Efficacy and safety of fortification with iron of maize flour in African children

Pauline EA Andang'o

Promotor

Prof. Dr. ir. Frans J. Kok

Hoogleraar Voeding en Gezondheid, Afdeling Humane Voeding, Wageningen Universiteit

Co-Promotoren

Dr. ir. Hans J.M. Verhoef

Post-Doc, Leerstoelgroep Celbiologie en immunologie, Wageningen Universiteit

Dr. ir. Saskia J.M. Osendarp

Onderzoeker, Unilever Food and Health Research Institute, Unilever R&D, Vlaardingen

Samenstelling promotiecommissie

Prof. Dr. ir. Ivonne M.C.M. Rietjens

Wageningen Universiteit

Prof. Richard F. Hurrell

Swiss Federal Institute of Technology, Switzerland

Prof. Bernard J. Brabin

Liverpool School of Tropical Medicine, United Kingdom

Dr. Martin W. Bloem

World Food Programme, Rome, Italy

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In memory of Clive West, who was instrumental in the inception of this work, but did not see its completion.

Abstract

Food fortification is an effective strategy for reaching populations with micronutrient deficiencies. Fortification with iron presents a major challenge because of the presence of phytates and other iron absorption inhibitors in diets of poor people. Addition of iron does not necessarily mean that it is absorbed. Because of its low cost and its relative stability in food vehicles, electrolytic iron has been the fortificant of choice in many national programmes, despite its low bioavailability compared to fortificants such as ferrous salts. NaFeEDTA may be a more effective iron fortificant in high-phytate foods. Concerns about its cost and safety, however, have delayed its application in fortification programmes.

This thesis is based on a 5-month randomised placebo-controlled trial conducted among school children (n = 516) aged 3-8 years in Kenya in May-November 2005. It aimed to measure the benefits of fortification with NaFeEDTA or electrolytic iron, and to assess the safety of using NaFeEDTA as an iron fortificant. The specific objectives were to assess a) the efficacy of fortifying whole maize flour with NaFeEDTA (28 mg iron/kg flour or 56 mg/kg) or electrolytic iron (56 mg/kg); b) the effect of the intervention on cognitive and motor function; c) response to the intervention of individuals with α^{+}-thalassaemia genotype relative to their counterparts with a normal genotype; d) and the effect of NaFeEDTA on the status of zinc, calcium, copper, magnesium and manganese.

Fortification with NaFeEDTA at high levels (56 mg iron per kg flour), or low levels (28 mg/kg) reduced the prevalence of iron deficiency anaemia by 89% (95% CI 49% to 97%) and 48% (-20% to 77%), respectively. High-dose fortification with NaFeEDTA led to larger gains in iron status than low-dose level, and was more efficacious in children with iron deficiency at baseline. By contrast, electrolytic iron did not improve iron status. There was no evidence that the iron intervention improved either cognitive or motor function of these children; or that children with the α^{+}-thalassaemia genotype responded differently to the iron intervention than their peers with a normal genotype. There was also no evidence that NaFeEDTA adversely affected the status of the nutritionally important mineral elements assessed.

The thesis concludes that in high-phytate flours, NaFeEDTA is more suitable than electrolytic iron for supplementation of iron in the diet. Governments should act and implement national programmes to fortify industrially processed flour, whereby NaFeEDTA should be considered the preferred fortificant in high-phytate food vehicles.

CONTENTS

Chapter 1

General Introduction

INTRODUCTION

The impact of improving micronutrient status on health outcomes and the attendant benefits are now recognised and are gaining increasing attention. In poorer regions of the world undernutrition continues to put individuals at risk of micronutrient malnutrition. This is mainly as a consequence of food shortages, lack of dietary diversity and food staples with poor micronutrient bioavailability. Greater access to a wider variety of micronutrient-rich and fortified foods, and better health have contributed to lowering the risk of micronutrient malnutrition in wealthier countries. Successful improvements in micronutrient status in the wealthier regions have served to inform strategies that may effectively improve the micronutrient status of poorer regions. This thesis will focus on iron deficiency, the most common micronutrient deficiency, and involves research findings of a study conducted in school-age children in Kenya. Although the prevalence of iron deficiency is difficult to quantify, because infections are common and influence iron status indicators; iron deficiency is estimated to be responsible for 50% of all anaemia cases[1]. Such estimates indicate that South East Asia has the highest prevalence of iron deficiency followed by Africa, Eastern Mediterranean countries and the Western Pacific[1]. In this chapter I will discuss factors contributing to poor iron status; the bioavailability of iron, and measurement of iron status, as a preamble to iron fortification as a strategy to reduce iron deficiency; eventually focusing on the use of NaFeEDTA as an iron fortificant. Factors will also be considered that may have influenced the results of our iron intervention in Kenya.

IRON DEFICIENCY AND BIOAVAILABILITY OF IRON

Iron is necessary for a number of body functions including the synthesis of haemoglobin that is required for oxygen transport; it is also present in myoglobin in muscle and in many iron-containing enzymes. When the dietary supply of iron exceeds the body's iron requirements, excess iron is stored in hepatocytes and macrophages of the mononuclear phagocyte system. Iron requirements are met by dietary iron or from existing body iron stores. Inadequate dietary iron intake eventually results in depleted body iron stores, which is presumed to indicate iron deficiency. The resulting shortage of iron for haemoglobin synthesis results in decreased haemoglobin concentrations, which eventually manifests as iron deficiency anaemia. *Thus an adequate supply of dietary iron is necessary to maintain body iron stores and ensure that there is adequate iron for normal body functioning and haemoglobin synthesis.*

Whilst food shortage can contribute to iron deficiency, it is generally recognised that the poor bioavailability of dietary iron is the key problem causing iron deficiency anaemia in many regions of the world. The two main determinants of low bioavailability are the form of dietary iron and the presence of substances such as phytates and polyphenols that bind iron and reduce its absorption. Dietary iron exists as either haem iron or non-haem iron. Haem iron is derived from animal foods and occurs in haemoglobin or myoglobin. It usually constitutes only 5-15% of the dietary intake of iron, but is relatively efficiently absorbed. Non-haem iron occurs in a wide variety of plant and animal foods. The efficiency of its absorption is low and markedly influenced by the dietary constituents that can either enhance or inhibit its absorption[2]. In plant-based diets, which predominate in developing countries, non-haem iron forms the bulk of the ingested iron. These diets, however, are mostly based on cereals and legumes that contain substantial amounts of iron absorption inhibitors. *Poor bioavailability of non-haem iron is one of the major contributing factors to the problem of iron deficiency, and its improvement is the focus of programmes to improve the iron status of deficient populations. This thesis will focus on non-haem iron.*

Iron absorption and metabolism

Bioavailability is a function of food digestibility, nutrient absorbability and the ability to use a nutrient for metabolic functions. The extent to which iron in the diet can improve iron status, therefore will reflect its bioavailability. Virtually all plant food-derived iron is in the ferric (Fe^{3+}) form, which must first be reduced to the ferrous (Fe^{2+}) form before it can be absorbed by enterocytes, a type of intestinal epithelial cells. The gastrointestinal environment is an important factor in determining how much iron will eventually be absorbed. In solutions with a pH greater than 3, ferric iron forms insoluble iron hydroxides and is precipitated from solution, hence is unavailable for absorption. This may be prevented in two ways: chelation of ferric iron, at low pH in the stomach by dietary and intestinal derived substances which keep iron in solution when it enters the less acidic duodenum[3]; or by reduction of ferric iron to ferrous iron. In normal circumstances the pH of the stomach is low therefore favourable for ferric iron chelation and solubilisation. Factors that reduce the acidity of the stomach can therefore reduce the bioavailability of non-haem iron. Some ferric iron is reduced by dietary constituents and intestinal secretions to ferrous iron which is soluble at neutral pH. However, in the absence of either continuous reduction or chelation to prohibit exposure of the iron to oxygen, ferrous iron is rapidly oxidized to ferric iron[4,5]. The stomach and to some extent the duodenal environment may also favour inhibition of iron absorption: other dietary

constituents such as phytates, which as already mentioned, are abundant in cereal and grain-based diets; polyphenols, calcium and some proteins, may form complexes with iron which render it unavailable for absorption. *To improve the bioavailability of non-haem iron, it must be reduced to Fe^{2+} or a sufficient amount of ligands must be present in order to maintain Fe^{3+} in the soluble phase.*

Enterocytes take up iron via the metal transporter, divalent metal transporter 1 (DMT1), although the existence of other uptake pathways cannot be precluded[3,6,7]. DMT1 transports iron exclusively in the Fe^{2+} form. Thus dietary ferric iron must first be reduced to its ferrous form before it can be absorbed. This is accomplished by duodenal cytochrome b (Dcytb), a ferric reductase that is located at the brush border of enterocytes and that presumably uses ascorbate to facilitate ferrireduction[7]. By contrast, supplemental iron, which is usually provided as a ferrous salt, is absorbed directly. Once inside the enterocyte, iron has two fates: it is either stored as ferritin within the enterocyte, or is transported across the enterocyte and exported into plasma at the basolateral end of the enterocyte[8]; by the transport protein ferroportin **(figure 1.1)**. Ferrous iron molecules are then oxidised to ferric iron by hephaestin, an enterocyte-bound reductase. This ferric iron is bound to the protein transferrin, and transported either to storage sites or to the erythron for haemoglobin production.

Regulation of iron absorption

Whilst increasing iron intake in deficient individuals improves iron status, there are concerns that it may also result in iron overload. Because no iron-excretory mechanisms exist, the body must tightly regulate the amount of iron that eventually enters the circulation, because excess iron resulting in iron overload is toxic[9]. Iron absorption is regulated at the basolateral membrane of enterocytes by hepcidin, a peptide hormone produced in the liver that circulates in plasma. Hepcidin is a negative regulator of plasma iron concentrations by inhibiting the export of iron by duodenal enterocytes into plasma. It acts by binding to the enterocyte iron exporter, ferroportin, and causing its degradation[10]. In addition, absorption of dietary ferric iron is possibly regulated at the apical end of enterocytes by duodenal cytochrome b: in iron deficiency, its activity is stimulated by enhanced protein expression[11,12]. Enterocytes have a life cycle of 2-3 days; thereafter, they are sloughed off and the iron still contained in them is lost through the gastrointestinal tract[8]. *Iron absorption into enterocytes and export from enterocytes into the circulation is tightly regulated and in normal circumstances will effectively prevent the risk of iron overload.*

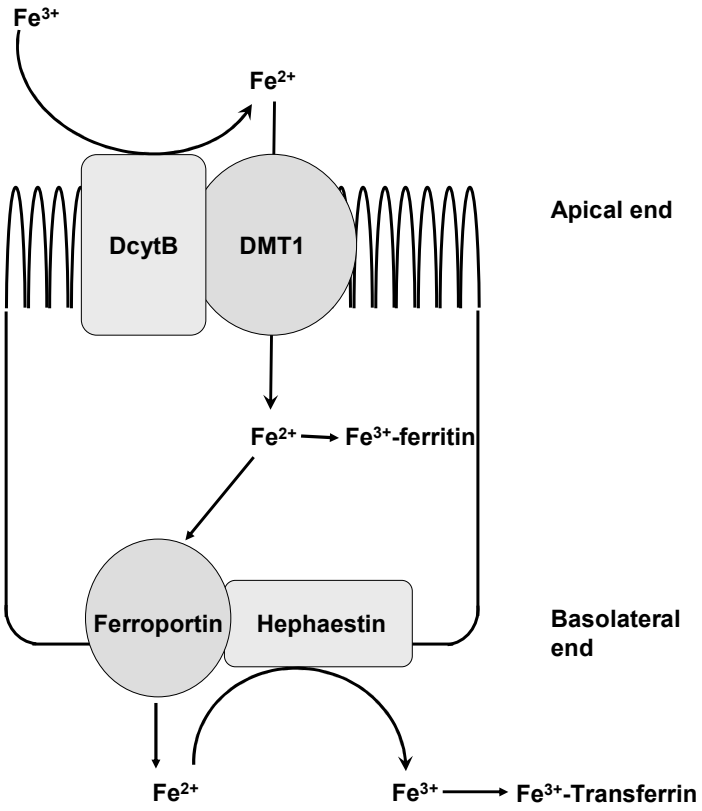

Figure 1.1 Iron uptake into and export from duodenal enterocytes. Adapted from Andrews 1999 and 2000 [8,13]. Iron must cross two membranes to be transferred across the absorptive epithelium. Each transmembrane transporter is coupled to an enzyme that changes the oxidation state of iron. The apical transporter has been identified as DMT1. The basolateral transporter requires hephaestin, a ceruloplasmin-like molecule, for the transfer of iron to the plasma. Iron within enterocytes is stored as ferritin.

