of their rapid growth and development. Although there are no statistics showing global

prevalence of micronutrient deficiencies, nearly 293 million children globally are estimated to have anaemia [4] either due to consumption of plant-based foods that are low in micronutrients or incapability to afford animal-rich sources of foods.

In 2011, WHO recommended home fortification with micronutrients including iron (12.5 mg, preferably as encapsulated ferrous fumarate) to improve iron status and reduce anaemia among infants and children aged 6-23 months. This recommended dose of 12.5 mg iron was established to meet almost 90 percent of the estimated total iron requirement of children aged 6-18 months [90] and in populations where the prevalence of anaemia is ≥20% in children under 5 years of age [4]. Following these recommendations, several trials conducted using home fortification with micronutrients containing iron (12.5 mg as encapsulated ferrous fumarate) showed the iron dose was efficacious in reducing the prevalence of iron deficiency anaemia. Conversely supplementation or food fortification with iron at this dose (12.5 mg iron ferrous salts) was shown to increase rates of hospital admissions [91], as well as diarrhoeal and respiratory diseases [27]. In another trial by Kortman et al. (2012), the recommended high iron dose was associated with potentially more pathogenic profile at the intestinal epithelial interface [92], leading to an increase in the faecal enterobacteria that has been correlated with gut inflammation in African children [36]. Ingestion of ferrous salts also frequently causes mild gastrointestinal adverse effects (e.g. constipation, nausea, vomiting, and epigastric discomfort) that may reduce adherence to treatment [93]. The frequency and severity of such effects depend on dose and dosage schedule [94, 95], may be due to oxidative stress [96], and appear to be reduced when iron is taken with food.

Thus an assessment of fortification with micronutrient powders containing low amounts of iron as 3 mg as NaFeEDTA was undertaken in an attempt to improve safety of iron fortification. The chelated form of iron as NaFeEDTA is highly absorbable even in high phytate meals and school children with iron deficiency and anaemia have been shown to benefit more from fortification with NaFeEDTA when compared to placebo or fortification with electrolytic iron [30]. In addition, iron as NaFeEDTA has been reported to cause less oxidative stress than an equimolar dose of iron as ferrous sulphate [97]. Thus, home fortification with a high dose of iron (12.5 mg) as ferrous fumarate when compared to a low dose of iron (3 mg) as NaFeEDTA may result in similar or higher amounts of absorbed iron [37], suggesting that it may have similar or superior efficacy in improving iron status. If this were the case, then low-dose iron as NaFeEDTA would be the preferred choice if the reduced amount of free iron in the intestinal tract is shown to be more tolerable and associated with better adherence, with less oxidative stress induced by free iron in the intestine, and with less intra-luminal iron available to promote propagation of potentially pathogenic bacteria with high iron needs. The safety of iron can be further improved when given in conjunction with measures to prevent malaria parasites and bacterial infections.

1.6 Conceptual framework

To maximise the efficacy of iron absorption (both low and high doses) and to prevent possible bacterial and parasitic infections associated with effects of iron [14-16,18,27], an iron intervention that is sustained for several weeks requires a protective effect from a long-acting anti-malarial and an anti-helminthic drug..

Administration of a curative dose of a slowly eliminated anti-malarial and anti-helminth drugs results in periods in which subsequent new parasitic infections are inhibited by persistent but declining concentrations of the drug (Figure 3 **Panel 1**). With time, blood concentrations of the drugs may fall below the minimal inhibitory concentration where parasite growth is possible, but the rate of parasite expansion is reduced because of residual drug effect. The iron doses can presumably be safely administered during the post-treatment prophylactic period, allowing for the restoration and building of iron stores. Upon withdrawal of the interventions, with time, body iron stores will become depleted and haemoglobin concentrations will start to drop (Figure 3: **Panel 2**). The duration of protection against anaemia is likely to depend on many factors, including the type of antimalarial and anti-helminth drugs, the dose and formulation of fortificant iron, duration of the iron intervention, initial iron status, and factors that influence iron absorption (e.g. inflammation and dietary intake of food compounds). A protection phase is exhibited when infections are inhibited by high concentration of the drugs and subsequently iron concentrations maintained through restoration and build-up of iron stores. To protect against malaria, participating children received a single 3-day therapeutic course of dihydroartemisinin-piperquine and to protect against severe anaemia, the children received benzimidazole anti-helminthic (albendazole) and praziquantel three days before randomization. Also, before randomization day, the medical staff conducted medical examinations on all children for parasitic and bacterial infections and treated them accordingly. On randomization day the children received the first dose of home fortificant with either iron or placebo, this was only administered after full completion of the drugs. The minimally desired re-treatment points (figure 3, **panel 2**) were determined based on the duration of protection against anaemia following the cessation of home iron fortification after 30 days.

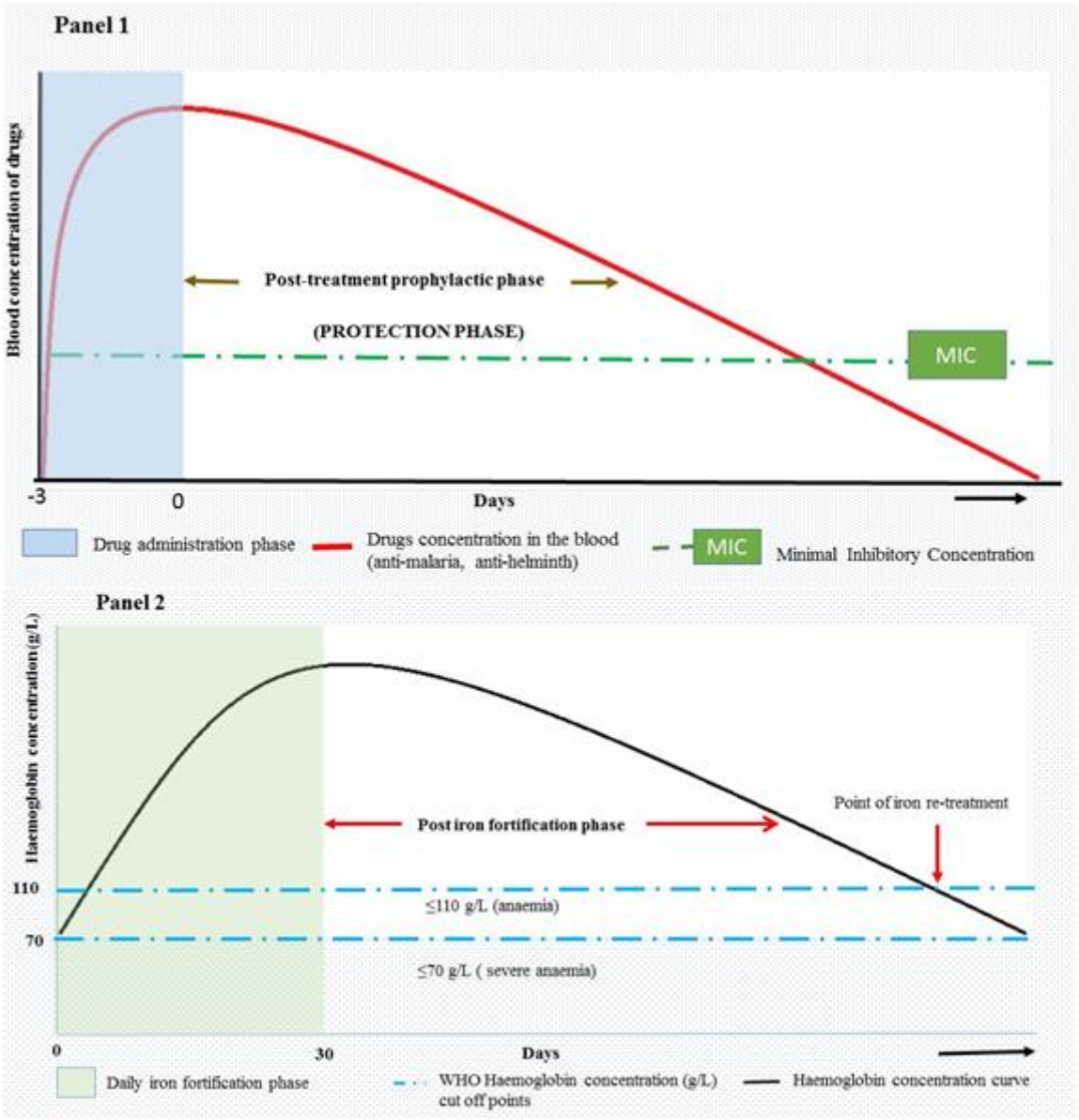

Figure 3: Theoretical framework for co-administering home iron fortification and intermittent prevention drugs.

Thus, the significance of this study is related to the importance of comparing the therapeutic effects (efficacy) of two iron formulations (3mg iron as NaFeEDTA compared with 12.5 mg iron as encapsulated ferrous fumarate) during time windows when children are protected against malaria and other parasitic infections. We anticipated that with successful drug treatments and home fortification with iron, haemoglobin concentration will substantially increase. Ideally, drug treatments and home fortification with iron should be co-administered at a frequency and with a duration that allows for the control of anaemia especially in malaria endemic areas.

1.7 Study objectives

Overall objective:
In children aged 12-36 months, to show non-inferiority of home fortification with 3mg iron as NaFeEDTA compared with 12.5 mg iron as encapsulated ferrous fumarate, with the response in haemoglobin concentration as the primary outcome.

Specific objectives

1. In children aged 12-36 months, compare daily home fortification for 30 days with two iron formulations (3mg iron as NaFeEDTA versus 12.5 mg iron as encapsulated ferrous fumarate) regarding:
   a. Haemoglobin concentration at the end of the 30-day fortification period ***(primary objective)***;
   b. Iron status at the end of the fortification period;
   c. Serum NTBI concentrations at 3 hours after ingesting the first dose of iron fortificant;
   d. Faecal calprotectin concentration at the end of the fortification period;
   e. *P. falciparum* infection at the end of the fortification period as indicated by whole blood density of asexual parasites or the presence of antigenaemia;
   f. Adherence to the daily home fortification with iron at the end of fortification period.

2. To compare daily home fortification for 30 days with iron (3mg iron as NaFeEDTA or 12.5 mg iron as encapsulated ferrous fumarate) versus placebo regarding:
   a. The indicators listed under 1a-f;
   b. Time to development of anaemia after cessation of iron fortification.

3. To evaluate the accuracy of self-reported and sachet counts in measuring adherence to home fortification and compare with medication events monitoring system as a gold standard.

4. To compare effects of iron on intestinal biota (conditional to sufficient resource being available).

5. To identify factors associated with ZPP measured in whole blood or erythrocytes from preschool children and assess the diagnostic performance and utility of ZPP (This objective was decided after field work was completed)

1.8 Study outcomes

1.8.1 Haemoglobin concentration (primary outcome)

Haemoglobin is an iron-binding metalloprotein compound. Over 300 million haemoglobin units are found in one red blood cell. The main function of haemoglobin is to transport oxygen, which binds to the iron, from the respiratory organs and release it to the body tissues where it will in turn collect carbon dioxide back to the respiratory organs for exhalation. When the body has insufficient robust red blood cells to carry oxygen needed to meet the body's physiologic needs, anaemia occurs [10]. It is estimated that 50% of anaemia worldwide is attributable to iron deficiency [100] and the most widely used test for anaemia is haemoglobin concentration [101]. Anaemia is not only caused by iron deficiencies, but also by infection, vitamin A deficiency, and genetic risk factors like alpha-thalassemia (an hereditary anaemic condition caused by impaired production of one or more of alpha globin chains that compose the haemoglobin) and sickle cell haemoglobin (a hereditary anaemic condition that occurs due to abnormal oxygen-carrying haemoglobin molecule that gives the red blood cell a crescent-like or moon like shape; these abnormal cells lack malleability and can block small blood vessels, impairing blood flow). Haemoglobin concentration in the peripheral blood can provide information about the severity of the iron deficiency [102] but should be used alongside other indicators of iron status because the classifications of haemoglobin values for persons with iron deficiency tend to overlap with those who are non-anaemic [102]. The only exception is when cost-related constraints for conducting bio-chemical assessments for iron deficiency anaemia exist; in that case, haemoglobin concentration is often used as a single marker to assess the prevalence of iron deficiency in a general population. The recommended WHO cut-off points for haemoglobin for children 6-59months are: 110g/l or higher = non-anaemic, 100-109g/l = mild anaemia, 70-99g/l = moderate anaemia and lower than 70g/l = severe anaemia [10]. We estimated the difference in haemoglobin concentrations at the end of the 30-day fortification period between groups of children allocated to different iron formulations. To determine the effect of treatment on haemoglobin concentration after 30 days of intervention we adjusted for study design, baseline iron status (haemoglobin concentration, plasma concentrations of ferritin and soluble transferrin receptor measured) and inflammation status that are prognosis for iron absorption.

1.8.2 Plasma ferritin concentration

Ferritin, a complex of iron protein, is the principal molecule in which iron is stored in the body. It can store intracellular iron safely to prevent the unbound iron from toxic interactions [103]. Plasma (or serum) ferritin concentration has been positively correlated with the size of the total body iron stores in the absence of inflammation [104] which makes it an important indicator of iron status in the body. Ferritin concentration varies with sex and age. At birth, ferritin concentration is high and

rises within the first two months of life then gradually falls. But at one year of age, concentrations begin to rise again and continue to increase into adulthood [105] (possible reasons are explained in Section 1.4.7). Elevated plasma ferritin concentrations have been found in many chronic inflammatory conditions related to infections and, under such circumstances do not reflect the size of iron stores [104] because infections stimulate synthesis of ferritin whenever bacteria produce endotoxins that up-regulate the gene coding for ferritin. Thus, any observed increased levels of ferritin may be interpreted as increased iron stores concealing the true iron deficiency status. In Gambian children (18-36 months), ferritin concentration was shown to increase with elemental iron supplementation in non-malaria children and decreased substantially in children had malaria infection because of the diminishing inflammatory effect of malaria [106].

Additionally, plasma ferritin by itself is not a reliable tool for assessing iron status during infancy or pregnancy because mean values are often close to the iron deficient range, as such should be coupled with other measurements which reflect more advanced degrees of iron deficiency [107]. The WHO threshold for diagnosing anaemia/depleted iron stores using plasma ferritin concentration remains at <15μg/l for individuals 5 years and above and <12 μg/l for children less than 5 years of age for both genders. But higher levels <30 μg/l are considered indicative of depleted iron in the presence of infections for children under five years of age. In this study, we categorised iron status using plasma ferritin as: deficient (plasma ferritin concentration < 12 μg/L), replete (plasma ferritin concentration ≥ 12 μg/L in the absence of inflammation) or uncertain (plasma ferritin concentration ≥ 12 μg/L in the presence of inflammation) [104].

1.8.3 Soluble Transferrin Receptor (sTfR)

Transferrin receptor is a membrane-bound, iron-related protein that can be expressed on the surface of most types of cells in the body, and that regulates the circulation and uptake of iron into all body cells. A soluble form of TfR circulates in human serum hence the term soluble transferrin receptor. Transferrin receptor levels in the serum are sensitive indicators of the degree of iron deficiency and anaemia and a more stable marker of iron levels in an inflammatory state than plasma ferritin [104]. In one study, serum sTfR level was shown to detect iron deficiency in inflammatory states and in anaemia of chronic disease such as type 1 diabetes [108]. Also, it can differentiate between anaemia of chronic diseases and iron deficiency anaemia [109]. However, its diagnostic ability is better when in combination with other related parameters like plasma ferritin and transferrin saturation. In areas with high infection pressure e.g. malaria-endemic areas, the TfR-F index is the best predictor of iron deficiency and has specifically been validated experimentally when iron stores are based on sTfR/ferritin ratio [110].

However, in this present study we only used plasma soluble transferrin receptor concentration (continuous variable) as one of the baseline factors that is associated with the primary outcome (haemoglobin concentration) to control for group imbalances that are likely to influence the effect estimates of the iron treatments.

1.8.4 Non-Transferrin Bound Iron (NTBI)

Transferrin is the β-globulin protein synthesized in the liver and located intra-vascularly. It binds and transports iron as the ferric ion (Fe^{+3}), a form of iron that is relatively insoluble at physiological pH and prevents iron from precipitating as ferric salts. Transferrin can control the level of free iron in biological fluids, thus enabling it to inhibit the growth of gram negative bacteria that need free iron for growth. When the iron-binding capacity of transferrin in the plasma of iron overloaded patients exceeded >100% transferrin saturation [111], non-transferrin bound iron is present in a chelatable low molecular weight form [112]. Again, under stress or some pathological conditions e.g. chronic infections such as malaria, a release of free iron or non-transferrin bound iron may occur, which is free from its protein-bound form [112] and may be toxic because it promotes the growth of existing pathogens. Also, the presence of excess oral iron supplementation may favour the growth of erythrocytic forms of the malaria parasite [103]. The study described in in this thesis originally aimed to examine which of the two daily home iron fortifications (3 mg iron as EDTA and 12.5mg as encapsulated ferrous fumarate) leads to the highest production of circulating non-transferrin bound iron. However, this data is not presented in this thesis because by the time of thesis submission, the bio-chemical analysis had not been done due to insufficient funds.

1.8.5. Zinc protoporphyrin-haem ratio

Zinc protoporphyrin has been used in several studies for measurement of iron deficiency. Most of the protoporphyrin in erythrocytes combines with iron to form haem but insufficient iron causes zinc to combine with protoporphyrin, forming zinc protoporphyrin (ZPP) that can be measured in samples of whole blood [102]. ZPP response occurs because iron and zinc interact as the enzyme ferro-chelatase substrates and zinc utilization increases whenever iron supply is suboptimal at the macrophages [113]. In recent studies, measurement of the ZPP-haem ratio in erythrocytes has been used to give the proportion of ZPP compared to the normal iron-containing haem in red blood cells and reflects the iron status in the bone-marrow [114,115]. Although ZPP has often been used in trials as a diagnostic tool for detecting iron deficiency, concerns have been raised regarding its ability to distinguish between individuals with or without iron deficiency especially in areas of low prevalence [116]. Conversely, the ZPP-haem ratio measured directly using ZPP-haematofluorometer has been shown to be a simple, accurate and sensitive test for detecting early iron depletion before the onset of anaemia but has a limitation of being affected by inflammation [117]. In this trial, zinc protoporphyrin-haem ratio was measured using ZPP-haematofluorometer (AVIV, model 206D, Lakewood NJ,

USA) in whole blood and in erythrocytes as a marker of iron-deficient erythropoiesis alongside other iron markers to detect iron deficiency. The ZPP-haem ratio as a diagnostic tool either alone or combined with haemoglobin concentration and factors that influence its diagnostic ability are presented elsewhere.

1.8.6 C-reactive protein and α_1-acid glycoprotein

Common markers for inflammation include C-reactive protein (CRP), α_1-acid glycoprotein (AGP), hepcidin and serum ferritin. In this study, we singled out CRP and AGP as inflammatory markers because they are acute phase proteins synthesised in the liver and respond to cytokines secreted by inflammatory cells. The concentrations of CRP and AGP vary with infections and increases rapidly with intensity of infection but decreases with disappearance of symptoms. We used a cut-off plasma concentrations of C-reactive protein and/or α_1-acid glycoprotein of >5mg/L [118] and >1.0g/L [119], respectively, as markers of the presence of inflammation. However, these cut-offs have not been validated in children under five years and it is possible that at lower levels of inflammation (CRP<5gm/L and AGP <1.0g/L) caused by either chronic infections or low loads of bacteria and parasites, hepcidin concentration can be elevated consequently blocking iron absorption (Rita Wegmuller, personal communication, May 2016). In our analysis, we used the recommended cut-off and considered lower inflammation levels as a possible cause for iron blockage to explain varied effects of iron fortification in young children.

1.8.7 Plasmodium infection

Lack of resources and technical expertise at local health facilities in many African countries and in a few other developing countries has resulted to a slow pace of detection for *Plasmodium* infections. As a result, the WHO recommended for the use of rapid diagnostic tests to diagnose malaria that has recently yielded tremendous benefits as the proportion of reported cases in Africa has risen substantially from less than 5% at the beginning of the decade to approximately 35% in 2009. [103]. Rapid Diagnostic Tests (RDT) to detect *P. falciparum* antigen in peripheral blood have high sensitivity and are accurate, low-cost, and easily available. Two types of dipsticks used in this trial respectively are: 1) Care Start G0151 for *P. falciparum*-specific lactate hydrogenase (pLDH)-based tests detect current infection [120-122] and can distinguish between *P. falciparum* and other *Plasmodium* species; 2) Care Start G0171 for histidine-rich protein-2 (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 [122], and is specific for *P. falciparum* only. These tests are among the best of 41 brands and types of dipstick tests that were evaluated under the auspices of WHO, and have excellent performance (>95%) in detecting low and higher parasite density (200 parasites/μL or 2000 parasites/μL, respectively) [123]. Also, in this study malaria parasitaemia (using conventional microscopic examination) analysis was conducted to detect malaria infection and intensity at

three different stages of the study; at baseline and after 30 days of intervention. During the post intervention period, blood was stored on DNA collection cards (FTA Mini Card) for subsequent assessment by PCR assay of *Plasmodium* parasites. The use of FTA mini cards was considered appropriate as they required less bleeding, are easy to use and convenient for storage and transportation for analysis later. Confirmation of malaria infection was necessary in supporting the inflammatory status at baseline and the end of 30 days of intervention and useful in determining the anti-malarial drug (dihydroartemisinin-piperaquine) post-treatment prophylactic effect.

1.8.8 Serum concentrations of vitamin B_{12} and albumin

Vitamin B_{12} (cobalamin) is one of the most important vitamins required for erythropoiesis alongside other nutrients like folate, vitamin B_2 (riboflavin), and iron. Measurement of plasma vitamin B_{12} is essential in this study because in the absence of folate (see reasons for omission of folate in Section 1.4.3), vitamin B_{12} deficiency can impair the production of tetrahydrofolate required for thiamine production which is fundamentally needed for DNA synthesis during red blood formation [102,124]. Whenever DNA synthesis is impaired then large immature and dysfunctional red blood cells are formed in the bone marrow thus inhibiting haemoglobin synthesis resulting to a condition known as megaloblastic anaemia. Megaloblastosis is not expected in children under five years old because it takes 5-10 years for it to appear due to the body's capacity to reutilise the vitamin [102]. Malabsorption of vitamin B12 is the secondary source of deficiency and is mostly caused by loss of parietal cells that produce intrinsic factor. Other causes are intestinal lesions, increased bacterial uptake and worm infestation. In this study, we assessed the concentration of vitamin B_{12} with a cut-off set at <150pmol/L [104] to determine its deficiency and its association to anaemia as measured by our primary outcome (haemoglobin concentration).

Albumin is the most abundant protein in blood plasma and synthesised in the liver. It is essential for transporting hormones, fatty acids and other compounds. More importantly and in relation to this study, it is a negative acute phase protein and is down regulated whenever there is an acute or chronic inflammatory response. This mechanism occurs because during inflammation the liver prioritises the synthesis of other proteins needed to combat inflammation thus causing the concentration of albumin to reduce in the plasma. The relationship between inflammation, serum albumin and other acute phase markers (CRP and ferritin) has been shown to have an inverse correlation with serum CRP level because C-reactive protein's concentration increases with inflammation (see details in Sections 1.8.6) and has a positive correlation with haemoglobin concentration (125). Fundamentally, in the absence of systemic inflammation albumin is up-regulated and more iron is likely to be absorbed resulting in more haemoglobin synthesis.

1.8.9 Measurements of adherence to daily home fortification

According to Brawley and Culos-Reed (2000), adherence implies that research participants are fully involved in behavioural plans and their contributions taken into consideration when adjusting research plans. Whereas compliance implies behaviour characterized by the extent to which research participants follow instructions or use prescriptions as assigned [126], these terms are often used interchangeably in clinical studies. Still, measuring adherence in a clinical study or treatment setting have potential risks as well as benefit, moreover study participants need to be informed about the adverse events, possible side effects, benefits and duration of the treatment [50].

There are several adherence measuring tools, most common are self-reporting, pill counts, visual analogue scale, pill identification tests, medication events monitoring systems (MEMS) and interviews [3,127] , most of these have been used for ambulatory care treatments for asthma, epilepsy, hypertension, HIV, tuberculosis, iron deficiency or diabetes among other chronic illnesses. Self- reporting questionnaires and sachet counts (either empty or full) are the most common praxis for measuring adherence to home-fortification powders because they are easy to use and relatively inexpensive, but their reliability and validity have been controversial because of various intrinsic factors: a) their sensitivity and specificity outside a gold standard is unknown; b) poor reporting is common, because it is either over-estimated resulting to unknown chances for suboptimal adherence; or it is under-reported causing it to be misinterpreted as low adherence to treatment [54], and subsequently rejection of a treatment that could have otherwise lowered incidences of diseases [126], denying an opportunity to decrease global burden of diseases. Adherence assessment to home fortification with micronutrients is still under investigation and existing evidence is weak [55], but an electronic monitoring and time-recording device (MEMS) is suggested to measure adherence for powdered micronutrients indirectly estimating frequency and dosage [128] of micronutrient intake. Adherence assessment using the MEMS devices is the reference standard [52, 129,], and is superior to medication pill counts and self-reported questionnaires [128,130]. In this study, we set to determine the accuracy of measuring adherence to daily home fortification of micronutrient powders using self-reporting questionnaires and empty sachet count method compared to the MEMS device.

1.9 Ethical statement

This study was conducted in accordance with the ethical guidelines as laid down in the revised Helsinki declaration [131] and the Council for International Organizations of Medical Sciences (CIOMS) in collaboration with the World Health Organisation [132, 133]. In addition, the Kenyan ethical procedures were used to obtain informed consent from communities and individuals participating in the study. Ethical clearance was obtained from London School of Hygiene and Tropical

Medicine Ethical Committee, UK (6503) and the Kenyatta National Hospital Ethical Review Committee, Kenya (KNH-ERC/A/402). The trial was registered with ClinicalTrials.gov (NCT02073149). LSHTM acted as the trial Sponsor. Implementation was only commenced after approval by both ethical committees and consent form signed by parents (Appendix 2). During the study implementation there were no major amendments to the protocol.

1.10 Candidate and Co- authors' contributions

I, Emily Teshome, wish to state that the original concept of this study was conceived by Dr. Hans Verhoef and with his guidance I planned the study design. I independently conducted all literature review, wrote chapters 1 and 6 and only received comments and technical inputs from Professor Andrew Prentice and Dr Hans Verhoef where needed. I coordinated the implementation of the research project and supervised all field work including staff recruitment, training, data collection, data entry into respective computer soft wares and data cleaning. I performed all the statistical analysis under the guidance of Dr. Hans Verhoef except where I shared responsibility as specified in the respective chapters below. I did not perform any laboratory analysis but shared this responsibility as specified in sections below.

Chapter 2 and chapter 3
In both chapters I am the first author, the co-authors to the manuscripts shared responsibility as follows: Dr. Pauline Andango assisted in implementing the study design; obtaining ethical permits and other research licences in Kenya; procurement of project materials and equipment; and assisted in recruitment and supervision of research staff. Dr.Walter Otieno was the research project paediatrician and was responsible of examining and treating all children with serious adverse events. He assisted in recruitment of technical staff and assisted in supervising the clinical officers, laboratory technicians and nurses; also, he provided technical support in the development of both medical examination and laboratory protocols. Ms. Sofie Terwel assisted in development and compiling the study protocol, training the research staff, procurement of materials and equipment, and assisted in supervising the research staff in the initial stages of implementation. Mr. Victor Osoti assisted in supervising human tissue collection, processing, storage, and conducted laboratory analysis, packaging and shipping to the Netherlands. In addition, he contributed towards procurement of laboratory materials and equipment. Dr. Ayşe Y. Demir was responsible for all biochemical analysis of blood samples conducted in the Netherlands. Professor Andrew Prentice reviewed the manuscripts and provided comments and technical inputs as needed. Dr. Hans Verhoef provided technical guidance as needed during study design, study protocol development, Statistical Analysis Plan, data analysis and comments during manuscript writing. He also analysed data using the metafor package in R software vs. 3.2.0; I was fully involved

in the interpretation of the analysed data and writing up of the results. All co-authors reviewed the manuscripts before submission.

Chapter 4
As the first author, I designed the study and data collection tools, coordinated field work, recruited and trained staff and supervised data collection collaboratively with the following: Veronica Oriaro assisted in designing the data collection tools, translated the participants' information brochure (appendix 3), self–reporting form (appendix 4) and follow- up checklists (appendix 5) into the local language. Also, she assisted in training and supervising research assistants. Dr. Pauline Andango offered technical guidance in designing the study design and data collection tools. Dr. Hans Verhoef provided technical input in study design and data analysis especially in the beta regression using the R- statistical software vs. 3.2.0. Professor Andrew Prentice provided comments and technical inputs as needed. All co-authors reviewed the manuscript before submission.

Chapter 5
I assisted in the study design, coordinated field work and data collection. Dr. Pauline Andango assisted in supervising field work. Dr. Hans Verhoef conceived the idea, provided technical input in the design, and overall supervised all aspects of the study. Dr. Ayşe Y. Demir supervised biochemical analyses. Dr. Hans Verhoef and I conducted statistical analyses and prepared the first draft manuscript. Professor Andrew Prentice reviewed the draft manuscript and provided technical inputs. All authors read and approved the final manuscript.

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CHAPTER 2: Research paper 1

Cover Sheet

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Principle Supervisor: Prof. Andrew Prentice

Title: Comparison of home fortification with two iron formulations among Kenyan children: rationale and design of a placebo-controlled non-inferiority trial

Authors: Emily M Teshome, Walter Otieno, , Sofie R.Terwel, Victor Osoti, Ayşe Y. Demir, Pauline E.A. Andango, Andrew M. Prentice, and Hans Verhoef

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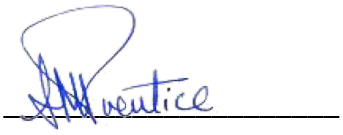
Supervisors Signature: (to confirm role as stated above): 

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Comparison of home fortification with two iron formulations among Kenyan children: Rationale and design of a placebo-controlled non-inferiority trial

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ABSTRACT

Introduction: Home fortification powders containing iron and other micronutrients have been recommended by World Health Organisation to prevent iron deficiency anaemia in areas of high prevalence. There is evidence, however, that home fortification at this iron dose may cause gastrointestinal adverse events including diarrhoea. Providing a low dose of highly absorbable iron (3 mg iron as NaFeEDTA) may be safer because the decreased amount of iron in the gut lumen can possibly reduce the burden of these adverse effects whilst resulting in similar or higher amounts of absorbed iron.

Objective: To show non-inferiority of home fortification with 3 mg iron as NaFeEDTA compared with 12.5 mg iron as encapsulated ferrous fumarate, with haemoglobin response as the primary outcome.

Design: 338 Kenyan children aged 12–36 months will be randomly allocated to daily home fortification with either: a) 3 mg iron as NaFeEDTA (experimental treatment), b) 12.5 mg iron as encapsulated ferrous fumarate (reference), or c) placebo. At baseline, after 30 days of intervention and within 100 days post-intervention, blood samples will be assessed for primary outcome (haemoglobin concentration), iron status markers, *Plasmodium* parasitaemia and inflammation markers. Urine and stool samples will be assessed for hepcidin concentrations and inflammation, respectively. Adherence will be assessed by self-reporting, sachet counts and by an electronic monitoring device.

Conclusion: If daily home fortification with a low dose of iron (3 mg NaFeEDTA) has similar or superior efficacy to a high dose (12.5 mg ferrous fumarate) then it would be the preferred choice for treatment of iron deficiency anaemia in children.

1. Introduction

Home fortification aims at supplementing local diets by adding micronutrient powders to semi-solid, ready-prepared foods (http://www.hftag.org/). The World Health Organisation (WHO) recommends daily universal home fortification with iron for children aged 6–23 months in populations where the prevalence of anaemia in children under 5 years of age is ≥ 20% [1]. Prevalence values within this range indicate a moderate-to-severe public health problem, which is the situation in virtually all developing countries [2].

The WHO-recommended iron dose for home fortification (10–12.5 mg iron as ferrous salt for children aged 6–23 months, [1]) corresponds to the dose that was previously established for iron supplementation in this age range [3]. There is evidence from randomised controlled trials among young children in low-income countries to suggest that home fortification with iron-containing micronutrients may cause an excess burden of diarrhoea, and increased numbers of potentially pathogenic enterobacteria, with a concurrent increase in gut inflammation [4]. Other gastrointestinal adverse effects of oral iron supplementation, such as epigastric discomfort, nausea and constipation, are common, are dose-dependent and are likely to reduce adherence [5].

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Compared to the conventional daily dose (12.5 mg as ferrous salt), home fortification or supplementation with a low dose of highly absorbable iron (3 mg iron as NaFeEDTA) may result in similar or higher amounts of absorbed iron [6,7] but the decreased amount of iron in the gut lumen can possibly reduce the burden of adverse gastrointestinal effects.

There is substantial evidence that iron interventions in young children can also increase rates of malaria and possibly respiratory disease [8–11]. Because adverse events associated with such systemic diseases are likely to depend on the absorbed amount of iron, the risks may be similar when comparing a daily dose of 3 mg iron as NaFeEDTA and 12.5 mg as ferrous salt. WHO has recommended that iron interventions should be implemented in conjunction with measures to control malaria [1].

We aimed to show non-inferiority of home fortification with 3 mg iron as NaFeEDTA compared with 12.5 mg iron as encapsulated ferrous fumarate in young Kenyan children protected for 3–4 weeks against malaria by chemoprevention.

2. Study methodology

2.1. Study site

The study will be conducted from January–December 2014 in the administrative units of Kanyawegi, Osiri and Ojolla in Kisumu-West District, a rural area at an altitude below 1,300 m, adjacent to Lake Victoria, Kenya. This area covers 395 square kilometres with a population of approximately 12,000 people, of whom 20% are children aged below five years. The majority of the population consists of subsistence farming families but inadequate and unreliable rainfall patterns have immensely affected agricultural activities in the area [12]. The local diet is mainly based on maize and vegetables. Animal foods, which are rich sources of iron, are rarely consumed and often sold in the urban markets to boost income. Malaria transmission is perennial and stable [13], with most infections being due to *Plasmodium falciparum* [14]. The prevalence of *P. falciparum* infection in children aged 1–4 years has been reported to range between 39% and 63% [15]. The area is endemic for *Schistosoma mansoni*, with a prevalence of infection in infants of 14% [16]. Hookworm and *Trichuris trichiura* infections are also common in young children [17]. Co-infection of hookworm, *T. trichiura* and *P. falciparum* has been associated with low haemoglobin concentrations in pre-school children [18].

2.2. Study design

This study concerns a randomised, double-blind, non-inferiority trial comparing daily home fortification for 30 days with 3 mg iron as NaFeEDTA (investigational intervention), 12.5 mg iron as encapsulated ferrous fumarate (reference) and placebo. We conceived it as an explanatory trial to evaluate the efficacy of daily home iron fortification under maximal compliance.

2.3. Sample size determination

Sample size calculations are based on procedures for non-inferiority trials as recommended by USA Food and Drug Administration [19,20].

1. Based on a meta-analysis [21] we estimated the expected effect of 12.5 mg iron as ferrous fumarate on haemoglobin concentration relative to placebo. The lower limit of the 95% CI thus obtained (9.3 g/L) was used as M_1, the minimum anticipated effect of 12.5 mg iron as ferrous fumarate (Fig. 1; left panel).
2. Next, we set M_2 as the margin specified to preserve 50% of the anticipated minimum effect of 12.5 mg ferrous fumarate. This margin (haemoglobin concentration of 4.7 g/L) can be interpreted as the largest loss of effect compared to 12.5 mg ferrous fumarate (inferiority) that would be acceptable, and is below an effect for 5 g/L iron as NaFeEDTA that we considered to be of minimum importance for public health.
3. We set the sample size at 339 children (estimating 113 children per intervention group) so that the lower limit of the 95% CI around the difference in haemoglobin concentration between the two iron formulations (i.e. 12.5 mg ferrous fumarate and 3.0 mg iron as NaFeEDTA) would lie above M_2 (Fig. 1; right panel).

2.4. Recruitment

The research assistants will hold meetings with local authorities, community health workers and parents to inform them about study aims and procedures. The community health workers will compile a list of parents with children aged 1–3 years residing within the three administrative units, and invite parents to bring these children for screening to the research clinic, where they will be asked to sign an informed consent form (Appendices 1, 2).

At the screening visit, research assistants will collect vital data and information on household characteristics: a) date of birth as recorded in the birth certificate or health card held by the mother or, if not available, from records of the Expanded Program of Immunization held by local clinics; b) anthropometric data that include weight measured to the nearest 100 g using a Salter scale (UNICEF, catalogue 0145555, Copenhagen, Denmark) that is calibrated daily using a 5 kg weight. During measurement, the child will wear neither clothes nor shoes; standing height (children $\geq$24 months or $\geq$ 85 cm) or recumbent length (children $\leq$24 months or $\leq$ 85 cm) will be measured within 0.1 cm using wooden measuring boards (UNICEF, catalogue 0114500); and mid-upper arm circumference, a marker of wasting, using a measuring tape (UNICEF, catalogue 145600) within 0.1 cm.

Medical staff will conduct medical examinations and collect the following data: a) a parent-reported 48-h history of illness including fever, diarrhoea, vomiting or breathing distress; b) parent-reported history of signs of major systemic disorders; c) parent-reported use of specific medicines (antiretroviral drugs, rifampicin, carbamazepine, phenytoin or phenobarbital); c) parent-reported drug allergies, or 30-day history of using drugs (antimalarials, benzimidazoles, praziquantel) that might interfere with the study treatment protocol.

Clinical officers will ask parents to bring children for re-screening two weeks later if the child has a 48 h history of antimalarial drug use, or has received treatment for malaria. Children with axillary temperature $\geq$37.5 °C plus demonstrated blood infection (rapid dipstick tests positive for malaria) or minor illnesses will be treated immediately and also asked to return after two weeks for re-screening.