METHODS OF ASSESSING IRON STATUS

Iron deficiency usually occurs in three stages: the depletion of iron stores; iron deficient erythropoiesis; and iron deficiency anaemia. In this section biomarkers are described for each of these stages.

Ferritin concentration

The concentration of ferritin in plasma is directly proportional to body iron stores in healthy individuals with 1µg/L increments corresponding to 8-10 mg or 120 µg storage iron/kg body weight[14]. Ferritin is currently the most important indicator for iron status. It is decreased in iron depletion, and is a sensitive indicator of the level of iron stores. Low concentrations

(<12μg/L in children < 5 years and <15 μg/L in individuals ≥ 5 years[1]) always indicate storage iron depletion[15]. In individuals with acute or chronic inflammation, malignancy or liver disease, however, plasma ferritin concentration is elevated independently of iron status[14]. In populations with a high prevalence of infection, iron status can be determined by excluding individuals with infection or inflammation, or by adjusting for the effects of infection or inflammation. The most commonly used plasma indicator for acute infection is C-reactive protein (CRP), whereas plasma concentration of α_1-acid-glycoprotein (AGP) has been proposed as a measure of chronic infection[15].

Soluble transferrin receptor (sTfR)

Cellular iron uptake occurs through interaction of Fe^{3+}-transferrin with a specific membrane receptor, transferrin receptor. A soluble form of transferrin receptor is found in circulation; its concentrations are proportional to the total number of membrane-bound receptors in the erythron. The expression of the cellular membrane-bound receptors, and thus plasma sTfR concentrations, are increased in iron deficiency. sTfR concentration is a reliable index of early tissue iron deficiency[16] and has the advantage of being minimally influenced by inflammation[15,17]. However, malaria-induced changes in erythropoiesis and other conditions that cause an expansion of the erythron will also produce elevated sTfR concentrations[17], so that it is currently impossible to determine iron status in individuals with these conditions. Study results of sTfR are difficult to compare because cut-off levels indicating iron deficiency depend on the commercial test that is deployed[18,19].

Haemoglobin

Anaemia is defined by haematocrit values or haemoglobin concentrations below normal values (<110 g/L in children < 5 years and <115 g/L in children ≥ 5 years [1]) in a reference population. Haemoglobin concentrations are influenced by a number of other factors therefore it is not considered a sensitive indicator of iron deficiency, but is however necessary to determine the presence of iron deficiency anaemia.

Plasma ferritin concentration, when used in combination with plasma CRP concentration and indicators of the presence of malaria infection, is the best available indicator of iron status in populations of African children. Plasma sTfR concentration can improve detection of iron deficiency in children with inflammation that is not due to malaria.

STRATEGIES TO REDUCE IRON DEFICIENCY ANAEMIA AND THE ASSOCIATED BENEFITS

The goal of iron interventions is to reduce the risks associated with iron deficiency. In children these risks include: increased morbidity due to decreased immunity; decreased activity, and decreased cognitive and motor function. Hence benefits in these areas are expected to occur with improved iron status. This thesis will address the effect of improved iron status on cognitive function. Changes that occur due to iron deficiency in early childhood, depending on the timing of iron deficiency, may be irreversible whilst those that occur after the weaning period may be reversed by iron repletion [20,21]. During childhood the frontal lobe is still maturing and iron deficiency at this stage may manifest in cognitive deficits. Studies show that school-aged children with iron deficiency anaemia score lower in IQ tests and tests of attention than their iron replete counterparts [22]. Improved iron status is associated with improvements in motor development and the ability to acquire language [23]. Although the exact mechanisms of the relationship between iron and cognitive and motor development are as yet unclear, it is plausible that a good iron status is beneficial. *Improving iron status may improve cognitive and motor function especially in children.*

Nutritional strategies to combat iron deficiency anaemia include food-based approaches such as food or dietary diversity, pharmaceutical approaches (supplementation), biofortification and food fortification.

Food diversity involves increasing the quantity and the variety of iron-rich foods consumed [24]. This may include promoting the production and consumption of foods rich in iron, and foods that contain substances that enhance iron absorption; and the use of food processing methods such as fermentation, that reduce the content of iron absorption inhibitors. Dietary diversity has the advantage of simultaneously improving the intake of multiple micronutrients.

Supplementation with pharmacological doses of iron can effectively achieve iron repletion within a short period, especially in groups at high risk of iron deficiency. Adherence may be poor, and wide-spread supplementation is logistically difficult to achieve.

In biofortification the iron content of plant foods may be increased through selection, classical breeding, genetic engineering or a combination of these methods. Breeding may however be

limited in achieving iron levels that are sufficiently high to substantially improve iron status. Genetic engineering may be a more feasible method to raise iron content or to enhance iron bioavailability by reducing phytate levels [25,26].

Food fortification aims at increasing iron intake through the addition of suitable iron fortificants to commonly consumed processed foods. It has potentially large benefits because it can reach a large proportion of deficient populations and improve iron status relatively rapidly [24].

Another main contributing factor to iron deficiency is infestation with helminths such as hookworm, *Trichuris trichura* and schistosomal worms. In addition to the food based strategies, therefore, helminth control is necessary for the improvement of iron status. Because of the focus of this thesis, factors will be reviewed in the subsequent sections that must be taken into consideration to fortify foods; particularly issues related to the use of NaFeEDTA as an iron fortificant.

Food fortification with iron

Most commonly recommended iron fortificants include elemental iron, ferrous sulfate, ferrous fumarate, and, more recently, NaFeEDTA. Elemental iron powders are manufactured by five different processes: H-reduction, CO-reduction, atomization, electrolytic and carbonyl processes [27]. Elemental iron powders were initially the iron fortificant of choice because of their stability in food vehicles, their low cost, and because they cause few sensory problems. Although this form of iron increases the overall iron content of the diet, the absorption of such iron, is highly dependent on meal composition. Of the elemental iron powders, electrolytic iron has reasonable efficacy in improving iron status when used in some food vehicles; and its stability and organoleptic properties favour its continued use. It is water insoluble and poorly soluble in dilute acid.

Ferrous sulfate and ferrous fumarate are highly bioavailable in the absence of iron absorption inhibitors [28]. Ferrous sulfate provides the reference to assess the relative bioavailability of other iron fortificants. Because it is water-soluble, however, it can induce oxidative processes under influence of moisture. This can cause rancidity of lipids contained in foods; and organoleptic changes. These effects limit the type of food vehicles to which ferrous sulfate can be added. By contrast, ferrous fumarate is poorly water soluble but is soluble in acidic

conditions prevailing in the stomach. It has low potential for adverse sensory changes, making it a suitable fortificant in food vehicles such as flour to which the use of ferrous sulfate cannot be applied. However, ferrous sulfate, ferrous fumarate and electrolytic iron are all susceptible to binding by iron absorption inhibitors, hence their bioavailability is substantially reduced in high-phytate diets. The bioavailability of these fortificants in high-phytate diets can be improved by concurrent use of iron absorption enhancers such as ascorbic acid or Na_2EDTA. Ascorbic acid enhances iron absorption in both water-soluble and water-insoluble compounds [29] in a dose-dependent way. However, its applicability is limited because it is expensive, susceptible to losses during storage, especially under hot, humid conditions, and cooking losses are high. Na_2EDTA, on the other hand, has the advantage of stability during storage and food preparation [28]. It enhances iron absorption from water-soluble compounds such as ferrous sulfate but not from water-insoluble compounds such as ferrous fumarate [29-32]. *Although highly bioavailable, the use of ferrous sulfate and ferrous fumarate is limited to low-phytate diets or diets rich in iron absorption enhancers. Ascorbic acid, although capable of enhancing iron absorption from a wide range of non-haem iron sources, has limited value as a food additive where such enhancing qualities are most required.*

Flour is an attractive food vehicle for fortification because it is widely consumed in large quantities by large population groups throughout the year. The high content of iron absorption inhibitors in flour, however presents special challenges. Thus an iron fortificant must be used that does not cause organoleptic changes and can supply iron despite the presence of phytates. NaFeEDTA has been shown to have high potential of being such a fortificant. Its ability to improve the bioavailability of both fortificant and intrinsic iron has been shown in isotope studies [33-36] and field trials [37,38]. In high-phytate diets, absorption of iron from NaFeEDTA is 2-3 times the absorption from ferrous sulfate; in the absence of phytates, its absorption is comparable to that of ferrous sulfate [27]. The application of NaFeEDTA in field trials, however, has been confined mainly to condiments such as fish sauce, soy sauce and curry powder. In these vehicles, it was shown to be efficacious in improving iron status [37,38]. Its application in high-phytate flour in a field trial had not been shown before the work for this thesis was started. *The use of NaFeEDTA as an iron fortificant has the potential to greatly enhance efforts to improve the iron status of populations consuming high-phytate cereal-based diets.*

Currently electrolytic iron is the preferred iron compound for low-phytate flour and was the form of iron legislated for fortification of flours in South Africa at the time the work for this thesis started; the use of ferrous fumarate is optional. NaFeEDTA is recommended for high extraction maize flour. It is also recommended for fortification of whole-wheat flour and condiments including fish sauce, sugar, curry powder [28] and soy sauce.

SAFETY OF NaFeEDTA

Central to the use of any food fortificant is the issue of safety. To establish safety of a food additive, assessments of acute toxicity, sub-acute toxicity and long-term toxicity are conducted in animals. Acute toxicity tests involve administration of single or repeated doses of the substance, orally or by intraperitoneal injection over 24 hours or less. These tests are used to determine the LD_{50}, or the amount of substance that kills 50% of test animals. Sub-acute toxicity tests involve feeding the substance in varying doses to groups of animals for a period of up to 10% of the expected lifespan. Doses are selected with the aim of producing an effect in at least one group and no effect in at least one other group. Results are used to establish any cumulative action of the substance and to find the maximum amount tolerated (highest level with no adverse effects) and the minimum toxic level (lowest level at which adverse effects are observed). Long-term tests involve feeding the substance at various dietary levels to groups of animals for periods equivalent to approximately 60 years in humans (2 years in rats and 80 weeks in mice). The tests aim to measure a no-effect level which would be expected to be at least 100 times that likely to be consumed by man. Carcinogenicity, effects on reproduction, lactation and spermatogenesis are established; and metabolic studies are conducted to assess the kinetics of absorption, storage and elimination; to measure effects on other dietary constituents; and to determine the nature and fate of the metabolic products in urine, faeces and other routes [39]. Only after safety is concluded in animal experiments can human trials be conducted.

Adverse effects attributed to EDTA concerned effects of zinc deficiency [40] that can theoretically be induced by high intakes of the chelate. When zinc was administered concurrently with high levels of EDTA the adverse effects were not observed. The chelating properties of EDTA and its potential to affect the absorption and or metabolism of other micronutrients became the main focus of EDTA safety for food fortification. Growth

depression and anaemia observed in rats fed 10-40 mg $CaNa_2EDTA$/kg diet was readily reversed by the addition of iron, copper and other mineral elements to the diet [28,41].

An Acceptable Daily Intake (ADI) is the endpoint of evaluations for food additives [42]. The ADI for $CaNa_2EDTA$ was established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1972, based on the highest no-effect-level (NOEL) available at the time with a safety factor to take into account the uncertainties of extrapolation of animal data to humans and for variation within the human species. The ADI of 2.5 mg/kg body weight per day referred to the compound $CaNa_2EDTA$. This value is however confusing when referring to the EDTA moiety of the compound; especially when using the ADI with reference to NaFeEDTA. Thus the value 2.5 mg/kg body weight has been quoted as the ADI for EDTA in early publications [43,44] and only recently has a value of 1.9 mg/kg body weight been explicitly stated specifically for EDTA [45].

Safety aspects of NaFeEDTA concern both its iron and its EDTA component. Iron from NaFeEDTA has been shown to have a similar level of toxicity to that of ferrous sulfate [46], and concentrations of up to 140 mg iron/kg from either ferrous sulfate or NaFeEDTA were not found to result in excessive loading in rats [47]. Early studies to assess the toxicity of EDTA have been reviewed comprehensively [44]. In animal studies, levels higher than used for fortification with NaFeEDTA did not affect growth, reproductive performance, or allergenicity; neither was there evidence of genotoxicity [28]. Although evidence of the potential usefulness of NaFeEDTA as an iron fortificant was available in the early 1970s, concerns about its safety stalled its use in fortification programmes. In the gastrointestinal tract, NaFeEDTA splits into iron and EDTA. Only a small fraction (<5%) of EDTA is absorbed; this absorbed EDTA is rapidly and completely excreted in the urine and does not accumulate in the body. Iron and EDTA are absorbed by separate and independent mechanisms when NaFeEDTA is added to a meal [28]. Thus a risk assessment for excess intake of NaFeEDTA should separately address the risks for excess intake of iron and EDTA. *Although there is growing evidence that NaFeEDTA may not influence the metabolism of other nutrient elements [48-54], evidence of its potential effect in young children is lacking.*

In addressing the safety of iron interventions it is important to take into account other factors that are related to iron status in ways that could increase risk of morbidity in some individuals.

Among these factors are infections and haemoglobin disorders. Because the setting of this thesis is within an area where the two factors are prevalent they will also be addressed here.

Iron and infection

In humans, one of the roles of iron is involvement in the effective mounting of an immune response [55]. Thus the host must effectively sequester iron from pathogens and simultaneously provide a supply of iron that is not limiting to its immune system. Iron supplementation therefore can theoretically increase the risk of infection or morbidity. Early studies showed conflicting results on the relationship between iron supplementation and infection risk [56], but a recent study in Pemba, which was published after the field work for this thesis had been completed, found that supplementation with iron and folic acid increased the rates of hospitalisation and mortality, presumably due to malaria [57]. Following these findings, the World Health Organization recommended that iron and folic acid supplementation for young children in settings with a high prevalence of malaria and a high incidence of other infectious diseases should target those who are anaemic and at risk of iron deficiency [58]. In the population studied within this thesis, malaria is a leading cause of morbidity and mortality and its reduction is central to public health measures to improve health. Whether or not iron will increase the risk of malaria morbidity may depend on transmission season, dose of iron, age, and the presence of haemoglobinopathies or other haemoglobin disorders[56]. There is currently no evidence that iron fortification produces malaria morbidity. This is however outside the scope of this thesis. The relationship examined in this thesis is the potential of infection to influence the effect of iron interventions. Infection may render iron supplementation inefficient: during the inflammatory process, iron absorption may be reduced, and much of the iron may be directed to reticulo-endothelial stores rather than to the erythron for haemoglobin synthesis [59]. Because hepcidin causes degradation of ferroportin, the transport protein necessary for iron export from enterocytes [10] absorption may be concurrently reduced during infection due to the action of hepcidin. *Hence iron supplementation could be detrimental during infection and, conversely, infection may reduce the efficacy of iron supplementation.*

Genetic haemoglobin disorders

Haemoglobinopathies and other haemoglobin disorders are common in malaria endemic areas and have been associated with protection against the effects of severe malaria [60-62]. These conditions include, among others, sickle cell anaemia, the thalassaemias and glucose-6-phosphate dehydrogenase deficiency. The most common in sub-saharan Africa are sickle cell

trait and α^+-thalassaemia. Few individuals with sickle cell anaemia, i.e. those who are homozygous, survive beyond childhood, and the prevalence is generally low. Sickle cell trait, the heterozygous genotype, is asymptomatic but may be characterised by mild anaemia. α^+-thalassaemia may occur at frequencies of up to 70% in Africa [57]. It involves a single deletion in one of the pair of α-globin genes (α-/αα). Homozygotes have two such deletions (α-/α-). Heterozygotes usually exhibit normal haemoglobin concentrations whilst in homozygotes, a mild microcytic hypochromic anaemia occurs that can easily be mistaken for iron deficiency. Individuals with α^+-thalassaemia are asymptomatic. The effect of sickle cell trait and α^+-thalassaemia on iron status should be taken into account when assessing iron status. It is important to note, however, that iron deficiency can exist in either condition [56]. In more severe forms of thalassaemia such as thalassaemia major, iron absorption is increased resulting in iron overload. *Although iron overload is not expected to occur in α^+-thalassaemia, it is not certain whether individuals with this condition may benefit more from iron interventions than individuals without thalassaemia, or whether they may load more iron than individuals without α^+-thalassaemia.*

II. AIMS AND OUTLINE OF THIS THESIS

Selection of study population

The demand for iron is greatest during pregnancy and periods of rapid growth such as infancy and adolescence. During childhood poor iron status is more likely to be due to environmental factors such as diet and infection than to normal physiological factors. Additionally, iron requirements at this time are not greatly influenced by sex [63]. *Because of the minimal influence of iron status by physiological factors during this period, it may be the best stage to obtain evidence of the efficacy of iron fortification over short periods.*

We chose to conduct the study among school children because they can be easily reached through schools for extended periods of time. Additionally, the children are old enough to consume target amounts of porridge but not yet at the stage of puberty with its attendant influences on iron status.

Objectives and outline of the thesis

As noted in the preceding sections, fortification has the potential to reach a substantial proportion of deficient populations; hence it is an efficient strategy to reduce iron absorption. An adequate supply of bioavailable iron in a suitable food vehicle that is commonly consumed will improve the impact of iron fortification programmes. It is also clear that one of the main hindrances to improving iron status is the presence of iron absorption inhibitors in cereal and legume-based diets of most deficient populations; and that the bioavailability of electrolytic iron, ferrous sulfate and ferrous fumarate is substantially reduced in the presence of iron inhibitors. An effective way of improving iron bioavailability in the mentioned circumstances, and the mechanism central to this thesis, is the chelation of iron, maintaining it in a soluble form to promote its bioavailability. This property is a characteristic of the fortificant, NaFeEDTA that doubles as an enhancer of iron absorption. Confirmation is necessary of its efficacy in high-phytate food vehicles as well as additional studies on its effect on the status of other nutrients. This thesis addresses the use of NaFeEDTA as an iron fortificant in a high-phytate food vehicle, maize flour. The benefit of NaFeEDTA will be assessed in two studies. First, it is assessed by comparing its efficacy in improving iron status to that of electrolytic iron, which is widely used due to its stability in food vehicles. Aditionally, evidence on the influence of iron on specific cognitive functions is still lacking. The thesis will therefore further assess the benefits of NaFeEDTA by exploring whether improvements in cognitive and motor function occurred after consumption of iron-fortified maize flour with the aim of adding information to this area of knowledge. The safety of NaFeEDTA will also be assessed in two studies. Because it is not certain whether individuals with α^+-thalassaemia may benefit more from iron interventions than individuals without thalassaemia or whether they could load more iron than individuals without α^+-thalassaemia, modification of the effect of the intervention by α^+-thalassaemia will be assessed in the first study addressing NaFeEDTA safety. The second study on safety will assess NaFeEDTA effect on the status of other micronutrients in our study population.

The immediate objectives of the thesis, therefore, are:

1. To assess the efficacy of consuming whole maize flour fortified with NaFeEDTA or electrolytic iron on the iron status of school-aged children
2. To assess the effect of iron fortification of whole maize flour with NaFeEDTA or electrolytic iron on cognitive function and fine motor skills

3. To assess modification of the effect of the intervention by alpha thalassaemia genotype
4. To assess the effect of the intervention on the status of nutritionally important mineral elements.

Because of the minimal influence on iron status by physiological factors during childhood, we chose to conduct our study in children aged 3-8 years on the basis that changes in iron status arising from an intervention would be more readily observed within a short period. A baseline survey and subsequent randomised trial was conducted between May and November 2004 in a sample of 516 children aged 3 to 8 years, to pursue the objectives 1 and 3. The results that address these objectives are discussed in chapters 2 and 4, respectively. At the end of the field trial, we conducted a study to assess cognitive and motor function in a sub-sample of 209 children aged 6 to 8 years, to pursue objective 2. The findings are addressed in chapter 3. Mineral element analysis of plasma from the 516 children in the randomised trial was conducted in 2007 to pursue objective 4 and is discussed in chapter 5 of this thesis. The main findings and conclusions of the studies within the thesis are discussed in chapter 6.

III. SETTING

Study site

We initially planned to conduct the study in schools located on two plantations in Thika, 60 km from Nairobi, Kenya. Because of the fixed schedules observed on the plantations, they are ideal for conducting a controlled trial because loss to follow-up due to migration is likely to be minimal. A cross-sectional survey revealed, however, that the anaemia prevalence was less than 5%; hence, it was not suitable for conducting an iron fortification trial because the prevalence of iron deficiency was also likely to be low. Subsequent cross sectional surveys of haemoglobin status in two adjacent districts on the Kenyan coast, Malindi and Kilifi, showed that the prevalence of anaemia was high in the two areas. Because the prevalence in the two areas was similar, we chose to conduct the study in Malindi district because the potential participating schools were in closer proximity of each other and were more readily accessible during the rainy season than schools in Kilifi. The study site was located in Marafa (**figure 1.2**).

Temperatures in Malindi are high, ranging between 25˚C and 34˚C. Despite its proximity to the coastal range, the area is semiarid, with 700-1000 mm rainfall annually. Malaria transmission is seasonal, and takes place mostly during or shortly after the two rainy seasons that occur between March and May, and between October and December. The main species causing malaria is *Plasmodium falciparum*. Marafa is one of three administrative areas in Malindi District and covers 1,617 square kilometers. The local population (approximately 50,000) mostly comprises the Mijikenda group of tribes living mainly from subsistence farming. There is minimal horticultural activity in most parts, and although the area supports livestock development, the diet is mainly cereal-based with low consumption of animal products with maize as the staple food. Maize may be replaced by dried cassava during periods of food shortage.