Phlebotomists will collect venous blood (4 mL) in tubes containing Li-heparin. We will determine haemoglobin concentration (HemoCue 301, Ängelholm, Sweden) and zinc protoporphyrin:haem ratio (AVIV, model 206D, Lakewood NJ, USA) in whole blood as a marker of iron-deficient erythropoiesis, each in triplicate. We will assay *Plasmodium* antigenaemia by rapid tests (see section 'Laboratory analyses' below). We will transfer aliquots of whole blood (125 μL) to DNA collection cards (FTA Mini Card, catalogue WB120055, Little Chalfont, UK) for storage at ambient temperature and subsequent detection by PCR of *Plasmodium* infection; and we will prepare thick and thin blood smears to allow for detection and counting of *Plasmodium* parasites.

An aliquot (1.0 mL) of blood will be centrifuged (600 × *g*, 10 min). Plasma (500 μL) will be transferred to a microtube, centrifuged (2000–3000 × *g*, 15 min), transferred to a cryovial, and stored immediately in liquid nitrogen (−196 °C). The erythrocyte sediment (500 μL) will be washed and centrifuged (600 × *g*, 8 min) three times with isotonic phosphate-buffered saline (Medicago, Uppsala, Sweden; catalogue 09-9400-100) to allow measurement in triplicate of the erythrocyte zinc protoporphyrin:haem ratio. Measurement of zinc protoporphyrin:haem ratio in washed erythrocytes is considered a more valid measurement when compared to whole blood because the

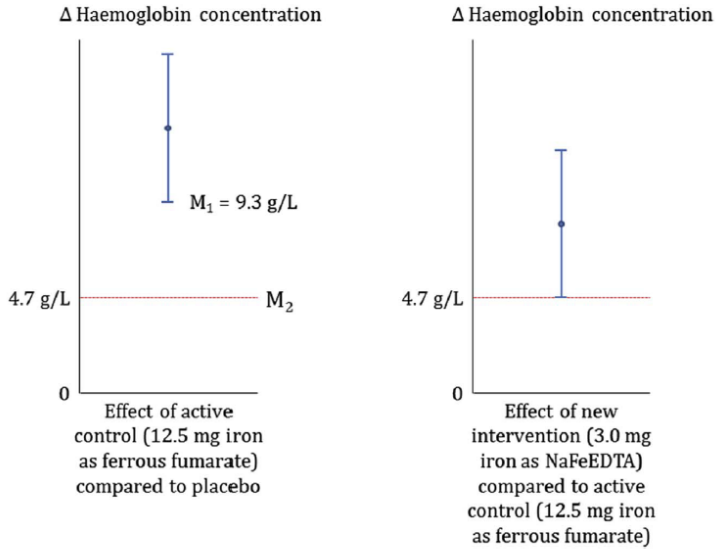

Fig. 1. Theoretical framework for sample size determination. The treatment effects are shown as 95% CIs around the estimates (shown by the blue lines). The estimated margin values are shown by the dotted red lines. **Left panel:** M_1 is the lower bound of 95% CI, estimated at 9.3 g/L and being the smallest effect of the 12.5 mg iron as ferrous fumarate (reference intervention) versus placebo. M_2 is the non-inferiority margin estimated as a 50% reduction of the minimum anticipated value effect (9.3%g/L) of reference intervention which corresponds to 4.7 g/L; **Right panel:** Success will be estimated as the difference in haemoglobin concentration between 12.5 mg ferrous fumarate and 3.0 mg iron as NaFeEDTA should lie above M_2, a conservative effect considered to be of minimum public health relevance. Using 113 children per group, the trial had 90% probability to detect superiority of the investigational arm over placebo and 95% probability showing non-inferiority relative to reference intervention given the following assumptions: effect of 5 g/L; equal group SDs of 10 g/L; 2-sided $\alpha = 0.05$; maximally 5% of children will drop out of the iron group, no 'drop-in' will occur of children crossing over from the placebo group to the iron group.

washing process removes substances dissolved in the plasma such as bilirubin that fluorescence in the same wavelength range as ZPP [22]. An aliquot of washed erythrocytes will be transferred to a cryovial for storage and subsequent measurement of folate concentration, and to a cryotube prefilled with 0.9% saline solution and a lysing agent (Celite, Sigma-Aldrich, catalogue 525235, St. Louis, MO) for subsequent acid extraction and measurement of metal-free protoporphyrin.

The remainder of the blood (2.75 mL) will be centrifuged (2000–3000 × *g*; > 15 min); aliquots of plasma will be stored in liquid nitrogen for subsequent measurement of iron markers (concentrations of ferritin, soluble transferrin receptor) and inflammation indicators (concentrations of C-reactive protein, *α*-1-acid glycoprotein). 125 μL buffy coat will be pipetted on DNA collection cards (FTA Mini Card), allowed to dry, stored and sealed in multi-barrier pouches containing 1 g desiccant for subsequent genotyping for host polymorphisms associated with susceptibility to malaria.

Research assistants will collect urine samples at the research clinic using 100 mL paediatric collection bags (Changzhou Huankang, Changzhou, Jiangsu, China). Prior to urine collection, we will clean and dry the area around the vulva or penis using disinfectant baby wipes or soap and water, apply the urine bags, and re-apply the child's diapers or pants. Parents will be asked to check regularly to ensure that the child does not remove the urine bag, and to inspect if the child has produced urine. Urine will be drained into a sterile 125 mL container. Samples (2 mL) will be stored immediately in liquid nitrogen for subsequent assessment of *Schistosoma* ova and hepcidin concentrations.

Research assistants will collect faecal samples at the research clinic on an aluminium sheet placed either inside a child's potty or directly onto the floor. Stool that is mixed with urine will be discarded. If a child is unable to produce stool, the parent will be asked to bring him/her again and retry on the subsequent 3 days until the stool is produced. A scoop (10 mL) will be transferred using a plastic spatula into a sterile disposable container that is placed immediately into a cool box and taken to the laboratory, where aliquots (2 mL) will be stored in liquid nitrogen for subsequent measurement of calprotectin concentration as an indicator of intestinal inflammation, and to assess for intestinal infections.

2.5. Premedication

Pre-medication will be administered to every eligible child during screening visit. We will give a therapeutic course of dihydroartemisinin-piperaquine (Sigma-tau, Rome, Italy; 40 mg dihydroartemisinin/320 mg piperaquine, administered as a daily dose for 3 consecutive days of ½ tablet and 1 tablet for children in weight ranges 7–12.99 kg and 13–24 kg, respectively) with the aim to protect children against malaria in the subsequent intervention period.

To protect against severe anaemia during the intervention period, we will administer two antihelminth drugs at the research clinic as per WHO recommendations [23]. Albendazole (Indoco Remedies, Mumbai, India) will be administered for 3 days at a daily dose of 200 mg or 400 mg for children aged 12–24 months and > 24–36 months, respectively. Praziquantel (Cosmos, Nairobi, Kenya; 600 mg tablets) will be administered as a single target dose of 40 mg per kg body weight (< 10 kg: ½tablet; 10–12 kg: ¾ tablet; > 13 kg: 1 tablet). The clinical officer will administer praziquantel and the first dose of albendazole and dihydroartemisinin-piperaquine at the research clinic, and instruct parents to administer the remaining doses at home. Albendazole and dihydroartemisinin-piperaquine will be given 1 h after consumption of food, while praziquantel will be administered after child has consumed a cup of *uji* (maize gruel, a common food locally given to young children) or after lunch to avoid adverse effects (e.g. nausea, vomiting, and abdominal pain). The clinical officer will crush and mix the tablets with clean drinking water and observe that the child swallowed them. If a child vomits the medicine within a period of 10 min, a repeat dose will be given immediately. The clinical officer will inform parents about the reasons why their child should complete the remaining two drug doses. Parents will be requested to observe any possible adverse reactions and report immediately to the field research workers. Parents will also be asked not to give their children foods based on maize or

sorghum flour 2–3-h before returning to the research clinic on the scheduled return date.

2.6. Eligibility criteria

We will include children if aged 12–36 months; resident in the study area and whose parents intended to stay in the area in the subsequent nine months; parental consent form signed by both parents; not acutely sick or febrile (axillary temperature ≥37 °C) at the time of recruitment; absence of reported or suspected major systemic disorder (e.g. HIV infection, sickle cell disease); no use of antiretroviral drugs against HIV, rifampicin, carbamazepine, phenytoin or phenobarbital; no twin sibling. Children will be excluded if: haemoglobin concentration < 70 g/L; severely wasted (weight-for-height z-score < –3 SD); known allergy to dihydroartemisinin-piperaquine, benzimidazoles or praziquantel; parent-reported history of using antihelminthic drugs in the 1-month period before the screening date; not at risk of malaria (e.g. children who received chemoprophylaxis against malaria because of HIV infection or sickle cell disease); after three days parent-reports child has not completed the 2nd and 3rd doses of dihydroartemisinin-piperaquine and benzimidazoles; has adverse effects associated with pre-medication; has fever (axillary temperature ≥37 °C); presents with any other illness.

2.7. Randomisation

To achieve group balance in size and baseline haemoglobin concentration, randomisation will be based on a stratified block design. A person not involved in the field work will create the randomisation scheme by assigning three treatment groups in a sequence of random permuted blocks of sizes 6 or 9 within two strata defined by baseline haemoglobin concentration class (< 100 g/L and ≥100 g/L), using tables with random numbers and random permuted blocks. Using this scheme, two other persons not involved in the field work will produce a set of labels with a child identification number that includes a letter for stratum (A or B) and a consecutive allocation number as indicated by the randomisation scheme. These labels will be stuck on a) sealed opaque envelopes each containing a paper slip with the word 'iron' or 'placebo'; and b) plastic bottles, each containing 30 sachets of one of the three types of micronutrient powders (see 'Interventions', below). The bottles will then be arranged in boxes according to stratum and sequential number as indicated in the randomisation scheme and handed over, together with the sealed envelopes, to the field team. All research staff (including trial coordinator) will not be allowed to open the envelopes until the end of the 30-day intervention period.

On the randomisation day visit, the trial coordinator will assign children successively to the next available allocation number within the appropriate stratum (indicated by haemoglobin concentration measured at the screening visit). This process will continue until the target sample size has been attained.

2.8. Interventions

We will use three types of micronutrient powders manufactured specifically for this trial by DSM Nutrition Products (Johannesburg, South Africa) and that contain 1 g sachets with either 3 mg iron as NaFeEDTA, 12.5 mg iron as encapsulated ferrous fumarate or without iron (placebo). The encapsulate consists of a thin coat of soy lipid. All powder types will contain thirteen micronutrients other than iron (Table 1), as recommended by the Home Fortification Technical Advisory Group except for folic acid, which we will omit because of our concerns that it may be utilized by *Plasmodium* parasites and increase the failure risk of anti-folate drugs, and because there is no evidence that folate deficiency is a public health problem among children in developing countries [24]. At the randomisation visit, research assistants will instruct parents on the use of the fortificants,

Table 1
Formulation of micronutrient powders.

Micronutrient	Content
Vitamin A, µg RE	300
Vitamin D, µg	5
Vitamin E, mg	5
Vitamin C, mg	30
Thiamin (vitamin B_1), mg	0.5
Riboflavin (vitamin B_2), mg	0.5
Niacin (vitamin B_3),mg	6
Vitamin B_6 (pyridoxine), mg	0.5
Vitamin B_{12} (cobalamine), µg	0.9
Iron	
EITHER iron as encapsulated ferrous fumarate, mg	12.5
OR iron as NaFeEDTA, mg	3
OR no iron (placebo)	0
Zinc, mg	5
Copper, mg	0.56
Selenium, µg	17
Iodine, µg	90

give them a supply of 30 sachets in a plastic bottle randomly assigned for each child by the trial coordinator, and ask them to daily add the contents of one sachet per child to semi-solid, ready-prepared foods for a period of 30 days. The assistants will also show them how to mix the content of the sachet (the first dose) with *uji*.

2.9. Blinding

Each type of micronutrient powders will be packed in identical plain white foiled sachets except for the batch number. Parents will receive 30 sachets for each child in a white plastic bottle that contains no other marker except the label with stratum, allocation number, name, start date and return date. The three types of micronutrient powders do not have apparent differences in taste, texture or colour of *uji*. Research assistants will observe consumption of each cup of *uji* during the administration of the first dose of treatment at the research clinic. Researchers, outcome assessors and parents will remain fully blinded to allocation and intervention until the 30-day intervention period has been completed. At that time, the trial coordinator will open the sealed envelopes to determine whether a child had received iron or placebo. Because the information in the envelope will not reveal the type of iron group, research assistants will be partially de-blinded; full de-blinding will be done after the statistical analysis plan has been completed and after crude intervention effects have been analysed without identification of the iron interventions.

2.10. Adherence monitoring

Adherence to intervention will be primarily monitored using an electronic monitoring and time-recording device (MEMS 6 TrackCap 45 mm without LCD display; http://www.mwvaardex.com/) that will be given for the duration of the study to parents of participating children. This battery-operated device consists of a cap that fits the bottle containing the micronutrient sachets, with a built-in microprocessor that records and stores date and time of all closings. Adherence assessment using these devices is considered the reference standard [25,26] and superior to medication counts and self-reported adherence, which are commonly used methods that tend to result in over-estimates [27,28]. Each bottle will be labelled with a child's identification number, serial number of the cap, name of child, start date and end date for ease of identification and tracking. Except for the trial coordinator and one field supervisor, neither parents nor research assistants will be informed about the function of the electronic device. Instead they will be informed that the MEMS cap is essential for maintaining the moisture content and good hygienic conditions of the micronutrient powder. Parents will be thoroughly instructed to close

the bottle after each opening, and will be shown how to use the storage bottle with the MEMS cap. In addition, parents will be requested to keep empty sachets in a zip-lock plastic bag marked with the child's name and identification number. These bags will be collected at the end of the study to allow adherence assessment by sachet count.

Parents will be taught how to fill out self-reporting forms written in their local language (*Dholuo*), and requested to daily record (by a tick) when the fortificant-containing food is given (morning, mid-morning, lunch, mid-afternoon or evening) during the 30-day intervention period. Lastly, parents will be instructed about the importance of immediately reporting any sickness or adverse reactions experienced by the child during the 30 days, and the date of reporting back to the research clinic.

2.11. Assessment of non-transferrin bound iron (NTBI)

Three hours after administering the first dose of home fortificants with *uji*, we will collect capillary blood (400–500 μL) by finger puncture in tubes without anticoagulant (Becton Dickinson, Breda, The Netherlands), using vinyl gloves to avoid contamination with trace elements, and avoiding finger squeezing. After clotting (30 min), serum will be transferred to a microtube, centrifuged (6000–15,000 × *g*, 10 min), and aliquots (300 μL) transferred to cryovials and stored in liquid nitrogen for subsequent NTBI analysis.

2.12. Follow-up during 30-day intervention period

Fig. 2 provides an overview of data and samples that will be collected in the course of the trial. Field workers will conduct weekly pre-announced home visits to check if the child is still in the study area, if parents are following protocol when administering the fortificants, and if parents are filling out forms and storing empty sachets. During these visits, field workers will discuss problems or clarify procedures, but they will not give parents instructions additionally to those given during the randomisation visit. All observations and problems experienced by parents will be recorded in a form and submitted to the field supervisor at the end of each day. Sick children will be referred to the research clinic. Clinicians and laboratory technicians will be available 24 h per day. Children with severe illness or serious adverse events will be referred to a nearby referral hospital (Kisumu town), and taken either by project vehicle or by local transport with refund of transport fees. Parents who withdraw children from the intervention will be asked for reasons and for permission to keep and analyse data and samples already collected.

2.13. Survey at 30 days of intervention

Parents will be asked to bring their children to the research clinic at 30 days post-randomisation. During this visit, clinicians will perform a medical examination and research assistants and phlebotomists will collect anthropometric data and samples (blood, urine and stool) as described for screening. Parents will be asked to return the plastic bottles with the MEMS cap, empty sachets and the self-reporting form. We will count the number of sachets and download information from the electronic device onto a computer. In addition, we will administer a standardised questionnaire to collect information on possible factors affecting adherence. Once all data and samples are collected, the trial coordinator will open the sealed brown envelope to determine child's intervention group (iron or placebo).

2.14. Post-intervention period

Children who received placebo will be retained in the study to observe adherence to home fortification during another 30-day period in the absence of regular monitoring visits by research assistants. Thus they will be given a 3-day therapeutic course of dihydroartemisinin-piperaquine and 30 sachets of 12.5 mg encapsulated ferrous fumarate in a bottle with the MEMS cap and again receive self-reporting forms, and instructions for use. Research assistants will conduct sporadic but pre-announced visits to their homes (one visit per child and additional visits as needed for a child with adverse events) to observe if the child is still resident and follows protocol and to check for sickness or adverse reactions. At the end of the 30-day post-intervention period, parents will be asked to returned to the clinic to submit the bottle with the

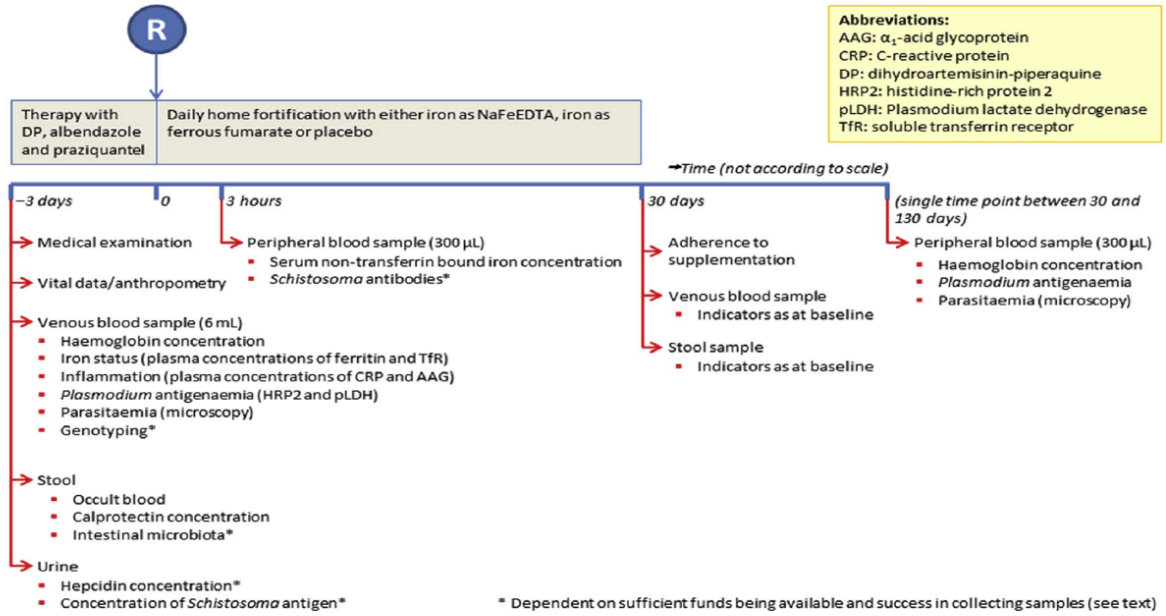

Fig. 2. Data collection timelines.

MEMS cap, self-reporting forms and empty sachets. Children will be medically examined, treated for incident illness as appropriate, and exit the study.

Children allocated to the iron group will not be given fortification powders but instead will be retained in the study to monitor the population decline in haemoglobin concentration over time in a 100-day follow-up period. During this period, haemoglobin concentrations are expected to decline exponentially (i.e. at a rate that is proportional to its current value), up to a point when it would be theoretically desirable to retreat the group with a new cycle of therapeutic course of antimalarial drugs with iron fortification. We aim to estimate the time point when ≥10% of children has developed severe anaemia haemoglobin concentration < 70 g/L; [29], taking into account our wish to restrict phlebotomies during the post-intervention period (for ethical reasons) to a single occasion per child. Thus we will phlebotomise each child on a single, randomly selected day in the 100-day follow-up period. We will use pre-programmed MS Excel software to randomly select a date of their return visit within a 100-day period. The date of this return visit will be concealed in the MS excel program until after the 30-day intervention return visit. Once the date is randomly calculated by the software, parents will be asked to take each child home and return on the randomly selected date. Parents will be requested to report immediately any sickness or adverse reactions experienced by the child during the post intervention period.

On the return visit, a laboratory assistant will collect capillary blood by finger puncture to measure haemoglobin concentrations in duplicate from a single drop, and to store DNA on a FTA Mini Card for subsequent assessment by PCR assay of *Plasmodium* parasites. Immediately following phlebotomy, half of these children will be withdrawn from further study (for ethical reasons) and will be given a therapeutic course of dihydroartemisinin-piperaquine and a supply of sachets for daily home fortification with 12.5 mg iron as encapsulated ferrous fumarate stored in silver blister pockets for another 30 days. The other half will be given a therapeutic course of dihydroartemisinin-piperaquine and a supply of sachets for daily home fortification with 12.5 mg iron as encapsulated ferrous fumarate in bottles with MEMS cap and a reporting format to determine the effect of interrupted fortification on adherence. These children will be requested to visit the research clinic after 30 days, the parents will be interviewed and informed on the three adherence tools processed similar to the end of 30-day intervention period. A summary of the flow of activities for the post intervention period is presented in Fig. 3.

2.15. Laboratory analyses

We will use two rapid tests (AccessBio, Somerset, NJ; CareStart G0151 and G0171) to detect *P. falciparum*-specific histidine-rich protein 2, *P. falciparum*-specific lactate dehydrogenase (LDH), and LDH specific for human *Plasmodium* spp. other than *P. falciparum*. The pLDH-based test was used to detect current infection [30–32]. We will use two commercially available tests (Hemoccult SENSA, Clindia Benelux, Almere, The Netherlands, catalogue no. 20000702; FOB advanced +, Ulti Med, Roeselare, Belgium, catalogue 010A210-20) to detect the presence of faecal occult blood, following assay instructions given by the manufacturers. Faecal occult blood will be interpreted as evidence of intestinal bleeding due to gastrointestinal helminths. Iron markers (plasma concentrations of ferritin, soluble transferrin receptor, transferrin), inflammation markers (plasma concentrations of C-reactive protein [CRP] and α_1-acid glycoprotein), albumin and vitamin B12 will be measured on an Abbott Architect C16000 and i2000 SR analyser as per manufacturer's instructions.

2.16. Study outcomes

We will use the following outcome definitions: *anaemia:* haemoglobin concentration < 110 g/L; mild, moderate and severe anaemia: haemoglobin concentration 100–109 g/L, 70–99 g/L and < 70 g/L, respectively [29]; iron status: *deficient* (plasma ferritin concentration < 12 μg/L), *replete* (plasma ferritin concentration ≥ 12 μg/L in the absence of inflammation) or *uncertain* (plasma ferritin concentration ≥ 12 μg/L in the presence of inflammation) [33]; *iron deficiency anaemia:* concurrent anaemia and iron deficiency; *inflammation:* plasma concentrations of C-reactive protein and/or α_1-acid glycoprotein of > 5 mg/L [34] and > 1.0 g/L [35], respectively; *Plasmodium* infection: presence or absence of parasites as indicated by histidine-rich protein-2, lactate dehydrogenase specific for *P. falciparum* or human *Plasmodium* spp. other than *P. falciparum* (i.e. *P. vivax*, *P. malariae* or *P. ovale*); high, medium and low *Plasmodium* parasite density: parasitaemia ≥10,000/μL, 1000–9999/μL and < 1000/μL, respectively. We will define high adherence (≥80%, 24 days or more) and low adherence (< 80%, 23 days or less) of scheduled fortification powders, as indicated by the MEMS device. This threshold is arbitrary, but is often used in published studies on medication adherence [28,36–39].

2.17. Statistical analysis

A statistical analysis plan will be finalised after data collection but before breaking the randomisation code.

Anthropometric indices will be calculated using WHO Anthro software vs.3.2.2 (World Health Organisation, Geneva, Switzerland). Data analysis will be done using SPSS 21 (IBM, Armonk, NY), CIA 2.2.0 (http://www.som.soton.ac.uk/research/sites/cia/), R software version 3.2.0 (www.r-project.org), and PowerView vs.3.5.2 (AARDEX Group ltd, Sion Switzerland; to analyse electronic adherence data). Since this is perceived to be an explanatory trial and as per recommendations by the European Medicine Agency for non-inferiority trials [40], we will pursue the primary (non-inferiority) objective by comparing results obtained by both intention-to-treat analysis and per protocol analysis, without formal adjustment for multiplicity (further details in discussion section).

Proportions and group means will be compared by conventional methods and expressed as absolute differences with corresponding 95% CIs; and with log-transformations as appropriate. We will estimate effects when possible; P-values, where reported, will be 2-sided. For primary analysis, we will estimate the difference in haemoglobin concentrations at the end of the 30-day fortification period between groups of children allocated to different iron formulations. Non-inferiority will be rejected if the difference between groups is less than the non-inferiority margin of 4.7 g/L.

2.18. Data and safety monitoring

We will appoint a trial monitor and an independent data and safety monitoring committee to review un-blinded data for safety purposes, monitor the progress of the trial, and to assess whether there were any safety issues that should be brought to participants' attention. No interim analyses will be conducted.

2.19. Ethical clearance

Ethical clearance has been obtained from London School of Hygiene and Tropical Medicine Ethical Committee, UK (6503) and the Kenyatta National Hospital/University of Nairobi Ethical Review Committee, Kenya (KNH-ERC/A/402). The trial is registered with ClinicalTrials.gov (NCT02073149).

3. Discussion

3.1. Duration of the intervention

We selected a relatively short 30-day intervention with iron in the expectation that premedication with dihydroartemisinin-piperaquine

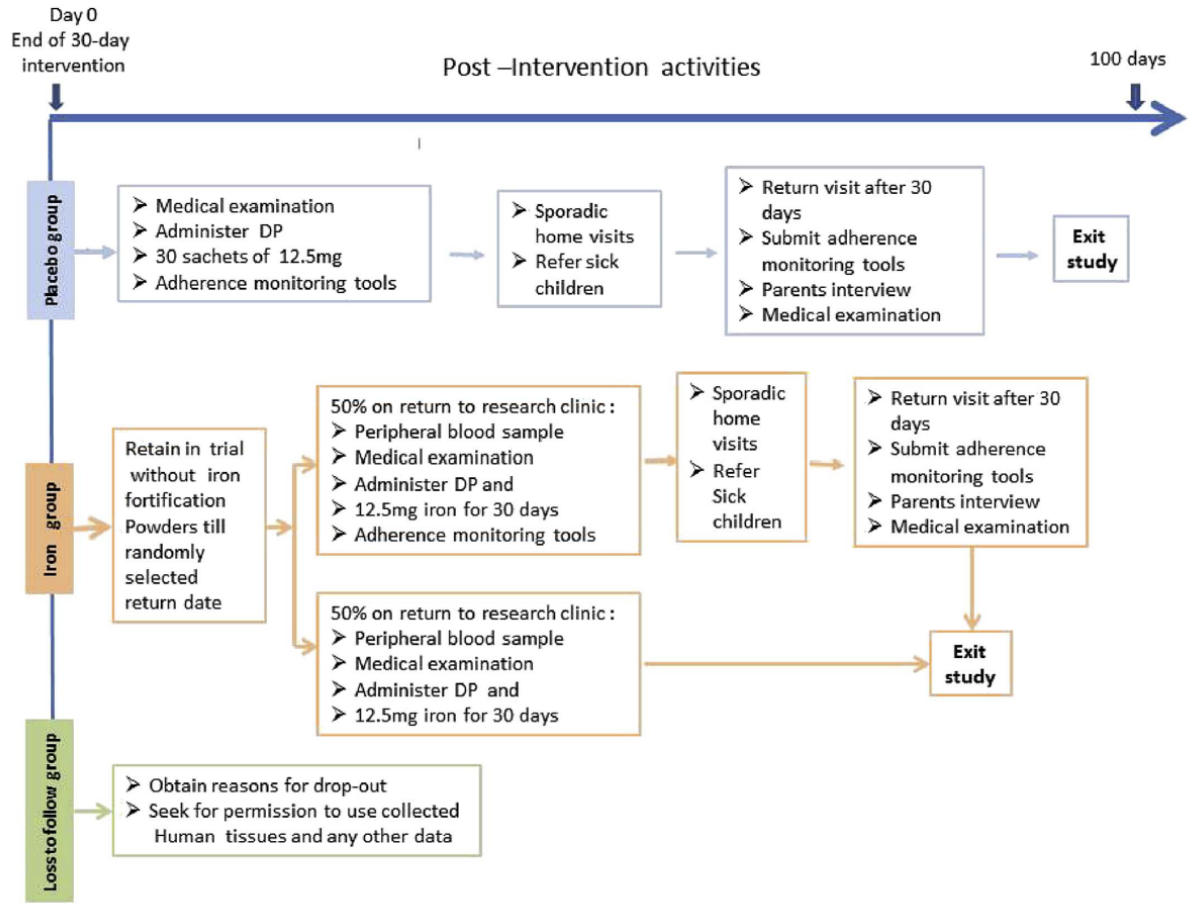

Fig. 3. Post intervention flow of activities.

will prevent malaria during this period, with a long-term view that the protection afforded by repeated chemoprevention with this combination drug would allow time windows for safe administration of short courses of iron intervention. In a recent study among preschool children in Burkina Faso, two cycles of chemoprevention with dihydroartemisinin-piperaquine, administered at the same target dose as in our study, resulted in a protection against malaria that persisted at a high level for 3–4 weeks and decreased rapidly thereafter, indicating that protection lasts at most 3–4 weeks [41]. In an earlier placebo-controlled randomised trial among Kenyan children aged 2–36 months, it was shown with smaller sample size (79 iron; 76 placebo) than the present study that weekly supplementation with 6 mg elemental iron as ferrous fumarate per kg bodyweight improved haemoglobin concentration at 4 weeks after the start of intervention [42].

3.2. Justification for use of a placebo

The use of placebo in non-inferiority trials is controversial. Opponents have argued that: a) the use of placebos as controls is unethical and mostly disregard the interest of patients [43]; b) placebo group are unnecessary where there is proof of the effect of the existing treatment and as such any new treatment should be tested against the existing treatment [44] and c) its use in trials should decline as medical knowledge increases [45].

Inclusion of a third arm (placebo) in this trial adheres to the guidelines for non-inferiority trials as stipulated by the European Medicine Agency [40] and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use [46]. We perceive a placebo arm to be ethical in the presence of an active control because in our study area there is no national policy for preventive, community-based supplementation or home fortification with iron in children and yet the children under five year are at a greater risk of iron deficiency anaemia. Because there is an on-going uncertainty about the safety of iron interventions in children living in malaria-endemic areas, our trial represents the only chance for eligible children to receive fortificants of iron with micronutrients for the iron arms and fortificants of micronutrients for the placebo arm with malaria chemoprevention. Thus prohibition of the trial on ethical grounds would be against the interest of eligible children and their guardians.

Use of a placebo is necessary in our explanatory trial because we aim to demonstrate that the experimental treatment (3 mg iron as NaFeEDTA) is non-inferior to the active control (12.5 mg iron as encapsulated ferrous fumarate). The demonstration of non-inferiority in a trial with only two arms can have two meanings only: both interventions are equally effective, or both interventions are equally ineffective against placebo. Furthermore, a placebo matches the comparative treatments in all ways except for the therapeutic components, and therefore the use of a standard treatment alone may not necessarily control for the same set of non-specific factors as a placebo [47]. Overall, the placebo group is useful for a) demonstration of superiority of home fortification with 3 mg iron as NaFeEDTA over

placebo (proof of efficacy); b) demonstration of superiority of the reference (12.5 mg iron as encapsulated ferrous fumarate) over placebo (proof of assay sensitivity) c) demonstration that home fortification with 3 mg iron as NaFeEDTA retains most of the efficacy of the reference over placebo (proof of non-inferiority) because failure for a test drug to demonstrate effectiveness does not necessarily mean it is not efficacious.

Increased medical knowledge has mistakenly been used to justify dropping the use of placebo in trials; conversely, increased medical knowledge has subsequently propell the production of new treatments. Thus dropping the use of placebos in a trial limits the determination of efficacy and safety of the new treatment [48] consequently denying physicians' opportunities to apply treatment options when needed.

3.3. Adjusting for multiplicity

It has been suggested that multiplicity adjustments may be necessary in non-inferiority tests especially in studies with multiple objectives [49]. European Medicine Agency regulatory guidelines [40] clearly state that when interpreting a non-inferiority trial for a potentially superior outcome there is no need to do multiplicity adjustment because a statistical significance test must be done to reject the non-inferiority. In line with these regulatory guidelines for non-inferiority trials we will not adjust for multiplicity for various reasons; first, our study has only one pre-defined primary variable (haemoglobin concentration at the end of the 30-day intervention period) that will be used to demonstrate the treatment effect. Second, as outlined in the preceding section, all three comparisons of treatment effects must show statistical significance of the haemoglobin concentration. We will therefore conduct multiple regression analysis to investigate evidence for group differences in the intervention effects and to determine the extent of bias due to irregularities between end points, because we believe that results from multiple comparisons should be mutually reinforcing, not mutually debasing [50] and hence no need for multiplicity adjustments. Third, any absence of treatment effect differences will be interpreted by confidence interval in the context of the set threshold for haemoglobin concentration (at a pre-set margin of 4.7 g/L) and all reported p-values will be 2-sided. The use of confidence intervals and statistical tests are of an exploratory nature and therefore no justification for a claim is anticipated. In addition, any multiple secondary endpoints analysis will provide supportive evidence related to our primary objective (proof of efficacy) and therefore no confirmatory conclusions are necessary. Fourth, non-inferiority will be rejected if the haemoglobin concentration differences between groups are less than the already set margin of 4.7 g/L and the results show statistical significance; thus the rest of the secondary outcomes will be considered supportive [51,52]; as such formal adjustments for the type 1 error will be considered irrelevant.

4. Funding and role of the funding agencies

The trial is funded by Sight and Life, a non-profit humanitarian initiative established by DSM Chemicals, Heerlen, The Netherlands. DSM Nutrition Products (Johannesburg, South Africa) manufactured the supplements with micronutrient powders. The International Nutrition Group of the Medical Research Council supported ET through a personal grant. Micronutrients other than iron will be included in the home fortificants at the request of Sight and Life; the funder will have no further role in study design, data collection and analysis, preparation of the manuscript, or decision to publish.

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We acknowledge all the institutions and their respective staff who collaborate with us on this study: MRC-ING, The Gambia; Wageningen University, The Netherlands; Maseno University, Kenya; Kenyatta Hospital Ethical Committee, Kenya, Amphia Hospital, Breda, The Netherlands,; Meander Medical Centre, Amersfoort, The Netherlands; Pharmacy and Poison Board of Kenya.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.conctc.2017.04.007.

Appendix 1. Information brochure

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 compare home fortification with two iron formulations. Many young children in Kenya have anaemia, a disorder that is characterized by blood with a light red colour, instead of a healthy dark red. The red pigment in blood is necessary to transport oxygen from inspired air to muscle. Children with anaemia often feel weak or tired, and may have difficulty learning. To prevent anaemia in children, medical doctors often prescribe supplements that contain 12.5 mg iron in a specific form (ferrous salts). A new form of iron has recently become available that can probably be given at lower doses, because the body is better able to absorb this type of iron.

In our study, we will divide young children into three groups and give each group a different treatment. Group 1 will receive the form of iron that has been used so far (12.5 mg iron as ferrous fumarate), Group 2 will receive the new form of iron (3 mg iron as sodium iron EDTA), and Group 3 will receive no iron. The results will be compared to determine if the new type of iron (3 mg iron as sodium iron EDTA) can prevent anaemia, and to determine if it is equally as good as the form of iron that has been used so far (12.5 mg iron as ferrous fumarate).

We will provide both forms of iron in sachets (little bags). Each day, the mother should empty and mix the contents of a single sachet into ready-prepared *uji* or some other type of food, before giving it to the child. This should be repeated every day for a period of 30 days. The child will be followed for some time afterwards.

Although iron is good to prevent anaemia, there are some concerns that it may increase the risk of malaria. For this reason, we will treat each child with a special medicine against malaria (dihydroartemisinin-piperaquine) at the start of the study, before the first dose of iron is provided. This medicine will protect the child against malaria for the time period in which the child will receive iron. In addition, all children will be dewormed at the start using two drugs (albendazole and praziquantel).

Do you have to participate?

We have asked you to take part because your child is within the age range suitable for our study. In total, we want to study 324 children. You can let your child join the study at your own free will, and withdraw your child at any moment, with or without giving reasons. . If you decide not to participate, or to withdraw from the study, this will not affect the normal care you receive in clinics or hospital elsewhere.

Read this information sheet and listen to our explanation of the study. We will then ask you to sign a consent form to show you have agreed to take part.

What will happen to you if you take part?

Screening visit: Our staff will invite you and your child to our research clinic to tell you about the aims and procedures of the study. If you agree for your child to participate, we will ask you to sign their fingerprint on a consent form. To decide whether your child can participate in the study, we will then ask you questions about your child, we will carry out a medical examination, and we will then collect samples of blood (6 mL, a volume equal to one table spoon) by arm prick, urine and stool from the child. This will take at least several hours. We will ask you to stay until if the child has produced stool. If necessary we will invite to and your child again the next day to try again. After sample collection, your child will be administered medicines (albendazole, praziquantel against worms, and dihydroartemisinin-piperaquine against malaria). You will be asked to bring your child again to the research clinic 3 days later.

Randomisation visit: At this visit, we will use the information collected so far to decide if your child can take part in the rest of the study. Participating children will receive sachets with powder. These sachets will be contained in a special dispensing bottle that you will receive with instructions for use. For one-third of children, these sachets will contain 12.5 mg iron as ferrous fumarate, one-third will contain 3 mg iron as sodium iron EDTA, and one-third contains no iron. For all three groups, the sachets also contain a mixture of other vitamins and minerals that are important for health. The allocation to group will be decided by chance (randomly). All sachets will look identical; we will not know which supplements contain iron until after the study. The first sachet will be given with food at the research dispensary. From 2 h before this point until 3 h afterwards, children will be allowed to drink but can only eat foods selected by the project team. We will then collect another blood sample (about 5–6 drops) by finger prick. From then on, community volunteers will daily supervise the supplementation in or close to your homestead.

During the 30-day intervention period: In the next 30 days, you should add and mix the contents of a single sachet to *uji* or any other food given to the child. Community volunteers will visit your home at least once a week to answer questions that you may have about the study. Children who become sick during this 30-day period will be referred to receive routine care by the regular health services. You may decide to withdraw your child at any point from the study. You may refuse to give reasons for your refusal, or to give permission for future collection of samples.

End-of-intervention survey: At the end of the 30-day period, we will collect the dispensing bottle and ask you some questions. We will again collect samples of blood (6 mL) by arm prick, stool and urine, using the same procedures as earlier. For each child, we will then break the randomisation code. Those who received placebo will be given antimalarial medicines (dihydroartemisinin-piperaquine) and 30-day supply of sachets with iron (12.5 mg iron as ferrous fumarate).

Follow-up after the 30-day intervention period: For children who received placebo, field staff will collect the dispensing bottle with the electronic device at the end of this 30-day period. Children who received iron will continue to be followed for a maximum of 100 days. In this period, we will collect a single sample of blood (5–6 drops, by finger prick). The time point for this collection will be decided by chance: for some children it may be as early as 1 week after home fortification was stopped; for others, it may be at the end of the 100-day period. Immediately following blood collection, children will be withdrawn from further study and will receive antimalarial medicines (dihydroartemisinin-piperaquine) and 30-day supply of sachets with iron (12.5 mg iron as ferrous fumarate). Field staff will also collect the dispensing bottle with the electronic device at this time.

To summarise, we will collect four blood samples from each child:

- *Randomisation visit:* 1 sample of 6 mL (a volume equal to one table spoon), to be collected by arm prick;
- *3 h later:* 1 sample of 5–6 drops, to be collected by finger prick;
- *At the end of the 30-day period:* 1 sample of 6 mL, to be collected by arm prick;
- *After the 30-day intervention period:* 1 sample of 5–6 drops, to be collected by finger prick.

We will store part of the blood samples in frozen condition, so that we can subsequently conduct tests to assess the success of the interventions. We may also check for hereditary factors that affect malaria and anaemia. Some of these tests may have to be done abroad.

Confidentiality: Results of this study will be shared with the public in a form of academic publication or presentation. The purpose of this publication or presentation is to create awareness and promote understanding of safely and efficiently treating anaemia in malaria endemic areas.

We will keep any information about your child confidential. Readers of the publication based on this research will not know that you gave this information. All personal information will be stored securely. This means that whenever we write or talk about anything we have been told, we never use your real name. The only information that we may have to pass on is if your child is at risk of serious harm.

Benefits and compensation: You will not receive financial benefits for participating in this study. If you need to stay more than 4 h, we will give you a small financial compensation to account for the lost hours.

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CHAPTER 3: Research paper 2

Cover Sheet

Student: Emily Mwadime Teshome

Principle Supervisor: Prof. Andrew Prentice

Title: Daily home fortification with iron as ferrous fumarate versus NaFeEDTA: a randomised, placebo-controlled, non-inferiority trial in Kenyan children

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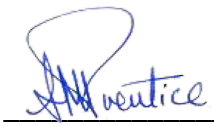
Supervisors Signature: (to confirm role as stated above): 

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RESEARCH ARTICLE Open Access

Daily home fortification with iron as ferrous fumarate versus NaFeEDTA: a randomised, placebo-controlled, non-inferiority trial in Kenyan children

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Abstract

Background: We aimed to show the non-inferiority of home fortification with a daily dose of 3 mg iron in the form of iron as ferric sodium ethylenediaminetetraacetate (NaFeEDTA) compared with 12.5 mg iron as encapsulated ferrous fumarate in Kenyan children aged 12–36 months. In addition, we updated a recent meta-analysis to assess the efficacy of home fortification with iron-containing powders, with a view to examining diversity in trial results.

Methods: We gave chemoprevention by dihydroartemisinin-piperaquine, albendazole and praziquantel to 338 afebrile children with haemoglobin concentration ≥70 g/L. We randomly allocated them to daily home fortification for 30 days with either placebo, 3 mg iron as NaFeEDTA or 12.5 mg iron as encapsulated ferrous fumarate. We assessed haemoglobin concentration (primary outcome), plasma iron markers, plasma inflammation markers and *Plasmodium* infection in samples collected at baseline and after 30 days of intervention. We conducted a meta-analysis of randomised controlled trials in pre-school children to assess the effect of home fortification with iron-containing powders on anaemia and haemoglobin concentration at end of intervention.

Results: A total of 315 children completed the 30-day intervention period. At baseline, 66.9% of children had inflammation (plasma C-reactive protein concentration >5 mg/L or plasma α_1-acid glycoprotein concentration >1.0 g/L); in those without inflammation, 42.5% were iron deficient. There was no evidence, either in per protocol analysis or intention-to-treat analysis, that home fortification with either of the iron interventions improved haemoglobin concentration, plasma ferritin concentration, plasma transferrin receptor concentration or erythrocyte zinc protoporphyrin-haem ratio. We also found no evidence of effect modification by iron status, anaemia status and inflammation status at baseline. In the meta-analysis, the effect on haemoglobin concentration was highly heterogeneous between trials (I^2: 84.1%; p value for test of heterogeneity: <0.0001).

Conclusions: In this population, home fortification with either 3 mg iron as NaFeEDTA or 12.5 mg iron as encapsulated ferrous fumarate was insufficiently efficacious to assess non-inferiority of 3 mg iron as NaFeEDTA compared to 12.5 mg iron as encapsulated ferrous fumarate. Our finding of heterogeneity between trial results should stimulate subgroup analysis or meta-regression to identify population-specific factors that determine efficacy.

Trial Registration: The trial was registered with ClinicalTrials.gov (NCT02073149) on 25 February 2014.

Keywords: Anaemia, Child, Pre-school, Ferric sodium EDTA, Home fortification, Iron, Non-inferiority, Meta-analysis

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Background

In 2011, the World Health Organisation (WHO) recommended daily home fortification with iron (12.5 mg as a ferrous salt) in populations where the prevalence of anaemia in children younger than 5 years of age is ≥20% [1], which covers most developing countries [2]. This recommendation was based on a meta-analysis of randomised controlled trials showing moderate quality evidence for an effect on anaemia and haemoglobin concentration [3].

The WHO-recommended dose of 12.5 mg iron was established to meet almost 90% of the estimated total iron requirement of children aged 6–18 months [4]. Several trials have shown, however, that supplementation or food fortification with iron at this dose can increase rates of hospital admissions [5] as well as diarrheal and respiratory diseases [6]. In addition, it can produce a potentially more pathogenic gut microbiota profile that is associated with gut inflammation [7, 8]. Ingestion of ferrous salts also frequently causes mild gastrointestinal adverse effects (e.g. constipation, nausea, vomiting and epigastric discomfort) that may reduce adherence to treatment [9]. The frequency and severity of such effects depend on dose and dosage schedule [10, 11], may be due to oxidative stress [12] and appear to be reduced when iron is taken with food.

By comparison, ingestion of a low dose of highly bioavailable iron (3 mg iron as ferric sodium ethylenediaminetetraacetate (NaFeEDTA)) may result in similar or even higher quantities of absorbed iron [13] and may be non-inferior in its effect on iron status. It may have the advantage that reduced amounts of ingested iron may reduce proliferation of pathogenic gut bacteria, produce less oxidative stress and increase tolerability and adherence [14]. In addition, iron as NaFeEDTA has been reported to cause less oxidative stress than an equimolar dose of iron as ferrous sulphate [15]. No study has so far compared the efficacy of daily home iron fortification with 12.5 mg iron as encapsulated ferrous fumarate versus 3 mg iron as NaFeEDTA.

In malaria-endemic areas, WHO has recommended that iron interventions should be implemented in conjunction with measures to control malaria [1], because there is substantial evidence that iron interventions can increase malaria rates in young children [16].

We aimed to show non-inferiority of home fortification with 3 mg iron as NaFeEDTA compared with 12.5 mg iron as encapsulated ferrous fumarate in children aged 12–36 months under cover of chemoprevention against malaria. We conducted a pre-specified analysis to explore to what extent efficacy depended on baseline iron markers (haemoglobin concentration, plasma concentrations of ferritin and soluble transferrin receptor measured), because iron absorption is known to depend on iron status. Lastly, we used results from our present study and other recent reports to update the meta-analysis by Salam et al. [5], with a view to examining diversity in study results.

Methods

Details of study methods are described in the statistical analysis plan (Additional file 1). During study implementation, there were no major amendments to the protocol.

Study setting and subjects

Fieldwork was conducted between January 2014 and December 2014 in Kisumu West District, Kenya. Malaria is highly endemic in the area [17, 18], with virtually all infections due to *Plasmodium falciparum*. In children aged 1–4 years, the prevalence of infections due to *P. falciparum* has been reported to range between 39% and 63% [19]. Previous trials in the area have shown that iron supplementation resulted in increased haemoglobin concentrations among pre-school children, suggesting that iron deficiency is common [20–22].

Study design

This was a randomised, double-blind, non-inferiority trial with three arms: 3 mg iron as NaFeEDTA (experimental treatment); 12.5 mg iron as encapsulated ferrous fumarate (active control treatment); and placebo. We included the placebo arm to demonstrate superiority of the investigational drug over placebo (proof of efficacy) [23]. The study was conceived as an explanatory trial to evaluate efficacy with maximal compliance.

Data collection timelines and field procedures

Community health workers invited parents of children aged 12–36 months old for screening at the research clinic. During screening, research assistants measured height and length within 0.1 cm using wooden measuring boards (UNICEF, Copenhagen, Denmark) and weight to the nearest 100 g using a Salter scale (UNICEF, Copenhagen, Denmark). They also administered a standardised form to collect vital data and household characteristics data. Medical staff conducted a medical examination and collected venous blood (4 mL) in tubes containing Li-heparin for subsequent determination of iron biomarkers and *Plasmodium* infection. Children who attained the eligibility criteria were given pre-medications 3 days before randomisation to treatment allocation.

Pre-medications

Medical staff administered drugs to prevent malaria and to control anaemia due to helminth infections in the subsequent intervention period. These pre-medications

comprised: (1) dihydroartemisinin-piperaquine (Sigma-Tau, Rome, Italy; tablets of 40 mg of dihydroartemisinin and 320 mg of piperaquine), for 3 days at a daily target dose of 4 mg/kg body weight [24]; (2) albendazole (Indoco Remedies, Mumbai, India), for 3 days at a daily target dose of 200 mg or 400 mg for children aged 12–24 months and >24 to 36 months, respectively; (3) praziquantel (Cosmos, Nairobi, Kenya; 600-mg tablets), as a single dose at a target dose of 40 mg/kg body weight [25]. Piperaquine is eliminated slowly (mean elimination half-life: 23 days in children in Burkina Faso) [26], resulting in a protective efficacy against malaria for at least 1 month [27, 28]. Medical staff observed that the child swallowed the first dose of the pre-medication drugs at the research clinic. Parents were instructed to administer the remaining two doses of dihydroartemisinin-piperaquine and albendazole at home on the subsequent 2 days.

Eligibility

Children were eligible for enrolment in the study after attaining the following eligibility criteria: aged 12–36 months; the child was expected to remain resident in the study area for the duration of the intervention and follow-up; no known or reported allergy to pre-medication drugs; not severely malnourished (weight-for-height z-score < –3 SD); absence of fever (axillary temperature <37.5 °C); absence of reported or suspected systemic disorders (e.g. HIV infection, tuberculosis, sickle cell disease); haemoglobin concentration ≥70 g/L; and at least one parent signed an informed consent form. Of the 433 children screened between April and July 2014, 338 children were randomised for intervention. In our sample size calculations [29–31], we specified a non-inferiority margin for haemoglobin concentration of 4.7 g/L, which we expected to preserve 50% of the reported and anticipated minimum effect of 12.5 mg ferrous fumarate (9.3 g/L, [32]; Additional file 1).

Randomisation

Three days after the screening visit, children who met the eligibility criteria were randomised at the research clinic. We used a stratified block design to achieve group balance in size and baseline haemoglobin concentration. A person not involved in the fieldwork assigned the three treatment groups to a sequence of random permuted blocks of sizes 6 or 9 nested within two strata defined by baseline haemoglobin concentration class (<100 g/L and ≥100 g/L), using tables with random numbers and random permuted blocks. Following this scheme, two other persons not involved in the fieldwork produced a set of labels with a child's identification number that included a letter for stratum (A or B) and a consecutive allocation number as indicated by the randomisation scheme. At the randomisation visit, the trial coordinator assigned children successively to the next available allocation number randomised for treatment and according to appropriate stratum.

Composition of fortificants

We used three types of micronutrient powders that contained vitamin A and zinc contents as per WHO recommendations [1], 11 other micronutrients (Table 1) at doses as recommended by the Home Fortification Technical Advisory Group [33] and, in addition, either 3 mg iron as NaFeEDTA, or 12.5 mg iron as encapsulated ferrous fumarate, or no iron (placebo). The micronutrient powders were packed in 1-g plain white foil single-serve sachets that were identical in appearance and that did not result in apparent differences in taste, texture or colour of *uji* (porridge made of maize flour). We excluded folic acid because of concerns that it may cause failure of antifolate drugs and because of the absence of evidence that folate deficiency anaemia is a public health problem among children in developing countries [34].

Blinding

Researchers, outcome assessors and parents remained blinded to the type of treatment allocated to each child until the 30-day intervention period had been completed. At this point they were partially unblinded to know if the child was in the placebo or iron group.

Table 1 Composition of home fortificants

Micronutrient	Content
Vitamin A	300 μg RE
Vitamin D	5 μg
Vitamin E	5 mg
Vitamin C	30 mg
Thiamin (vitamin B_1)	0.5 mg
Riboflavin (vitamin B_2)	0.5 mg
Niacin (vitamin B_3)	6 mg
Vitamin B_6 (pyridoxine)	0.5 mg
Vitamin B_{12} (cobalamin)	0.9 μg
Iron	
EITHER iron as encapsulated ferrous fumarate	12.5 mg
OR iron as NaFeEDTA	3 mg
OR no iron (placebo)	0 mg
Zinc	5 mg
Copper	0.56 mg
Selenium	17 μg
Iodine	90 μg

RE retinol equivalents

Investigators were fully unblinded after the fieldwork was complete and the statistical analysis plan written.

Adherence monitoring

Adherence to intervention was monitored using an electronic monitoring and time-recording device (Medication Events Monitoring Systems, MEMS, 6 TrackCap 45 mm without LCD display; WestRock, Sion, Switzerland; http://www.medamigo.com/) that is considered to be the reference standard and superior to medication counts and self-reported adherence methods [35, 36]. The battery-operated device consists of a cap with a built-in microprocessor that fits the bottle with the micronutrient sachets. It records and internally stores dates and times of all openings. Parents were taught how to use the bottle with the electronic device without knowing that the device was monitoring the openings of the bottle and instructed to return the electronic device with any remaining sachets at the end of 30 days of intervention.

Intervention period

On the first day of intervention, research assistants gave parents a supply of 30 sachets in a plastic bottle with a MEMS cap and instructed them to add the contents of a single-serve sachet to the child's semi-solid, ready-prepared foods every day for a period of 30 days. The main staple food consumed by pre-school children was *uji* made from locally milled flour from either maize or sorghum grains. The grains are not de-germed and sifted and so have high contents of phytic acid and phenolic compounds [37, 38]. At the research clinic, the assistants showed parents how to mix the contents of the first sachet with *uji*. This first dose was consumed at the research facility, and trained research assistants closely observed that each child consumed all the *uji*. Parents were given a mosquito net and instructed to immediately inform research assistants whenever a child fell sick. Research assistants conducted weekly pre-announced home visits to check if parents were adhering to instructions given at randomisation. Sick children found in the homes were referred to the research clinic. Children with fever (≥37 °C) and who tested positive for *Plasmodium* infection by microscopic examination of blood smears were treated immediately with artemisinin-lumefantrine; during treatment, these children were temporarily discontinued from the intervention treatment until medication was completed and subsequent microscopic examination of blood smears conducted after 7 days showed negative for malaria parasites.

Parents who withdrew children from the intervention were asked for reasons and permission to keep and analyse data and samples already collected. After 30 days of intervention, the phlebotomist collected venous blood (4 mL) and processed samples as stated above (Data collection time lines and field procedures). Medical staff examined every child, and fieldworkers collected anthropometric data, plastic bottles with the MEMS cap and empty sachets and administered a questionnaire to parents to collect additional information on possible factors affecting adherence. Once all data and samples were collected, the trial coordinator opened the sealed brown envelope to find out the child's intervention group (either iron or placebo).

Post-intervention period

For ethical reasons, children in the placebo group were given a 3-day course of dihydroartemisinin-piperaquine and a subsequent 30-day course of home fortification with 12.5 mg iron as encapsulated ferrous fumarate. Children in the iron group were retained without fortification powders to monitor the population decline in haemoglobin concentration over time in a 100-day follow-up period. Parents were requested to take each child home and bring them back to the research clinic on a date generated by a pre-programmed Microsoft Excel software that randomly selected a date of their return visit within a 100-day period. On the return visit, a capillary blood sample was collected by finger puncture to measure haemoglobin concentrations in duplicate from a single drop and to store DNA on collection cards for subsequent assessment by PCR assay of *Plasmodium* parasites. Immediately, these children were withdrawn from further study and received appropriate medication if sick, a therapeutic course of dihydroartemisinin-piperaquine and a supply of sachets for daily home fortification with 12.5 mg iron as encapsulated ferrous fumarate for another 30 days.

Laboratory analysis

We determined haemoglobin concentration (HemoCue 301, Ängelholm, Sweden) and zinc protoporphyrin (ZPP)-haem molar ratio (AVIV, model 206D, Lakewood NJ, USA) in whole blood and in erythrocytes as a marker of iron-deficient erythropoiesis, each in triplicate. We assayed *Plasmodium* antigenaemia by histidine-rich protein 2 (HRP2) and lactate dehydrogenase (LDH) tests and transferred aliquots of whole blood (125 μL) on DNA collection cards (FTA Mini Card, catalogue WB120055, GE Healthcare, Little Chalfont, UK) for storage at ambient temperature and subsequent detection by PCR of *Plasmodium* infection; we also prepared thick and thin blood smears to allow for detection and counting of *Plasmodium* parasites. Iron markers (plasma concentrations of ferritin, soluble transferrin receptor and transferrin), inflammation markers (plasma concentrations of C-reactive protein (CRP) and α_1-acid glycoprotein), albumin and vitamin B12 were measured at Meander Medical Centre, Amersfoort, The Netherlands, on an Abbott Architect C16000 and i2000 SR analyser as per manufacturer's instructions.

Statistical analysis

Details of the statistical analysis are presented in a supplementary paper (Additional file 1). Data were double entered, checked for completeness and verified for possible entry errors using Microsoft Excel. Anthropometric indices and electronic adherence data were analysed using WHO Anthro software v.3.2.2 (World Health Organisation, Geneva, Switzerland) and PowerView v.3.5.2 (AARDEX Group Ltd, Sion, Switzerland), respectively. The final statistical analysis was conducted using SPSS 21 (IBM, Armonk, NY, USA) and CIA 2.2.0 (https://eprints.soton.ac.uk/393017/). To assess EDTA intake at home fortification levels of 3 mg iron as NaFeEDTA, we calculated the intake of iron per kilogram of body weight for all children in the trial, both at baseline and at 30 days after intervention. The corresponding intake of NaFeEDTA ($NaFeC_{10}H_{12}N_2O_8$) was calculated as intake iron × (molecular $weight_{EDTA}$/molecular $weight_{Iron}$) = intake iron × (288.21/55.88). From these results, we assessed the prevalence of EDTA intake exceeding the upper level of the acceptable daily intake (ADI) (i.e. the amount of a food additive, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk [39]) to be 1.9 mg/kg body weight [40].

The definitions we used were anaemia: haemoglobin concentration <110 g/L [41]; iron deficiency: plasma ferritin concentration <12 μg/L [42]; *Plasmodium* infection: presence of parasites and gametocytes of any *Plasmodium* species [43]; inflammation: plasma CRP concentration >5 mg/L [44] or plasma α_1-acid glycoprotein concentration >1.0 g/L [45], respectively. Group adherence was defined as the proportion of children who consumed ≥24 home fortification doses, corresponding to ≥80% of the 30 scheduled doses [46], with exclusion of children who were lost to follow-up because they moved out of the study area.

Variation of outcomes with a log-normal distribution was expressed as geometric standard deviation (GSD), i.e. a multiplicative factor such that division or multiplication of the geometric mean by this ratio indicates a variation that is equivalent to subtraction or addition of one standard deviation on a log-transformed scale [47].

Plasma ferritin concentration can be elevated by inflammation independently of iron status. Thus, when estimating the prevalence of iron deficiency, we accounted for inflammation by two methods. First, we restricted the analysis to children without inflammation. This method has the disadvantages that it leads to a reduced statistical precision (because of the reduced sample size) and it may produce biased results (if the true prevalence of iron deficiency differs between those with and without inflammation). Second, we used linear regression to adjust plasma ferritin concentrations for plasma concentrations of CRP and α_1-acid glycoprotein concentration. We used the following formula for adjustment: $\log_e(\text{ferritin}_{\text{adjusted}}) = \log_e(\text{ferritin}_{\text{unadjusted}}) - \beta_1[\log_e(\text{CRP}_{\text{observed}}) - \log_e(\text{CRP}_{\text{reference}})] - \beta_2[\log_e(\text{AGP}_{\text{observed}}) - \log_e(\text{AGP}_{\text{reference}})]$; the results were then exponentiated to express ferritin concentrations in their natural units. The formula given here is similar to what was recently proposed [48]; however, we used log-transformed values because of the linear relationship that has been observed between log-ferritin and log-CRP, as well as between log-ferritin and log-α_1-acid glycoprotein (Suchdev, personal communication, 2016; and also the present study (not shown)). We used 15 mg/L and 2.59 g/L as reference values for CRP and α_1-acid glycoprotein, respectively, because these values were reported as the upper limits of the 95% reference range in healthy French children aged 3–5 years [49]. We implicitly assumed that higher upper values for reference ranges, as can be observed in their peers in developing countries, are due to infections and other inflammation-inducing disorders.

The objective of non-inferiority was determined by comparing end points obtained by both intention-to-treat and per protocol analyses. We visually inspected histograms to assess whether outcome variables were normally distributed within intervention groups. Outcome variables with a log-normal distribution were log-transformed; exponentiation of group differences in log-transformed outcomes resulted in associations being expressed as relative differences. Outcomes that were not normally distributed, even after log-transformation, were compared using non-parametric tests. We estimated effects; *p* values, where reported, are two-sided.

For the primary analysis, we estimated the difference in haemoglobin concentrations at the end of the 30-day fortification period between groups of children allocated to different iron formulations. Analysis was done using analysis of variance (ANOVA) and multiple linear regression analysis. As pre-planned, we accepted non-inferiority only when all of the following conditions were met: (1) home fortification with 3 mg iron as NaFeEDTA was superior to placebo (proof of efficacy); (2) home fortification with 12.5 mg iron as encapsulated ferrous fumarate was superior to placebo (proof of assay sensitivity); and (3) the lower limit of the 95% confidence interval (CI) around the difference in haemoglobin concentration between the groups who received home fortification with the two different iron formulations excluded the non-inferiority margin of 4.7 g/L, in both intention-to-treat and per protocol analyses (proof of efficacy).

For the secondary analysis, we used stratified analysis and multiple linear regression models to control for group imbalances of baseline factors that were strongly

or moderately associated with primary outcome and were likely to influence the effect estimates. These baseline factors were plasma ferritin concentration, plasma soluble transferrin receptor concentration and age.

Subgroup analysis

Because iron absorption is known to depend on iron status, we considered iron markers at baseline (anaemia, iron status) as potential modifiers for intervention effects on haemoglobin concentration and plasma ferritin concentration at the end of the 30-day intervention period. Stratified analysis was used to measure effect sizes within subgroups; evidence for group differences in intervention effects was formally investigated in multiple linear regression models that included intervention and each baseline factor as main terms, as well as their product term. We conducted all three possible paired group comparisons (12.5 mg iron as ferrous fumarate versus 3 mg iron as NaFeEDTA; 12.5 mg iron as ferrous fumarate versus placebo; 3 mg iron as NaFeEDTA versus placebo). In these analyses, we adjusted for plasma concentrations of ferritin and transferrin receptor at baseline (both as continuous variables); in the analysis of plasma ferritin concentration at 30 days after start of intervention, these baseline variables were log-transformed because this gave a better model fit than the untransformed variables.

Meta-analysis

We conducted a meta-analysis of randomised controlled trials in pre-school children to assess the effect of home fortification with iron-containing powders on anaemia and haemoglobin concentration at end of intervention. The methods are described in Additional file 2.

Results

Flow of participants

Of 433 children who were invited for screening, 366 children met the criteria for pre-medication administration and 338 children were randomised. Of these, 315 (93.5%) completed 30 days of the intervention period and were included in the per protocol analysis (Fig. 1). Reasons for loss to follow-up were refusal by parents ($n = 8$), moving out of the study area ($n = 6$) and unknown ($n = 9$).

A total of 80 children became sick during the 30-day intervention period. We found no evidence that the proportion of sick children varied between intervention groups ($p = 0.13$). The most common infections were uncomplicated malaria with upper respiratory tract infections (24), severe malaria (14), upper respiratory tract infections (11) and uncomplicated malaria (8). Overall, 200 (60.6%) of the children consumed more than 80% (>24) of 30 scheduled sachets, and there was no evidence that this percentage differed between intervention groups ($p = 0.99$).

Baseline characteristics

The intervention groups were similar with respect to baseline characteristics (Table 2). Overall, the mean age was 23.6 months; 62.1% of children had anaemia. Inflammation as indicated by elevated concentrations of CRP or α_1-acid glycoprotein occurred in 66.9% (226/338) of children. The prevalence of iron deficiency was 17.1% (57/333) or 42.5% (57/134), depending on whether or not children with inflammation were excluded from analysis. With ferritin concentrations adjusted for inflammation by linear regression, the prevalence of iron deficiency was 53.2% (177/333). Of 366 blood samples assessed for the presence of *P. falciparum* by microscope or rapid dipstick tests (HRP2 or *P. falciparum*-specific pLDH), 40.7% and 36.4% of children were positive, respectively, with a median (25^{th}, 75^{th} percentiles) density of asexual parasites of 1410 (207, 682) μ/L. The prevalence of being stunted, wasted and underweight was 30.1%, 3.3% and 13.9%, respectively.

EDTA intake

Home fortification with 3 mg iron as NaFeEDTA resulted in a mean intake (SD) of 1.48 (0.24) EDTA/kg body weight and 1.44 (0.23) EDTA/kg body weight at baseline and at 30 days after the start of intervention, respectively (Fig. 2). Correspondingly, assuming that this intake follows a normal distribution, 4.0% and 2.0% of children would exceed the ADI for EDTA of 1.9 mg/kg body weight.

Intervention effects

In the per protocol analysis, there was no evidence that home fortification for 30 days, whether using a daily dose of 3 mg iron as NaFeEDTA or 12.5 mg iron as ferrous fumarate, was efficacious in improving haemoglobin concentration, plasma ferritin concentration, plasma transferrin receptor concentration or erythrocyte ZPP-haem ratio (Table 3). Adjustment for baseline factors that were prognostic for haemoglobin concentration did not substantially change intervention effects (haemoglobin concentration difference of 1.3 g/L (−1.8 g/L to 4.3 g/L). Similarly, the intention-to-treat analysis (Additional file 3: Table S2) led to virtually identical results as obtained by per protocol analysis, and restriction of the analysis to children without inflammation led to similar effect estimates on haemoglobin concentration (0.9 g/L, −4.1 to 5.9 g/L and 2.3 g/L, −3.1 to 7.8 g/L for 3 mg iron as NaFeEDTA and 12.5 mg iron as ferrous fumarate, respectively). Given these results, we conducted no further analysis to demonstrate non-inferiority of 3 mg iron as NaFeEDTA.

Compared to placebo, home fortification with 3 mg iron as NaFeEDTA seemed to reduce the prevalence of iron deficiency by 20.2% (Table 4); one-quarter of children who

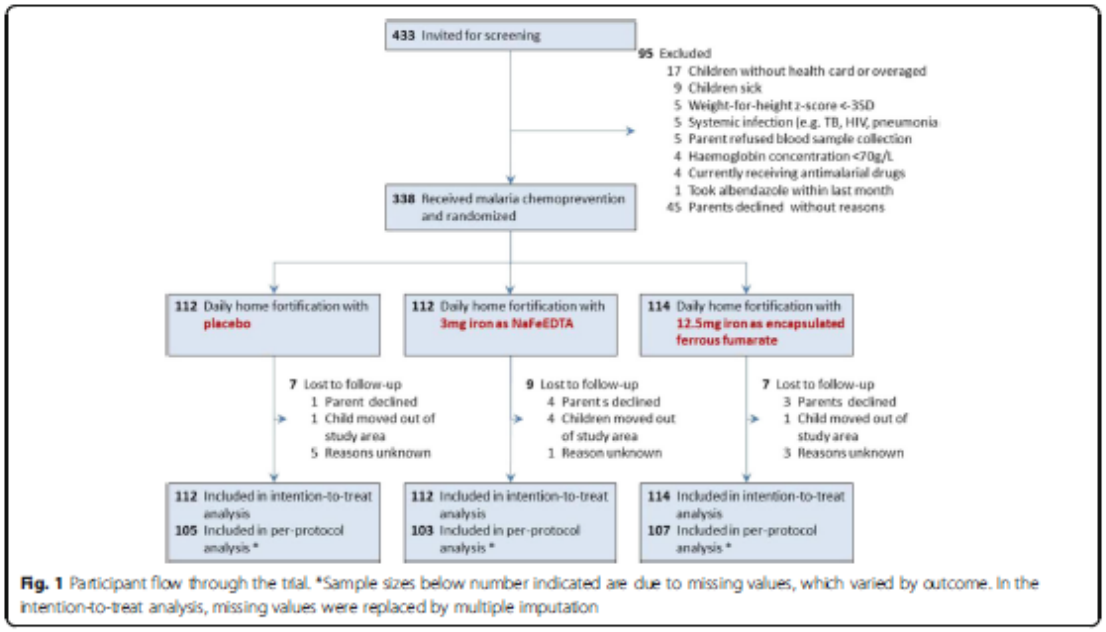

Fig. 1 Participant flow through the trial. *Sample sizes below number indicated are due to missing values, which varied by outcome. In the intention-to-treat analysis, missing values were replaced by multiple imputation

received this iron formulation nonetheless remained anaemic after 30 days. Home fortification with 12.5 mg iron as ferrous fumarate seemed to reduce the prevalence of iron deficiency by 14.1% (95% CI: −3.5 to 30.7%), but the statistical evidence for such a reduction, as judged by the 95% CI, was weak. There was no evidence that either of the iron interventions affected the prevalence of anaemia, which affected one-half of children at the end of the 30-day intervention period, or the prevalence of *Plasmodium* infection, which remained at 16.4–19.1%, depending on the method of assessment.

Subgroup analysis

There was no evidence that the effect of home fortification with iron, whether administered at a daily dose of 3 mg iron as NaFeEDTA or 12.5 mg iron as encapsulated ferrous fumarate, on haemoglobin concentration or plasma ferritin concentration was modified by iron status or anaemia status at baseline (see Figs. 3 and 4). The effect of home fortification with 12.5 mg iron as ferrous fumarate on increased plasma ferritin concentration seemed larger in children without inflammation than in their peers with inflammation at baseline (89% versus 3%), but the statistical evidence of such an effect modification was weak (p interaction: 0.17; Fig. 4).

Meta-analysis

The effect on haemoglobin concentration was highly heterogeneous (I^2: 8.1%; p value for test of heterogeneity: <0.0001; Additional file 2: Figure S4). The pooled effect on haemoglobin concentration was 3.9 g/L (95% CI: 2.2–5.50 g/L), indicating that in a random sample of a hypothetically infinite number of trials, each estimating a different true underlying effect, one may on average expect an increase in haemoglobin concentration by 3.9 g/L, with the 95% CI excluding an effect beyond 5.5 g/L.

Discussion

We found no evidence that daily home fortification for 30 days with a daily dose of either 3 mg iron as NaFeEDTA or 12.5 mg iron as encapsulated ferrous fumarate was efficacious in improving haemoglobin concentration or iron markers (plasma ferritin concentration, plasma soluble transferrin receptor concentration or erythrocyte ZPP-haem ratio). Compared to placebo, however, home fortification with 3 mg iron as NaFeEDTA reduced the prevalence of iron deficiency by 20.2% (44.6% versus 24.5%; Table 4). Meta-analysis of trial results indicates a small, heterogeneous effect of home fortification with iron-containing powders on haemoglobin concentration.

Dichotomising a continuous outcome variable has the disadvantages that individuals close to but on opposite sides of the cut-off value are characterised as being very different rather than very similar [50], and that the prevalence difference depends on the cut-off value used for dichotomisation of the outcome [51]. Thus, in our study, a small group difference in the distribution of plasma ferritin concentration may misleadingly result in

Table 2 Baseline characteristics, by intervention group

Characteristic	Placebo	Iron, 3 mg as NaFeEDTA	Iron, 12.5 mg as ferrous fumarate
Number (*n*)	112	112	114
General characteristics			
Sex, male	69 (61.6%)	61 (54.5%)	56 (49.1%)
Age, months	22.8 (6.8)	23.2 (6.2)	24.9 (6.4)
Age class			
12–23 months	61 (54.5%)	57 (50.9%)	44 (38.6%)
24–36 months	51 (45.5%)	55 (49.1%)	70 (61.4%)
Nutritional markers			
Haemoglobin concentration, g/L	104.4 (13.2)	105.9 (13.3)	104.7 (13.3)
Anaemia			
Moderate (haemoglobin concentration 70–99.99 g/L)	33 (29.5%)	34 (30.4%)	36 (31.6%)
Mild (haemoglobin concentration 100–109.99 g/L)	39 (34.8%)	31 (27.7%)	37 (32.5%)
No anaemia (haemoglobin concentration ≥110 g/L)	40 (35.7%)	47 (42.0%)	41 (36.0%)
ZPP:haem ratio, μmol:mol[a]			
In whole blood	170 (119; 305)	172 (102; 260)	196 (137; 283)
In erythrocytes	130 (84; 301)	141 (76; 223)	160 (101; 246)
Plasma ferritin concentration, μg/L[a,b]	37.7 (17.3; 74.0)	31.4 (16.0; 56.6)	36.9 (17.6; 68.8)
Iron status[b]			
Deficient (plasma ferritin concentration <12 μg/L)	17 (15.2%)	20 (18.3%)	20 (17.9%)
Replete (plasma ferritin concentration ≥12 μg/L in the absence of inflammation)	26 (23.2%)	32 (29.4%)	19 (17.0%)
Uncertain (plasma ferritin concentration ≥12 μg/L in the presence of inflammation)	69 (61.6%)	57 (52.3%)	73 (65.2%)
Iron deficiency, based on adjusted ferritin concentrations[b,c]	52.7% (59/112)	57.8% (63/109)	49.1% (55/112)
Plasma soluble transferrin receptor concentration, mg/L[a]	2.41 (1.75; 3.46)	2.39 (1.82; 3.16)	2.60 (1.93; 3.41)
Plasma albumin concentration, g/L	34.6 (3.9)	35.0 (3.5)	34.7 (4.1)
Vitamin B_{12} concentration, pmol/L	391 (291; 557)	409 (311; 569)	401 (315; 554)
Infection and inflammation markers			
Plasma C-reactive protein concentration, mg/L[a]	2.5 (0.7; 7.6)	2.5 (0.6; 7.8)	4.5 (1.3; 11.0)
Plasma α_1-acid glycoprotein concentration, g/L[a]	1.20 (0.90; 1.57)	1.08 (0.81; 1.47)	1.17 (0.97; 1.63)
Inflammation			
Plasma C-reactive protein concentration >5 mg/L	40 (35.7%)	35 (31.3%)	53 (46.5%)
Plasma α_1-acid glycoprotein concentration >1 g/L	71 (63.4%)	63 (56.3%)	81 (71.1%)
Plasma C-reactive protein concentration >5 mg/L or plasma α_1-acid glycoprotein concentration >1 g/L	77 (68.8%)	65 (58%)	84 (73.7%)
Plasmodium antigenaemia, by rapid dipstick tests[d]			
P. falciparum (either HRP2 or *P. falciparum*-specific pLDH)	39 (35.1%)	40 (36.0%)	43 (38.1%)
Plasmodium species other than *P. falciparum* (pLDH specific for *P. malariae*, *P. ovale* or *P. vivax*)[e]	1 (0.9%)	2 (1.8%)	0
Any *Plasmodium* species	39 (35.1%)	41 (36.9%)	43 (38.1%)
Blood smear tests, by microscopy			
Asexual or sexual forms of *P. falciparum*	47 (41.9%)	49 (43.8%)	55 (48.2%)
Asexual forms of both *P. falciparum* and human *Plasmodium* spp. other than *P. falciparum* (i.e. *P. malariae*, *P. ovale* or *P. vivax*)	2 (1.8)	1 (0.8)	2 (1.8)
Asexual parasite density for *P. falciparum*, μ/L[a]	757 (172; 3972)	3340 (297; 20,023)	1048 (207; 6820)

Table 2 Baseline characteristics, by intervention group *(Continued)*

Low (<1000 μ/L)	17 (15.2%)	9 (8.0%)	19 (16.7%)
Medium (1000–9999 μ/L)	12 (10.7%)	8 (7.1%)	15 (13.2%)
High (≥10,000 μ/L)	3 (2.7%)	7 (6.3%)	5 (4.4%)
Gametocyte density, μ/L[a]	326 (49; 732)	82 (37; 120)	1126 (57; 2679)
Anthropometric markers			
Body height, cm	80.9 (5.9)	82.1 (5.7)	82.4 (5.1)
Body weight, kg	10.6 (1.8)	10.8 (2.0)	10.9 (1.7)
Height-for-age z-score, SD	−1.42 (1.47)	−1.15 (1.43)	−1.43 (1.31)
Weight-for-height z-score, SD	−0.10 (1.05)	−0.16 (1.07)	−0.14 (0.93)
Weight-for-age z-score, SD	−0.81 (1.18)	−0.71 (1.22)	−0.86 (1.09)
Stunted (height-for-age z-score < −2 SD)	34 (30.4%)	31 (27.7%)	37 (32.5%)
Wasted (weight-for-height z-score < −2 SD)	3 (2.7%)	5 (4.5%)	2 (1.8%)
Underweight (weight-for-age z-score < −2 SD)	19 (17.0%)	12 (10.7%)	15 (13.2%)

Values indicate *n* (%), mean (SD) or [a]median (25th and 75th percentiles). *HRP2* histidine-rich protein-2, *pLDH P. falciparum*-specific lactate dehydrogenase
[b]Due to missing values for ferritin concentrations in 5 children, *n* was 112, 109 and 112 for groups that received placebo, NaFeEDTA and ferrous fumarate, respectively; [c]based on ferritin concentrations adjusted for inflammation by linear regression (see text)
[d]Due to missing values in 3 children, *n* was 111, 111 and 113 for groups that received placebo, NaFeEDTA and ferrous fumarate, respectively
[e]One child in placebo group and 1 child in NaFeEDTA group with antigenaemia due to *P. malariae* or *P. ovale* or *P. vivax* also had antigenaemia to *P. falciparum* (mixed infection)

a relatively large difference in prevalence of iron deficiency. For this reason, group differences in the prevalence of iron deficiency should be interpreted with caution, and more weight should be given to group differences in plasma ferritin concentrations.