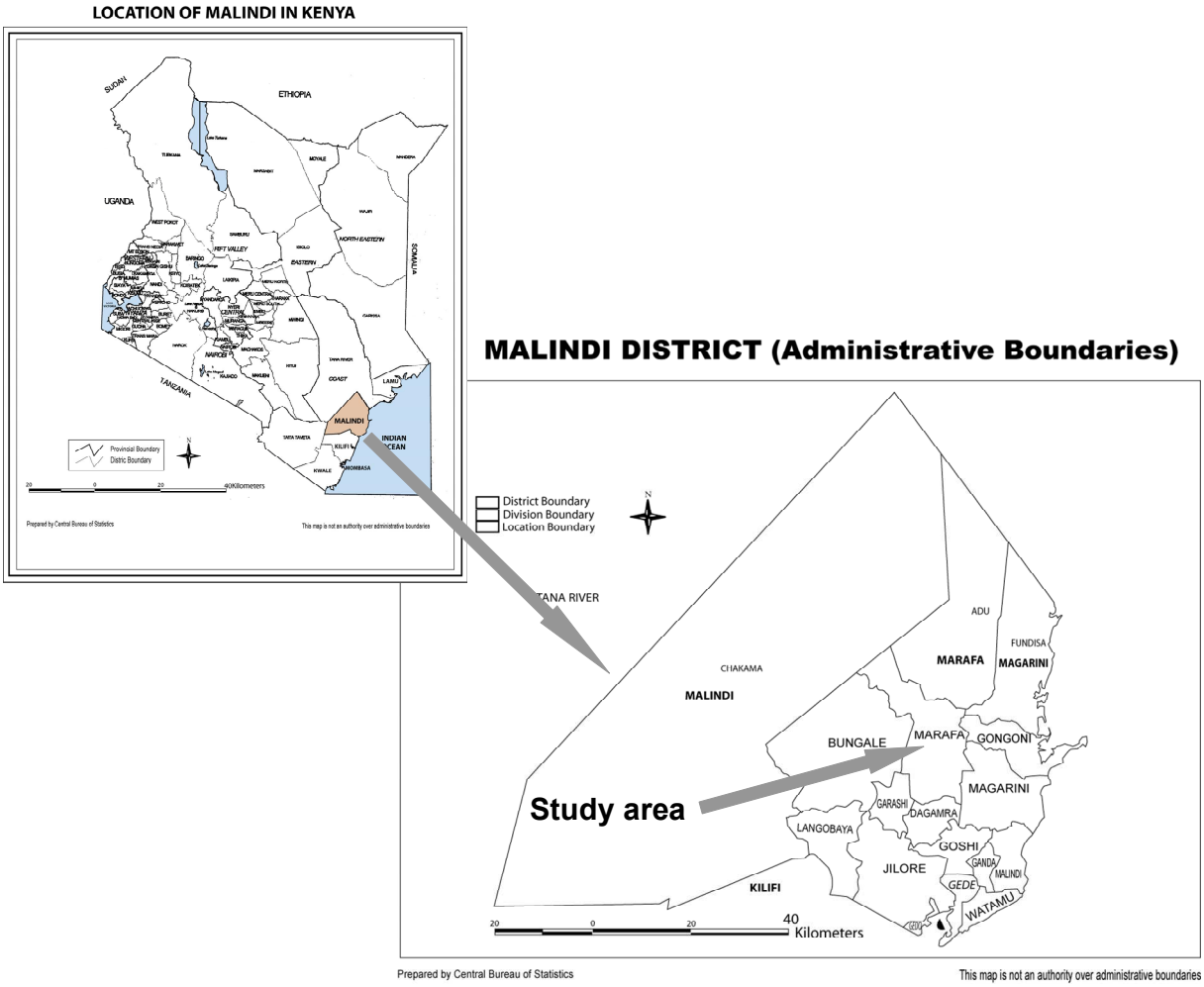

Figure 1.2 Location of the study area.

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Chapter 2

Efficacy of iron-fortified whole maize flour on iron status of school children in Kenya: a randomised controlled trial

Pauline EA Andang'o, Saskia JM Osendarp, Rosemary Ayah
Clive E West[†], David L Mwaniki, Corine A De Wolf
Rob Kraaijenhagen, Frans J Kok
Hans Verhoef

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[†] Deceased

Abstract

Sodium iron edetic acid (NaFeEDTA) might be a more bioavailable source of iron than electrolytic iron when added to maize flour. We aimed to assess the effect, on children's iron status, of consumption of whole maize flour fortified with iron as NaFeEDTA or electrolytic iron.

516 children, aged 3-8 years, from four schools in Marafa, Kenya, were randomly assigned to four groups. All were given the same amount of porridge, five times a week. The porridge for one group was made from unfortified whole maize flour; for the other three groups it was fortified with either high-dose NaFeEDTA (56 mg iron/kg flour), low-dose NaFeEDTA (28mg/kg), or electrolytic iron (56 mg/kg). All children received malaria chemotherapy 14 days before final blood collection to avoid inflammation-induced effects on iron status indicators. Concentrations of haemoglobin, plasma ferritin, and transferrin receptor were analysed in samples taken at baseline and at the end of the 5-month intervention. The primary outcome was iron-deficiency anaemia. We analysed data on an intention-to-treat basis. This trial is registered with ClinicalTrials.gov, NCT00386704.

The prevalence of iron-deficiency anaemia in children given unfortified flour was 10%. Compared with placebo, the prevalence of iron-deficiency anaemia in children given flour fortified with high-dose NaFeEDTA, low-dose NaFeEDTA and electrolytic iron changed by -89% (-97% to -49%), -48% (-77% to 20%) and 59% (-18% to 209%), respectively. Consumption of high-dose NaFeEDTA improved all measured iron status indicators. Low-dose NaFeEDTA decreased the prevalence of iron deficiency but did not noticeably change the prevalence of anaemia. Electrolytic iron did not improve any of these iron-status indicators. Children who were iron deficient at baseline benefited more from high-dose and low-dose NaFeEDTA than those with sufficient iron at baseline.

Consumption of whole maize flour fortified with NaFeEDTA caused modest, dose-dependent improvements in children's iron status. Fortification with electrolytic iron did not improve their iron status. Therefore, in high-phytate flours, NaFeEDTA is more suitable than electrolytic iron for supplementation of iron in the diet.

Introduction

Fortification of staple cereal flours may be a cost-effective, sustainable way to improve iron status in developing countries. It can be achieved by addition of a suitable substance (or fortificant), either on a large-scale to centrally processed flour, or on a small-scale, in communities.[1] International efforts to advocate flour fortification are now beginning to move the process forward. 49 countries routinely add iron to flour (compared with two countries in 1990)[2]. Thus about 15% of output from the world's flour mills is fortified with iron.[2] In sub-Saharan Africa, Nigeria and South Africa have made fortification of flour with iron mandatory, and countries such as Cape Verde, Côte d'Ivoire, Guinea, Ghana, and Kenya are planning flour fortification programs.[2] In eastern Africa, maize and wheat flours are suitable fortification vehicles because they are consumed as part of the typical diet.

Elemental iron powders are the most widely used nutrient vehicle for fortification of flour. In an authoritative review, Hurrell and colleagues[3] concluded that electrolytic iron is the only form of elemental iron that can be recommended as an iron fortificant in cereal flours. Fortification of wheat and maize flour with electrolytic iron is mandatory by law in South Africa (at minimum concentrations of 44·28 mg/kg and 50·40 mg/kg for sifted and unsifted flour).[4] However, although electrolytic iron is inexpensive, its bioavailability is questionable because it can easily bind to phytates in cereals. Only two randomised trials have assessed whether consumption of sifted, low-phytate flour, fortified with electrolytic iron, affects iron status.[5,6] The first trial[5] was not conclusive; in the second trial, the iron status of participants improved.[6]

In developing countries, however, the typical diet includes whole grain flour which has a much higher content of phytate than low-extraction white flour. In such cases, NaFeEDTA may be a better fortificant than electrolytic iron for supplementation of iron, because EDTA chelates iron, and might prevent it from binding to phytates. Isotope studies suggest that iron absorption from NaFeEDTA might be two to three times higher than from electrolytic iron.[7] This substance might not only improve absorption of added iron, but also of non-haem iron in food.[8] When given in fortified condiments, NaFeEDTA improved iron status.[9-12] It is well absorbed when added to corn masa flour,[13] which contains inhibitors of iron absorption. Therefore NaFeEDTA is potentially suitable for iron fortification in high-phytate foods. We aimed to assess the effect of consumption of whole maize flour fortified with high and low

doses of NaFeEDTA, and with electrolytic iron, on children. We expected that the efficacy of iron fortification might be affected by children's iron status at baseline.

Subjects and methods

Participants

Our study was based at four schools in Marafa, in the hinterland of Malindi district, in the semiarid coastal lowlands of Kenya. We did all the fieldwork between May and November, 2004, to reduce non-compliance due to holidays in April and August, and from December to early January. Because of this constraint our trial period coincided with the two rainy seasons (March-May and October-December), in which malaria transmission is highest. Most malaria episodes are due to *Plasmodium falciparum.*[14]

Local families are mostly poor subsistence farmers from Mijikenda tribal groups. Their diet is monotonous, and predominantly based on maize, with a low content of animal products. Children sometimes receive one daily meal through government-funded feeding programmes that are operated by schools in periods of drought.

Recruitment and screening

We selected children who were enrolled in nursery and the first year of primary school. Four weeks before the intervention we began to give children a target daily amount of cooked unfortified flour to assess the acceptability of the fortification vehicle and compliance with consumption of the target quantity. Three weeks before the trial we screened 528 children for eligibility. Our criteria for inclusion were age 3-8 years and consumption of at least 50% of the target amount of cooked unfortified flour during the month-long run-in period. Children with chronic disease, overt severe malnutrition, mental disability, or haemoglobin concentrations <70 g/L were excluded. All children were examined by a clinical officer. Those who had fever or other signs of malaria, and who tested positive for malaria were given sulfadoxine-pyrimethamine before the start of the intervention. Children who became ill during the intervention period were treated or referred according to guidelines from the Kenyan Ministry of Health. Ethical approval was granted by the ethical review committee of Kenya Medical Research Institute (KEMRI). We obtained signed informed consent from the parents of all enrolled children.

We took samples of venous blood at baseline into containers with Na-heparin (Becton-Dickinson; Temse, Belgium), and transferred blood into bottles with EDTA for haematological analysis and to detect P *falciparum* malaria antigenemia by dipstick test. Heparinised blood was separated in the field within one hour of collection; plasma was kept and transported in a cool box for 2-6 hours, and subsequently stored in liquid nitrogen (-196ºC) and below -70ºC. We also preserved blood sediment in buffer (AS1; Qiagen, Valencia, CA, USA) at ambient temperatures for subsequent α-thalassaemia genotyping. We measured childrens' height to the nearest 1 mm and weight to the nearest 0.1 kg (Leicester stadiometer and Tanita BWB800 scale, respectively; Chasmors, London, UK). The scales were calibrated daily with standard weights.

Procedures

The fortification vehicle consisted of *uji*, a porridge of maize flour cooked in water, and sweetened with sugar. The target daily intake was 700 mL *uji* (containing 100 g flour) for children aged 3-5 years, and 1,000 mL *uji* (containing 150 g flour) for children aged 6-8 years. This target amount was provided daily to each child in two equally divided portions. We estimated that addition of iron at a low dose of 28 mg/kg flour and a high dose of 56 mg/kg flour would provide 20% and 40%, respectively, of the daily iron requirements for children aged 3-5 years; and 18% and 36%, respectively, of the requirements for children aged 6-8 years. In this calculation, we assumed that 5% of the fortificant iron would be absorbed.

Both fortified and unfortified whole maize flour for *uji* were produced and coded at Unilever Kenya, Nairobi. Flour was fortified with NaFeEDTA (Akzo Nobel, Netherlands) at 56 mg or 28 mg iron per kg flour, or electrolytic iron (Food Chemicals Codex V-grade; Industrial Metal Powders, Pune, India) at 56 mg/kg flour. The electrolytic iron fortificant was identical to that specified for mandatory addition to flour in South Africa. Flour fortified with iron was also supplemented with a nutritional premix (Roche, now DSM Nutritional Products, Basel, Switzerland) containing vitamin A (2,500 µg/kg), thiamin (3·5 mg/kg), riboflavin (4·0 mg/kg) and niacin (45·0 mg/kg). We measured the mean particle diameter of electrolytic iron particles along three orthogonal axes by laser beam diffraction (Rhodos, Sympatec, Clausthal-Zellerfeld, Germany); the median particle size was 34 µm (10th-90th-percentiles:

14-62 µm). The morphology of the electrolytic iron particles was examined by scanning electron microscope is shown in **figure 2.1**.