Adherence to intervention was suboptimal, with a substantial proportion of children (~40%) having consumed <80% of the scheduled sachets. We found no evidence of bias in effect estimates due to differences in baseline factors that were prognostic for haemoglobin concentration. Our failure to show efficacy for either compound was found in both the per protocol and intention-to-treat analyses, was consistent across subgroups that were defined by anaemia and iron status and precluded further assessment of the non-inferiority of 3 mg iron as NaFeEDTA compared to 12.5 mg iron as encapsulated ferrous fumarate.

Despite a course of chemoprevention with dihydroartemisinin-piperaquine, a substantial proportion of children carried *Plasmodium* parasites at the end of the 30-day intervention period. When assessed by rapid dipstick tests, this proportion may be overestimated because the *P. falciparum* HRP2 protein can persist in circulation for several weeks after parasite clearance [52, 53]. We nonetheless found a similar prevalence estimate by microscopy, suggesting recrudescence of infections or the occurrence of new infections. In 2015, WHO revised the recommended target oral doses for malaria treatment with dihydroartemisinin and piperaquine in children, in recognition that the previously recommended dosage schedule (which was used in the present study) may be inadequate and may predispose children to an increased risk of treatment failure [54]. In addition, poor adherence to the second and third doses of dihydroartemisinin-piperaquine administered by parents at home may also have contributed to the recurrence of malaria. Future studies should consider direct observation of adherence to all doses during the entire course.

We selected a relatively short 30-day intervention with iron in the expectation that pre-medication with dihydroartemisinin-piperaquine would prevent malaria during this period, with a long-term view that the protection afforded by repeated chemoprevention with this combination drug would allow time windows for safe administration of short courses of iron intervention. The duration of protection of a single course of dihydroartemisinin-piperaquine is likely to vary between individuals and populations, depending, among other things, on variance in absorption and disposition of piperaquine and levels of acquired immunity (and thus on age and frequency and duration of exposure to *Plasmodium* infection). In a recent study among pre-school children in Burkina Faso, two cycles of chemoprevention with dihydroartemisinin-piperaquine, administered at the same target dose as in our study, resulted in a protection against malaria that persisted at a high level for 3 to 4 weeks and decreased rapidly thereafter, highlighting the importance of strict timing to ensure that children receive treatment at monthly intervals [55].

Our data show that the fortification dose of 3 mg iron as NaFeEDTA cannot be increased without a substantial proportion of children in this age range exceeding the ADI for EDTA. It has been argued, however, that this ADI may have been set too low [56], and a recent trial

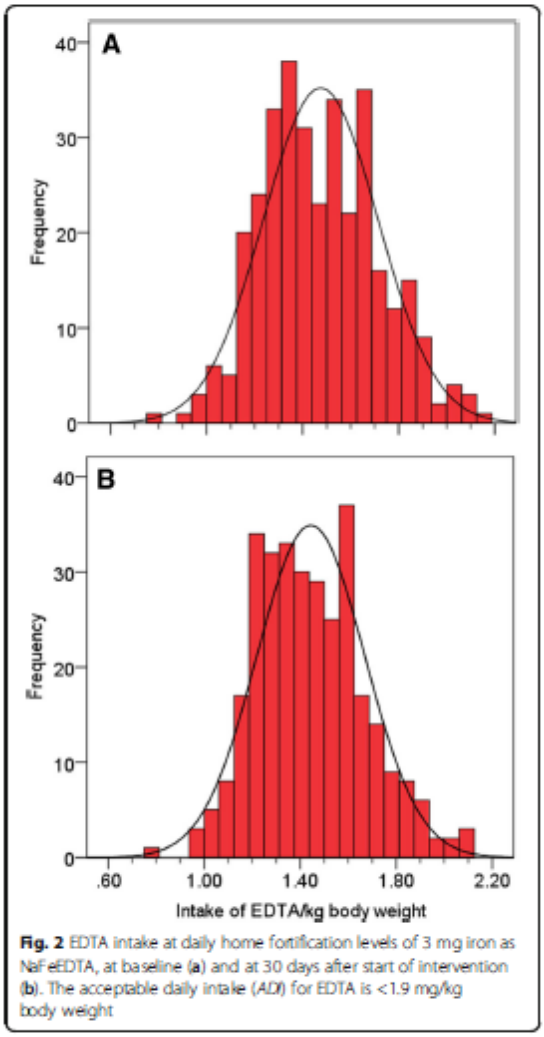

Fig. 2 EDTA intake at daily home fortification levels of 3 mg iron as NaFeEDTA, at baseline (**a**) and at 30 days after start of intervention (**b**). The acceptable daily intake (*ADI*) for EDTA is <1.9 mg/kg body weight

in Moroccan children has shown that daily oral intake of EDTA can reduce blood lead concentrations [57], which is important in view of the enormous public health burden due to lead exposure in developing countries.

The question may be raised whether our intervention period of 30 days was too short to show an effect on haemoglobin concentrations. In an earlier placebo-controlled randomised trial among Kenyan children aged 2–36 months, it was shown with a smaller sample size (79 iron; 76 placebo) than the present study that weekly supplementation with 6 mg elemental iron as ferrous fumarate per kilogram body weight improved haemoglobin concentration at 4 weeks after the start of intervention [58]. Several other trials with longer intervention periods of home fortification also failed to demonstrate haematological response to home fortification with iron. For instance, a randomised trial in 6-month old Kenyan infants showed no effect of daily home fortification with 2.5 mg iron as EDTA on haemoglobin concentration after 6 and 12 months of intervention [59]. Similarly, a trial conducted among Ghanaian children aged 8–20 months failed to show an effect on haemoglobin concentration after 6 months of daily home fortification with 40 mg elemental iron as microencapsulated ferrous fumarate [60]. These findings suggest that there are other underlying factors that may cause a lack of effect of iron interventions on haemoglobin concentration.

Inflammation was highly prevalent and is known to reduce iron absorption. In our subgroup analysis, however, there was no evidence that inflammation at baseline influenced the magnitude of the effect of iron interventions on haemoglobin concentration, and only weak evidence ($p = 0.17$) that it decreased the effect of home fortification with 12.5 mg iron as ferrous fumarate on plasma ferritin concentration. However, the cut-off definition for inflammation levels has not been validated in children, so there is a possibility that iron absorption was impaired at levels of inflammatory markers within the normal range (plasma CRP concentration <5 mg/L or plasma α_1-acid glycoprotein concentration <1.0 g/L). Chronic infections caused by either viruses or low bacterial and parasitic loads can increase the inflammatory cytokine-mediated production of hepcidin, thus blocking iron absorption, in the absence of evident inflammation (Rita Wegmuller, personal communication, 25 May 2016). The notion that infection-induced inflammation can reduce iron absorption is supported by the findings of a study conducted among Gambian children aged 18–36 months, which showed elevated serum CRP concentrations and impaired absorption of orally supplemented iron in children with post-malarial anaemia compared with those with non-malarial anaemia, but serum CRP concentrations had reversed and iron absorption was recovered at 2 weeks after antimalarial treatment [61]. Another study among Kenyan infants showed that both low doses (2.5 mg iron as NaFeEDTA) and a high dose (12.5 mg iron as ferrous fumarate) increased the pathogenic profile of gut bacteria and gut inflammation [7].

Other factors that may have contributed to our failure to show efficacy are high contents of iron-inhibiting factors such as phytates and polyphenolic compounds in the food vehicles used for home fortification and suboptimal adherence to the daily home fortification with iron-containing powders.

The use of placebo in non-inferiority trials is controversial. Some argue that it is unethical to use placebos in the context of established interventions that have been shown to be efficacious [62, 63]. The Declaration of Helsinki (2013) asserts that 'the benefits, risks, burdens

Table 3 Effect of daily home fortification with 3 mg iron as NaFeEDTA and 12.5 mg iron as encapsulated ferrous fumarate on continuous outcomes at 30 days after start of intervention, per protocol analysis

Outcome/intervention group	No. (*n*)	Estimate[a]	Effect (95% CI) relative to placebo[b]	Effect (95% CI) relative to standard[b]
Haemoglobin concentration				
Placebo	105	106.9 g/L (13.3 g/L)	Reference	Not applicable
Iron, 3 mg as NaFeEDTA	103	110.0 g/L (12.5 g/L)	3.0 g/L (−0.2 g/L to 6.2 g/L)[c]	1.3 g/L (−1.8 g/L to 4.3 g/L)[c]
Iron, 12.5 mg as ferrous fumarate	107	108.6 g/L (12.0 g/L)	1.6 g/L (−1.6 g/L to 4.8 g/L)[c]	Reference
Plasma ferritin concentration				
Placebo	104	29.7 μg/L [3.47]	Reference	Not applicable
Iron, 3 mg as NaFeEDTA	102	33.7 μg/L [2.53]	16.2% (−14.3% to 57.7%)[d]	2.5% (−22.4% to 35.4%)[d]
Iron, 12.5 mg as ferrous fumarate	105	32.6 μg/L [3.00]	12.3% (−17.1% to 52.0%)[d]	Reference
Plasma soluble transferrin receptor concentration				
Placebo	105	2.24 mg/L [1.61]	Reference	Not applicable
Iron, 3 mg as NaFeEDTA	103	2.15 mg/L [1.47]	−4.3% (−13.5% to 5.9%)[d]	3.6% (−5.5% to 13.6%)[d]
Iron, 12.5 mg as ferrous fumarate	106	2.07 mg/L [1.38]	−7.3% (−16.2% to 2.6%)[d]	Reference
Erythrocyte ZPP-haem ratio				
Placebo	104	136 μmol/mol [2.17]	Reference	Not applicable
Iron, 3 mg as NaFeEDTA	103	127 μmol/mol [1.97]	−6.5% (−23.5% to 14.2%)[d]	-5.3% (-21.7% to 14.5%) [d]
Iron, 12.5 mg as ferrous fumarate	106	134 μmol/mol [2.00]	−0.7% (−18.6% to 21.0%)[d]	Reference

[a]Mean (SD) or geometric mean [geometric standard deviation]
[b]Effects were adjusted for study design (blocks nested within strata of haemoglobin concentration <100 g/L and ≥100 g/L)
[c]Effects were calculated as absolute difference in means
[d]Exponentiation of group differences with log-transformed outcomes resulted in associations being expressed as relative differences

Table 4 Effect of daily home fortification with 3 mg iron as NaFeEDTA and 12.5 mg iron as encapsulated ferrous fumarate on categorical outcomes at 30 days after start of intervention, per protocol analysis

Outcome/intervention group	Prevalence	(*n*/*n*)	Effect (95% CI) relative to placebo
Anaemia			
Placebo	53.3%	(56/105)	Reference
Iron, 3 mg as NaFeEDTA	43.7%	(45/103)	−9.6% (−22.7% to 3.9%)
Iron, 12.5 mg as ferrous fumarate	51.4%	(55/107)	−1.9% (−15.1% to 11.3%)
Iron deficiency[a]			
Placebo	44.6%	(25/56)	Reference
Iron, 3 mg as NaFeEDTA	24.5%	(12/49)	−20.2% (−36.4% to −18.5%)
Iron, 12.5 mg as ferrous fumarate	30.5%	(18/59)	−14.1% (−30.7% to 3.5%)
Plasmodium infection, by dipstick test[b]			
Placebo	16.2%	(17/105)	Reference
Iron, 3 mg as NaFeEDTA	18.4%	(19/103)	2.3% (−8.1% to 12.6%)
Iron, 12.5 mg as ferrous fumarate	22.6%	(24/106)	6.4% (−4.3% to 17.0%)
P. falciparum infection, by microscopy			
Placebo	18.5%	(19/103)	Reference
Iron, 3 mg as NaFeEDTA	15.5%	(15/97)	−3.0% (−13.4% to 7.6%)
Iron, 12.5 mg as ferrous fumarate	15.2%	(15/99)	−3.3% (−13.6% to 7.2%)

[a]Analysis restricted to children without inflammation (see text)
[b]Presence of HRP, pLDH specific to *P. falciparum* or pLDH due to human *Plasmodium* species other than *P. falciparum*

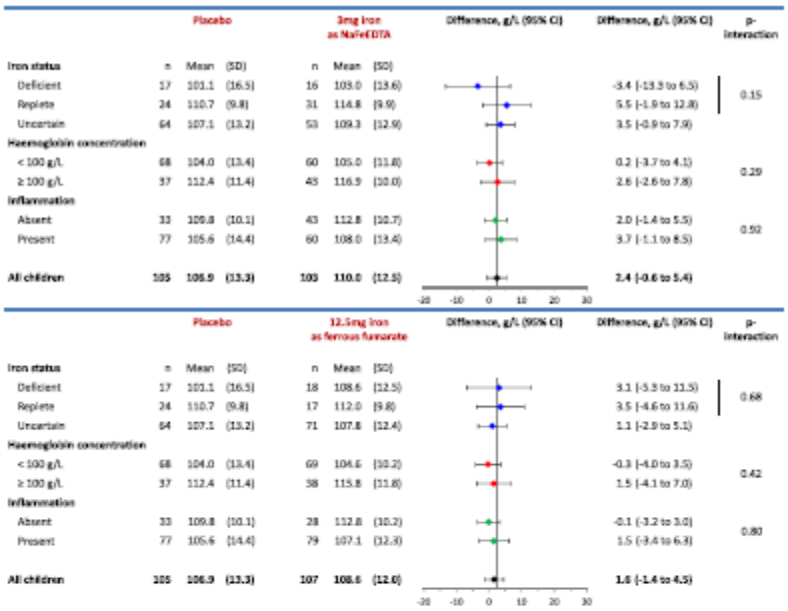

Fig. 3 Effect of interventions on haemoglobin concentration (g/L) at 30 days after start of intervention, by subgroups of iron status and haemoglobin concentration class at baseline. Inflammation was defined to be absent when plasma C-reactive protein (*CRP*) concentration was ≤5 mg/L and plasma α_1-acid glycoprotein concentration was ≤1.0 g/L, and present when either plasma CRP concentration was >5 mg/L or plasma α_1-acid glycoprotein concentration was >1.0 g/L. Group means were obtained by one-way ANOVA. Intervention effects were adjusted for stratified block design as well as plasma concentrations of ferritin and soluble transferrin receptor at baseline (both continuous variables). The *p* values indicate the two-sided probability that group effects are as different as observed or more extreme when assuming that they are identical

and effectiveness of a new intervention must be tested against those of the best proven intervention(s)' [64]. In our case, there was an established WHO recommendation for daily home fortification with 12.5 mg iron as ferrous salt, based on a meta-analysis of six randomised controlled trials [3] that showed an overall reduction of the prevalence of anaemia.

We included a third arm with placebo, however, because we were concerned that established efficacious interventions do not consistently demonstrate superiority in placebo-controlled studies. Notably, one-third of meta-analyses that demonstrate a protective effect from interventions are not supported by subsequent large randomised controlled trials [65, 66]. In addition, in the meta-analysis that formed the basis for the WHO recommendation for daily home fortification, three of six trials included could not exclude the absence of an effect on haemoglobin concentration. A trial among Ghanaian children, published after the meta-analysis but before we started our study, with a larger sample size than all previous studies combined, also failed to showed an effect of daily home fortification with 12.5 mg iron as ferrous fumarate on either change in haemoglobin concentration or the prevalence of anaemia, despite 45% of children being iron deficient at baseline and good adherence to intervention [61]. Our use of a three-armed non-inferiority trial that includes a placebo arm is consistent with international recommendations [67] and is validated by our results: in the absence of a placebo group, we would erroneously have concluded that 3 mg iron as NaFeEDTA was non-inferior to 12.5 mg iron as encapsulated ferrous fumarate.

In our meta-analysis, we found a high level of heterogeneity in effects across trials. How should we interpret this finding? In the absence of evidence for an effect in single trials, meta-analysis is often understood to be the continuation of the pursuit of statistical significance by other means. A high level of heterogeneity indicates, however, that there is no single true effect in a single, common population that underlies the trials included in the meta-analysis. Heterogeneity may reflect methodological differences between trials (dosage, formulation and duration of intervention, adherence, study quality, etc.), but it may also indicate that there are different types of populations, each with different true underlying effects. Thus, the pooled random effect may not reflect the actual effect in any particular population being studied, and has little value other than perhaps providing some

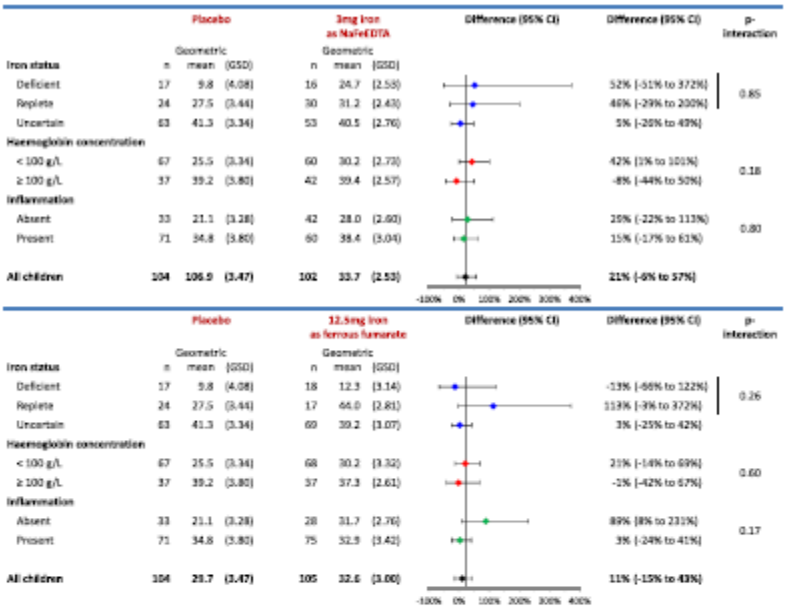

Fig. 4 Effect of interventions on plasma ferritin concentration (μg/L) at 30 days after start of intervention, by subgroups of iron status and haemoglobin concentration class at baseline. Inflammation was defined to be absent when plasma CRP concentration was ≤5 mg/L and plasma α_1-acid glycoprotein concentration was ≤1.0 g/L, and present when either plasma CRP concentration was >5 mg/L or plasma α_1-acid glycoprotein concentration was >1.0 g/L. Group geometric means were obtained by exponentiation of results of one-way ANOVA. Intervention effects were adjusted for stratified block design as well as plasma concentrations of ferritin and soluble transferrin receptor at baseline (both log-transformed continuous variables). Exponentiation of group differences with log-transformed outcomes resulted in associations being expressed as relative differences. *GSD* geometric standard deviation. The *p* values indicate the two-sided probability that group effects are as different as observed or more extreme when assuming that they are identical

evidence to inform policy decisions. Our meta-analysis (Additional file 2: Figure S4) suggests a small gain in haemoglobin concentration in most trials, indicating that home fortification with iron-containing micronutrient powders provides some benefit across different settings. This gain may be insufficient to recommend home fortification in all settings, as illustrated by the main results of our trial. Our finding of heterogeneity between trial results should stimulate subgroup analysis or meta-regression to identify population-specific factors that determine efficacy (e.g. differences in prevalence of iron deficiency and inflammation, food content of compounds that inhibit iron absorption). Such approaches may become possible as evidence is accrued from a variety of studies in different settings.

Conclusions

In this population, home fortification with either 3 mg iron as NaFeEDTA or 12.5 mg iron as encapsulated ferrous fumarate was insufficiently efficacious to assess non-inferiority of 3 mg iron as NaFeEDTA compared to 12.5 mg iron as encapsulated ferrous fumarate. Our finding of heterogeneity between trial results should stimulate subgroup analysis or meta-regression to identify population-specific factors that determine efficacy.

Additional files

Additional file 1: Statistical analysis plan. (DOCX 109 kb)

Additional file 2: Effect of home fortification with iron-containing powders on anaemia and haemoglobin concentration in pre-school children: meta-analysis of randomised controlled trials. (DOCX 61 kb)

Additional file 3: Effect of daily home fortification with iron on haemoglobin concentration by intention-to-treat analysis. (DOCX 16 kb)

Abbreviations

ADI: Acceptable daily intake; AGP: α_1-acid glycoprotein; CRP: C-reactive protein; EDTA: ethylenediaminetetraacetate; EMA: European Medicine Agency; HF-TAG: Home Fortification Technical Advisory Group; HRP2: histidine-rich protein 2; MEMS: Medication Events Monitoring System; pLDH: *Plasmodium* lactate dehydrogenase

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
ET was involved in the study design and coordinated the implementation of the research project. PEA was involved in the study design and implementation. ST compiled the study protocol; VO conducted the laboratory analysis in Kenya, WO assisted in clinical aspects of the field work, and AYD conducted the biochemical analysis in The Netherlands. AMP reviewed the manuscript and provided technical inputs. HV conceived the idea and study design. Both ET and HV conducted the statistical analysis. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Ethical clearance was obtained from the London School of Hygiene and Tropical Medicine Ethical Committee, UK (reference 6503) and the Kenyatta National Hospital Ethical Review Committee, Kenya (reference KNH-ERC/A/402). The parents or primary guardians signed the consent form.

Publisher's Note

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Online additional file 1: Statistical analysis plan

Emily Teshome, Hans Verhoef

1. Preamble

This plan was developed to analyse data from the SEICK (Safe and Effective Iron for Children in Kenya) trial. The study received ethical clearance in Kenya (Kenyatta University National Hospital/University of Nairobi, #KNH-ERC/A/402)) and in England (London School of Hygiene and Tropical Medicine, #6503)), and was registered at ClinicalTrials.gov (NCT02073149). The principal features of the planned analysis were established as part of the trial proposal. The current plan provides an update, was finalised after data collection but before breaking the randomization code, and should take precedence over the trial proposal.

2. Trial objectives

The study was primarily conceived to assess non-inferiority of daily home fortification with 3mg iron as NaFeEDTA compared with 12.5 mg iron as encapsulated ferrous fumarate, with haemoglobin concentration at the end of the 30-day intervention period as the primary outcome.

The study proposal states the following objectives:

1. In children aged 12-36 months, to compare daily home fortification for 30 days with two iron formulations (3mg iron as NaFeEDTA versus 12.5 mg iron as encapsulated ferrous fumarate) regarding:
    a. Haemoglobin concentration at the end of the 30-day fortification period ***(primary objective)***;
    b. Iron status at the end of the fortification period;
    c. Serum NTBI concentrations at 3 hours after ingesting the first fortificant dose;
    d. Faecal calprotectin concentration at the end of the fortification period;
    e. *P. falciparum* infection at the end of the fortification period as indicated by whole blood density of asexual parasites or the presence of antigenemia;
    f. Adherence to intervention.

2. To compare daily home fortification for 30 days with iron (3mg iron as NaFeEDTA or 12.5 mg iron as encapsulated ferrous fumarate) versus placebo regarding:
    a. The indicators listed under objective 1a-f;
    b. Time to development of anaemia after cessation of iron fortification.

3. To evaluate the accuracy of self-reported adherence with home fortification, using medication events monitoring system as a gold standard.

4. To assess associations between major intestinal parasites and haemoglobin concentration at baseline.

5. To compare intervention effects of iron on intestinal biota.

Objectives 1c, 1d, 4 and 5 are conditional to sufficient resources still being found to conduct the laboratory analyses required, and will not be further discussed in this plan.

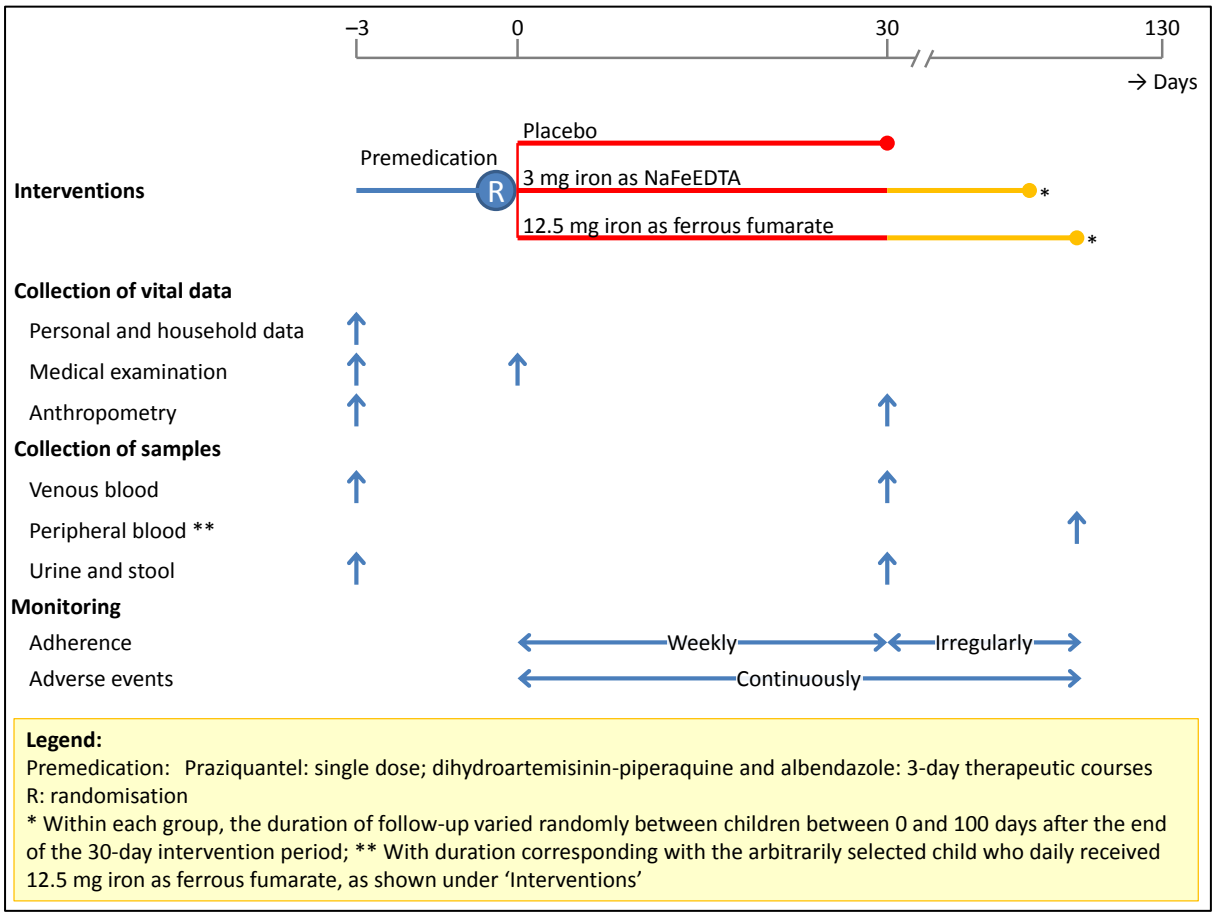
3. Study type and key design features

The study concerns an individually-randomised, controlled non-inferiority trial with three parallel arms, with participants randomised to daily home fortification for 30 days with sachets containing either a) 3 mg iron as NaFeEDTA (experimental treatment); b) 12.5 mg iron as encapsulated ferrous fumarate (positive control); or c) placebo (negative control).

Demonstration of non-inferiority in a trial with two arms can have two meanings only: both interventions are equally effective, or both interventions are equally ineffective against placebo. Thus, as per guidelines of the European Medicine Agency [1] and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use [2], we included a third arm with placebo, which allows a) demonstration of superiority of home fortification with 3mg iron as NaFeEDTA over placebo (proof of efficacy); b) demonstration of superiority of the reference (12.5 mg iron as encapsulated ferrous fumarate) over placebo (proof of assay sensitivity); and c) demonstration that the home fortification with 3mg iron as NaFeEDTA retains most of the efficacy of the reference over placebo (proof of non-inferiority).

Haemoglobin concentration at baseline was strongly expected to be prognostic for haemoglobin concentration at the end of intervention. To achieve group balance in size and baseline haemoglobin concentration, randomisation was based on a stratified block design, with strata defined by baseline haemoglobin concentration class (<100 g/L and ≥100 g/L) and randomly sized permuted blocks of 6 or 9. Stratification at the stage of randomisation and adjustment for this stratification in the analysis was also expected to improve the efficiency of the analysis and the precision of the estimated intervention effect. Because of the short recruitment period, we did not expect blocks to be associated with haemoglobin concentration, even though we could not entirely exclude this possibility (e.g. due to seasonal changes in diet or malaria transmission). We did not conduct interim analyses and had no stopping guidelines.

Figure 1: **Summary of study design (not to scale)**.



4. Sample size calculations

The target sample size was 108 participants per group, which was determined using procedures and terminology for sample size calculations in non-inferiority trials as recommended by US Food and Drug Administration [3,4]

a. Based on a meta-analysis [5], we estimated the effect of 12.5 mg ferrous fumarate on haemoglobin concentration relative to placebo. The lower limit of the 95% CI thus obtained (9.3 g/L) was used as M_1, the minimum anticipated effect of 12.5 mg ferrous fumarate in our trial (**Figure 2;** left panel).

b. Next, we set M_2 as the margin specified to preserve 50% of the anticipated minimum effect of 12.5 mg ferrous fumarate anticipated reduction in the 3mg NafeEDTA test treatment. This margin (haemoglobin concentration of 4.7 g/L) can be interpreted as the largest loss of effect compared to 12.5 mg ferrous fumarate (inferiority) that would be acceptable, and also is below an effect for 5g/L haemoglobin concentration that we consider to be of minimum importance for public health.

c. We set the sample size so that the lower limit of the 95% CI around the difference in haemoglobin concentration between the two iron formulations (i.e. 12.5 mg ferrous fumarate and 3.0 mg iron as NaFeEDTA) would lie above M2 (Figure 1; right panel).

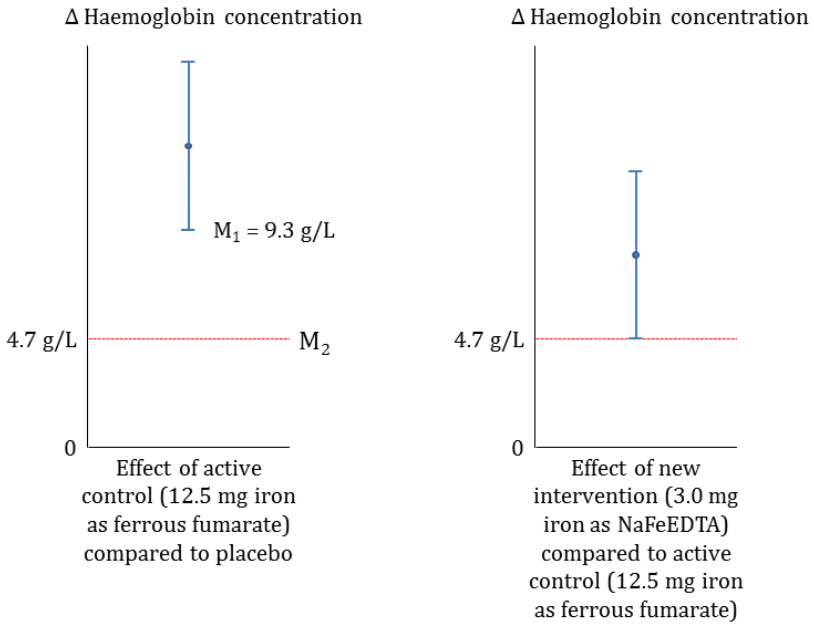


Figure 2: Theoretical framework for sample size determination

Peer reviewers or editors of scientific journals may demand that we report sample size calculations and statistical power. Although such calculations are critically important in planning a study, we consider statistical power to be an exclusively pre-trial concept, which is irrelevant and conceptually wrong in the interpretation of study results, based on assumptions that are not usually met during trial implementation, and prone to be misinterpreted by most readers [6,7]. Thus we intend to refrain from reporting sample size calculations, but we will report the method used to determine the non-inferiority margin.

5. Software

Anthropometric indices will be calculated using WHO Anthro software vs.3.2.2 (World Health Organisation, Geneva, Switzerland). Data will be double entered, checked for completeness and verified for possible entry errors using Microsoft Excel. Data cleaning and analysis will be done using SPSS 21 (IBM, Armonk, NY); R-software version 3.2.0 (www.r-project.org) and CIA 2.2.0 (http://www.som.soton.ac.uk/research/sites/cia/and PowerView vs.3.5.2 (AARDEX Group ltd, Sion Switzerland) to analyse electronic adherence data.

6. Intention-to-treat analysis versus per protocol analysis

Intention-to-treat (ITT) analysis: Per definition, ITT analysis includes all randomised children. It generally is the preferred strategy for data analysis in superiority trials (objective 2 above) because a) it tends to avoid over-optimistic estimates of effect in real-life conditions, because non-compliers included in the ITT set will generally reduce the estimated treatment effect;[8] b) ITT analysis with multiple imputation yields valid results when missingness of data is random. Most trials aim to estimate the effects of allocating an intervention in real-life conditions, not the effects in the subgroup of the participants who adhere to it.

In a non-inferiority trial, however, intention-to-treat analysis is generally not conservative because protocol violation and non-adherence to treatment may cause the results of the treatment groups to appear similar [9].

Per protocol analysis: Per protocol analysis is restricted to participants who have adhered closely to what is prescribed in the study protocol in the terms of eligibility, interventions, and outcome assessment. It does not show the practical value of the experimental intervention, but rather envisages determining the biological effect of the intervention that can be obtained under ideal circumstances, with participants who adhere to trial instructions. However, missing outcome data due to attrition (drop-out) during the study or exclusions from the analysis raise the possibility that effect estimates obtained by per protocol analysis is biased. Per protocol analysis may also raise concerns about data manipulation.

In our study, we will include children in per protocol analysis who received at least one dose of home fortification, with exclusion of those not meeting inclusion criteria, poorly adhered to intervention, crossed over between intervention groups or ingested iron-containing preparations acquired outside the trial, and excluding those with missing outcome data. We will define adherence as the number of days that home fortificants have been made available relative to the scheduled 30-day intervention period, with availability indicated by the electronic device for storage of home fortificants having been opened. Poor adherence will be defined as adherence < 80% (23 days). This threshold is somewhat arbitrary, but it has been used in most published studies on medication adherence [10,11] and we believe that adherence above this threshold to be associated with improved iron status. Group adherence will be reported as the proportion of participants for whom home fortificants were available ≥80% (≥24 days).

Relative importance of intention-to-treat analysis and per protocol analysis: We conceived the SEICK trial primarily to be an explanatory trial (i.e. to assess intervention effects under optimal conditions) rather than a pragmatic trial (i.e. to assess intervention effects under real-life conditions). As per recommendations [12] we will pursue the

primary (non-inferiority) objective by comparing results obtained by both intention-to-treat analysis and per protocol analysis. Non-inferiority will be accepted only if efficacy (objective #2), assay sensitivity (objective #2) and non-inferiority (objective #1) have all been shown. In our trial report, we will include results of the per protocol analysis in the main text, and results of the intention-to-treat analysis as supplementary material.