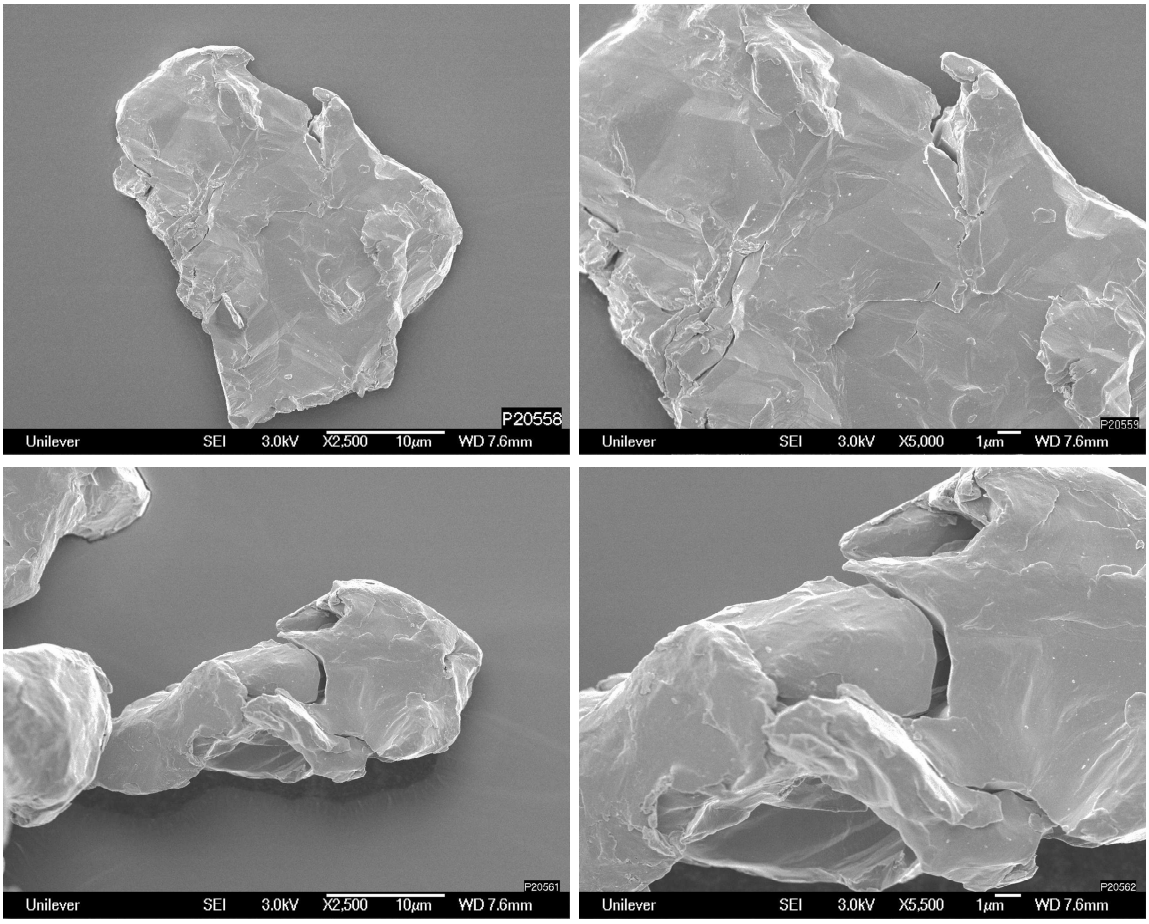

Figure 2.1

Morphology of electrolytic iron used in this study, as determined by scanning electron microscope.
Panels on the right are details from panels on the left, taken with greater magnification.

Each flour type was labelled with colour-coded packaging. This code was withheld from participants and investigators until all data had been collected and statistical analyses had begun. Flour types could not be visually distinguished, even in the cooked product. Eligible children were randomly allocated to one of four treatment groups, which were colour-coded to correspond to the flour packaging. The allocation code was generated by simple randomisation, using a table of random numbers by one of us (HV) who was not involved in the screening and enrolment.

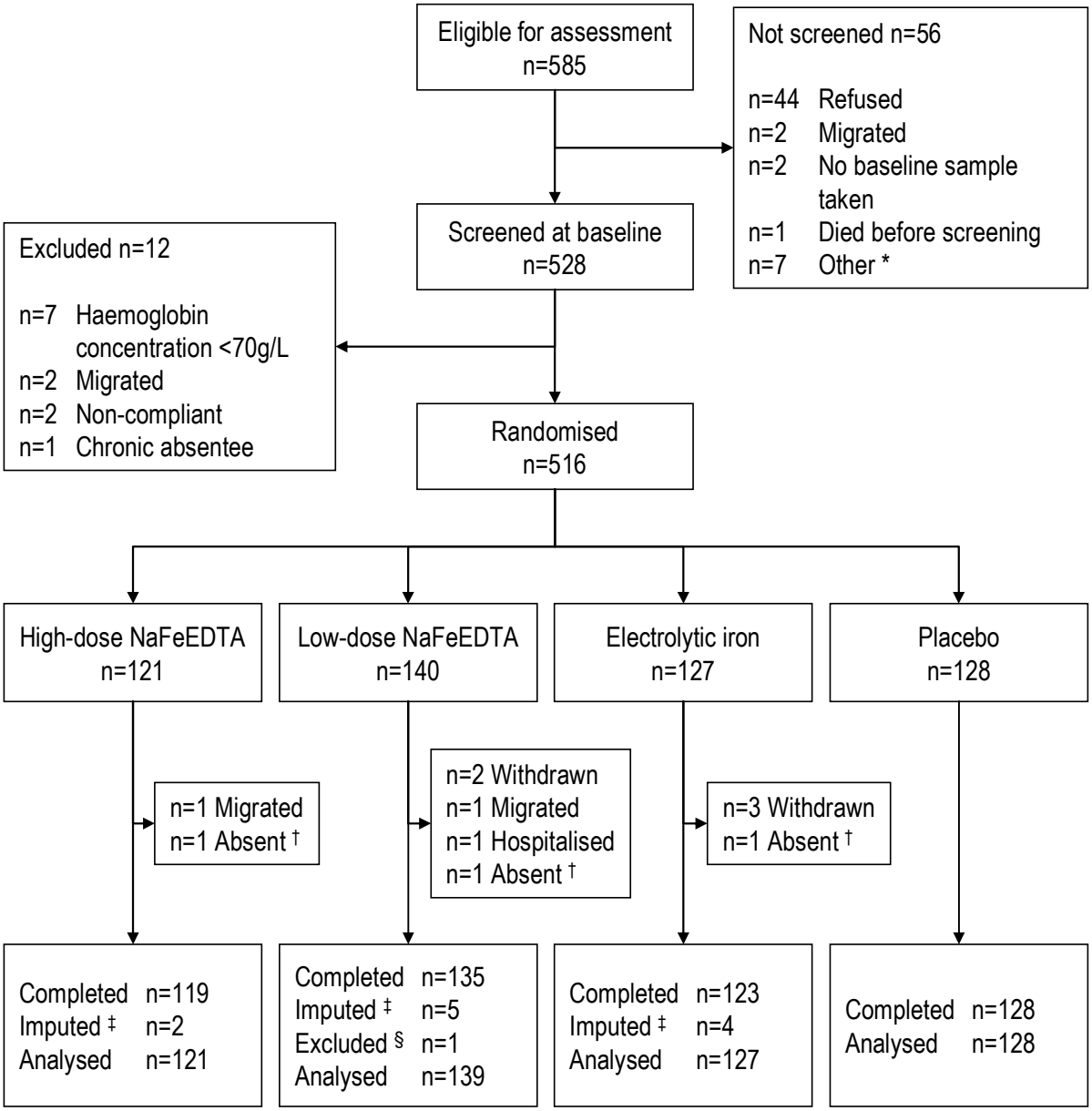

Figure 2.2 Study profile

*One child refused to attend school, three children were absent at screening, one child's parent was absent at screening, two children were absent during the school vacation; † absent at time of collection of final blood specimen; ‡ group mean or median values; § abnormal iron status indicators associated with sickle cell anaemia.

Table 2.1 Baseline characteristics of study participants, by intervention group

	High-dose NaFeEDTA	Low-dose NaFeEDTA	Electrolytic iron	Placebo
n	121	140	127	128
Age, years	5·9±1·4	5·9±1·5	5·8±1·4	6·0±1·4
Sex M/F, %/%	46/54	50/50	50/50	52/48
Haemoglobin concentration, g/L	109·9±11·6	110·8±10·9	110·9±11·3	114·0±10·7
Plasma ferritin concentration, µg/L				
All children [*]	30·0 [20·5, 45·0]	28·2 [16·0, 40·8]	26·0 [16·0, 44·0]	34·0 [20·0, 53·8]
Children without inflammation [*,†]	28·0 [19·5, 44·0]	27·0 [15·0, 39·0]	24·0 [15·0, 41·5]	32·0 [19·0, 53·0]
Plasma soluble transferrin receptor concentration, mg/L [*]	2·7 [2·2, 3·4]	2·7 [2·2, 3·3]	2·6 [2·2, 3·3]	2·5 [2·1, 3·1]
Plasma C-reactive protein concentration, mg/L[*]	1·0 [0·0, 3·0]	1·0 [0·0, 3·0]	0·0 [0·0, 2·0]	0·5 [0·0, 3·0]
Inflammation[‡], n [%]	8 [6·6]	13 [9·3]	6 [4·7]	9 [7·0]
Anaemia, n [%] [§]	71 [58·7]	84 [60·4]	75 [59·1]	60 [46·9]
Iron deficiency, n[%][‖]	18 [14·9]	27 [19·3]	21 [16·5]	12 [9·4]
Iron deficiency anaemia, n [%] [¶]	12 [9·9]	19 [13·6]	15 [11·8]	8 [6·3]
Malaria antigenemia, n [%]	62 [51·2]	65 [46·4]	62 [48·8]	65 [50·8]

(Table 2.1 continued)

	High-dose NaFeEDTA	Low-dose NaFeEDTA	Electrolytic iron	Placebo
Helminth infestation, n [%]				
Ascaris	3 [2·5]	1 [1·0]	2 [2·0]	0 [0·0]
Trichuris	0 [0·0]	0 [0·0]	2 [2·0]	2 [2·0]
Hookworm	14 [12·0]	16 [11·0]	11 [9·0]	13 [10·0]
Any infection	16 [13·2]	17 [12·1]	14 [11·0]	15 [11·7]
Positive sickling test result, n [%]	21 [17·4]	31 [22·1]	26 [20·5]	21 [16·4]
α- thalassaemia genotype, n [%]				
Data not available	3 [2·5]	10 [7·2]	6 [4·7]	6 [4·7]
Normal	42 [34·7]	42 [30·0]	29 [22·8]	37 [28·9]
Heterozygous	57 [47·1]	66 [47·1]	71 [55·9]	66 [51·6]
Homozygous	19 [15·7]	22 [15·7]	21 [16·5]	19 [14·8]
Height for age, z-score	-1·4±1·4	-1·5±1·3	-1·1±1·3	-1·4±1·2
Weight for height, z-score	-0·8±0·7	-0·8±0·7	-0·7±0·8	-0·7±0·6
Weight for age, z-score	-1·5±1·0	-1·5±1·0	-1·3±0·9	-1·4±0·9

Mean ± SD unless indicated otherwise; [*]median and interquartile range; [†]n=113, 126, 121 and 119, respectively; [‡]plasma C-reactive protein concentration >10 mg/L; [§]haemoglobin concentration<110 g/L and <115 g/L for children aged <5 years and ≥5 years, respectively; [‖]plasma ferritin concentration <12µg/L and <15 µg/L for children aged <5 years and ≥5 years, respectively; [¶]concurrent anaemia and iron deficiency

The intervention started for all children on the same day, immediately after the one-month run-in period. Children consumed *uji* in graduated mugs, at school, 5 days a week for 5 months. Teachers and fieldstaff supervised preparation of the porridge, and ensured that there was no cross-over between groups and that children did not share *uji*. The total amount of *uji* consumed by individuals was recorded after every meal.

We measured haemoglobin concentration in blood samples in a haematology analyzer (KX-21, Sysmex Corporation, Japan). At baseline, we used a sickling test, with the slide method and sodium metabisulphite as a reducing agent. We tested for current or recent malaria infection with a rapid, qualitative assay (Parachek Pf; Orchid Biomedical Systems, Goa, India) that detects *P. falciparum*-specific histidine-rich protein-2. We chose this test in preference to conventional microscopic examination of blood-films because it has a reported sensitivity and specificity of >90%.[15-17] Plasma concentrations of C-reactive protein, ferritin and soluble transferrin receptor were measured in the Netherlands (Meander Medical Centre, Amersfoort) on a Behring nephelometer (BN-Prospec, Dade-Behring, Marburg, Germany), with Behring kits and calibration and assay procedures. We used C-reactive protein as a marker of current inflammation, since infection-induced inflammation can increase ferritin concentrations independently of iron status.[18] Genotyping for α-thalassaemia was done by PCR.[19]All children received malaria chemotherapy 14 days before final blood collection to avoid inflammation-induced effects on iron status indicators.

The primary outcome was iron-deficiency anaemia. Secondary outcomes were iron deficiency; anaemia; and difference in haemoglobin concentration and plasma concentrations of ferritin and soluble transferrin receptor in the two treatment groups. We used WHO Guidelines[20] to define anaemia as haemoglobin concentration less than110 g/L or115 g/L for children younger or older than 5 years, respectively; iron deficiency as plasma ferritin concentration of less than 12 µg/L or 15µg/L for children younger or older than years respectively; iron-deficiency anaemia as concurrent anaemia and iron deficiency; and inflammation as 10 mg/L or more of C-reactive protein in plasma.

Statistical analysis

We analysed anthropometric Z-scores for all children with Epi-Info 2005 software (version 3·3·2), and analysed other data using SPSS software (version 12·0). The total amount of *uji*

consumed per group was calculated as a proportion of the amount assigned to every group during the intervention period.

Analysis was by intention-to-treat, except that we retrospectively excluded one child with abnormal iron status and sickle cell anaemia. Data were assessed for normality by visual examination of distribution plots, and were normalised as appropriate by log transformation. We obtained geometric means and corresponding CIs for absolute concentrations by calculation of exponents for log-transformed data.

Missing data were imputed blindly, before we started primary analysis, as the mean or median values from children without missing values in the same group. Because numbers of missing values were low, results from this simple approach are comparable with better but more complicated methods of imputation.[21] The method assumes that children whose data are imputed are representative for their group and not systematically different from children for whom a full set of data was obtained.[22]

We estimated treatment effects as group differences at the end of intervention relative to placebo. We used multiple linear regression to adjust for baseline concentrations of haemoglobin, ferritin, soluble transferrin receptor and C-reactive protein, and malaria antigenaemia. We decided *a priori* that adjusted estimates would be more relevant than crude estimates because even minor differences in prognostic factors at baseline could still lead to confounding.

For binary outcomes, we estimated prevalence ratios and corresponding CIs using Cox regression analysis with constant time at risk.[23,24] This method is generally used for survival analysis; we used it to produce valid point estimates of prevalence ratios when adjusting for baseline factors. However, we are aware that it can produce inflated estimates of SEs and CIs.[25] We converted prevalence ratios obtained by Cox regression, and effect estimates obtained from log-transformed data, to percentage differences relative to placebo. We analysed subgroups to assess whether the magnitude of the treatment effect was affected by iron deficiency or iron-deficiency anaemia at baseline, malaria antigenaemia at baseline, α-thalassaemia genotype, or the result of the sickling test. This trial is registered with ClinicalTrials.gov, number NCT00386074.

Table 2 .2 Effect on iron status of consuming flour fortified with electrolytic iron (56mg/kg), and 28 mg/kg NaFeEDTA (low dose) and 56 mg/kg NaFeEDTA (high dose).

	High dose NaFeEDTA	Low dose NaFeEDTA	Electrolytic iron	Placebo
n	121	139	127	128
Haemoglobin concentration, g/L [*]	117·2±8·5	114·7±8·8	112·2±9·9	115.7±9.7
Effect of intervention:				
Crude	1·6 [-0·7 to 3·8]	-0·7 [-2·9 to 1·5]	-3·5 [-5·7 to -1·2]	Reference
Adjusted [†]	4·0 [2·3 to 5·6]	1·3 [-0·3 to 2·8]	-1·3 [-2·9 to 0·3]	Reference
Plasma ferritin concentration, µg/L [‡]	35·0 [24·5, 47·0]	28·0[20·0, 39·0]	23·0 [13·0, 32·0]	23·0 [16·0, 36·0]
Effect of intervention: [§]				
Crude	54% [33% to 78%]	23% [7% to 41%]	-3% [-16% to 12%]	Reference
Adjusted [†]	67% [49% to 89%]	36% [21% to 53%]	6% [-5% to 20%]	Reference
Plasma soluble transferrin receptor concentration, mg/L [‡]	2·3 [1·9, 2·7]	2·4 [2·0, 2·8]	2·6 [2·1, 3·2]	2·5 [2·1, 3·1]
Effect of intervention: [§]				
Crude	-11% [-17% to -5%]	-8% [-14% to -2%]	4% [-3% to 11%]	Reference
Adjusted [‡]	-15% [-19% to -11%]	-12% [-16% to -8%]	0% [-4% to 5%]	Reference
Anaemia, n [%] [?]	38 [31·4]	58 [41·7]	65 [51·2]	48 [37·5]
Effect of intervention: [¶]				
Crude	-16 [-45 to 28]	11 [24 to 63]	36 [-6 to 98]	Reference
Adjusted [†]	-36 [-58 to -1]	-2 [-33 to 44]	12 [-23 to 63]	Reference

(Table 2.2 continued)

	High dose NaFeEDTA	Low dose NaFeEDTA	Electrolytic iron	Placebo
Iron deficiency, n [%] **	3 [2·5]	14 [10·0]	33 [26·0]	27 [21·1]
Effect of intervention: ¶				
Crude	-88 [-96 to -61]	-52 [-75 to -9]	23 [-26 to 105]	Reference
Adjusted †	-91 [-97 to -49]	-70 [-85 to -40]	1 [-40 to 69]	Reference
Iron deficiency anaemia, n [%] ††	2 [1·7]	12 [8·6]	27 [21·3]	13 [10·2]
Effect of intervention: ¶				
Crude	-84 [-96 to -28]	-15 [-61 to 86]	109 [8 to 306]	Reference
Adjusted †	-89 [-97 to -49]	-48 [-77 to 20]	59 [-18 to 209]	Reference

Mean [95%CI] unless indicated otherwise. Treatment effects are measured as group differences (continuous outcomes) relative to placebo. All analyses are by intention to treat.

* Mean ± SD

† Adjusted for baseline factors: haemoglobin concentration, plasma concentrations of ferritin, soluble transferrin receptor and C-reactive protein, and malaria antigenaemia

‡ Median, interquartile range

§ Values indicate difference between groups, expressed as a percentage relative to the placebo group, obtained by exponentiation of effect estimates from log-transformed data

‖ Haemoglobin concentration <110 g/L and <115g/L for children aged <5 years and ≥5 years, respectively

¶ Values indicate percentage difference in prevalence as compared with placebo [95%CI], obtained by conversion of prevalence ratios from Cox regression with constant time at risk (see Subjects and methods)

** Plasma ferritin concentration <12 µg/L and <15µg/L for children aged <5 years and ≥5 years, respectively

†† Concurrent anaemia and iron deficiency

Results

505 completed the study (**figure 2.2**). Values for iron status indicators were imputed for one child whose baseline plasma sample was lost and for 11 children who either discontinued the intervention or were absent at the end of intervention. We did not know the α-thalassaemia genotype for 25 children, because two blood samples were spilled during transportation, five samples were lost, seven were not clearly labelled, and 11 samples were not obtained because children were absent at the final survey.

Table 2.1 shows the baseline characteristics of children in the four groups. Haemoglobin concentrations were highest in the placebo group. Almost half the children (49%) had current or recent malaria infection. 290 (56%), 78 (15%), and 54 (11%) children had anaemia, iron deficiency and iron-deficiency anaemia, respectively. The prevalence of children who were heterozygous and homozygous for α-thalassaemia was 260 (49%) and 81 (16%), respectively. 19% of all study participants had a positive result for the sickling test. Of the three worm infections assessed, hookworm was the most often detected.

At baseline, both malaria antigenaemia and a homozygous genotype for α-thalassaemia were associated with low haemoglobin concentrations (mean difference -5·2 g/L, 95%CI -3·4 to -7·0 g/L) and (-8·8 g/L, -6·0 to -11·6 g/L), respectively. A heterozygous genotype for α-thalassaemia was not associated with noticeably low concentrations of haemoglobin (data not shown).

Groups assigned to receive *uji* fortified with high-dose NaFeEDTA, low-NaFeEDTA, electrolytic iron and unfortified flour consumed 92%, 89%, 90% and 93%, respectively, of the total amount given to them during the intervention period. At the end of the intervention few children had inflammation, which was of low degree: the median concentration of C-reactive protein in all groups was 0·0 mg/L with interquartile ranges of 0·0-1·0 mg/L in the two NaFeEDTA groups, 0·0-2·0 mg/L in the electrolytic iron group and 0·0-0·8 mg/L in the placebo group.

The prevalence of iron-deficiency anaemia in children who consumed flour fortified with high-dose NaFeEDTA was almost 90% lower than in controls (**table 2.2**). The prevalence of iron-deficiency anaemia in children given low-dose NaFeEDTA was about half that in those given placebo, although the 95% CI was compatible with a 20% increase in iron-deficiency anaemia.

Iron-deficiency anaemia did not change in the group assigned flour fortified with electrolytic iron.

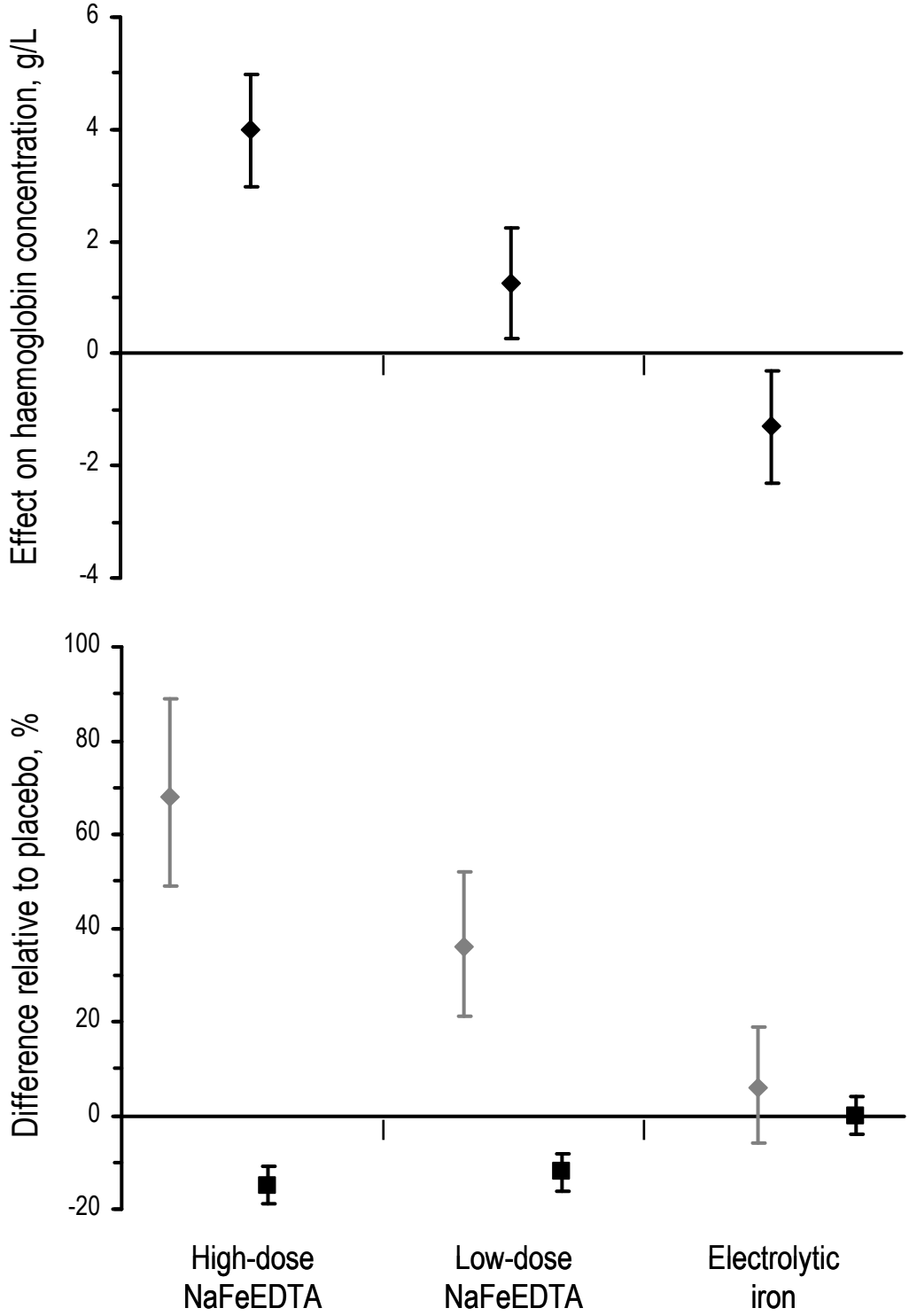

Figure 2.3

Effect on concentrations of haemoglobin (top panel), plasma ferritin (bottom panel; black marker) and plasma soluble transferrin receptor (bottom panel; grey marker) of consuming flour fortified with NaFeEDTA (56 mg/kg and 28 mg/kg), and electrolytic iron (56 mg/kg). Line bars indicate 95% CIs.