7. Handling of outliers and missing data

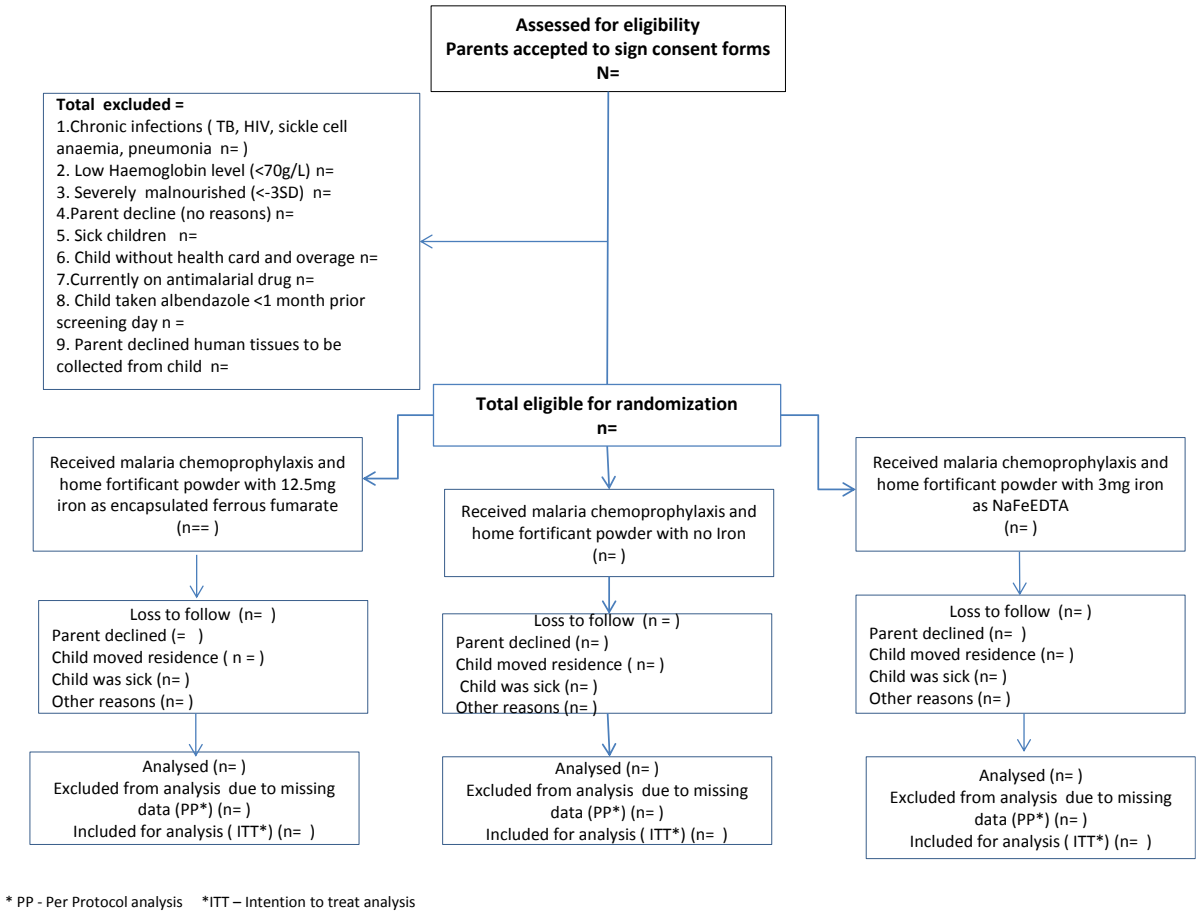
Outliers will be identified by examining a) ranges of continuous variables; b) frequency tables of categorical variables; c) visual inspection of scatter plots of pairs of continuous variables. Outliers will be dropped only if it is obvious that they are due to incorrectly entered or measured data.

Reasons for missing outcome data will be given where possible, coded and reported quantitatively as either a) withdrawal of consent; b) adverse events or withdrawn for medical reasons (reasons specified); c) no show for follow-up visit(s); d) moved away from the study area; e) refused intervention; f) refused to give human tissue samples; g) excluded from analysis due to not meeting inclusion criteria; h) excluded from analysis due to poor adherence to intervention; i) excluded from analysis due to crossing over between intervention groups; j) excluded from analysis due to taking iron-containing preparations acquired outside the trial; k) other reasons (specified).

For ITT analysis, missing data will be replaced under a missing-at-random assumption by multiple imputation (SPSS, 'automatic' imputation method; 20 iterations), with log-transformed variables as necessary to normalize distributions. Imputations will be done separately for each of the three intervention groups, with datasets derived with these imputed values being merged to allow subsequent analysis. All variables used in regression models will be included in the imputation model; these will include both baseline factors and outcomes. A list of these variables will be reported as supplementary material.

8. Participant flow

Figure 3 shows the format for presenting the flow of children during the four main stages of the trial (enrolment, allocation, follow-up and analysis). For children who were excluded before randomisation, reasons will be given where possible, coded and reported quantitatively as either a) no show; b) refused to participate; c) no written informed consent obtained; d) unlikely to stick to protocol; e) ineligible (with reasons specified if possible); f) medical conditions (with reasons specified if possible); g) other reasons; h) not known.



* PP - Per Protocol analysis *ITT – Intention to treat analysis

Figure 3: Flow of study participant (incomplete)

9. Description of group characteristics at baseline

For continuous variables, we will visually inspect histograms to assess whether they are normally distributed within intervention groups, with log-transformation of variables with a lognormal distribution. Population characteristics (**Table 1**) will be reported by intervention group as proportions, means (SD), geometric mean (geometric SD), and median (25^{th}- and 75^{th} percentiles) as appropriate, with corresponding group size. We will not test for group differences for reasons given by Assmann et al. (2000)[13].

Table 1: Description of baseline characteristics

Variable	Type	Units/categories
Age	Continuous	months
Age class	Binary	12-23 months, 24-36 months
Sex	Binary	%males : %females
Height-for-age z-score	Continuous	SD
Weight-for-height z-score	Continuous	SD
Weight-for-age z-score	Continuous	SD
Haemoglobin concentration	Continuous	g/L
Anaemia status	Ordinal	*Severe to moderate anaemia:* haemoglobin concentration <89g/L; *mild anaemia:* haemoglobin concentration 90-109g/L; *non-anaemic:* haemoglobin concentration ≥110g/L
Plasma ferritin concentration	Continuous	µg/L
Iron status	Nominal	*Deficient:* plasma ferritin concentration <12 µg/L regardless of presence or absence of infection or inflammation; *replete:* plasma ferritin concentration ≥12 µg/L in the absence of infection or inflammation; *uncertain:* plasma ferritin concentration ≥12 µg/L in the presence of infection or inflammation
Plasma soluble transferrin receptor concentration	Continuous	mg/L
Plasma C-reactive protein concentration	Continuous	mg/L
Plasma α_1-acid glycoprotein concentration	Continuous	mg/L
Inflammation	Binary	Plasma concentrations of C -reactive protein (CRP) > 5mg/L and/or α_1-acid glycoprotein (AGP) > 1g/L
Whole blood ZPP concentration	Continuous	µmol/mol haem
Erythrocyte ZPP concentration	Continuous	µmol/mol haem

***Plasmodium* parasite density (asexual and sexual forms)**	Continuous	μL^{-1}
***Plasmodium* infection, by rapid dipstick tests**	Nominal	No infection; *P. falciparum* (by HRP2 or *P. falciparum*-specific pLDH), other human *Plasmodium* spp. (by pLDH), mixed infection
Urinary hepcidin concentration *	Continuous	nmol/mmol creatinine
Plasma folate concentration *	Continuous	nmol/L
Plasma vitamin B_{12} concentration	Continuous	pmol/L

* Funds permitting

10. General issues in the analysis of intervention effects

Effects are measured by comparing outcomes measured after a particular intervention with those measured if an alternative intervention has been given (counterfactual argument), thus focusing on comparison of end points (instead of before-after measurements within groups). We will estimate effects when possible; P-values, where reported, will be 2-sided. We will avoid hypothesis testing and interpretation of results as 'significant' or $p<0.05$ for reasons reported elsewhere [14,15].

We will describe outcome variables by intervention groups as proportions, means (SD), geometric means (geometric SD), and median (25^{th}- and 75^{th} percentiles) as appropriate, with corresponding group sizes. For continuous outcomes, these estimates will be obtained by ANOVA.

Intervention effects will be assessed as absolute differences (proportions and means) or relative differences in geometric means (variables with lognormal distributions) between groups, with accounting for the stratified block design. To facilitate interpretation, odds ratios resulting from these analyses will be converted to differences in proportions. To allow extrapolation of results to other settings, we will also consider reporting relative effects for binary outcomes (i.e. proportion ratios). Outcomes that are not normally distributed, even after log-transformation, will be compared using non-parametric tests (e.g. Mann-Whitney U test, or Wilcoxon rank sum statistics)

11. Analysis of the primary outcome (objective 1a and 2)

Primary analysis: We will estimate the difference in haemoglobin concentrations at the end of the 30-day fortification period between groups of children allocated to different iron formulations. Non-inferiority will be rejected if the difference between groups is less than the non-inferiority margin.

Secondary analysis of the primary outcome will be as follows:

a. *Adjustment for selected baseline variables:* Biased effect estimates would occur if there are group imbalances in baseline factors that are prognostic for outcome. For baseline factors that are known to be strongly, or at least moderately, associated with the primary outcome, or for which there is a strong biological rationale for such an association, i.e. iron status, plasma soluble transferrin receptor concentration, and age, we will evaluate the role of such bias using stratified analysis and directly by multiple linear regression models. We also expect haemoglobin concentration at baseline to be prognostic for outcome but, because this will already be accounted for by adjusting for study design. In stratified analysis, we will estimate intervention effects in subgroups formed by these baseline factors (for iron status: see definition in Table 1; plasma soluble transferrin receptor concentration dichotomised with the median as cut-off value;

and age in classes of children aged 12-23 months and 24-30 months) to allow an assessment of the validity of model assumptions. In the regression models, we will compare effect estimates with and without adjustment by baseline factors (plasma concentrations of ferritin and soluble transferrin receptor and age; as continuous variables). In the interpretation of data, we will give priority to the unadjusted effect, unless the adjusted effect is markedly different (>15%), in which case the adjusted effect will be considered the primary analysis.

Subgroup analysis: Because iron absorption is known to depend on iron status, we will consider proxy markers for iron status (haemoglobin concentration, plasma concentrations of ferritin and soluble transferrin) at baseline as potential modifiers for intervention effects.

Stratified analysis will be used to measure effect sizes within subgroups. Evidence for group differences in intervention effects will be formally investigated in a multiple regression model with main effects (for baseline factors and the intervention) and multiplicative interaction terms.

To define subgroups, baseline factors will be categorized (haemoglobin concentration class <100 g/L and ≥100 g/L; iron status [see classification above] and plasma soluble transferrin receptor concentration [dichotomised, with the median as threshold]). For iron status, we will restrict the analysis to a comparison of iron-deficient and iron-replete subgroups.

Categorisation of baseline factors has the advantage that results can be conveniently presented in a forest plot. It has the disadvantage, however, that the precision of the interaction depends on the number and position of the cut-off values used to categorise baseline factors [16]. We will also explore interactions with baseline factors as continuous covariates, using fractional polynomials [16]. Such a strategy is more powerful, especially at towards extreme ends of the covariate distribution where limited numbers of observations generally preclude detection of interaction, and it may reveal more about the nature of possible interactions. We will inspect scattergrams and conduct sensitivity analysis as needed to examine the influence of possible outliers on results.

Results of subgroup analyses will be interpreted cautiously for reasons reported elsewhere [13].

b. *Intention-to-treat analysis:* For binary outcomes, pooled estimates from multiple iterations may result in non-integer values. This precludes computation of confidence intervals for differences in proportions, and may be counterintuitive to readers not well versed in multiple imputation techniques. To handle this problem, we will calculate differences in means under the assumption of binary

outcome variables having a Bernoulli distribution. Pooled chi-squared statistics for cross-tabulated data and associated p-values will be calculated as described by Van Buuren (2012), thus the p-values obtained are only crude estimates of the true values [17].

c. *Paired comparisons of iron groups with placebo (objective 2):* This analysis will be done as for the comparison between iron groups (see above). Superiority will be established if no effect is excluded from the 95% CI.

12. Analysis of secondary outcomes (objectives 1b and 2)

To assess intervention effects, we will conduct all three possible paired comparisons between the intervention groups. We will use plasma ferritin concentration as an outcome indicator of iron status, and soluble transferrin receptor as an outcome indicator of the iron demand by the erythrocytes (both measured at the end of the 30-day intervention period). We will conduct analysis with and without adjustment for baseline factors, as well as intention-to-treat analysis (see methods above).

Plasma ferritin concentration has the limitation that it can be enhanced by inflammation independently of iron status. Because our interest in plasma ferritin concentration is limited to its value as a marker of iron status, we will adjust all analysis for inflammation markers (plasma concentrations of C -reactive protein and α_1-acid glycoprotein; both as continuous variables) measured at the end of the 30-day intervention period. Thus we will effectively measure effects on plasma ferritin concentration assuming no inflammation.

Similarly, plasma soluble transferrin receptor concentration can be elevated by an increased erythropoiesis under influence of malaria-induced haemolysis. Because our interest in plasma soluble transferrin receptor concentration is limited to its value as a marker of tissue iron demand, we will adjust all analysis for inflammation markers (plasma concentrations of C -reactive protein and α_1-acid glycoprotein; both as continuous variables) measured at the end of the 30-day intervention period.

Stratified analysis will be used to measure effect sizes within subgroups. To assess effect modification directly, we will compare multiple logistic regression models with and without product terms, with categorical baseline factors entered as dummy-coded variables.

13. Multiplicity

Multiple comparisons to evaluate non-inferiority (section 6) or to measure effects on multiple secondary outcomes may raise concerns about multiplicity (i.e. occurrence of false-positive results). We consider statistical solutions (e.g. Bonferroni correction) to be inappropriate in our trial because a) the concept of multiplicity is rooted in hypothesis testing, which is not our framework for making inferences; b) we will not formally adjust for multiplicity because any absence or present effect differences will be interpreted by the confidence interval in the context of the set thresholds as defined in section 6 [14,15,12]; c) for an intervention that leads to multiple changes in related outcomes, results from multiple comparisons are mutually reinforcing, not mutually debasing [18] .

In case of substantial discordance in results from analyses to evaluate non-inferiority, we will consider the results to be indeterminate.

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Online additional file 2: Effect of home fortification with iron-containing powders on anaemia and haemoglobin concentration in preschool children: meta-analysis of randomised controlled trials.

Objective: We conducted a meta-analysis of randomised controlled trials in preschool children to assess the effect of home fortification with iron-containing powders on haemoglobin concentration at the end of intervention.

Methods: This study is an update of the meta-analysis by Salam et al. [1], which included trials that provided iron-containing micronutrient powders either in the home or at designated centres, with different dosages and duration of intervention. Studies that included supporting interventions such as nutrition education were included only if the supporting interventions were given to both the intervention and comparison groups, so that the difference between the two groups was solely of micronutrient powders. We did not follow a review protocol.

To identify studies that were published after the review by Salam et al. [1], we conducted a search on Pubmed (https://www.ncbi.nlm.nih.gov/pubmed) with the following terms: (Micronutrient* OR "multiple micronutrient*" OR "multi-vitamin*" OR "multi-mineral*" OR "micronutrient powder*" OR MNP OR sprinkle*) AND (Fortifi* OR "food fortifi*" OR "point of use" OR "home fortification") AND (hemoglobin OR haemoglobin OR anemia OR anaemia) and with filters: Randomized Controlled Trial; Publication date from 2012/11/01 to 2017/03/14; Humans; Infant: 1-23 months; Preschool Child: 2-5 years. This procedure yielded 21 papers. Upon screening titles and abstracts, 15 papers were rejected because they did not meet the criteria for inclusion in the present review. We perused the full text of the remaining 6 papers, and restricted ourselves to trials that used randomisation at the individual or cluster level. We included studies that reported home fortification in children aged < 5 years for at least 5 days/week with powders containing ≥10mg iron as ferrous salt (i.e. 80% of the 12.5mg iron recommended by the WHO for home prevention), or ≥2.0 mg iron as NaFeEDTA (considering that fractional absorption of iron added to foods as NaFeEDTA may be 4-5-fold higher than when added as ferrous salts [2,3]). We excluded trials with fortificants that were lipid-based or in liquid formulations. Authors were contacted to request additional data as necessary.

For each study, we restricted the analyses to haemoglobin concentration measured at the end of intervention or change of haemoglobin concentration over time, without adjustment for group differences in baseline factors that were predictive for outcome, and without adjustments for multiplicity. Differences in means with corresponding standard errors were calculated as described by Higgins et al. [4]. Cut-off points to define anaemia were as reported by the investigators (haemoglobin concentration < 110 g/L). For cluster-randomised trials, standard errors were inflated

by the square root of the estimated design effect, D. Such estimates were obtained from published reports; if not reported, we used the formula $D = 1 + (m - 1) \cdot ICC$, where m and ICC are the reported average cluster size and the intra-cluster-correlation coefficient, respectively. The ICC was estimated at 0.13 [5]). The analysis was implemented using the 'metafor' package [6] in R software vs. 3.2.0 (www.r-project.org). We used random-effects models, with Hartung and Knapp adjustment to account for the relatively small number of trials included in the analysis [7], and a restricted maximum-likelihood estimator of the between-study variance, τ^2. We assessed potential reporting bias by visual inspection for asymmetry of the funnel plot, and Egger's regression test.

Results: From the meta-analysis by Salam et al. [1], we excluded two studies that were conducted in school children older than 5 years [8,9]. For the study by Giovanni et al. [10], we pooled we the two groups that received iron. In the study by Sharieff et al. [11], we included the group that received micronutrients with heat-inactivated *Lactobacillus acidophilus*, which we pooled with the group that received these micronutrients without *L. acidophilus*.

From our Pubmed search, we excluded three papers because the intervention concerned a specially prepared, pre-fortified complementary food [12], children received only 120 sachets with micronutrient powders for flexible consumption over a supplementation period of 11 months [13], or infants in control group received a liquid iron preparation [14]. Thus we identified and included 16 eligible trials (including our own) in our meta-analysis [10,11,15-27].

The effect on haemoglobin concentration was highly heterogeneous (I^2: 84.1%; p-value for test of heterogeneity: < 0.0001; Figure 1). The pooled effect on haemoglobin concentration was 3.9 g/L (95% CI: 2.2-5.5 g/L), indicating that, in random sample of a hypothetically infinite number of trials, each estimating a different true underlying effect, one may on average expect an increase in haemoglobin concentration by 3.9 g/L, with the 95% CI excluding an effect beyond 5.5 g/L. Visual inspection of the funnel plot and Egger's regression test (p=0.44) did not yield evidence for reporting bias.

Discussion: We found a high level of heterogeneity in effects across trials. How = should we interpret this finding? In the absence of evidence for an effect in single trials, meta-analysis is often understood to be the continuation of the pursuit of statistical significance by other means. A high level of heterogeneity indicates, however, that there is no single true effect in a single, common population that underlies the trials included in the meta-analysis. Heterogeneity may reflect methodological differences between trials (dosage, formulation and duration of intervention, adherence, study quality, etc.) but it may also indicate that there are different types of populations, each with different true underlying effects. Thus the

pooled random-effect may not reflect the actual effect in any particular population being studied, and has little value other than perhaps providing some evidence to inform policy decisions. Reassuringly, our meta-analysis (Figure 1) suggests a small gain in haemoglobin concentration in most trials, indicating that home fortification with iron-containing micronutrient powders provides some benefit across different settings. Our trial results illustrate, however, that the evidence may be insufficient to recommend home fortification in all settings. Our finding of heterogeneity should stimulate subgroup analysis or meta-regression to identify population-specific factors that determine efficacy (e.g. differences in prevalence of iron deficiency and inflammation, food content of compounds that inhibit iron absorption). Such approaches may become possible as evidence is accrued from a variety of studies in different settings.

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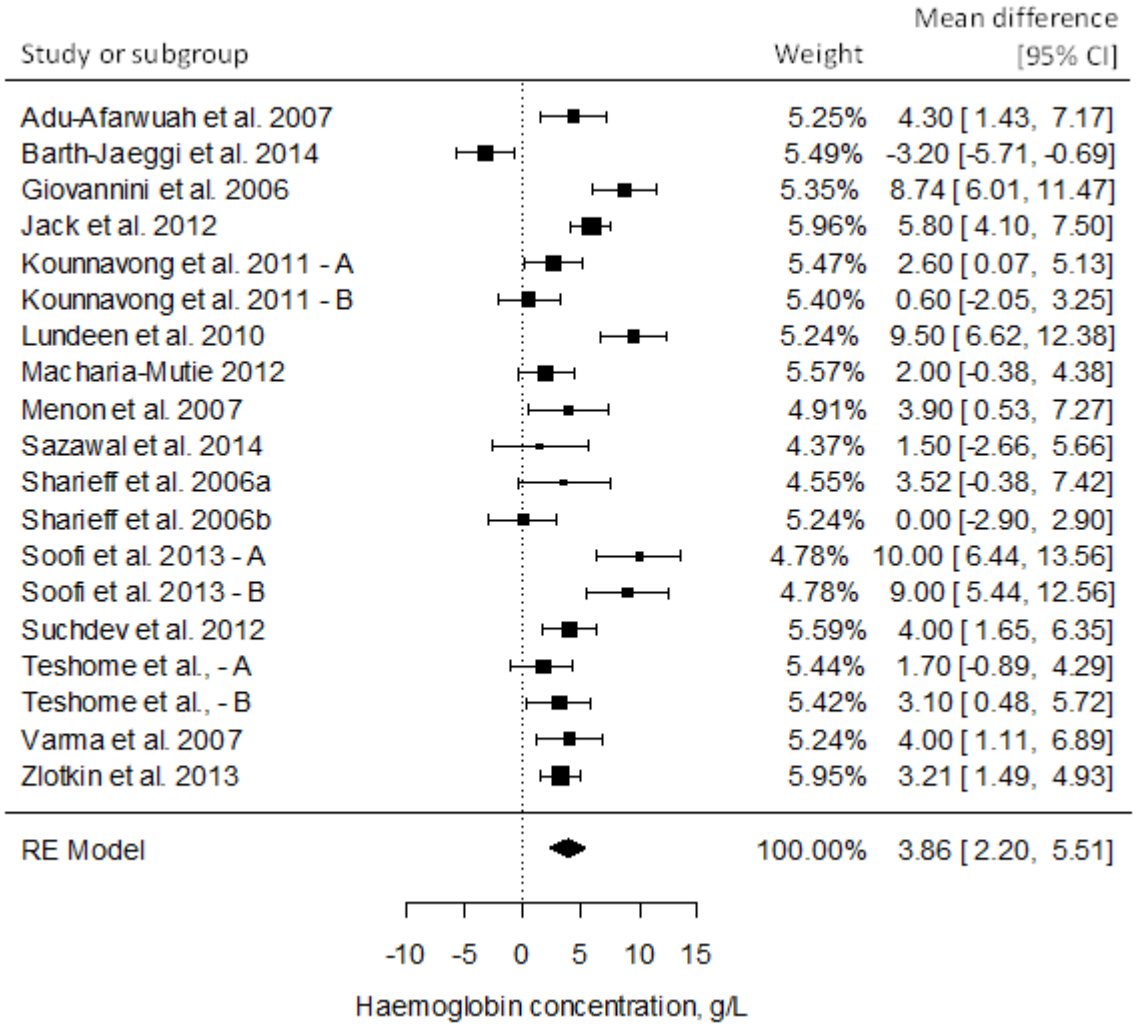
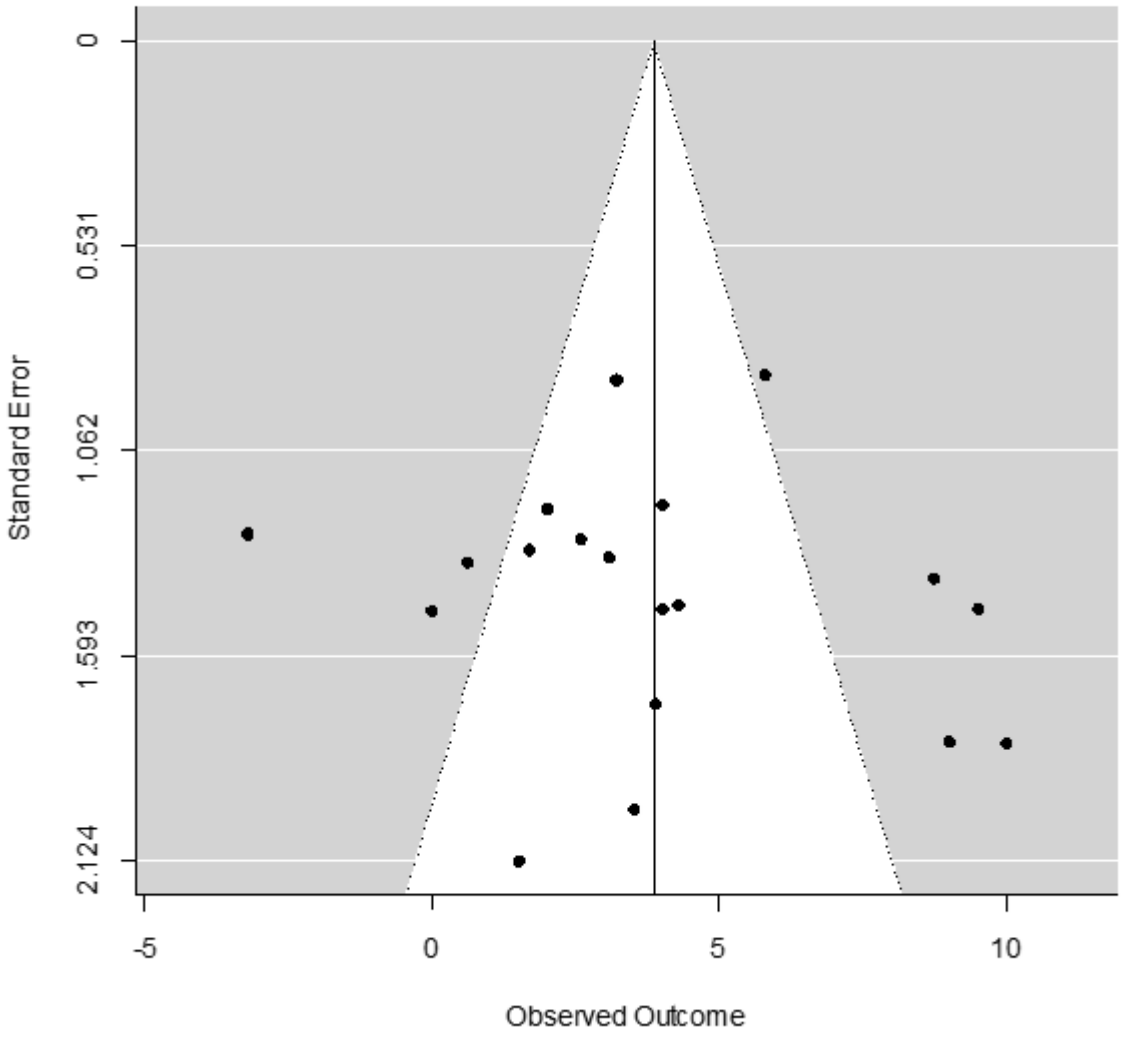
Figure 1: Effect of home fortification with iron-containing powders on haemoglobin concentration at end of intervention: meta-analysis of randomised controlled trials in preschool children [1]



Kounnavong et al. (2011) A and B refer to daily or twice-weekly home fortification with 10 mg iron, respectively. Soofi et al. (2013) A and B refer to home fortification with micronutrient powders excluding and including zinc. Teshome et al. A and B refer to effects of 12.5 mg iron as ferrous fumarate and 3 mg iron as NaFeEDTA, respectively, as reported by the present study.

[1] τ^2 (estimated amount of total heterogeneity): 9.55 (95% CI: 4.57, 23.86); I^2 (total heterogeneity / total variability): 84.1% (95% CI: 71.7%, 93.0%); H^2 (total variability / sampling variability): 6.29 (95% CI: 3.53, 14.21). Test for heterogeneity: Q (df = 18) = 102.61, $p < 0.0001$.

Figure 2: Effect of home fortification with iron-containing powders on haemoglobin concentration at end of intervention: funnel plot of randomised controlled trials in preschool children



Egger's regression test for funnel plot asymmetry: p = 0.44

Table 1. Effect of daily home fortification with 3mg iron as NaFeEDTA and 12.5mg iron as encapsulated ferrous fumarate on continuous outcomes at 30 days after start of intervention, by intention-to-treat analysis

Outcome/intervention group	n	Estimate [a]	Effect (95% CI) relative to placebo [b]	Effect (95% CI) relative to standard [b]
Haemoglobin concentration				
Placebo	112	107.0 g/L (1.3 g/L)	Reference	Not applicable
Iron, 3 mg as NaFeEDTA	112	110.0 g/L (1.2 g/L)	3.0 g/L (-0.1 g/L to 6.2 g/L) [c]	1.3 g/L (-1.8 g/L to 4.3 g/L) [c]
Iron, 12.5 mg as ferrous fumarate	114	108.6 g/L (1.2 g/L)	1.6 g/L (-1.4 g/L to 4.7 g/L) [c]	Reference
Plasma ferritin concentration				
Placebo	112	29.5 µg/L	Reference	Not applicable
Iron, 3 mg as NaFeEDTA	112	33.1 µg/L	16.2% (-14.3% to 57.7%) [d]	2.5% (-22.4% to 35.4%) [d]
Iron, 12.5 mg as ferrous fumarate	114	32.5 µg/L	12.3% (-17.1% to 52.0%) [d]	Reference
Plasma soluble transferrin receptor concentration				
Placebo	112	2.25 mg/L	Reference	Not applicable
Iron, 3mg as NaFeEDTA	112	2.14 mg/L	-4.3% (-13.5% to 5.9%) [d]	3.6% (-5.5% to 13.6%) [d]
Iron, 12.5mg as ferrous fumarate	114	2.09 mg/L	-7.3% (-16.2% to 2.6%) [d]	Reference
Erythrocyte ZPP-haem ratio				
Placebo	112	137 µmol/mol [2.17]	Reference	Not applicable
Iron, 3mg as NaFeEDTA	112	127 µmol/mol [1.97]	-6.5% (-23.5% to 14.2%) [d]	-5.3% (-21.7% to 14.5%) [d]
Iron, 12.5mg as ferrous fumarate	114	135 µmol/mol [2.00]	-0.7% (-18.6% to 21.0%) [d]	Reference

[a] Mean (SE) or geometric mean; [b] Effects were adjusted for study design (blocks nested within strata of haemoglobin concentration <100 g/L and ≥100 g/L). [c] Effects were calculated as absolute difference in means. [d] Exponentiation of group differences with log-transformed outcomes resulted in associations being expressed as relative differences.

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Student	Emily Mwadime Teshome
Principal Supervisor	Andrew Prentice
Thesis Title	Adherence to home fortification with micronutrient powders in Kenyan pre-school children: self-reporting and sachet counts compared to an electronic monitoring device

If the Research Paper has previously been published please complete Section B, if not please move toSection C

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Stage of publication	**Submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I designed the study, conducted literature review, planned for the field work, developed study tools, coordinated and implemented the research field work, recruited and trained research staff; supervised all data collection, cleaned data and under my co-supervisor's(Dr. Hans verhoef) technical
	guidance I partially analysed the data, wrote the manuscript and received technical input as needed from both my supervisors (Prof. Andrew Prentice and Dr. Hans verhoef) .

Student Signature: _____ Date: 26/09/2014

Supervisor Signature: _____ Date: _____

CHAPTER 4: Research Paper 3

Title: Adherence to home fortification with micronutrient powders in Kenyan pre-school children: self-reporting and sachet counts compared to an electronic monitoring device

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Abstract

Introduction: The efficacy of home fortification with iron-containing micronutrient powders varies between trials, perhaps in part due to population differences in adherence. We aimed to assess to what extent of adherence measured by sachet count or self-reporting forms is in agreement with adherence measured by electronic device. In addition, we explored to how each method of adherence assessment (electronic device, sachet count, self-reporting forms) is associated with haemoglobin concentration measured at the end of intervention; and to what extent baseline factors were associated with adherence as measured by electronic device.

Methods: 338 rural Kenyan children aged 12-36 months were randomly allocated to three treatment arms (home fortification with two with different iron formulations or placebo). Home fortificants were administered daily by parents or guardians over a 30 day-intervention period. We assessed adherence using an electronic device, self-reporting and sachet counts. We also assessed haemoglobin concentration at the end of intervention.

Results: Adherence, defined as having received at least 24 sachets (≥80%), during the 30-day intervention period was attained by only 60.6% of children. The corresponding values were higher when adherence was assessed by self-report (83.9%; difference: 23.3%, 95% CI: 18.8% to 27.8%) and sachet count (86.3%; difference: 25.7%, 95% CI: 21.0% to 30.4%). Among children who received iron, each 10 openings of the sachet storage container were associated with an increase in haemoglobin concentration at the end of intervention by 1.15 g/L (95% CI: 0.04 to 1.92). For each increment in age of the parent or guardian by 1 year, the logit of the fractional response increased by 4% (95% CI: 1% to 6%, p=0.002).

Conclusions: Compared to adherence monitoring by electronic device, self-reporting and sachet count over a 30-day intervention period was associated with over-reporting. Adherence to home fortification was associated with the age of the parent or guardian.

Key words:
Adherence; child; preschool; compliance; home- fortification; iron; Kenya; medication events monitoring system, sachet count; self-reporting

4.1 Introduction

In 2011, WHO recommended home fortification with iron-containing micronutrient powders (1). The basis of this recommendation was a meta-analysis of randomised controlled trials showing moderate quality evidence for an effect on anaemia and haemoglobin concentration (2). In a recent placebo-controlled trial, however, we failed to find effects of home fortification with iron-containing micronutrient powders on either anaemia or haemoglobin concentration. In addition, in an updated meta-analysis, we showed that the magnitude of the effect on haemoglobin concentration varied between trials (Teshome, et al, submitted). Such variability in trial results may be due in part to population differences in adherence to home fortification. Adherence has been assessed in several trials by self-reporting (or proxy reporting, in the case of guardian reporting for children in their care) and sachet counts. Although these assessment methods are easy to use and relatively inexpensive, their reliability and validity are controversial (3). Both frequently result in overestimation of adherence because study participants respond in a manner that they believe is socially desirable but conceals their actual behaviour, or participants may consciously or unconsciously fail to return unused medication. Poor recall, particularly over long periods, can also lead to underreporting and underestimation of adherence (3-5).

Medication event monitoring system (MEMS) although expensive is superior to medication counts and self-reporting adherence method [6-7] and has been considered to be the reference standard in several trials [8-9]. We are not aware of studies that used MEMS devices to measure adherence to home fortification with micronutrient powders.

We aimed to assess to what extent of adherence measured by sachet count or self-reporting forms is in agreement with adherence measured by electronic device. In addition, we explored to how each method of adherence assessment (electronic device, sachet count, self-reporting forms) is associated with haemoglobin concentration measured at the end of intervention; and to what extent baseline factors were associated with adherence as measured by electronic device.

4.2 Subject and Methods

Study design

This report concerns a secondary analysis of an explanatory, individually-randomised, placebo-controlled non-inferiority trial to assess the effect of daily home fortification on haemoglobin concentration and iron status. We measured adherence over the 30 day-intervention period using MEMS, self-reporting and sachet counts. Details regarding study methodology are available in the study protocol (Teshome et al, submitted).

Study area and participants

The study was conducted in 3 administrative areas of Kisumu West, Kenya in the period between January and December 2014. A total of 433 children aged 12-36 months were invited by field workers to the research clinic for screening and their parents signed informed consent forms. During the screening period, medical staff verified their ages by birth certificates or health cards, conducted medical examination, took anthropometric measurements (height and weight), and collected venous blood (4mL) in tubes containing Li-heparin after 30 days of intervention for subsequent determination of haemoglobin concentration (HemoCue 301, Ängelholm, Sweden).

Children who attained the following admission criteria were randomised: aged 12–36 months; resident in the study area and parents intending to stay in the area in the subsequent nine months; child had no twin; parental consent form signed by both parents; not acutely sick or febrile (axillary temperature ≤37°C) at the time of recruitment; absence of reported or suspected major systemic disorder; haemoglobin concentration ≥70 g/L; not severely wasted (weight-for-height z-score <–3 SD); no known allergy to dihydroartemisinin-piperaquine, benzimidazole or praziquantel; no reported history of using anti-helminth drugs in the 1-month period before the screening date; not at risk of malaria (e.g. because children had received chemoprophylaxis).

To protect children against malaria and severe anaemia during the intervention period, we administered pre-medications to every eligible child at the end of the screening visit. We gave a therapeutic course of dihydroartemisinin-piperaquine (Sigma-tau, Rome, Italy; target dose: 40mg dihydroartemisinin/320mg piperaquine), albendazole (Indoco Remedies, Mumbai, India) and praziquantel (Cosmos, Nairobi, Kenya) [1, 10].

Interventions and follow-up

Three days after starting pre-medication, parents were asked to bring their child again for a visit to the research clinic, where children were randomly allocated to receive daily home fortification for 30 days with micronutrient powders containing: a) 3mg iron as NaFeEDTA versus, or b) 12.5 mg iron as encapsulated ferrous fumarate, or c) placebo (no iron). The micronutrient powders were manufactured specifically for this trial by DSM Nutrition Products (Johannesburg, South Africa) and packaged in 1g sachets. The home fortificant powders for all three groups contained the same multiple micronutrients other than iron (table 1) as recommended by the Home Fortification Technical Advisory Group, except folic acid, which we omitted because of our concerns that it may be utilized by *Plasmodium* parasites and increase failure risk of antifolate drugs [11,12]

The micronutrient powders were packed in 1-gram plain white foil single-serve sachets that were identical in appearance and that did not result in apparent differences in taste, texture or colour of *uji* (maize gruel, a local food commonly given to young children). The MEMS device comprised of a white plastic bottle with an electronic cap and had no other marker except the serial number, label with the child's identification number, date of start and end of intervention period. Except for the trial coordinator and one field supervisor, neither parents nor research assistants were informed about the function of the electronic device. Instead they were informed that the MEMS cap is essential for maintaining the moisture content and good hygienic conditions of the micronutrient powders.

The research assistants gave parents a supply of 30 sachets in a plastic bottle with a MEMS cap and instructed them to daily add the contents of one sachet to the child's semi-solid, ready-prepared foods for a period of 30 days. The first dose of the treatment was administered at the research clinic. Parents were shown how to mix the micronutrient powder with *uji*. Parents were trained by the research assistants to fill out the self–reporting form (Appendix 4) and were instructed to fill out the form each day after the child completed consuming a meal containing the micronutrient powders. They were shown how to pack the empty sachet in empty zip-lock plastic bag provided to them and instructed to immediately report any sickness or adverse reactions experienced by the child during the intervention period. Each child was issued with a plate, a spoon and a mug to minimise sharing of the sachet content. A return date to the research clinic was written on the self-reporting forms, plastic bag and MEMS bottle, and parents were verbally informed about this return date.

Research assistants conducted weekly pre-announced home visits (one visit per child per week) making a total of 3 visits per child, these visits were denoted as "regular monitoring" to check if the child was still in the study area, if parents were following protocol when administering the fortificants, if sachets were still in MEMS device (research assistants were instructed not to open the bottle during the home visits but to gently shake the bottle to ascertain the contents), and to check if parents were filling out forms and storing empty sachets. Also, the research assistants discussed problems or clarified procedures, but they did not give parents instructions additional to those given during the randomisation visit. All observations and problems experienced by parents were recorded in a home-visit report form and submitted to the field supervisor at the end of each day. Sick children were referred to the research clinic.

Parents were asked to bring their children again to the research clinic at the end of the 30-day intervention period, where phlebotomists collected blood and performed point-of-care tests (including haemoglobin concentration) using the same procedures as in the screening visit. Research assistants collected all the adherence-measuring tools.

Description of adherence measuring tools

Medication events monitoring system (MEMS): Electronic monitoring system provides detailed information in the timing of the events e.g. day, correct time intervals and per cent prescribed doses taken [13]. We used an electronic monitoring and time-recording device (MEMS 6 TrackCap 45mm without LCD display; http://www.mwvaardex.com/) that was given for the duration of the study to parents of participating children. This battery-operated device consists of a cap that fit the bottle, with a built-in microprocessor that records and stores date and time of all openings. Each bottle had a capacity of holding 30 micronutrient sachets, and was labelled with a child's identification number, serial number of the electronic cap, name of child, start and end date for ease of identification and tracking. Parents were thoroughly instructed to open the bottle only when removing the sachet and to close it immediately after each opening. The research assistant demonstrated this instruction when the first dose of powder was administered at the research clinic. The information recorded in the MEMS cap was downloaded and stored electronically in the computer.

Sachet counts: We defined sachet count as the number of empty sachets stored after intervention period. The empty sachet is a proxy indicator that child has consumed the content of a full sachet. Research assistants instructed parents to securely keep empty sachets in a zip-lock plastic bag marked with the child's name and identification number. On their return visit to the research clinic, parents returned the empty sachets contained in zip-lock plastic bag plus any left-over full sachets in the MEMS device. The research assistant immediately counted and recorded the empty sachets into the Excel spread sheet. The leftover full sachets remained in the MEMS bottle and were only removed after the MEMS data had electronically been transferred to power-view software. The number of full sachets was manually recorded in the MEMS software power view data sheet in order to verify if the number of times MEMS cap was not opened matched the number of full sachets.

Self-reporting form: We used a 1-page self-reporting form that was simple and easy to fill out by a parent, even with low level of primary education. The form had a simple chart that allowed a parent to easily tick a box whenever a full sachet of micronutrient powder was administered to the child, either in the morning, mid-morning, lunch, mid-afternoon or evening. The form was translated into the local language (*dholuo*).

4.3 Definition of endpoints

Adherence to treatment was defined as the number of days within the intervention period that the electronic monitoring device was opened, the number of empty sachets within the intervention period, or the number of days within the intervention period that the child received home fortification as reported by the mother or guardian. High adherence was defined as adherence ≥80% (24 days or more). This threshold is arbitrary, but is often used in published studies on medication adherence [7, 13-16]. Low-adherence was defined as adherence < 80% (23 days or less) [17].

4.4 Statistical analysis

Analysis was conducted using SPSS 21 (IBM, Armonk, NY), WHO Anthro software vs.3.2.2 (World Health Organisation, Geneva, Switzerland), Power View vs.3.5.2 (AARDEX Group ltd, Sino, Switzerland) and R-software version 3.2.0 (www.r-project.org). CIA (https://www.som.soton.ac.uk/research/sites/cia/download/), For children who did not complete intervention because parents refused supplementation after randomisation, or because of unknown reasons, we retained 30 days of intervention or 30 sachets as denominators of these proportions. For children who were lost to follow-up during the intervention period because they moved out of the study area, we initially intended to censor the observation period at the day that the child was lost and to use proportional weights to account for differences in observation time between children. However, since we could not establish the day that these children moved, we excluded them from the analysis.

Differences in proportions of children with high adherence were compared by Newcomb's method for paired samples. To evaluate agreement in adherence, measured as continuous variables between various assessment methods, we used scatter plots with corresponding Pearson's product-moment correlation coefficient, r. We used t-tests and linear regression analysis to model haemoglobin concentration at the end of 30 days of intervention as a function of adherence. In these analyses, we excluded children who received placebo.

Lastly, we used simple beta regression analysis with a logit link to identify variables that were associated with adherence as measured by MEMS device. In this analysis, we transformed the adherence outcome variable so that fractional response values of 0 or 1 were replaced using the formula $y' = [y + (n - 1) + 0.5]/n$ [17] with y being the fractional response, and n being the sample size. The variables examined included experimental treatment, baseline characteristics of the child (sex, age, being infected by *P. falciparum*, wasting, stunting), the child being sick during the 30-day intervention period, and characteristics of the parent or guardian (age, gender, education level).

. 4.5 Results

Participant flow and baseline characteristics
The profile of trial participants and reasons for loss to follow are presented in Figure 1. Of the 433 children invited for screening at the research clinic 339 were eligible but within the first few days of the intervention one child was excluded because she had a twin sister. 23 children were lost to follow-up: parents or guardians from eight children refused further cooperation, six children moved residence, and nine children were lost for unknown reasons. 15 participants failed to submit their self-reporting forms, 13 participants did not return empty sachets, one electronic cap was damaged and another was lost. All parents who declined to continue with the study submitted their measuring instruments and gave permission for data collected to be included in the study.

Table 2 presents the baseline characteristics. We included more boys than girls (186 versus 152), mean age (SD) was 23.6 ±6.5 months and the distribution of children by age class was evenly distributed between the intervention groups. At baseline, the mean (SD) haemoglobin concentration was 105 (3.2) g/L and 62.1% (209/338) of the children were anaemic. The prevalence of being stunted, wasted and underweight were 30.2% (102/338), 2.9% (10/338) and 13.6% (46/338), respectively. Mean (SD) age of primary guardians was 27.4 (6.3) years ranging from 16 to 50 years. Interviews conducted at end of study (post intervention) showed that about 80.2% (158/197) of the mothers administered the sachets at home; in other cases, the child's older siblings (9.1%), extended family members (6.1%) or father (4.1%) took up this role. Most primary guardians 71.2% (141/197) attained upper primary education level followed by secondary education level 20.8% (41/197), lower primary education level 5.5% (11/197) and very few 2.5% (5/197) attained tertiary level of education. At the end of 30 days of intervention, the mean (SD) haemoglobin concentration was 108.5 (12.7) g/L and the prevalence of anaemia was about 46%.

Adherence as measured by self-reporting, sachet counts and MEMS device
MEMS data indicated that 60.6% of children received at least 24 sachets during the 30-day intervention period (Table 3). Such high adherence was more frequently indicated by both self-report (83.9%; difference: 23.3%, 95% CI: 18.8% to 27.8%) and sachet count (86.3%; difference: 25.7%, 95% CI: 21.0% to 30.4%) than recorded by MEMS (Table 3). This over-reporting by self-report and sachet count was apparent when inspecting the scatterplots of results obtained by these methods with those obtained MEMS data (Figure 2). The value for Pearson's r expressing the strength of the correlation between adherence by self-report and MEMS device was 0.39 ($p<0.001$), whilst the corresponding value for the correlation between adherence by sachet count and MEMS device was 0.38 ($p<0.001$).

Adherence and haemoglobin concentration at the end of iron intervention
Among 228 children who received home fortification with iron, formulated either as ferrous fumarate or NaFeEDTA, the mean haemoglobin concentration at the end of intervention was 109.3 g/L (SD: 12.2 g/L), with a corresponding prevalence of anaemia of 44.3% (n=101).

Within children who received iron, we could identify an association between haemoglobin concentration at the end of the 30-day intervention period and adherence as assessed by MEMS (p=0.04), but not when assessed by self-report or sachet count (p=0.11 and p=0.14, respectively) (Table 4). Each 10 openings of the sachet storage container were associated with an increase in haemoglobin concentration of 1.15 g/L (95% CI: 0.04 to 1.92).

When adherence was assessed by MEMS, we found that children with high adherence to home fortification with iron had higher haemoglobin concentration at the end of the 30-day intervention period than their peers with low adherence (111.1g/L versus 106.6g/L; difference: 4.6g/L, 95% CI: 1.2g/L to 8.0g/L). There was no evidence for such an association when adherence was assessed by self-report (109.7g/L versus 107.0g/L; difference: 2.7g/L, 95% CI: -1.6g/L to 7.0g/L) or sachet counts (109.6g/L versus 107.3g/L; difference: 2.2g/L, 95% CI: -2.6g/L to 7.1g/L).

Factors associated with adherence measures using MEMS device
Adherence to home fortification was associated with the age of the parent or guardian: for each increment in age by 1 year, the logit of the fractional response increased by 4% (95% CI: 1% to 6% p=0.002; Figure 3). Compared to adherence with home fortification with placebo, there was no evident difference in adherence with home fortification with iron, either as NaFeEDTA or as ferrous fumarate. We also found no evidence that adherence was affected by baseline characteristics of the child (sex, age, being infected by *P. falciparum*, wasting, stunting), the child being sick during the 30-day intervention period, or characteristics of the parent or guardian (gender, education level) (Table 5).

4.6 Discussion

Compared to adherence monitoring by electronic device, we found that self-reporting and sachet count over a 30-day intervention period was associated with over-reporting. Among children who received iron, haemoglobin concentration at the end of the 30-day intervention period was associated with adherence as assessed by MEMS, but there was no evidence of it being associated with adherence as assessed by self-report or sachet count. Each 10 openings of the electronic device were associated with an increase in haemoglobin concentration of 1.15 g/L (95% CI: 0.04 to 1.92). Adherence to home fortification was associated with the age of the

parent or guardian, but we found no evidence that it was associated with home fortification with iron or any other baseline characteristics for child or parent.

We assume that assessment of adherence by electronic device is more reliable than by self-report or sachet count. This seemed confirmed by our finding that adherence assessed by MEMS was associated with a haemoglobin concentration response in children who received iron, whereas such a response could not be shown when adherence was assessed by self-report or sachet count. Our findings are similar to results of a randomized trial that showed MEMS caps opening data positively reflected actual adherence and a positive effect to hypertensive treatment among the African-Americans [14]. However, we cannot exclude the possibility that there were parents who administered missed doses on the next day in addition to the scheduled dose. Compared to ingestion of a single dose of non-haem iron, multiple dosing leads to a decrease in the proportion of iron absorbed, but an increase in the absolute amount of iron absorbed [17]. Thus the efficacy of missing a dose and doubling it on the next day is probably in between the efficacy of regular daily dosing and skipping a missed dose altogether. In that case, assessment of adherence by sachet count may theoretically be better than by electronic device.

As reported elsewhere, we failed to find an effect of home fortification with either 3mg iron as NaFeEDTA or 12.5mg iron as ferrous fumarate on haemoglobin concentration, erythrocyte zinc protoporphyrin or plasma iron markers (Teshome, et al, submitted). This failure may have been due in part to the relatively short intervention period, consumption of diets containing high phytate and phenolic compounds and in part to infection-associated inflammation, which was highly prevalent in our study population. In the present study, we found that adherence, as assessed by MEMS, showed only 60.6% of children consumed at least 80% (≥24) sachets during the 30-day intervention period. These data, coupled with our finding that adherence was associated with haemoglobin concentration at the end of the 30-day intervention period; indicate that sub-optimal adherence may also have contributed to our failure to show efficacy.

The association between age of the parent or guardian and adherence to home fortification should be interpreted with caution, because this finding was the result of exploratory analyses. Nonetheless, this finding supports results of a trial conducted among HIV-infected adults in Los-Angeles, where older age was associated with higher adherence rates of anti-retroviral adherence [18] compared to the younger patients. Therefore, it is possible that in our present study the older parents are better carers and attempted to observe the instructions given for the use of MEMS device, potentially improving the adherence measurement when compared to the younger carers. Further trials are needed that are specifically designed to identify and understand determinants of adherence to home fortification of micronutrient powders. In such trials, latent class analysis [19] may be helpful to

define subgroups on the basis of beliefs and attitudes about home fortification at baseline.

A strong point of our study is the use of beta regression analysis to model adherence expressed as a fractional response. Linear regression analysis of the untransformed fractional response is commonly used but has limitations: a) the effect of explanatory variables tend to be non-linear, particularly with responses towards the extremes of the range [0, 1]; b) a proportion is not normally distributed (even though a normal approximation may be reasonable if all observations are reasonably close to 0.50); and c) the variance is not constant but varies with the outcome, meaning it is maximised at a population proportion of 0.50 and it shrinks when the population proportion approaches one of the boundaries. Similar problems are likely to arise when estimating adherence as the mean fractional response, or when comparing groups by the mean difference in fractional response using ANOVA or assuming t-distributions.

In conclusion, compared to adherence monitoring by electronic device, self-reporting and sachet count over a 30-day intervention period was associated with over-reporting. Adherence to home fortification as measured by MEMS device was associated with the age of the parent or guardian

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Table 1: Formulation of micronutrient powders (study formulation)

Micronutrient	Content
Vitamin A, µg RE	300
Vitamin D, µg	5
Vitamin E, mg	5
Vitamin C, mg	30
Thiamin (vitamin B_1), mg	0.5
Riboflavin (vitamin B_2), mg	0.5
Niacin (vitamin B_3),mg	6
Vitamin B_6 (pyridoxine), mg	0.5
Vitamin B_{12} (cobalamine), µg	0.9
Iron	
EITHER iron as encapsulated ferrous fumarate, mg	12.5
OR iron as NaFeEDTA, mg	3
OR no iron (placebo)	0
Zinc, mg	5
Copper, mg	0.56
Selenium, µg	17
Iodine, µg	90

Table 2: Baseline characteristics

Characteristic	Placebo	Iron, 3mg as NaFeEDTA	Iron, 12.5mg as ferrous fumarate
n	112	112	114
General characteristics			
Sex, male	69 (61.6%)	61 (54.5%)	56 (49.1%)
Age, months	22.8 (6.8)	23.2 (6.2)	24.9 (6.4)
Age class			
12-23 months	61 (54.5%)	57 (50.9%)	44 (38.6%)
24-36 months	51 (45.5%)	55 (49.1%)	70 (61.4%)
Nutritional markers			
Haemoglobin concentration, g/L	104.4 (13.2)	105.9 (13.3)	104.7 (13.3)
Anaemia			
Moderate (haemoglobin concentration 70–99.99 g/L)	33 (29.5%)	34 (30.4%)	36 (31.6%)
Mild (haemoglobin concentration 100–109.99g/L)	39 (34.8%)	31 (27.7%)	37 (32.5%)
No anaemia (haemoglobin concentration ≥ 110g/L)	40 (35.7%)	47 (42.0%)	41 (36.0%)
Anthropometric markers			
Body height, cm	80.9 (5.9)	82.1 (5.7)	82.4 (5.1)
Body weight, kg	10.6 (1.8)	10.8 (2.0)	10.9 (1.7)
Stunted (height-for-age z-score < –2 SD)	34 (30.4%)	31 (27.7%)	37 (32.5%)
Wasted (weight-for-height z-score < –2 SD)	3 (2.7%)	5 (4.5%)	2 (1.8%)
Underweight (weight-for-age z-score < –2 SD)	19 (17.0%)	12 (10.7%)	15 (13.2%)

Values indicate n (%), mean (SD)

Table 3: Adherence as indicated by various methods during the 30-day intervention period

Adherence	MEMS (n=338)	Self-reporting (n=338)	Sachet count (n=338)
Coverage [a]	330 (97.6%)	317 (93.8%)	313 (92.6%)
Missing [b]	8 (2.4%)	21 (6.2%)	19 (5.6%)
High (≥ 80% or ≥ 24 sachets consumed)	200 (60.6%)	266 (83.9%)	270 (86.3%)
Low (<80% or 1-23 sachets consumed)	130 (39.4%)	51 (16.1%)	43 (13.7%)

Values indicate n (%), **MEMS**= Medication Events Monitoring System

[a] Number of children that consumed at least one of the 30 sachets scheduled during the intervention period.

[b] Missing data as follows: **MEMS** = data from 6 participants were excluded because they moved residence, 1 participant damaged electronic device and another lost the electronic devices; **Self-reporting** = data from 6 participants were excluded because they moved residence and 15 others failed to submit their self-reporting forms; **Sachet counts** = data from 6 participants was excluded because they moved resident and 13 others failed to return empty sachets.

Table 4: Association between adherence and haemoglobin concentration in children who received daily home fortification with iron as ferrous fumarate or NaFeEDTA

Adherence assessment method	Difference in haemoglobin concentration, g/L (95% CI) [a]	P-value
MEMS	1.15 (0.04 to 1.92)	0.04
Self-reporting	2.33 (-0.50 to 5.16)	0.11
Sachet count	2.69 (-0.88 to 6.27)	0.14

MEMS: Medication Events Monitoring Systems

[a] Association expressed as the mean difference in haemoglobin concentration for each increment in adherence by 10 MEMS openings, or 10 sachets consumed as indicated by self-report or sachet count.

Table 5: Baseline characteristic associated with measures of adherence by MEMS device

Baseline Characteristic	Estimate	SE	p-value	95%CI-L	95%CI-H
Intervention					
Placebo	1.24797	0.11100	Reference		
NaFeEDTA	0.11084	0.14720	0.451	-0.177672	0.399352
Ferrous fumarate	0.07503	0.14651	0.609	0.2121296	0.3621896
Sex					
Male	1.25617	0.08981	Reference		
Female	0.12123	0.12076	0.315	0.1154596	0.3579196
Age					
Age of child (days)	0.00000	0.00003	0.922		
Parents Age (years)	0.03761	0.01218	**0.002**	0.0137372	0.0614828
Morbidity					
Sick	0.00886	0.13993	0.950	0.2654009	0.28311292
P. *falciparum* infection	-0.15645	0.12415	0.208	-0.399784	0.086884
Nutritional indicators					
Wasting	0.06404	0.05851	0.274	0.0506396	0.1787196
Stunting	0.05756	0.04326	0.183	0.0272296	0.1423496
Underweight	0.08083	0.05200	0.120	-0.02109	0.18275
Education level					
None	1.76250	0.33190	Reference		
Upper primary	-0.54270	0.33790	0.108	-1.204984	0.119584
Secondary	-0.26150	0.36530	0.474	-0.977488	0.454488
Tertiary	-0.19690	0.63060	0.755	-1.432876	1.039076

Figure 1: Participants flow chart

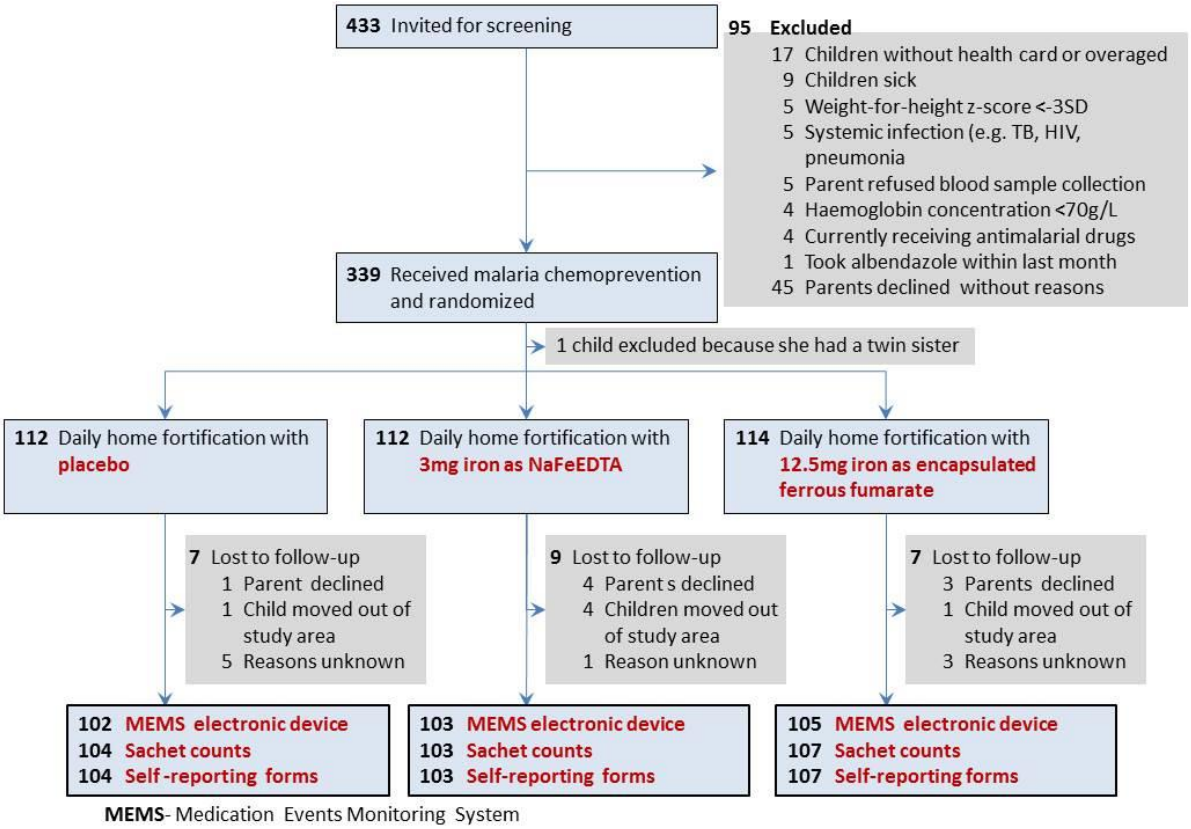
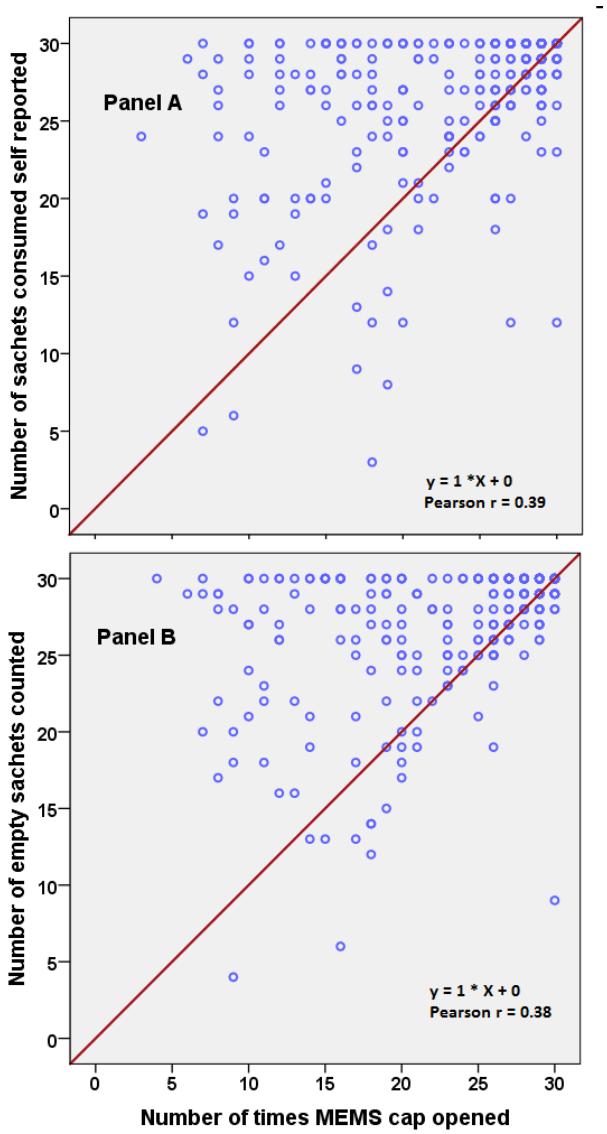
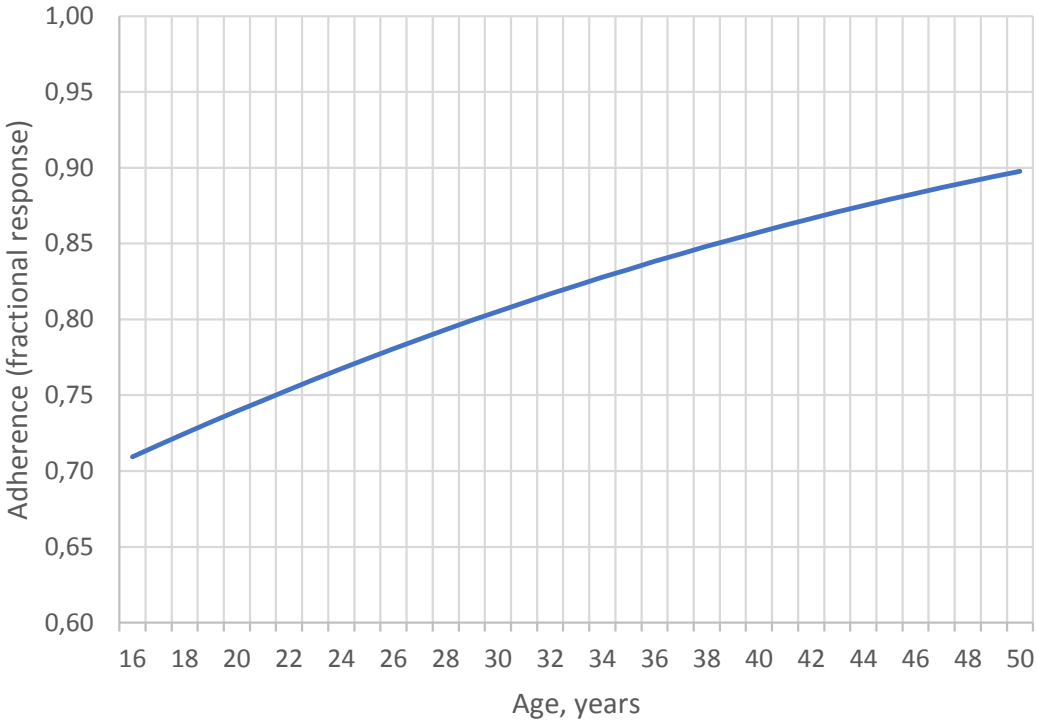


Figure 2: Adherence measurements as assed by self-reporting and sachet tools compared with MEMS device.



Panel A: Data were not available for 17 children because 15 self-reporting forms were either missing or not filled out and records of 2 MEMS devices missing because 1 device was damaged and another was not returned at end of 30 days after randomization**. Panel B:** Data were not available for 15 children because 13 children lost or did not returned empty sachets and 2 MEMS devices were unavailable. Not all data points in **Panel A** and **Panel B** are visible due to over-lapping of adherence counts. The red line indicates perfect agreement in adherence counts between pairs of assessment methods. For each panel, data points presented above the reference line indicate overestimation of adherence assessed by self-report or sachet count, respectively, as compared to adherence measured by MEMS, whereas data points presented below the reference line indicate underestimation of adherence measured by MEMS.

Figure 3: Age of parent as predictor of measure of adherence to daily home fortification



The blue diagonal line shows for each increment in age by 1 year, the logit of the fractional response increased by 4%.

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SECTION A – Student Details

Student	Emily Mwadime Teshome
Principal Supervisor	Andrew Prentice
Thesis Title	Diagnostic utility of zinc protoporphyrin to detect iron deficiency in Kenyan preschool children

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
When was the work published?			
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	BMC-Public Health
Please list the paper's authors in the intended authorship order:	Emily M Teshome, Andrew M Prentice, Pauline E.A. Andango, Hans Verhoef
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SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I conducted literature review, planned for the field work, developed study tools, coordinated and implemented the research field work, recruited and trained research staff; supervised all data collection, cleaned the data. The data analysis was conducted by Dr. Hans verhoef, but I was involved in interpreting the results and drafting the manuscript. Both (Prof. Andrew Prentice and Dr. Hans Verhoef) provided technical inputs as needed.

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Chapter 5: Research Paper 4

Title: Diagnostic utility of zinc protoporphyrin to detect iron deficiency in Kenyan preschool children

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Abstract

Zinc protoporphyrin has often been used in trials as a diagnostic tool for detecting iron deficiency, however, concerns have been raised regarding its ability to distinguish between individuals with or without iron deficiency especially in areas of low prevalence and little is known regarding its diagnostic utility either alone or combined with haemoglobin concentration in young children. We aimed to a) identify factors associated with zinc protoporphyrin (ZPP) in children aged 1-3 years; b) assess the diagnostic performance and utility of ZPP, either alone or in combination with haemoglobin, in the identification of iron deficiency.
Methods: We used baseline data from 338 Kenyan children enrolled in a community-based randomisedtrial , To identify factors related to ZZP measured in whole blood or erythrocytes we used multiple linear regression analysis. To assess diagnostic performance, we excluded children with elevated plasma concentrations of C-reactive protein α_1-acid glycoprotein , and *Plasmodium* infection. In addition, we analysed receiver operating characteristics (ROC) curves, with plasma ferritin concentration <12µg/L as the reference for iron deficiency. We also developed models to assess the diagnostic utility of ZPP and haemoglobin concentration when used to screen for iron deficiency.
Results: Whole blood ZPP and erythrocyte ZPP were independently associated with haemoglobin concentration, *Plasmodium* infection and plasma concentrations of soluble transferrin receptor, ferritin, and C-reactive protein. In children without inflammation or *Plasmodium* infection, the prevalence of true iron deficiency was 32.1%, compared to prevalence of 97.5% and 95.1% when assessed by whole blood ZPP and erythrocyte ZPP with conventional cut-off points (70µmol/mol and 40µmol/mol haem, respectively). Addition of whole blood ZPP or erythrocyte ZPP to haemoglobin concentration increased the area-under-the-ROC-curve (84.0%, p=0.003, and 84.2%, p=0.001, respectively, versus 62.7%). A diagnostic rule (0.038689[haemoglobin concentration, g/L] + 0.00694[whole blood ZPP, µmol/mol haem] >5.93120) correctly ruled out iron deficiency in 37.4%-53.7% of children screened, depending on the true prevalence, with both specificity and negative predictive value ≥90%.
Conclusions: In young children, whole blood ZPP and erythrocyte ZPP have added diagnostic value in detecting iron deficiency compared to haemoglobin concentration alone. A single diagnostic score based on haemoglobin concentration and whole blood ZPP can rule out iron deficiency in a substantial proportion of children screened.

Trial registration: NCT02073149 (25 February 2014)

Key words: Erythrocyte protoporphyrin; inflammation; iron deficiency; Kenya; malaria; *Plasmodium;* child, preschool; zinc protoporphyrin

5.1 Background

Zinc protoporphyrin (ZPP) is formed in erythrocytes when the iron supply for erythropoiesis is less than required, or when iron utilization is impaired. In such conditions of iron-deficient erythropoiesis, protoporphyrin IX, the immediate precursor of haem, incorporates an atom of zinc rather than iron, resulting in the formation of ZPP instead of haem. Thus depleted iron stores or a decrease in circulating iron in the bone marrow lead to elevated ZPP concentrations in whole blood or erythrocytes [1, 2].

ZPP can be determined rapidly and at low assay cost by haematofluorometer. It has been used to screen and manage iron deficiency in individual children, but at a population level, it has also been a recommended marker for iron status in cross-sectional studies, together with haemoglobin concentration [1, 3].

We found earlier, however, that both whole blood ZPP and erythrocyte ZPP have little diagnostic utility as a screening marker to manage iron deficiency in pregnant women, whether used as single tests or combined with haemoglobin concentration [4].

The present study aimed to identify factors associated with ZPP measured in whole blood or erythrocytes from preschool children. We also assessed the diagnostic performance and utility of ZPP, either alone or in combination with haemoglobin concentration, in detecting iron deficiency defined as plasma ferritin concentration <12 μg/L in children without inflammation or *Plasmodium* infection.

5.2 Methods

The study was conducted from January–December 2014 in Kisumu-West District, Kenya, and received ethical clearance both in Kenya (Kenyatta University National Hospital, reference KNH-ERC/A/402) and in England (London School of Hygiene and Tropical Medicine, reference 6503). In order to recruit the children, community health workers compiled a list of parents with children aged 1-3 years in the study area and invited parents to bring these children for screening to the research clinic, where they were asked to sign an informed consent form. Weight and height were determined using Salter Scale (UNICEF, catalogue 0145555, Copenhagen, Denmark) and height/recumbent length boards (UNICEF, catalogue 0114500, Copenhagen, Denmark) within 100g and 1 mm, respectively.

A total of 338 children aged 12-36 months were recruited into the study. We included children if: resident in the study area; parental consent form signed by both

parents; not acutely sick or febrile (axillary temperature ≥37.5°C) at the time of recruitment; absence of reported or suspected major systemic disorder (e.g. HIV infection, sickle cell disease); no use of antiretroviral drugs against HIV, rifampicin, carbamazepine, phenytoin or phenobarbital and no twin sibling. Children were excluded if: haemoglobin concentration <70 g/L; severely wasted (weight-for-height z-score <–3 SD); known allergy to dihydroartemisinin-piperaquine, benzimidazole or praziquantel; parent-reported history of using antihelminthic drugs in the 1-month period before the screening date; not at risk of malaria (e.g. children who received chemoprophylaxis against malaria because of HIV infection or sickle cell disease); did not complete the second and third doses of dihydroartemisinin-piperaquine.

Phlebotomists collected venous blood in tubes containing Li-heparin. We assessed haemoglobin concentration (HemoCue 301, Ängelholm, Sweden) and zinc protoporphyrin: haem ratio (AVIV haematofluorometer, model 206D, Lakewood NJ, USA) in whole blood as a marker of iron-deficient erythropoiesis, each in triplicate. An aliquot of blood was centrifuged, plasma was transferred to a microtube, centrifuged, and stored immediately in liquid nitrogen (-196 °C). The erythrocyte sediment was washed and centrifuged three times with isotonic phosphate-buffered saline to allow measurement in triplicate of the erythrocyte zinc protoporphyrin: haem ratio. Rapid diagnostic tests were used to detect *Plasmodium falciparum*-specific lactate hydrogenase (pLDH), and *P. falciparum*-derived histidine-rich protein-2 (HRP2). Further details are reported elsewhere.

Plasma iron indicators (concentrations of ferritin and soluble transferrin receptor), inflammation indicators (concentrations of C-reactive protein, α-1-acid glycoprotein) and other nutritional markers (concentrations of albumin and vitamin B_{12}) were measured on an Abbott Architect C16000 and i2000 SR analyser with reagents from and as per instructions of the manufacturer.

5.3 Statistical analysis

Anthropometric indices were calculated by comparing measurements with the standards WHO Growth Standards [5]. For whole blood and erythrocyte ZPP, we used a cut-off value of 70 µmol/mol haem (2.7 µg/g haemoglobin), corresponding to the 95% upper limit of the reference values for women and children participating in the US National Health and Nutrition Examination Survey (NHANES) II, from which individuals with anaemia, low transferrin saturation and elevated blood lead concentrations had been excluded [1-3]. For erythrocyte ZPP, we also used a cut-off point of 40 µmol/mol haem, which is based on several small studies comparing iron-deficient and iron-replete individuals [6, 7].

We defined iron deficiency as the absence or near-absence of storage iron, indicated by plasma ferritin concentration <12 µg/L [8]. Because this definition is

recommended by WHO to measure population iron status except where inflammation is prevalent [2], we considered it to be valid only in children without inflammation, *Plasmodium* infection, or HIV infection. In addition, we used the following definitions: anaemia = haemoglobin concentration < 110 g/L [9]; inflammation = plasma concentrations of CRP > 5 mg/L [10] and/or AGP > 1 g/L [11]; being stunted or wasted = height-for-age or weight-for-height z-score < -2 SD; [5] *P. falciparum* infection = presence in blood of HRP2 or *P. falciparum*-specific pLDH; any *Plasmodium* infection = presence of HRP2 or pLDH specific to either *P. falciparum* or human *Plasmodium* species other than *P. falciparum*; low vitamin B_{12} status = plasma vitamin B_{12} concentration <150pmol/L.

Description of the study population
We calculated prevalence values for binary variables, means with corresponding SDs for variables with an approximately normal distribution, and quartiles for continuous variables that were not normally distributed. Because some of the plasma markers used (ferritin, albumin, vitamin B_{12}) can act as acute phase reactants we also described these characteristics in children without inflammation or *Plasmodium* infection.

Factors associated with ZPP
We explored associations between ZPP and personal characteristics (age, sex), inflammation markers, iron markers, *Plasmodium* infection and other plasma markers (albumin and vitamin B_{12} concentrations). Groups were compared assuming t-distributions of ZPP values that were normalised by log-transformation. Exponentiation of results yielded group differences that were expressed as relative differences.

We inspected scatterplots and used simple linear regression analysis to assess associations between ZPP (log-transformed) and explanatory variables with continuous outcomes. Some explanatory factors were untransformed, with the implicit assumption that ZPP values can increase or decrease exponentially with an absolute increment in the explanatory variable; in other cases, log-transformation of the explanatory factor yielded a better model fit, indicating that ZPP values and explanatory variables change at rates that are proportional to their current values. In such cases, variation in the independent variable was expressed as geometric standard deviation, i.e. a dimensionless, multiplicative factor such that dividing or multiplication of the geometric mean by this ratio indicates a variation that is equivalent to subtraction or addition of one standard deviation on a log-transformed scale [12].