Consumption of flour fortified with high-dose and low-dose NaFeEDTA caused modest improvements in haemoglobin concentration compared with placebo (**figure 2.3**). In both groups given NaFeEDTA, plasma concentrations of ferritin increased and soluble transferrin receptor decreased (figure 2.3). By contrast, fortification with electrolytic iron did not affect any of these three iron-status indicators (figure 2.3). Prevalence of anaemia was reduced only for the group given high-NaFeEDTA (**figure 2.4**). The prevalence of iron deficiency was 91% lower in the high-dose NaFeEDTA group and 70% lower in the low-dose NaFeEDTA group, but did not change for children given electrolytic iron. Prevalence of iron-deficiency anaemia was reduced by 89% in the high-dose NaFeEDTA group and by 48% in the low-dose NaFeEDTA group, but was more prevalent in children given electrolytic iron than those given placebo.

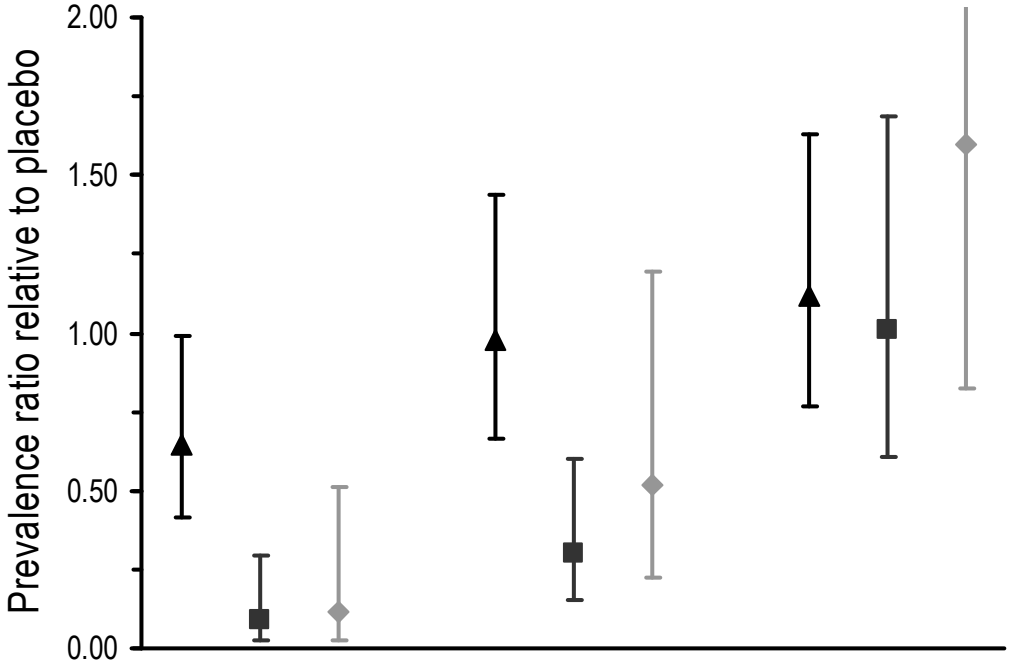

Figure 2.4

Effect on anaemia, iron deficiency and iron deficiency anaemia of consuming flour fortified with high- and low-dose NaFeEDTA (56 mg/kg and 28 mg/kg; black triangular and square markers, respectively), and electrolytic iron (56 mg/kg; grey marker). Upper confidence limit for iron deficiency anaemia: 3·1%.

Children with iron deficiency or iron-deficiency anaemia at baseline benefited more from fortification with NaFeEDTA than children without such deficiencies (**figure 2.5**). In children with iron-deficiency anaemia at baseline, consumption of high-dose NaFeEDTA, low-dose NaFeEDTA and electrolytic iron changed haemoglobin concentration by 11·7 g/L,

8·5 g/L and -0.4 g/L, respectively, whereas for other children haemoglobin changed by 3·4 g/L, 0.5 g/L and -1.2 g/L (differences in effect estimates, 95%CI; 8.4 g/L, 2.3 g/L to 14.4 g/L; 8.0 g/L, 2.5 g/L to 13.6 g/L; 0.7 g/L, -5.1 g/L to 6.6 g/L). We noted similar effect modification for plasma ferritin and soluble transferrin receptor. Neither α-thalassaemia genotype, sickling test result nor malaria antigenaemia at baseline seemed to alter the effect of fortified flour consumption. We did not record any adverse events associated with NaFeEDTA consumption.

Results of an additional per-protocol complete-case analysis did not differ from those obtained with imputed values as reported here (data not shown).

Discussion

Consumption of whole maize flour fortified with high-dose NaFeEDTA reduced iron-deficiency anaemia, iron deficiency, and anaemia in Kenyan children. Our findings show that low-dose NaFeEDTA conferred protection against iron deficiency but not against iron-deficiency anaemia or anaemia. Electrolytic iron did not confer protection against any of these disorders. Consumption of both high- and low-dose NaFeEDTA improved the iron status of children, as indicated by their increased concentrations of haemoglobin and plasma ferritin, and decreased concentrations of soluble transferrin receptor. Treatment effects were most pronounced in children who had iron deficiency and iron-deficiency anaemia at baseline.

Haematological measurements at the end of intervention were valid indicators of iron status because all children received malaria chemotherapy 2 weeks before assessment to avoid inflammation-induced effects on iron-status indicators. We also regarded our effect estimates as accurate indicators of the true values, since we achieved good precision, small loss to follow-up (2%), good compliance and low likelihood of cross-over of treatments.

The lower than expected prevalence of iron deficiency in our study population did not bias our results. Because only a small proportion of anaemic children in our study were iron-deficient, protection against ID and IDA were more pronounced than protection against anaemia. Malaria and α-thalassaemia at baseline were also associated with reduced

haemoglobin concentrations, although these factors, in combination with iron deficiency, still do not fully account for the high prevalence of anaemia.

Iron status at baseline modified the effect of NaFeEDTA. In children with iron-deficiency anaemia, the effect of high-dose NaFeEDTA on haemoglobin concentration was more than three times greater than the effect in iron-replete children. Our findings accord with stable isotope studies that suggest that iron absorption becomes more effective as iron stores become depleted.[26] By contrast, even in the subgroup analysis, the treatment effect of electrolytic iron on iron status was negligible.

Malaria could have affected our findings in three ways. First, we regarded baseline infection as a potential confounder of treatment effects. Evidence for such confounding was absent, however, because adjustment for malaria antigenaemia did not change effect estimates. Second, baseline infection could have modified the magnitude of the treatment effect. Although we had no evidence for such effect modification, our study design did not allow us to exclude this possibility. Third, malaria episodes during the intervention could have mediated treatment effects -iron interventions can increase susceptibility to malaria,[27] and conversely malaria might reduce absorption of iron because of inflammation[28,29] If malaria reduces iron absorption, then iron interventions would improve iron status more in populations without malaria, and be less effective in populations with high endemic malaria.

We concluded that fortification of whole maize flour with electrolytic iron does not improve iron status, at least in the concentration and form used in this study. But this does not necessarily contradict the finding that electrolytic iron can improve iron status when consumed with low-extraction wheat flour.[6] Extraction reduces the phytate content of flour,[30] which might increase bioavailability of iron. Discrepancies in study results may furthermore be explained by differences in the morphology, but not the size of iron particles. We used iron particles with a median diameter of 34 μm (10th- 90th-percentile: 14-62 μm); those used by Zimmermann and co-workers[6] were of a similar size (28 μm [10-56 μm]; Zimmermann, Swiss Federal Institute for Technology and Wageningen University; personal communication). Although iron particles used in our study had similar morphological characteristics to those used by Zimmermann and co-workers (figure 2.1), we cannot exclude the possibility that the surface area to weight ratio was different.

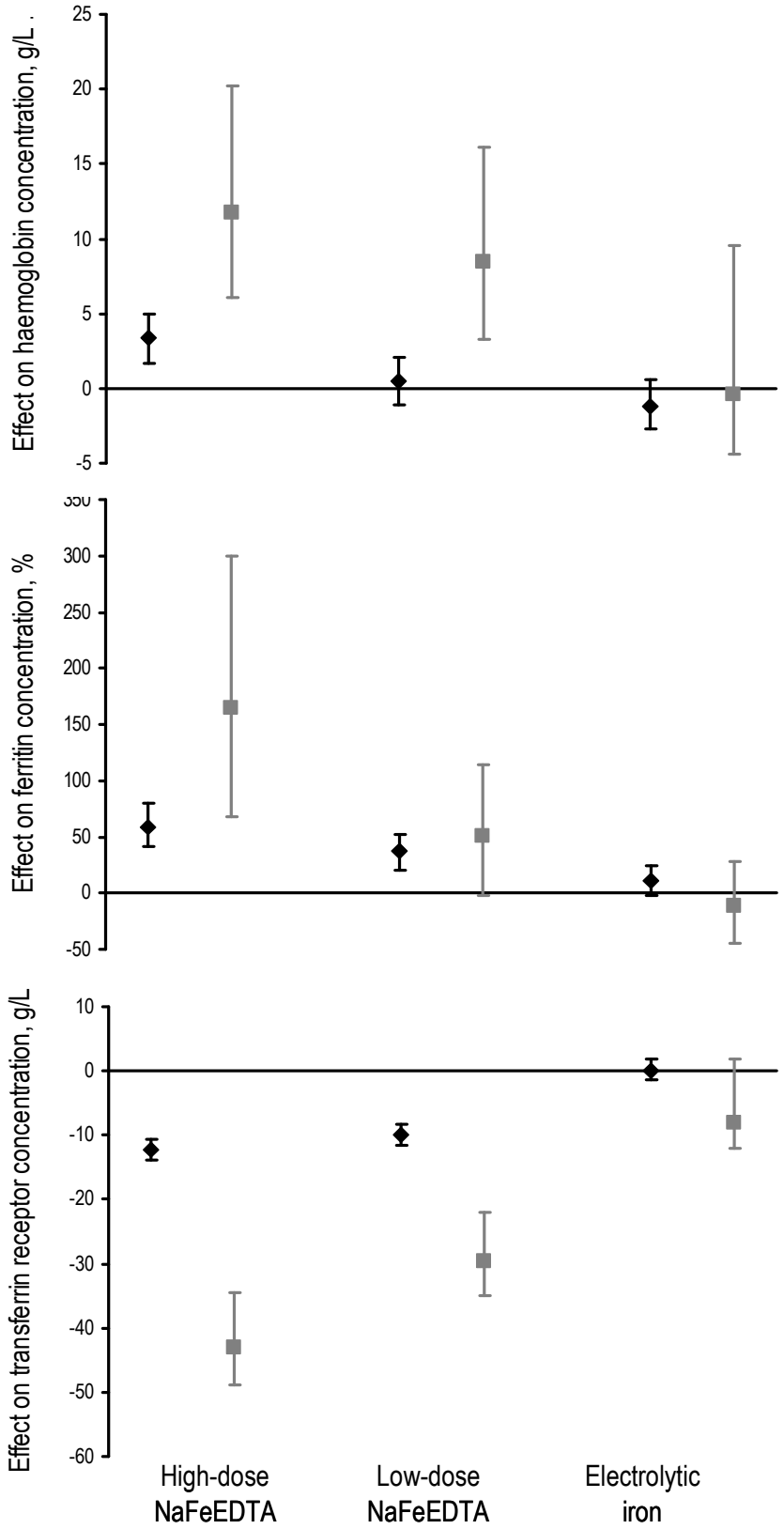

Figure 2.5

Effect of whole maize flour fortification with high- and low-dose NaFeEDTA (56 mg/kg and 28 mg/kg) and electrolytic iron (56mg/kg), in children who were iron sufficient at baseline (grey markers) or had iron deficiency anaemia at baseline (black markers); line bars indicate 95% CIs.