We subsequently used multiple linear regression analyses to identify factors that were independently associated with ZPP. Given a linear association between continuous variables, dichotomisation generally results in loss of statistical precision

[13]. Thus we preferred to use continuous variables that were shown to be linearly associated with ZPP in the bivariate analyses. Our analysis started with a full model that included haemoglobin concentration, *Plasmodium* infection, and plasma concentrations of ferritin, soluble transferrin receptor, CRP, AGP, albumin, vitamin B_{12}, sex (binary) and age class (binary). All plasma markers except albumin and vitamin B_{12} were log-transformed. Factors were manually eliminated using a backward elimination process with a removal criterion of $p>0.05$.

Diagnostic performance of ZPP to detect iron deficiency
This part of the analysis was restricted to children without inflammation (i.e. plasma concentrations of CRP > 5 mg/L and/or AGP > 1 g/L) and without *Plasmodium* infection. We used logistic discriminant analysis to model the probability of iron deficiency as a function of continuous explanatory variables, either alone or combined.

We used the pROC package [14] within R vs. 3.2.0 (www.r-project.org) to produce and analyse receiver operating characteristics (ROC) curves, with comparison of areas-under-the-curve (AUCs) by DeLong's test for paired curves. Partial AUCs were computed with a correction to achieve a maximal value of 1.0 and a non-discriminant value of 0.5, whatever the range of specificity or sensitivity values. Confidence intervals of estimates for partial areas-under-the-curve (pAUCs) were computed by stratified bootstrapping with 10,000 replicates.

Diagnostic utility of ZPP to estimate prevalence of iron deficiency
First, we assessed the diagnostic performance of ZPP to estimate the prevalence of iron deficiency, using two commonly used cut-off points for ZPP, namely whole blood ZPP >70 μmol/mol haem and erythrocyte ZPP >40 μmol/mol haem (see preceding paragraphs). We used Wilson's method to calculate confidence intervals around proportions [15, 16]; for sensitivity and specificity, and for the pair of predictive values, we calculated 97.5% univariate CIs. The cross-product of these univariate CIs, considered together, form a joint 95% confidence region for both population parameters [17].

Second, as an example, we used our data to explore the utility of using the combination of haemoglobin concentration and ZPP to screen for iron deficiency (Additional file 1), with cut-points chosen to ensure a high sensitivity so that most cases are detected, at the cost of false positives that could be eliminated by further diagnostic tests. Children can be excluded from further testing if negative test results correctly identify children without iron deficiency in the vast majority of cases. Such a strategy may be desirable in community-based surveys with relatively low prevalence of iron deficiency, but also in medical practice with higher prevalence values (because of self-selection). Thus we estimated the proportion of children who could be eliminated from further testing in settings with a prevalence range for iron

deficiency of 0%-50%, which probably covers the vast majority of community settings, with arbitrarily selected sensitivity values and negative predictive values of >90%. Because of its ease of measurement, we limited this assessment with ZPP being measured in whole blood.

5.4 Results

Description of the study population

The prevalence of iron deficiency, measured in children without inflammation or *Plasmodium* infection, was 32.1%; when measured in all children (Table 1), this value was 17.1%. Whole blood ZPP > 70 μmol/mol haem occurred in 97.9% and erythrocyte ZPP > 40 μmol/mol occurred in 96.7% of children. The prevalence of anaemia was 62.1%, but there were virtually no children with low vitamin B_{12} status. Inflammation, assessed by plasma concentrations of C-reactive protein and α_1-acid glycoprotein, occurred in 66.9% of children, and was mostly mild. Of 226 children with inflammation, 98 (43.4%) had elevated concentrations of α_1-acid glycoprotein with normal C-reactive protein concentrations, 11 (4.9%) had elevated C-reactive protein concentrations with normal concentrations of α_1-acid glycoprotein, and 117 (51.8%) had elevated concentrations of both markers. Of children who carried *Plasmodium* parasites (36.4%=123/338), only 1 was infected with *Plasmodium* species other than *P. falciparum*.

Factors associated with ZPP: crude analysis

In bivariate analysis, both whole blood and erythrocyte ZPP were strongly elevated in iron deficiency, anaemia and *Plasmodium* infection; they declined with increasing haemoglobin and plasma ferritin concentrations; and they increased with plasma transferrin receptor concentration (Table 2). Whole blood ZPP was also associated with increased plasma concentrations of α_1-acid glycoprotein and C-reactive protein concentration; a higher prevalence of inflammation as defined by α_1-acid glycoprotein > 1 g/L; decreased plasma albumin concentration, and increased plasma vitamin B_{12} concentration. Although erythrocyte ZPP was reduced in children aged 24-36 months and, seemed higher in boys than in girls, there was no evidence that it was associated with inflammation, however defined, or with either of the two plasma inflammation markers. We found no evidence that ZPP, whether measured in whole blood or erythrocytes, was associated with z-scores for height-for-age or weight-for-height, or with being stunted or wasted.

Factors associated with ZPP: multiple linear regression analysis

In multivariate analysis, there was no evidence that ZPP, whether measured in whole blood or erythrocytes, was associated with plasma concentrations of α_1-acid glycoprotein and vitamin B_{12}, or with sex or age class. Thus these factors were eliminated from the models shown in Table 3.

Whole blood ZPP was independently associated with decreased concentrations of haemoglobin and ferritin and with increased plasma concentrations of transferrin receptor and C-reactive protein (Table 3). Of all biochemical markers, plasma transferrin receptor concentration showed the strongest association with whole blood and erythrocyte ZPP. Similar results were found with erythrocyte ZPP as dependent variable. *Plasmodium* infection was associated with elevated whole blood ZPP, and an even more pronounced elevation in erythrocyte ZPP values. There was a mild association between plasma albumin concentration and erythrocyte ZPP, but retention of this factor in the model for whole blood ZPP did not appreciably change the magnitude of the association for other factors.

Diagnostic performance of ZPP to detect iron deficiency
ROC curve analysis showed that whole blood and erythrocyte ZPP had similar diagnostic performance (AUC values: 79.1% and 81.2%, respectively; p=0.36) in detecting iron deficiency, but either marker performed better than haemoglobin concentration (AUC: 62.7%) (Figure 1A; Table in Figure 1). The diagnostic accuracy was further improved by combining either whole blood or erythrocyte ZPP with haemoglobin (Figure 1B, p=0.003; and Figure 1C, p=0.001, respectively).

Overall, there was no evidence that the diagnostic accuracy differed between the combination of haemoglobin concentration with erythrocyte ZPP and the combination of haemoglobin concentration with whole blood ZPP (AUCs: 84.2% versus 84.0%; p=0.91). The ROCs for these markers crossed (Figure 1D) at a sensitivity of 81.5%, corresponding to $0.07195[Hb] + 0.01449[ZZP_{whole}] = 10.39334$ (where Hb and ZPP_{whole} indicate haemoglobin concentration in g/L and whole blood ZPP:haem ratio in μmol/mol, respectively; in this equation, parameter estimates are shown with 5 decimals to avoid misclassification due to multiplication of rounding errors). At all sensitivity values above this cut-off (Figure 1D, red rectangle), the diagnostic accuracy of the combination of erythrocyte ZPP with haemoglobin concentration was superior to the combination of whole blood ZPP with haemoglobin concentration (corrected pAUCs: 76.3% versus 70.4%, p=0.04).

Diagnostic utility of ZPP
When whole blood ZPP was considered without additional markers to detect iron deficiency, a conventional threshold of 70 μmol/mol haem resulted in the following estimates **(**Table 4**)**: sensitivity: 100%; specificity: 3.7%, positive predictive value: 34.2%; prevalence: 97.5% (as compared to a 'true' prevalence of 32.1%; Table 1). Corresponding values for an erythrocyte ZPP threshold of 40 μmol/mol haem were: 100%, 7.4%, 35.1% and 95.1%.

Within a prevalence range of iron deficiency of <14.1%, a diagnostic rule of haemoglobin concentration > 122 g/L would rule out iron deficiency in 14.1%-14.8% of children tested, depending on the actual prevalence, with both sensitivity and

negative predictive value > 90% (Figure 2). Similarly, within a prevalence range of iron deficiency of < 28.6%, whole blood ZPP > 99 μmol/mol haem would rule out iron deficiency in 28.6%-36% of children tested; and within a prevalence range of 37.4%, 0.038689 [Hb] + 0.00694 [whole blood ZPP] > 5.93120 would rule out iron deficiency in 37.4%-53.7% of children. At all prevalence values exceeding these ranges, these diagnostic tests would not be able to rule out children with the predefined diagnostic criteria (i.e. both sensitivity and negative predictive value should be 90%), and all children would need to undergo further diagnostic work-up using more advanced tests.

5.5 Discussion

In our population, virtually all children had whole blood ZPP values exceeding conventional cut-off points of 70 μmol/mol haem, resulting in very low specificity and gross overestimates of the 'true' prevalence of iron deficiency of 32.1%, whether assessed in the overall population or when restricted to those without inflammation and without *Plasmodium* infection. A similar problem was noted with erythrocyte ZPP > 40 μmol/mol haem. Both whole blood ZPP and erythrocyte ZPP were independently associated with *Plasmodium* infection and plasma C-reactive protein concentration. ZPP, whether measured in whole blood or erythrocytes, yielded higher diagnostic accuracy in detecting iron deficiency than haemoglobin concentration alone, and also improved this diagnostic accuracy when used in combination with haemoglobin concentration. When applied in a screen-and-treat strategy to control iron deficiency in paediatric populations with a prevalence of iron deficiency of <37.4% (which covers most settings in developing countries), our data suggest that a diagnostic rule of 0.038689 [Hb] + 0.00694 [whole blood ZPP] > 5.93120 can correctly identify 90% of children with iron deficiency, and correctly rule out iron deficiency in 37.4%-53.7% of children who are tested, depending on the true prevalence.

We found a particularly strong relationship between ZPP, whether measured in whole blood or erythrocytes, and plasma transferrin receptor concentration. This is not surprising because both are markers for iron-deficient erythropoiesis. Consistent with our data, ZPP is known to be increased in iron deficiency, inflammation and other causes of an inadequate iron supply to erythroblasts. The increase in whole blood ZPP that was associated with *Plasmodium* infection may be due in part to the formation of bilirubin and other haemoglobin breakdown products in plasma that result from haemolysis and that fluoresce in the same wavelength range as protoporphyrins. *Plasmodium* infection was also associated with an even larger increase in erythrocyte ZPP, independently of inflammation. This was unexpected because erythrocytes lack plasma constituents, which are removed by washing. A possible explanation is that haemolysis-induced increase in erythropoietin activity under influence of *Plasmodium* infection drives up the demand for iron in the

erythron. We have not been able to find previous reports of an association between erythrocyte ZPP and plasma albumin concentration, which could be a spurious finding.

One limitation of our study is the difficulty of measuring iron status in the presence of inflammation. Throughout the remainder of this discussion, it should be noted that we assessed the diagnostic performance and ZPP in children without inflammation and without *Plasmodium* infection, because plasma ferritin concentration can be elevated in the presence of infection-induced inflammation independently of iron status. We used this approach in favour of other biomarkers and approaches that have been proposed.

In one method [18,19], fixed correction factors for serum ferritin concentrations are computed based on geometric mean values in groups that are defined by cross-classification of individuals by two inflammation markers (serum concentrations of C-reactive protein > 5 g/L and α_1-acid glycoprotein > 1.0 g/L). The resulting ratios in geometric mean values are used to adjust individual values, and deficiency is determined on the basis of these adjusted values. This method has the disadvantages, however, that it is not validated using a reference standard, its validity depends on untested and perhaps invalid assumptions, and it does not take into account likely between-person variability in the response of ferritin concentration to inflammation. Recently, it has been proposed to adjust values using regression analysis [11]. Although this approach offers several theoretical advantages, its validity remains to be investigated by comparison with a reference standard. The ratio of concentrations of soluble transferrin receptor/log ferritin has been suggested as a marker of body iron content but its use to detect iron deficiency remains problematic for reasons discussed elsewhere [4].

Our results clearly show that, in the absence of inflammation or *Plasmodium* infection, ZPP has added diagnostic value in detecting iron deficiency over haemoglobin concentration alone. This added value applied both to ZPP measured in whole blood or erythrocytes. By contrast, we found that the diagnostic performance of haemoglobin concentration in detecting iron deficiency was similar in pregnant women (AUC: 61%, [4]) as in children (AUC: 63%, present study); in pregnant women, however, replacement of haemoglobin concentration by ZPP, or addition by ZPP to haemoglobin concentration, whether measured in whole blood or in erythrocytes, had little diagnostic value [4].

When combined with haemoglobin concentration, whole blood ZPP or erythrocyte ZPP yielded AUC values for the ROC curves of approximately 84%. Whether this accuracy is satisfactory depends on the purpose of testing. Conventional cut-off points for whole blood ZPP and erythrocyte ZPP are clearly inappropriate and produce gross overestimates of prevalence and a very low positive predictive value

(Table 5 in this paper; see also [4]) due to their low specificity. Mwangi et al 2014 [4], reported how cut-off points can be manipulated to minimize bias in the estimation of population prevalence. In the present paper, we have shown how cut-off points for haemoglobin and whole blood ZPP, either alone or in combination, can be calibrated in a screen-and-treat strategy to identify individuals with iron deficiency at community level. Screening generally requires the selection of a cut-off point to ensure a high sensitivity for the test or combination of tests to be employed. Such high sensitivity ensures that most cases are detected, at the cost of false positives that can be eliminated by further diagnostic tests. Although the accuracy of this approach is insufficient to give a final diagnosis in all individuals, the combination of haemoglobin concentration and whole blood ZPP can constitute a rapid and convenient method to rule out iron deficiency in a substantial proportion of children screened. Although we considered the example of screening at community level, a similar strategy can be employed in clinical practice to identify children who may need referral to higher levels of care for further testing. When $0.07195[Hb] + 0.01449[ZZP_{whole}] < 10.39334$, the diagnostic accuracy may be improved by using a combination of erythrocyte ZPP with haemoglobin concentration, but the improved diagnostic accuracy thus achieved must be weighed against the procedure of washing red cells, which may be cumbersome in practice.

One problem that has not been solved in our study is the difficulty in detecting iron deficiency in the presence of inflammation. The associations found between ZPP and *Plasmodium* infection and between ZPP and C-reactive protein, underscore the importance of this issue. Further research is required to extend and validate our approach with appropriately selected reference standard for iron deficiency in a population that included individuals with inflammation and infections. Because point-of-care tests are rapidly developing, with quantitative tests already being commercially available for plasma concentrations of ferritin and C-reactive protein, and for various infections, this is likely to be a fruitful area of future research.

Conclusion

In Kenyan preschool children, ZPP, whether measured in whole blood or in erythrocytes, has added diagnostic value in detecting iron deficiency over haemoglobin concentration alone. When used in a screen-and-treat approach, combination of haemoglobin concentration and whole blood ZPP in a single diagnostic score can be used as a rapid and convenient testing method to rule out iron deficiency in a substantial proportion of children screened.

Abbreviations

AGP: α_1-acid glycoprotein; AUC: Area-under-the-curve; CRP: C-reactive protein; EP: Erythrocyte protoporphyrin; FEP: Free erythrocyte protoporphyrin; Hb: haemoglobin concentration; HRP2: Histidine-rich protein-2; IPT: Intermittent preventive treatment; pLDH: *Plasmodium* lactate dehydrogenase; ROC: Receiver

operating characteristics; WHO: World Health Organization; ZPP: Zinc protoporphyrin

Competing interests
The authors declare that they have no competing interests.

Authors contributions
ET assisted in the study design and coordinated field work. PEAA supervised field work. AYD supervised biochemical analyses. HV conceived, designed, and supervised all aspects of the study. ET and HV conducted statistical analyses and prepared the first draft manuscript. All authors read and approved the final manuscript.

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On line additional file 1: The utility of haemoglobin concentration and ZPP as diagnostic tests to screen and rule out iron deficiency

Consider the example of a community survey in which children are screened for iron deficiency, with treatment being restricted to those who are iron deficient.

In the absence of inflammation, serum ferritin concentration < 12 μg/L is recommended by WHO to measure iron status in paediatric populations [2]. Although commercial point-of-care tests for serum ferritin concentration have recently become available, its measurement is often difficult under field conditions and relatively expensive. A screening strategy whereby haemoglobin concentration and whole blood ZPP are used to rule out iron deficiency would be useful and could possibly lead to substantial cost savings.

Let the result of a diagnostic test (i.e. haemoglobin concentration and ZZP, used alone or in combination) be dichotomized. Table 1 shows the cross-tabulated data that might be obtained.

Table 1. Cross-tabulated data for true iron status and diagnostic test results

Diagnostic test result	Iron deficient (reference)		Total
	Yes	No	
Positive	A	B	U
Negative	C	D	V
Total	R	T	N

Capital letters in each cell indicate the number of individuals in that cell

The aim of screening is to detect most cases of iron deficiency, i.e. with a cut-point of the test result selected to ensure a high sensitivity (e.g. >90%). Because a high sensitivity comes at the cost of a reduced specificity, this will inevitably result in false positives that could be eliminated by further diagnostic tests. The advantage of screening with a high sensitivity, however, is that iron deficiency can be effectively and with near-certainty be ruled out in those with a negative test result [20].

Let the negative predictive value (NPV = D/V) be set to ensure that a high proportion of negative test results (e.g. >90%) are correct. The NPV can be rewritten as a function of the prevalence, sensitivity and specificity:

$$NPV = \frac{D}{V} = \frac{T \cdot Spec}{C + D} = \frac{(N - R) \cdot Spec}{R \cdot (1 - Sens) + (N - R) \cdot Spec}$$

Dividing both numerator and enumerator by *N* yields:

$$NPV = \frac{(1-P)\cdot Spec}{P\cdot(1-Sens)+(1-P)Spec}$$

Thus, at a sensitivity of 90% and corresponding specificity derived from the ROC curves (Figure 1), a *NPV* > 90% can be obtained only within a certain prevalence range.
Similarly, the positive predictive value (*NPV* = A/U) can be formulated as a function of the prevalence, sensitivity and specificity:

$$PPV = \frac{P\cdot Sens}{P\cdot Sens+(1-P)\cdot(1-Spec)}$$

The fraction with a negative test result (*F* = V/N) for whom iron deficiency can be ruled out can similarly be rewritten as a function of the prevalence or pre-test probability (*P* = R/T), sensitivity (*Sens* = A/R) and specificity (*Spec* = D/T) as follows:

$$F = \frac{V}{N} = \frac{(C+D)}{N} = \frac{R\cdot(1-Sens)+T\cdot Spec}{N} = \frac{R\cdot(1-Sens)}{N} + \frac{(N-R)\cdot Spec}{N}$$

Thus:

$$F = P\cdot(1-Sens)+(1-P)\cdot Spec = Spec + P\cdot(1-Sens-Spec)$$

As indicated by this formula, when the sensitivity is arbitrarily set at >90%, and the corresponding specificity is derived from the ROC curves (Figure 1), *F* varies only with the prevalence of iron deficiency.

In summary, by screening with a cut-point for the diagnostic test result that is preselected to yield a high sensitivity (e.g. > 90% or > 95%), one can determine a prevalence range in which the negative predictive value that is within an acceptably high range (e.g. > 90% or > 95%) and that produces a variable fraction of persons in whom iron deficiency can be ruled out (Figure 2).

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TABLE 1. Characteristics of the study population

	All children		Children without inflammation or *Plasmodium* infection	
n		338		84
Age				
12-23.99 months	53.6%	(181)	54.8%	(46)
24-36 months	46.4%	(157)	45.2%	(38)
Sex, male	55.0%	(186)	47.6%	(40)
Height-for-age z-score, SD	-1.33	(1.4)	-0.91	(1.5)
Stunted (height-for-age z-score < -2 SD)	30.2%	(102)	14.3%	(12)
Weight-for-height z-score, SD	-0.14	(1.0)	-0.15	(0.97)
Wasted (weight-for-age z-score <-2 SD)	3.0%	(10)	2.4%	(2)
Whole blood ZPP, µmol/mol haem	181	[124-282]	131	[98-239]
Whole blood ZPP > 70 µmol/mol haem	97.9%	(331)	97.6%	(82)
Erythrocyte ZPP, µmol/mol haem	142	[86-246]	117	[69-215]
Erythrocyte ZPP > 70 µmol/mol haem	83.1%	(281)	72.6%	(61)
Erythrocyte ZPP > 40 µmol/mol haem	96.7%	(327)	95.2%	(80)
Haemoglobin concentration, g/L	105.0	(13.2)	111.7	(10.0)
Anaemia (haemoglobin concentration < 110 g/L)	62.1%	(210)	42.9%	(36)
Plasma ferritin concentration, µg/L	35.3	[17.1-67.2] [a]	17.3	[10.3-28.8] [b]
Iron status [c]				
Deficient	17.1%	(57/333)	32.1%	(27/81)
Replete	23.1%	(77/333)	64.3%	(54/81)
Uncertain	59.8%	(199/333)	Not applicable	

Plasma sTfR concentration, mg/L	2.43	[1.84-3.30]	2.11	[1.50-2.94]
Plasma albumin concentration, g/L	34.8	(3.9)	37.4	(2.2)
Plasma vitamin B_{12} concentration, pmol/L	400	[307-559] [d]	356	[283-452] [e]
Plasma vitamin B_{12} concentration < 150 pmol/L	1.2%	(4/332)	1.2%	(1/80)
Plasma CRP concentration, mg/L	2.9	[0.8-9.2]	Not applicable	
Plasma AGP concentration, g/L	1.16	[0.9-1.55]	Not applicable	
Inflammation				
Plasma CRP concentration > 5 mg/L	37.9%	(128)	Not applicable	
Plasma AGP concentration > 1.0 g/L	63.6%	(215)	Not applicable	
Plasma CRP concentration > 5 mg/L, or plasma AGP concentration > 1.0 g/L	66.9%	(226)	Not applicable	
Plasmodium infection				
P. falciparum	36.1%	(122)	Not applicable	
Plasmodium spp. other than *P. falciparum*	0.3%	(1)	Not applicable	
Missing	0.9%	(3)	Not applicable	

Values indicate mean (SD), median [25th and 75th percentile] or % (n)
AGP: α_1-acid glycoprotein; CRP: C-reactive protein; sTfR: soluble transferrin receptor; ZPP: zinc protoporphyrin

Missing values, due to insufficient plasma volumes for analysis, resulted in [a] n=333, [b] n=81; [c] *Deficient:* plasma ferritin concentration < 12 µg/L, regardless of the presence or absence of inflammation; *replete:* plasma ferritin concentration ≥ 12 µg/L, in the absence of inflammation; *uncertain:* plasma ferritin concentration ≥ 12 µg/L, in the presence of inflammation defined as plasma concentrations of CRP > 5 mg/L or AGP > 1.0 g/L. Missing values, due to insufficient plasma volumes for analysis, resulted in [d] n=332 and [e] n=80.

TABLE 2. Factors associated with ZPP-haem ratio measured in whole blood or erythrocytes, crude analysis [a]

Factor	n	Whole blood ZPP-haem ratio			Erythrocyte ZPP-haem ratio		
		Geometric mean	Δ [b]	(95% CI)	Geometric mean	Δ [b]	(95% CI)
Age							
12-23.99 months	162	199	Ref		163	Ref	
24-36 months	176	178	-10.8%	(-21.2% to 0.9%)	134	-18.1%	(-4.3% to -29.9%)
Sex							
Girls	152	179	Ref		135	Ref	
Boys	186	195	9.4%	(-3.4% to 23.8%)	158	16.4%	(-0.5% to 36.1%)
Inflammation (CRP concentration > 5 mg/L)							
No	210	180	Ref		144	Ref	
Yes	128	201	11.4%	(-1.9% to 26.5%)	153	6.6%	(-9.3% to 25.4%)
Inflammation (AGP concentration > 1 g/L)							
No	123	166	Ref		136	Ref	
Yes	215	201	21.4%	(7.0% to 37.9%)	154	13.3%	(-3.7% to 33.3%)
Inflammation (CRP concentration > 5 mg/L or AGP concentration > 1 g/L)							
No	112	166	Ref		137	Ref	
Yes	226	199	20.0%	(5.4% to 36.7%)	153	11.4%	(-5.6% to 31.6%)
Iron deficiency (ferritin concentration < 12 µg/L)							
No	276	173	Ref		132	Ref	
Yes	57	278	60.4%	(36.9% to 87.8%)	255	93.6%	(58.8% to 136.0%)
Anaemia							
No	128	129	Ref		98	Ref	
Yes	210	236	82.3%	(63.3% to 103.5%)	189	92.0%	(65.9% to 122.1%)
Plasmodium infection, any species							

No	212	167	Ref		131	Ref	
Yes	123	230	37.5%	(21.5% to 55.6%)	180	38.1%	(18.6% to 60.8%)
Plasma ferritin concentration, 5.11-fold change [d, e]			-16.7%	(-24.6% to -7.9%)		-18.1%	(-24.1% to -11.6%)
Plasma sTfR concentration, 1.43-fold change [d]			38.3%	(34.0% to 42.7.0%)		66.8%	(57.7% to 76.4%)
Plasma CRP concentration, 2.72-fold change [d]			3.5%	(0.8% to 6.2%)		6.8%	(-1.2% to 15.5%)
Plasma AGP concentration, 1.60-fold change [d]			23.7%	(10.0% to 39.1%)		7.1%	(-1.0% to 15.8%)
Haemoglobin concentration, change by 13.2g/L [f, g]			-29.8%	(-33.1% to -26.2%)		-31.5%	(-35.9% to -26.7%)
Plasma albumin concentration, change by 3.9g/L [f]			-7.0%	(-12.5% to -1.1%)		-1.4%	(-8.9% to 6.7%)
Plasma vitamin B_{12} concentration, change by 213pmol/L [f]			7.0%	(0.6% to 13.9%)		6.1%	(-2.0% to 14.8%)

AGP: α_1-acid glycoprotein; CRP: C-reactive protein; GSD: geometric standard deviation; Ref: reference; SD: standard deviation; sTfR: soluble transferrin receptor; ZPP: zinc protoporphyrin

[a] ZPP values were normalised by log-transformation; exponentiation of results yielded associations being expressed as relative differences; [b] Difference; [c] Based on HRP2- and pLDH-based dipstick test results; [d] Corresponding to 1 geometric standard deviation; [e] For example, a 5.11-fold increase in plasma ferritin concentration, which corresponds to a variation that is equivalent to addition of 1 SD on a log-transformed scale, is associated with a reduction in the whole blood ZPP-haem ratio by 16.7%; [f] Corresponding to 1 SD; [g] For example, an increase in haemoglobin concentration by 13.2g/L, which corresponds to an increase by 1 SD, is associated with a reduction in the whole blood ZPP-haem ratio by 29.8%.

TABLE 3. Factors associated with ZPP-haem ratio measured in whole blood or erythrocytes, multiple linear regression analysis [a]

	Whole blood ZPP-haem ratio (model 1)		Whole blood ZPP-haem ratio (model 2)		Erythrocyte ZPP-haem ratio	
	Δ [b]	**(95% CI)**	**Δ [b]**	**(95% CI)**	**Δ [b]**	**(95% CI)**
Plasma ferritin concentration, 5.11-fold change [c]	-10.1%	(-14.3% to -5.6%)	-9.6%	(-14.0% to -4.9%)	-15.5%	(-21.1% to -9.5%)
Plasma sTfR concentration, 1.43-fold change [c]	34.8%	(28.3% to 41.7%)	34.9%	(28.3% to 41.8%)	45.3%	(35.7% to 55.6%)
Plasma CRP concentration, 2.72-fold change [c]	5.0%	(0.1% to 10.0%)	5.4%	(0.4% to 10.5%)	9.8%	(2.8% to 17.2%)
Haemoglobin concentration, change by 13.2g/L [d]	-13.9%	(-18.3% to -9.4%)	-14.9%	(-19.4% to -10.1%)	-14.5%	(-20.7% to -7.9%)
Plasmodium infection, any species [e]	17.3%	(7.8% to 27.7%)	16.8%	(7.4% to 27.2%)	93.3%	(23.8% to 202.0%)
Plasma albumin concentration, change by 3.9g/L [d]	—	(eliminated)	3.0%	(-1.7% to 8.0%)	2.2%	(0.5% to 4.0%)

CRP: C-reactive protein; sTfR: soluble transferrin receptor; ZPP: zinc protoporphyrin

[a] ZPP values were normalised by log-transformation; exponentiation of results yielded associations being expressed as relative differences. Plasma concentrations of α_1-acid glycoprotein (log-transformed), vitamin B_{12} (log-transformed), sex (binary) and age class (binary) were eliminated from all models through a manual stepwise backward elimination process with an removal criterion of p>0.05; [b] Difference; [c] Corresponding to 1 geometric standard deviation; [d] Corresponding to 1 SD; [e] Based on HRP2- and pLDH-based dipstick test results

TABLE 4. Diagnostic performance of zinc protoporphyrin-haem ratio, with dichotomised test results, to detect iron deficiency

Whole blood ZPP > 70 µmol/mol haem	n/n	Estimate (CI)
Sensitivity	27/27	100.0% (84.4%–100.0%) [a]
Specificity	2/54	3.7% (0.9%–14.4%) [a]
Positive predictive value	27/79	34.2% (23.5%–46.7%) [a]
Negative predictive value	2/2	100.0% (28.6%–100.0%) [a]
Prevalence	79/81	97.5% (91.4%–99.3%) [b]
Erythrocyte ZPP > 40 µmol/mol haem		
Sensitivity	27/27	100.0% (84.4%–100.0%) [a]
Specificity	4/50	7.4% (2.6%–19.5%) [a]
Positive predictive value	27/77	35.1% (24.2%–47.8%) [a]
Negative predictive value	4/4	100.0% (44.4%–100.0%) [a]
Prevalence	77/81	95.1% (88.0%–98.1%) [b]

[a] 97.5% CI; [b] 95% CI

FIGURE LEGENDS

FIGURE 1. Ability of zinc protoporphyrin-haem ratio, either alone or in combination with haemoglobin concentration, to discriminate between children with and without iron deficiency

Panel A: Receiver operating characteristics (ROC) curves for various blood markers, used alone, to discriminate between iron-deficient and iron-replete children.

Panel B: As in Panel A, with haemoglobin concentration and whole blood ZPP, alone and in combination.

Panel C: As in Panel A, with haemoglobin concentration and erythrocyte ZPP, alone and in combination.

Panel D: As in Panel A, with combined haemoglobin concentration and whole blood ZPP, versus combined haemoglobin concentration and erythrocyte ZPP.

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

FIGURE 2. Application of a diagnostic strategy to rule out iron deficiency

[Hb]: haemoglobin concentration, expressed in g/L; [whole blood ZPP]: whole blood ZPP content, expressed in μmol/mol haem.

The diagnostic strategy in a screen-and-treat survey is based on two criteria: a) the probability of correctly diagnosing iron deficiency should exceed 90%; and b) iron deficiency can be ruled out if the probability of a negative test result being correct (negative predictive value) exceeds 90%.

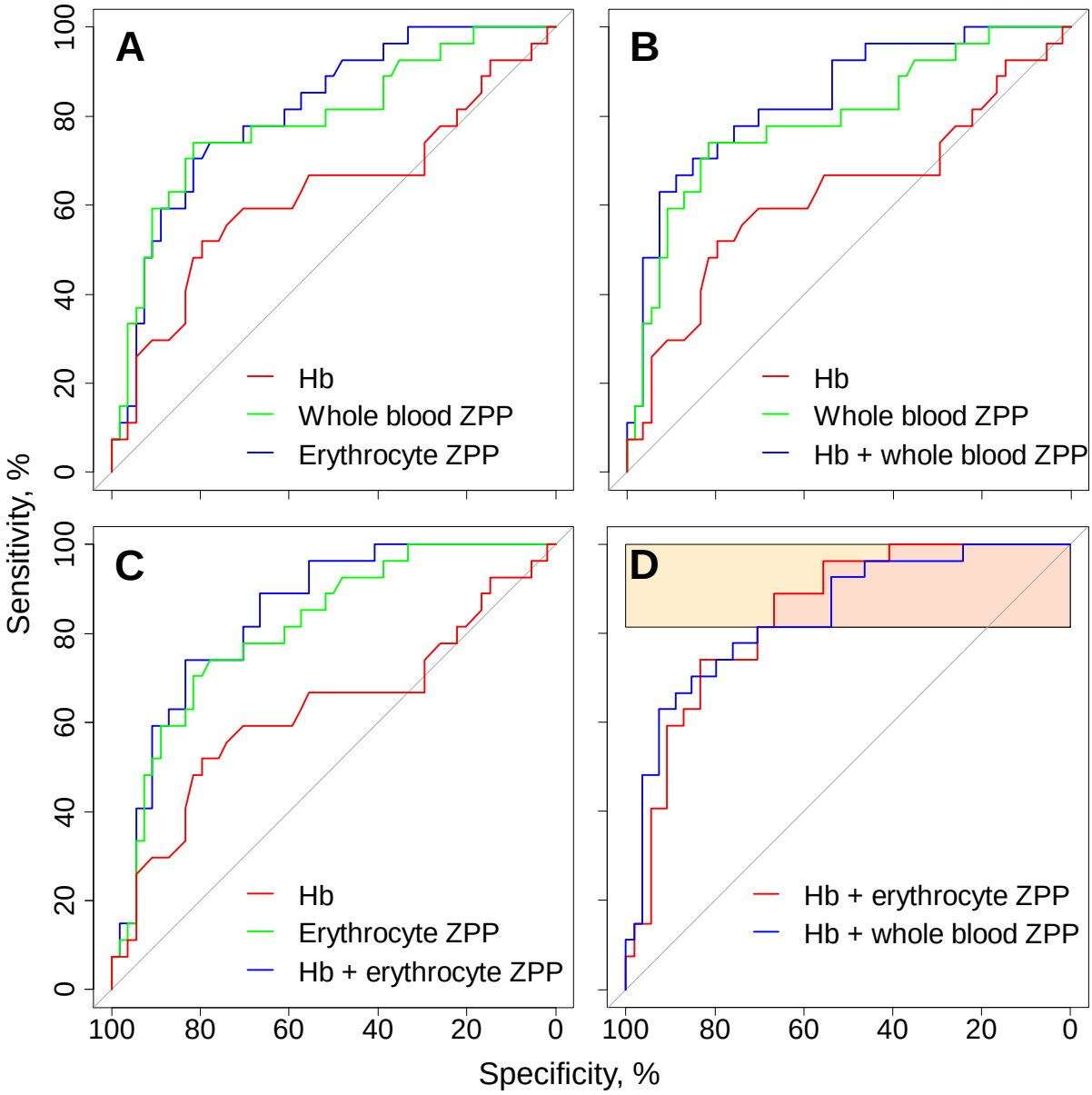
To meet the first requirement, a cut-off point for each diagnostic test result (top) is selected to yield a sensitivity of 90%; the corresponding specificity value is obtained from the ROC curves (Figure 1). For haemoglobin concentration and whole blood ZPP, the cut-off points were 122 g/L and 99 μmol/mol haem; the corresponding specificity values were 14.8% and 36.0%, respectively. When these markers were combined in a single diagnostic rule, 0.038689 [Hb] + 0.00694 [whole blood ZPP] > 5.93120 had a specificity of 53.7%.

The negative predictive value (top panels, blue lines) depends on sensitivity and specificity values thus fixed, and the prevalence of iron deficiency. The second diagnostic criterion, i.e. the negative predictive value should exceed 90%, applies only within a limited prevalence range (top panels, red rectangle); at prevalence values exceeding this range, the negative predictive value will be below 90% and iron deficiency cannot be ruled out with diagnostic test applied (top).

The percentage of children with a negative test result declines linearly with the prevalence of iron deficiency (middle panels, blue lines). The percentage of children for whom iron deficiency can be ruled out (middle panels, Y-intercepts of red rectangles) depends on the prevalence range in which the negative predictive value exceeds 90% (top panels, red rectangles).

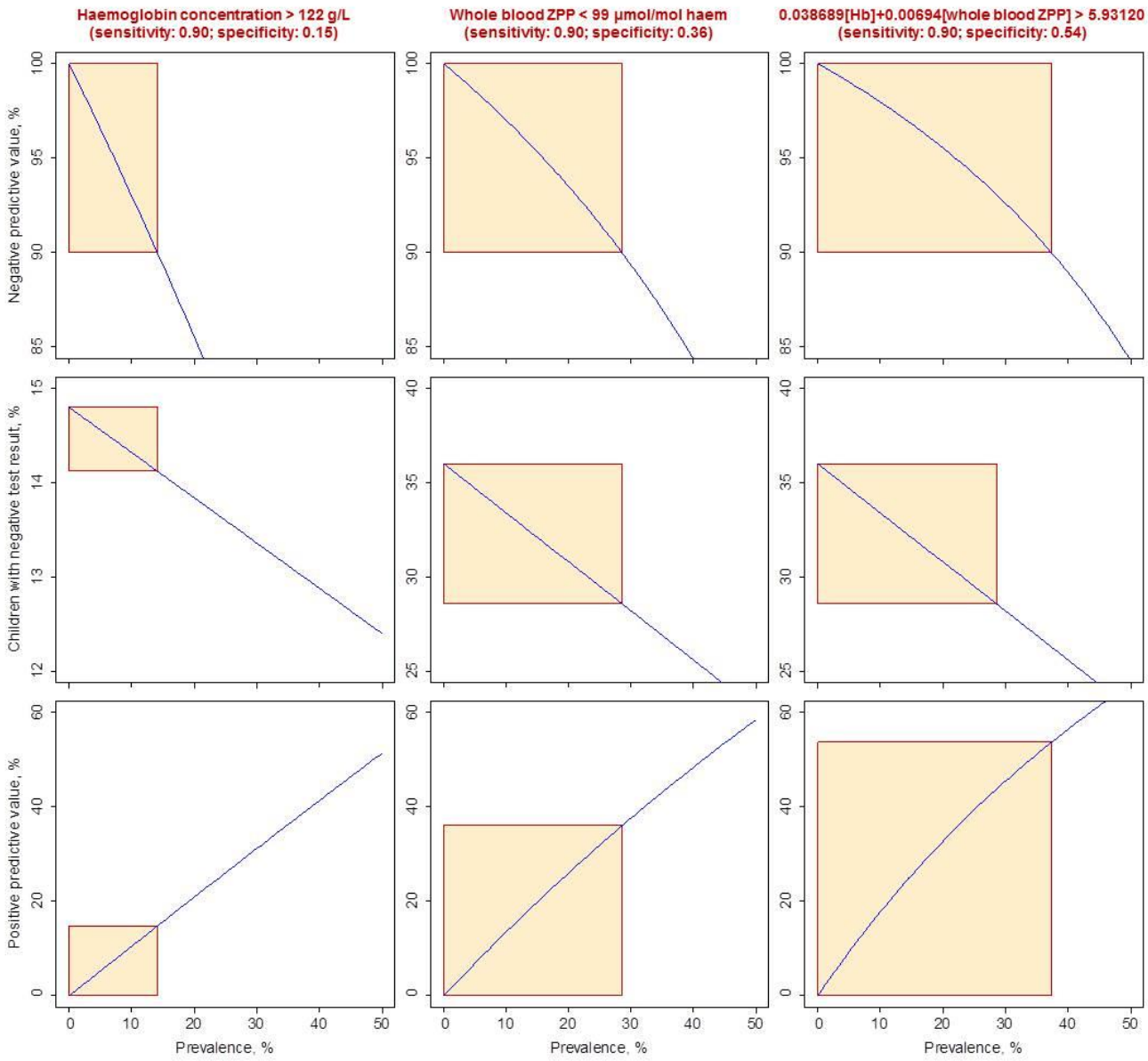
With fixed sensitivity and specificity values, the positive predictive value (bottom panels, blue lines) increase monotonically with the prevalence of iron deficiency. Within the prevalence range in which the negative predictive value exceeds 90% (top panels, red rectangles), the highest positive predictive value is 54% (for combined use of haemoglobin concentration and whole blood ZPP), indicating that additional tests (i.e. other than haemoglobin concentration and whole blood ZPP) are required to accurately determine iron status.

For example, haemoglobin concentration > 122 g/L (left panels) has 90% sensitivity of detecting iron deficiency; at a true prevalence of iron deficiency < 14.1%, a negative test result obtained with this decision rules out iron deficiency with a probability of 90% (upper left panel, red rectangle). Depending on the true prevalence of iron deficiency, such a cut-off for haemoglobin concentration would result in iron deficiency being ruled out in 14.1%-14.8% of children who are tested (middle left panel, red rectangle). Similarly, within a prevalence range < 28.6%, whole blood ZPP > 99 μmol/mol haem rules out iron deficiency in 28.6%-36% of children tested. Within a prevalence range of < 37.4%, 0.038689 [Hb] + 0.00694 [whole blood ZPP] > 5.93120 rules out iron deficiency in 37.4%-53.7% of children.



Marker	AUC (95% CI)	*P* [a]
Haemoglobin concentration	62.7% (48.7%–76.6%)	–
Whole blood ZPP	79.1% (68.2%–90.1%)	0.001
Erythrocyte ZPP	81.2% (71.6%–90.8%)	<0.001
Haemoglobin concentration + whole blood ZPP	84.0% (74.7%–93.2%)	0.003
Haemoglobin concentration + erythrocyte ZPP	84.2% (75.7%–92.8%)	0.001

[a] Relative to haemoglobin concentration alone


Haemoglobin concentration > 122 g/L
(sensitivity: 0.90; specificity: 0.15)
Whole blood ZPP < 99 µmol/mol haem
(sensitivity: 0.90; specificity: 0.36)
0.038689[Hb]+0.00694[whole blood ZPP] > 5.93120
(sensitivity: 0.90; specificity: 0.54)
Negative predictive value, %
Children with negative test result, %
Positive predictive value, %
Prevalence, %

Chapter 6: Discussion and implications

The WHO recommendation to use home fortification with multiple micronutrient powders containing 12.5 mg iron as ferrous fumarate for children aged 6-23 months [1] has been shown to reduce iron deficiency in children, but it has also raised public health concerns even when such fortification in malaria-endemic areas is accompanied by malaria control measures. Evidence from clinical trials has shown that administration of the high dose of 12.5 mg iron as ferrous salts increased the risks of morbidity and hospital admission among infants and young children [2-5]. These concerns created the motivation for our study and we proposed that providing a low dose of iron (3 mg as NaFeEDTA) may be safer and more tolerable because it is stable, with less oxidative stress, more tolerable, and is highly absorbable in high phytate diets resulting to fewer risks of morbidity and a likely improved adherence [6-7].

Thus, we set out to compare 3mg iron as NaFeEDTA (investigational treatment) with 12.5 mg iron as encapsulated ferrous fumarate (active control), with haemoglobin concentration response as the primary outcome. The study was conducted among 12-36 month old children living in a malaria endemic area of western Kenya from January to December 2014. This being a non-inferiority study**,** we included a third arm (placebo) to adhere to regulatory guidelines for non-inferiority trials [8,9]. However, inclusion of a placebo in a non-inferiority trial is controversial. In our study we considered it ethical in the presence of the active control for various reasons: 1) in our study area, there is no national policy for preventive, community-based supplementation or home fortification with iron in children under five years who are at a greater risk of iron deficiency anaemia; 2) our trial represents the only chance for eligible children to receive micronutrients powders with iron (for the iron arms) and micronutrients powders (for the placebo arm); 3) proof of efficacy for the iron treatments can be done through the use of the placebo arm, because failure for an investigational treatment to demonstrate effectiveness does not necessarily mean it is not efficacious, thus precluding any chance of making erroneous conclusions; and 4) it has also been argued that as medical knowledge increases, the use of a placebo in trials should decline. Conversely, increased medical knowledge has subsequently propelled the production of new treatments. Dropping the use of a placebo in a trial where a new treatment is being investigated limits the determination of efficacy and safety of the new treatment [10] consequently denying physicians opportunities to apply better treatment options when needed.

The results of this study showed no evidence that daily home fortification for 30-days with either a daily dose of 3 mg iron as NaFeEDTA or 12.5 mg iron as encapsulated ferrous fumarate were efficacious even after adjusting the treatment

effect for study design relative to placebo in both per protocol and intention to treat analysis. Also, in the sub-group analysis we found no change of intervention effects after adjusting for baseline factors that are prognostic for haemoglobin concentration. Following our failure to demonstrate efficacy, no further analysis was required to show non-inferiority. Though disappointing, these research results are informative and timely and are consistent with other subsequent studies that have shown lack of iron intervention effect on haemoglobin concentration. Such studies include three of the six trials in a meta-analysis that formed the basis for the WHO recommendation for daily home fortification with 12.5 mg iron as ferrous salts [11]. Nonetheless, our lack of efficacy for the iron treatments may elicit several queries regarding the study design, adherence to treatment, dosage, regimen and formulation, duration of intervention and other population-level factors such as chronic infections, inflammation status and consumption of inhibitory iron absorption compounds in food vehicles.

Adherence to the two iron interventions as measured by the medication events monitoring system (the reference standard) showed that only 60.6% of the children consumed ≥ 24 sachets after 30 days of intervention meaning that there was a substantial proportion (~40%) of children that did not adhere to daily home fortification with micronutrient powders . Our evidence show that home-fortification with 3 mg iron as NaFeEDTA failed to show efficacy as measured by comparison with placebo, but there are known benefits for administering fortification with 3mg iron as NaFeEDTA (outlined above). In addition, children with iron deficiency and anaemia have been shown to benefit more from iron fortification with NaFeEDTA when compared to placebo or fortification with electrolytic iron in high phytate diets [12]. We similarly recorded a reduction of iron deficiency in the absence of inflammation, this effect being absent in the group that received 12.5 mg iron as encapsulated ferrous fumarate. For safety reasons, we maintained the daily dose of NaFeEDTA within the Acceptable Daily Intake (ADI) for EDTA (0-1.9 mg kg^{-1} day^{-1}) even though our study children consumed complementary food with high contents of phytate and phenolic compounds throughout the study period. Moreover, our data show that we could not increase the daily NaFeEDTA dose without a substantial proportion of children exceeding the ADI for EDTA. Accordingly, our findings are not unusual because they are consistent with other studies that show no effect on haemoglobin concentration where low iron with EDTA was consumed with diets containing high phytate compounds [4]. Thus researchers and technical experts may find these results useful in supporting a proposal to evaluate the amount of iron with EDTA when administered with high phytate diets [13]. Technical experts may also consider advocating the use of exogenous phytase (produced by *Aspergillus niger*) that has been shown to substantially increase iron absorption when consumed just before consumption of high phytate diet in primary school children [14].

On the other hand, the reference treatment, 12.5mg iron as ferrous fumarate not only failed to show efficacy in this present study, but it is associated with increased risks of morbidity and hospitalisation after administration to infants and young children (risks discussed above). These results elicit concerns as to whether 12.5mg iron as ferrous fumarate should remain an option for iron in home-fortification powders as recommended by the World Health Organisation, or whether other efficacious iron alternatives should be further sought.

The intervention duration of 30 days was within the time period during which piperaquine, the long-acting anti-malarial drug administered at the start of iron treatment, was expected to protect against malaria. Nonetheless, at the end of the 30-day intervention period, a substantial proportion of children carried *Plasmodium* parasites. This can be explained by: a) some children may not have completed the full therapeutic regimen, particularly the second and third doses that were to be administered unsupervised at home, prompting the need for direct observation of anti-malarial dose intake in children in future studies; b) an over-estimation due to persistence of *P. falciparum* HRP2 protein in circulation for several weeks after parasite clearance. It may be necessary to advocate for an increased duration after treatment of plasmodium infections to allow for reduction of HRP2 protein in circulation ; c) we used the manufacturer's dihydroartemisinin-piperaquine dosage regimen that was recently shown to offer sub-optimal performance in children younger than five years. After we started our study, WHO revised the therapeutic dosage regimen [15]; the new recommendation is likely to yield a longer protective phase when dihydroartemisinin-piperaquine is administered for chemoprevention in future studies. Again, our 30 days duration may have been adequate for iron fortification because earlier trials conducted in Kenya among infants and young children supplemented with iron as ferrous fumarate improved haemoglobin concentration within 4 weeks [16]. Trials conducted in malaria-endemic areas with the same iron formulations but for a longer duration (6-18 months) showed no effect on haemoglobin concentration [4,5], meaning that there may be other factors that caused lack of efficacy for the iron interventions.

The prevalence of inflammation was high in both iron groups. There was no evidence that inflammation at baseline influenced the effect of iron treatment on haemoglobin concentration. This study supports findings from a recent study conducted in Kenya that showed lower doses of iron with EDTA (2.5 mg iron as NaFeEDTA) and a high dose of 12.5 mg iron as ferrous fumarate increased the pathogenic profile and gut inflammation in infants and young children, even though no serious adverse events were reported [4]. The cut-off points for inflammation markers used in our study have been used in previous studies but they have not been validated in children. Thus it is possible that there was substantial blockage of iron absorption by elevated hepcidin concentration at lower inflammatory markers due to chronic infections caused by either viruses or low bacterial and parasitic

loads. Although our study children underwent a thorough medical examination prior randomization, we cannot rule out that there were still a substantial proportion of children with chronic infections and enteric inflammation. This, together with consumption of high amounts of iron-inhibiting factors in the food vehicles used for home fortification, and suboptimal adherence to daily home-fortification with iron may explain the lack of efficacy in both iron interventions.

In our meta-analysis, we found a high level of heterogeneity in effects on haemoglobin concentrations across 15 trials, which may reflect methodological differences in iron dosage, formulation, duration of intervention, adherence, study quality, but, even more interestingly, may be due to population-level factors that determine efficacy (e.g. differences in prevalence of iron deficiency, chronic infections, inflammation status and inhibitory iron absorption compounds in food vehicles). Decisions made by policy makers regarding iron interventions mostly rely on results from meta-analyses; yet, information from our meta-analysis showed that the effect of iron treatment on haemoglobin concentration was highly heterogeneous and the pooled random-effect obtained may not reflect the actual effect due to differences in study methodology and populations. The finding from our updated meta-analysis showing a pooled effect of home fortification with iron-containing powders on haemoglobin concentration by 3.9g/L suggests a benefit in haemoglobin concentration across different settings, but does not allow prediction of efficacy in specific settings.

The efficacy of home fortification with iron-containing micronutrient powders varies between trials, perhaps in part due to population differences in adherence. However, concerns have been raised regarding the most common methods of assessing adherence to home-fortifications with micronutrient powders, namely self-reporting or empty sachet counts. It has been argued that these tools either over-estimate or under-estimate adherence measurements of intervention treatments [16, 17]. Thus we set out to determine their accuracy and compare to the MEMS electronic device (which we nominated as the gold standard). Our results confirmed that both self-reporting and sachet counts lead to over-estimates of adherence and may lead to biased conclusions. It also denies the investigator a chance to ascertain whether the correct regimen was followed. Adherence to iron supplementation has been associated with improved anaemia cure rates [19] but in our study there was no evidence of an association between haemoglobin response and adherence as measured either by self-reporting or sachet count. This may be due to over-reporting when in reality there was no consumption of micronutrient powders as scheduled. The strong association between MEMS caps opening and increase in haemoglobin concentrations supports findings of another trial that showed MEMS caps opening data positively reflected actual adherence and a positive effect to hypertensive treatment among the African-Americans [20]. We used beta-regression analysis to model adherence expressed as a fractional response of adherence to daily home-

fortification because linear regression analysis of the untransformed fractional response although commonly used has several limitations (for example; the effect of explanatory variables tend to be non-linear , proportions are not normally distributed and the variance is not constant but varies with the outcome). Our results showed that age of the parent or guardian was the only baseline factor that influenced adherence measures using MEMS device but **we** found no evidence that adherence was affected by other baseline characteristics of the parent or guardian (gender, education level) or child's characteristics (sex, age, being infected by *P. falciparum*, wasting, stunting, sickness during intervention period). It has been shown elsewhere that older patients tend to adhere better to anti-retroviral treatment compared to their younger counterparts. Presumably older parents or guardians are better carers and attempt to observe medication instructions better. Hence, future studies assessing adherence to home-fortification of micronutrient powders in young children should consider using the MEMS device either alone or alongside self-reporting and sachet counts to ensure accuracy of measurement and to complement adherence data respectively.

Finally, in a recent trial, both whole blood ZPP and erythrocyte ZPP were shown to have little diagnostic utility as a screening marker to manage iron deficiency in pregnant women, whether used as single tests or combined with haemoglobin concentration [21]. But in this present study, whole blood ZPP and erythrocyte ZPP in the absence of inflammation added diagnostic value in detecting iron deficiency compared to haemoglobin concentration alone in young children. Also, a single diagnostic score based on haemoglobin concentration and whole blood ZPP ruled out iron deficiency in a substantial proportion of children screened. However, we found it difficult to detect iron deficiency in the presence of inflammation. The increase in whole blood ZPP that was associated with *Plasmodium* infection and between ZPP and plasma C-reactive protein, can be explained partly due to the formation of bilirubin and other haemoglobin breakdown products in plasma that result from haemolysis and that fluoresce in the same wavelength range as protoporphyrins, also because plasma ferritin concentration can be elevated in the presence of infection-induced inflammation independently of iron status. *Plasmodium* infection was also associated with an even larger increase in erythrocyte ZPP, independently of inflammation, this result was unexpected because erythrocytes lack plasma constituents, which are removed by washing. Therefore, a possible explanation is that haemolysis-induced increase in erythropoietic activity under influence of *Plasmodium* infection that drives up the demand for iron in the erythron. The information presented here is extremely useful to technical experts and researchers and can be applied in a screen-and-treat approach. Specifically, a combination of haemoglobin concentration and whole blood ZPP in a single diagnostic score can be used as a rapid and convenient testing method to rule out iron deficiency in young children screened.

Study limitations

Due to lack of funds we were unable to conduct biochemical analysis for urine and stool samples needed for assessment of hepcidin concentration and intestinal biota profile respectively. For the same reason, analysis of serum NTBI concentrations at 3 hours after ingesting the first dose of iron fortificant and faecal calprotectin concentration at the end of the fortification period was not conducted. Assessments of urine hepcidin concentration would have provided us with substantial evidence for or against our arguments regarding iron blockage caused by increased hepcidin concentration at low levels of inflammation. Our study *Objective 4* required us to analyse stool samples in order to compare effects of the two iron interventions on intestinal biota. Assessment of intestinal biota would have informed us which of the two iron interventions adversely increased the profile of pathogens and subsequently would increase gut inflammation.

Recommendations for further research

This was an explanatory type of study, therefore opportunities for future studies were deemed necessary to further elaborate, refine or validate our results.

1. We conducted our study within 30 days in view of maintaining our daily iron administration within the estimated duration of the protective phase of antimalarial drugs. To rule out limited duration of intervention as an underlying factor for lack of efficacy for the iron intervention, a similar study design (non-inferiority) may be considered with a longer duration, perhaps 6 or 12 months.

2. Our data shows that the fortification dose of 3 mg iron as NaFeEDTA cannot be increased without a substantial proportion of children in the age range studied exceeding the ADI for EDTA (0-1.9 mg kg^{-1} day^{-1}). It has been argued, however, that this acceptable daily intake may have been set too low, but more information is needed regarding the safe levels of EDTA for children when the amount of NaFeEDTA is increased for areas with high consumption of diets containing high phytate and phenolic compounds living in malaria endemic areas.

3. In our study we defined inflammation as plasma C-reactive protein concentration >5 mg/L or plasma α_1-acid glycoprotein concentration >1.0 g/L. These cut-off points, although used in many trials to determine inflammation status have not been validated in young children. Yet, there is a possibility that during chronic infections production of hepcidin increases in response to inflammatory cytokines and a substantial proportion of iron absorption can

be blocked at lower levels of inflammatory markers (plasma C-reactive protein concentration <5 mg/L or plasma α_1-acid glycoprotein concentration <1.0 g/L).

4. Evidence in our trial may be insufficient to recommend home fortification in all settings, but the high heterogeneity observed in the meta-analysis should stimulate subgroup analysis or meta-regression to identify population-specific factors that determine efficacy.

5. The association between age of the parent or guardian and adherence to home fortification was the result of exploratory analyses. In order to identify and understand the determinants of adherence to home fortification of micronutrient powders there is need to specifically design a trial that will use latent class analysis which may be helpful in defining subgroups that are prognostic to home-fortification adherence.

6. Point-of-care tests are rapidly developing, with quantitative tests already being commercially available for plasma concentrations of ferritin and C-reactive protein, and for various infections. Therefore, further research is required to extend and validate our diagnostic utility of ZPP approach with appropriately selected reference standard for iron deficiency in a population that include individuals with inflammation and infections.

Way forward

In our conceptual framework (chapter 1), we anticipated a substantial increase in haemoglobin concentration with co-administration of drug treatments and home-fortification with the two iron formulations at a frequency and duration that may control anaemia. We administered the daily home fortification within a duration of 30 days after providing a post treatment prophylactic phase by administering dihydroartemisinin-piperaquine, praziquantel and Albendazoles to the study children, 3 days' prior randomization to the intervention groups. In this study, home fortification with 3mg iron as NaFeEDTA and 12.5mg iron as encapsulated ferrous fumarate had a mean (SD) haemoglobin concentration (g/L) of 110g/L (12.5g/L) and 108.6g/L(12,0) respectively at the end of 30 days. Several factors, discussed above, may have led to this suboptimal increase of haemoglobin concentration (figure 1, panel 1). We selected a relatively short 30-day intervention with iron in the expectation that premedication with dihydroartemisinin-piperaquine will prevent malaria during this period, with a long-term view that the protection afforded by repeated chemoprevention with this combination drug would allow time windows for safe administration of short courses of iron intervention. In Burkina Faso pre-school children, two cycles of chemoprevention with dihydroartemisinin-

piperaquine, administered at the same target dose as in our study, resulted in a protection against malaria [22]. Thus if we maintain the duration of 30 days and increase the frequency of co-administering the anti-malarial treatment using the recent WHO revised therapeutic dosage regimen [15], closely monitor the uptake of 2nd and 3rd dosage at the research clinic, and treat all children with common bacterial infections and any other enteric infections as determined by hepcidin concentrations alongside other inflammation markers, test and eliminate children with blood disorders, then we anticipate a substantial increase in the haemoglobin concentration as demonstrated in figure 1 panel 2.

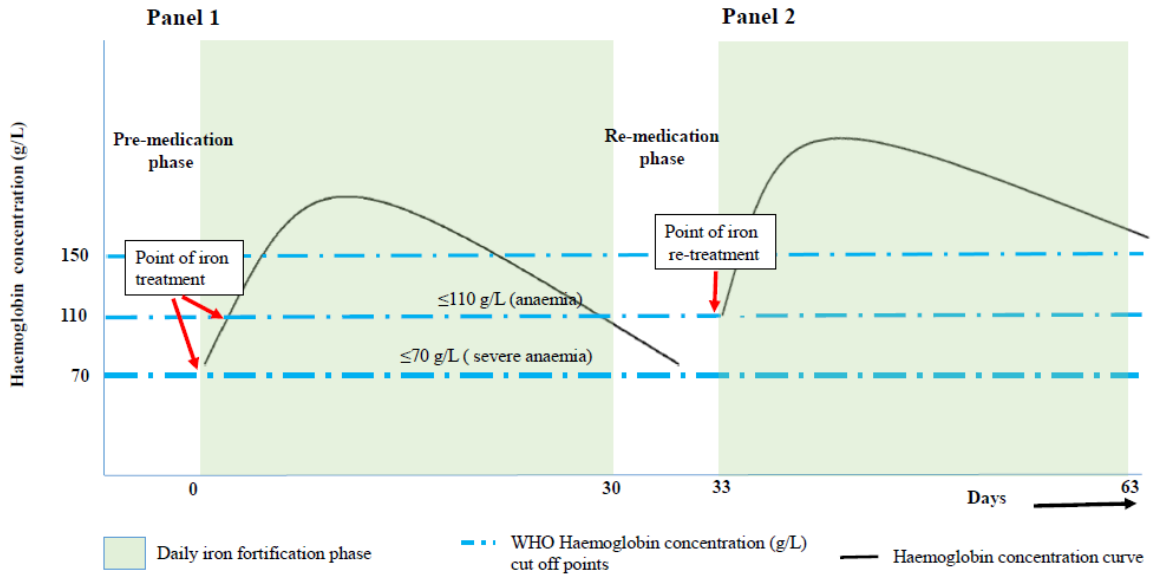


Figure 1: Frequency and duration of co-administering drug treatments and daily home fortifications

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23. **Appendix 1: SEICK research staff list**

Security guard (Employee code no. SEICK/W)

No	Full Name
W05	Willington Omondi Ang'ienda
W11	Richard Onyuro Ochweda

Cleaner (Employee code no. SEICK/CL)

No	Full Name
Cl04	Mercy Achieng Wadegu
Cl17	Lilian Akoth Onduru

Cook (Employee code no. SEICK/CK)

No	Full Name
Co09	Milicent A Odhiambo

Driver (Employee code no. SEICK/DR)

No	Full Name
D02	James Odhiambo Ouko

Store keeper (Employee code no. SEICK/SK)

No	Full Name
S2	Peter Odiko Akech
S2	Joram Nyange Mwakala

Office Administrator (Employee code no. SEICK/OA)

No	Full Name
O06	Belynder Akoth Ogone

Nurse (Employee code no. SEICK/NE)

No	Full Name
N 01	Rebecca Ogone Odongo
N 06	Lilian Beatrice Otieno

Research Paediatrician (Consultant code no. SEICK /CP)

No	Full Name
CP 01	Dr. Walter Otieno

Clinical Officer (Employee code no. SEICK/CO)

No	Full Name
CO 01	Fredrick Obosi Ochara
CO 02	Jack Obiero Jalang'o

Research Assistant (Employee code no. SEICK/RA)

No	Full Name
RA 02	Bonventure Otieno Onyango
RA 03	Teresia Akumu Atieno
RA 06	Helen Atieno Abach
RA 09	Claris Winnie Onyango
RA 10	Barrack Charles Okumu Were
RA 13	Linet Akinyi Ouma
RA 16	Sylvia Aketch Akoko
RA 24	Morris omondi Ochuka
RA 33	Dyphine Ndenga
RA 36	Manase Ouma Oukoh
RA 41	Susan Anyango Okoth
RA 46	Jackline Achieng Ojodo
RA	Millicent Atieno Obiero
RA82	Beryl Akinyi Okoth
RA84	Mercy N. Adhiambo
RA88	Owino Awino Philister

Lab Technologist (Employee code no. SEICK/LT)

No	Full Name
LB03	Eunice Anyango Abunde
LB04	Tabitha Amondi Otieno
LB06	Victor Kenyanyasi Osoti

Data Clerk (Employee code no. SEICK/DC)

No	Full Name
DC 01	Kennedy Ouma Ondijo
DC 02	Joram Nyange Mwakala

Appendix 2: Informed consent form

A study to assess the safety and efficacy of home fortification with iron in Kenyan children

General

1. 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.

   Signature/thumb print of parent/guardian:_____

Screening and enrolment

2. I understand that I will be asked information about my child, and consent for my child to undergo medical examination and to donate samples of blood (by arm prick; 6 mL, a volume equal to one table spoon), urine and stool.
3. I consent that my child will be administered medicines (albendazole, praziquantel against worms, and dihydroartemisinin-piperaquine against malaria).
4. I understand that 3 days after screening, I am expected to bring my child back to the health facility where I will be given the outcome of the medical examination. Depending on the outcome of the medical examination my child may or may not be enrolled in the study. If my child successfully passes the screening process, I consent to continue with the randomization process.

Signature/thumb print of parent/guardian:_____

Randomisation

5. I understand that my child will be allocated by chance ('randomly') to one of 3 groups, and that, depending on the group, my child will receive sachets containing either 12.5 mg iron as ferrous fumarate, or 3 mg iron as sodium iron EDTA, or no iron.
6. I consent for the contents of the first sachet to be given at the research dispensary.
7. I understand that my child is allowed to drink and eat food given by the research team in the period starting 2h before the first dose is given, and 3h afterwards. I agree to <u>not</u> give any other food to my child in this time period.
8. I consent that, 3 hours after the first dose is given, another blood sample (about 5-6 drops) will be collected by finger prick from my child.

   Signature/thumb print of parent/guardian:_____

Intervention

9. I understand that I will be given a dispensing bottle with 29 sachets, and that this bottle will remain property of the project. I agree to keep the dispensing bottle for the duration of the study in a safe place.
10. I consent to each day add and mix the contents of a single sachet to a small amount of *uji* or other food, and to ensure that my child will consume all of this food.
11. I understand and consent that community volunteers will visit my home at least once a week to answer questions that I may have about the study.
12. I understand that, if my child becomes sick during the 30-day study period, I am expected to take my child for routine care to the regular health services.

Signature/thumb print of parent/guardian: _____

At 30 days after start of intervention

13. I consent for samples of blood (6 mL) by arm prick, stool and urine to be collected at 30 days after the start of the study.
14. I understand that, at 30 days after the start of the study, the study team and I will be informed whether the sachets given to my child contained iron or not.
15. I understand that, *if my child received* ***no iron***, he or she again will be given antimalarial medicines (dihydroartemisinin-piperaquine) and 30-day supply of sachets with iron (12.5 mg iron as ferrous fumarate) in the dispensing bottle.
16. I understand that, ***if my child received iron***, field staff will collect the dispensing bottle with the electronic device

Signature/thumb print of parent/guardian: _____

Follow-up after 30 days of intervention

17. I understand that, *if my child received* ***no iron***, he or she will leave the study at 30-days after the intervention was stopped (thus 60 days after the start of the study), and that field staff will collect the dispensing bottle with the electronic device.
18. I understand that, ***if my child received iron,*** he or she will continue to be followed for a maximum of 100 days, and that the end date will be decided by chance. I consent that, at the end date, two small blood samples (total: 5-6 drops) will be collected by finger prick. I understand that my child will then be given antimalarial medicines (dihydroartemisinin-piperaquine) and 30-day supply of sachets with iron (12.5 mg iron as ferrous fumarate). I understand that my child will then leave the study.
19. I understand that, if my child becomes sick during the 30-day study period, I am expected to take my child for routine care to the regular health services.

Signature/thumb print of parent/guardian: _____

Storage and future use of samples

20. I understand that samples of blood, stool and urine will be used for laboratory tests, and to check for hereditary factors that may affect malaria and anaemia. I consent to these samples being retained for unspecified use during or after the conclusion of the research project.
21. I consent for small units of blood, stool and urine samples may be taken abroad for specialized analysis that cannot be easily done in Kenya.
22. I understand that the results of these tests will remain confidential, regardless of whether the tests will be conducted within or outside Kenya.

Signature/thumb print of parent/guardian:_____

Benefits and compensation

23. I understand that I will be reimbursed for transport expenses made to take my child to the health facility for the purpose of the study.
24. I understand that, when requested to stay at the health facility for more than 6 hours for the purposes of the study, I will receive food (breakfast, lunch or snacks) free of charge.
25. I understand that there will be no other compensation for participation in the study, whether financial or otherwise.

Signature/thumb print of parent/guardian:_____

Voluntary participation and confidentiality

26. I know that all my personal information will remain confidential.
27. 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.
28. 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, and received a signed copy of this agreement.

Date: __ __ / __ __ 2014

I am the father/mother/legal guardian of (name of the child):_____

Screening number: _____

Name of parent/guardian:_____

Signature/thumb print of parent/guardian:_____

This form has been read by / I have read the above parent/guardian in a language that (s)he understands. I believe that (s)he has understood what I have explained and (s)he has made the free choice to participate in this study.

Name of nurse/fieldworker: _____

Signature of nurse/fieldworker:_____

Name of witness: _____

Signature of witness:_____

Contacts:

1. Emily Teshome, Trial Coordinator
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   College of Health Sciences
   P.O. Box 19676 code 00200
   Tel +254 20 2726300 Ext 44355 email: uonknh_erc@uonbi.ac.ke

Appendix 3: Participants information brochure

A study to compare the efficacy and safety of different iron formulations in Kenyan children

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 compare home fortification with two iron formulations. Many young children in Kenya have anaemia, a disorder that is characterized by blood with a light red colour, instead of a healthy dark red. The red pigment in blood is necessary to transport oxygen from inspired air to muscle. Children with anaemia often feel weak or tired, and may have difficulty learning. To prevent anaemia in children, medical doctors often prescribe supplements that contain 12.5 mg iron in a specific form (ferrous salts). A new form of iron has recently become available that can probably be given at lower doses, because the body is better able to absorb this type of iron.

In our study, we will divide young children into three groups and give each group a different treatment. Group 1 will receive the form of iron that has been used so far (12.5 mg iron as ferrous fumarate), Group 2 will receive the new form of iron (3 mg iron as sodium iron EDTA), and Group 3 will receive no iron. The results will be compared to determine if the new type of iron (3 mg iron as sodium iron EDTA) can prevent anaemia, and to determine if it is equally as good as the form of iron that has been used so far (12.5 mg iron as ferrous fumarate).

We will provide both forms of iron in sachets (little bags). Each day, the mother should empty and mix the contents of a single sachet into ready-prepared *uji* or some other type of food, before giving it to the child. This should be repeated every day for a period of 30 days. The child will be followed for some time afterwards.

Although iron is good to prevent anaemia, there are some concerns that it may increase the risk of malaria. For this reason, we will treat each child with a special medicine against malaria (dihydroartemisinin-piperaquine) at the start of the study, before the first dose of iron is provided. This medicine will protect the child against malaria for the time period in which the child will receive iron. In addition, all children will be dewormed at the start using two drugs (albendazole and praziquantel).

Do you have to participate?
We have asked you to take part because your child is within the age range suitable for our study. In total, we want to study 324 children. You can let your child join the study at your own free will, and withdraw your child at any moment, with or without giving reasons. If you decide not to participate, or to withdraw from the study, this will not affect the normal care you receive in clinics or hospital elsewhere.

Read this information sheet and listen to our explanation of the study. We will then ask you to sign a consent form to show you have agreed to take part.

What will happen to you if you take part?

Screening visit: Our staff will invite you and your child to our research clinic to tell you about the aims and procedures of the study. If you agree for your child to participate, we will ask you to sign their fingerprint on a consent form. To decide whether your child can participate in the study, we will then ask you questions about your child, we will carry out a medical examination, and we will then collect samples of blood (6 mL, a volume equal to one table spoon) by arm prick, urine and stool from the child. This will take at least several hours. We will ask you to stay until if the child has produced stool. If necessary, we will invite you and your child again the next day to try again. After sample collection, your child will be administered medicines (albendazole, praziquantel against worms, and dihydroartemisinin-piperaquine against malaria). You will be asked to bring your child again to the research clinic 3 days later.

Randomisation visit: At this visit, we will use the information collected so far to decide if your child can take part in the rest of the study. Participating children will receive sachets with powder. These sachets will be contained in a special dispensing bottle that you will receive with instructions for use. For one-third of children, these sachets will contain 12.5 mg iron as ferrous fumarate, one-third will contain 3 mg iron as sodium iron EDTA, and one-third contains no iron. The allocation to group will be decided by chance (randomly). All sachets will look identical; we will not know which supplements contain iron until after the study. The first sachet will be given with food at the research dispensary. From 2h before this point until 3h afterwards, children will be allowed to drink but can only eat foods selected by the project team. We will then collect another blood sample (about 5-6 drops) by finger prick. From then on, community volunteers will daily supervise the supplementation in or close to your homestead.

During the 30-day intervention period: In the next 30 days, you should add and mix the contents of a single sachet to *uji* or any other food given to the child. Community volunteers will visit your home at least once a week to answer questions that you may have about the study. Children who become sick during this 30-day period will be referred to receive routine care by the regular health services. You may decide to withdraw your child at any point from the study. You may refuse to give reasons for your refusal, or to give permission for future collection of samples.

End-of-intervention survey: At the end of the 30-day period, we will collect the dispensing bottle and ask you some questions. We will again collect samples of blood (6 mL) by arm prick, stool and urine, using the same procedures as earlier. For each child, we will then break the randomisation code. Those who received placebo will be given antimalarial medicines (dihydroartemisinin-piperaquine) and 30-day supply of sachets with iron (12.5 mg iron as ferrous fumarate).

Follow-up after the 30-day intervention period: For children who received placebo, field staff will collect the dispensing bottle with the electronic device at the end of this 30-day period. Children who received iron will continue to be followed for a maximum of 90 days. In this period, we will collect a single sample of blood (5-6 drops, by finger prick). The time point for this collection will be decided by chance: for some children it may be as early as 1 week after home fortification was stopped; for others, it may be at the end of the 90-day period. Children who become sick during this follow-up period will be referred to receive routine care at the health facility. Immediately following blood collection, children will be withdrawn from further study and will receive antimalarial medicines (dihydroartemisinin-piperaquine) and 30-day supply of sachets with iron (12.5 mg iron as ferrous fumarate). Field staff will also collect the dispensing bottle with the electronic device at this time.

To summarise, we will collect four blood samples from each child:

- *Randomisation visit:* 1 sample of 6 mL (a volume equal to one table spoon), to be collected by arm prick;
- *3 hours later:* 1 sample of 5-6 drops, to be collected by finger prick;
- *At the end of the 30-day period:* 1 sample of 6 mL, to be collected by arm prick;
- *After the 30-day intervention period:* 1 sample of 5-6 drops, to be collected by finger prick.

We will store part of the blood samples in frozen condition, so that we can subsequently conduct tests to assess the success of the interventions. We may also check for hereditary factors that affect malaria and anaemia. Some of these tests may have to be done abroad.

Confidentiality: Results of this study will be shared with the public in a form of academic publication or presentation. The purpose of this publication or presentation is to create awareness and promote understanding of safely and efficiently treating anaemia in malaria endemic areas.

We will keep any information about your child confidential. Readers of the publication based on this research will not know that you gave this information. All personal information will be stored securely. This means that whenever we write or talk about anything we have been told, we never use your real name. The only information that we may have to pass on is if your child is at risk of serious harm.

Benefits and compensation: You will not receive financial benefits for participating in this study. If you need to stay more than 4 hours, we will give you a compensation gift to account for the lost hours.

Appendix 4: Self reporting form

SEICK PROJECT 2014

Self- Reporting Sheet

Date of start	
ID No of Child	
Location	

To be filled by caregiver immediately after adminstering contents in the sachets

End of reporting date

Tick the time child consumes warm *Uji o* r any warm meal with Micronutrient powder

Day	Morning	Mid-Morning	Lunch	Mid-afternoo	Night	Did not consume
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						
21						
22						
23						
24						
25						
26						
27						
28						
29						
30						

Appendix 5: Follow-up checklist

Follow-up visit form

Sub-Location_____ Village Name _____ ID No. of child_____
Name of Caregiver_____ Research Assistant _____ _____

Week	Date of Visit DD/MM/YY	Study Child Present Yes/No	Caregiver Present Yes/No	If caregiver is not present who else was present? Indicate name and relation to child	Problems /challenges raised by Caregiver	Observe if the following are still in good use. (Please do not open the MEMS device) Tick where present
1					1. 2. 3. 4.	MEMS Self- reporting
2					1. 2. 3. 4.	MEMS Self- reporting
3					1. 2. 3. 4.	MEMS Self- reporting
4					1. 2. 3. 4.	MEMS Self- reporting