With continuous iron interventions, iron stores increase rapidly and after 2-3 years reach final plateau values that depend on the absorbable iron that is supplied.[31] Thus we think that continued intervention beyond 5 months would eventually have led to an even greater discrepancy between the treatment effects associated with NaFeEDTA and electrolytic iron.

The most relevant potential adverse effect of protracted administration of large quantities of an EDTA complex is zinc deficiency,[32] as reported in studies with daily intakes exceeding 1g EDTA/kg body weight as Na_2EDTA.[33] However, fortification of food with NaFeEDTA seems to have no detrimental effect on the metabolism of zinc, copper, calcium, or magnesium, and might even enhance zinc status by improving zinc absorption. [32,34,35] The safety of NaFeEDTA at the doses used in our trial will need to be confirmed.

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Chapter 3

The effect of iron fortification on cognitive and motor function of school-aged Kenyan children

Andang'o Pauline EA, Holding PA, Verhoef H
Kok FJ, Ayah R, Mwaniki DL
Clive E West[†], Osendarp SJM

Submitted for publication

[†] Deceased

Abstract

Cognitive function in school-aged children is reportedly compromised by iron deficiency and may be corrected by large doses of supplemental iron. It is possible that lower doses given through fortification are beneficial.

The objective of this study was to assess the effect of daily consumption for a 5-month period of iron-fortified food on cognitive and motor function outcomes in Kenyan school children.

Children consumed whole maize flour either unfortified or fortified with iron as NaFeEDTA at 56 (high-dose) or 28 (low-dose) mg/kg flour or electrolytic iron at 56 mg/kg flour. Outcomes were assessed in 146 anaemic and 63 non-anaemic children aged 6-8 years and comprised spatial working memory, attention; and fine motor control and co-ordination as indicated by self-ordered pointing test, visual search and bead threading, respectively. Multiple linear regression was used to assess test scores relative to the control, adjusted for potential confounders measured at baseline.

No treatment effect on any of the test scores was evident. Consumption of high-dose NaFeEDTA, resulted in the following effects (95% CIs): self-ordered pointing test, total errors: -0.50 (-3.8, 2.8); visual search, efficiency: -18.9 (-34.2, 0.2); bead threading, total beads: -1.49 (-3.47, 0.50). Consumption of low-dose NaFeEDTA or electrolytic iron similarly showed no treatment effect on test scores.

We found no evidence that iron fortification, in the type and dosage levels used in our trial, improved attention, working memory or fine motor dexterity and control regardless of anaemia status at baseline.

Introduction

Iron deficiency is widespread in developing countries, and has been associated with poor cognitive function.[1,2] This suggests that a substantial proportion of children may not reach their full potential. The evidence indicates that iron interventions are most likely to improve outcomes in individuals with iron deficiency anaemia.[1] Mechanisms responsible for developmental delays or deficits are possibly a direct effect from anaemia causing a low oxygen delivery to the brain,[3,4] or specific effects of iron deficiency resulting in altered neurotransmitter function and myelination.[5-7]

Tasks involving memory, attention, problem solving and motor control are controlled by areas of the brain that are sensitive to iron.[5,6,8,9]. Most of the evidence of improvements in cognitive and motor function is based on therapeutic doses of iron. Daily supplementation doses as low as 15 mg, were shown to improve skills related to information processing in pre-school children.[10] Fortification interventions, which usually involve much lower doses, are a more cost-effective approach to improve the iron status of large proportions of populations. Reports from South Africa that fortification improved cognition in older children[11] and improved motor function in infants[12] are promising. These findings need to be replicated in studies specifically designed to assess functions affected by iron deprivation. Tests that assess specific aspects of cognitive and motor development may be more sensitive than tests of global function and thus provide better information on the effects of iron interventions on developmental functions.[2,10]

We aimed to assess cognitive and motor function in anaemic and non-anaemic children who participated in a five-month iron-fortification intervention trial to assess the efficacy of NaFeEDTA and electrolytic iron when used as fortificants in whole maize flour. In that study the iron status of children who received iron as NaFeEDTA improved whereas that of children who received electrolytic iron did not improve [13]. We expected, therefore, that children who received iron as NaFeEDTA would have better test scores than their peers who received electrolytic iron or unfortified flour.

Subjects and Methods

Subjects and study design

We assessed cognitive and motor function from November to December 2004 at the end of a trial with iron-fortified whole maize flour. Methods and results of this study have been described elsewhere.[13] The study was conducted among children aged 3-8 years attending four schools in Marafa, an administratively defined area in the hinterland of Malindi district in the coastal lowlands of Kenya. Marafa has the highest prevalence (22%) of underweight children (weight-for-age z-score < 2SD below reference weight of children of a similar age) in Malindi district.[14] The local population mostly comprises poor families from the Mijikenda group of tribes, living mainly from subsistence farming. Ethical approval was granted by Kenya Medical Research Institute Ethical Review committee.

Eligibility criteria

Children participating in our trial were eligible for assessment of cognitive and motor function if aged ≥ 6 years at the time of assessment; and consent for participation was given by parents or guardians. The age limit was set because the psychometric tests used were designed for children aged 6 years and above in this population (Holding, unpublished results). Taking into account that an effect of treatment on cognitive and motor function test scores was likely to be small in the given time period, and that effects in other trials were more pronounced in children who were anaemic, presumably due to iron deficiency,[1] we selected children based on their anaemia status at baseline. Thus a total of 154 anaemic children were eligible. A random sample of children (n=64) without anaemia was additionally selected to determine if the tests results depended on the anemia status at baseline.

Age was determined from official records such as birth certificates, birth notification or clinic cards. When none of these documents was available, a calendar of events was used to determine age or to verify age as reported by a relative. Trained research assistants measured height to the nearest 1 mm and weight to the nearest 0.1 kg (Leicester stadiometer and Tanita scale, model BWB800, respectively; Chasmors Ltd, London, UK). The scales were calibrated daily using standard weights

For 5 months prior to the assessment, the children consumed porridge prepared from whole maize flour that was unfortified (control) or fortified with NaFeEDTA at 56 mg iron/kg flour or 28 mg/kg, or with electrolytic iron at 56 mg/kg flour. Iron-fortified flour was fortified additionally with vitamin A (2500 µg/kg), vitamin B_1 (3.5 mg/kg), vitamin B_2 (4.0 mg/kg) and niacin (45.0 mg/kg). Two weeks before the end of intervention, children were treated for malaria to eliminate possible effects of malaria on iron status indicators.

Laboratory analyses

Haemoglobin concentration was measured in a haematology analyzer (KX-21, Sysmex Corporation, Japan). Plasma concentrations of C-reactive protein, ferritin and soluble transferrin receptor (sTfR) were measured in The Netherlands (Meander Medical Centre, Amersfoort) on a nephelometer (model BN-Prospec, Dade-Behring, Marburg, Germany), using Behring kits and calibration and assay procedures according to the manufacturer's instructions. Plasma concentration of C-reactive protein was determined as a marker of current inflammation, to take into account that infection-induced inflammation is known to increase ferritin concentrations independent of iron status [15]. Genotyping for α^+-thalassemia was done by PCR.[16] Current or recent infection with *Plasmodium falciparum* was assessed by dipstick test (Paracheck; Orchid Biomedical, Goa, India). This test has a reported sensitivity and specificity of more than 90% as compared to conventional microscopical examination of blood films.[17-19]

Cognitive and motor function tests

Self Ordered Pointing Test: In this test, participants are presented with a booklet of 36 pages divided into four sections – 6 pages with 6 pictures each, 8 pages with 8 pictures each, 10 pages with 10 pictures each, and 12 pages with 12 pictures each. The overall layout of each page remains constant, with each picture appearing only once on each page, although the position of the pictures vary randomly on each page. In each section a child is instructed to touch a picture on each page, to touch each picture not more than once, and to not touch the same position more than twice. Three consecutive trials of each section are administered. Pictures included were prepared locally and tested in a pilot exercise involving children attending local primary and nursery schools in neighbouring Kilifi District. Test scores were computed as the total number of errors across all trials. This test is a measure of spatial working memory.[20] It assesses the capacity to organise visually presented information, retain

the information, initiate a sequence of responses, retain the responses and monitor the consequences of each response. The assessment was found to be associated with the development of frontal lobe function both in adults and in children.[21,22] The test was highly reliable (r = .73) in a study population of Kenyan children similar to ours (Holding, unpublished results).

Visual search: This task was adapted from one applied to school children in Jamaica [21]. In that population, the mean number of errors was 0.3%, and the test-re-test reliability was measured as r = .82. In Kenyan children the test-re-test reliability was .84 [23]. The task requires the child to identify a target picture embedded in rows of a selection of different pictures. Silhouette drawings are used, taken from the Word Order task,[23], which have been screened for familiarity and clarity. Performance was measured by both the accuracy and speed with which these pictures are marked with a pencil. A visual search efficiency score was calculated using the formula: [(1/time)/(√errors +1)]*100. The target skills of this task include: speed of information processing, spatial processing, sustained attention, selective attention, planning, and impulsivity.

Bead threading: The design is adapted from Henderson and Sugden[24] and has been used in various forms across many cultural groups.[25-28] The task requires the child to pick up one bead at a time and to thread it onto a string or shoe lace. Three trials are given, each lasting 30 seconds, in which the child is required to thread as many as s/he can in the time available. The thread is cleared between each trial. The score was calculated per child as the total number of beads that were threaded in three trials. This task measures bi-manual dexterity, fine motor control, and motor co-ordination.

The tests were administered by assessors who were trained to teach children at the primary education level. All assessors were fluent in both the local language (*Kigiriama*) and English, and underwent one week of training in test administration. In a pilot test, the assessors administered the tests under supervision to children not participating in the study.

Because hunger may affect test scores,[29] participants consumed a mug of porridge just before the tests were conducted. Tests were administered to individual children in quiet classrooms, with no visible visual distractions. The assessment environment was similar in all rooms

where testing was conducted. For each test, assessors provided detailed instructions to children in *Kigiriama*. Test sessions on average took 30 minutes per child. All children took the tests in the same order: self-ordered pointing test, visual search and finally bead threading. Assessors awarded scores as specified for each test at the time of test administration and tallied each child's score after the test session was completed. Tests were conducted over a period of 8 days, with each assessor administering tests to 5 children per day.

Data analysis

We defined anaemia as haemoglobin concentration <110 g/L and <115 g/L for children aged <5 years and ≥5 years, respectively; [30] iron deficiency: plasma ferritin concentration <12 µg/L or < 15µg/L for children ≤5 aged years or >5 years, respectively;[30] and iron deficiency anaemia: concurrent anaemia and iron deficiency; inflammation: concentrations of plasma C-reactive protein >10 mg/L. Z-scores for stunting, wasting and underweight were calculated using Epi-Info 2005 version 3·3·2 (CDC, Atlanta, GA, http://www.cdc.gov/epiinfo), and data was analysed using SPSS (v12·0 for Windows, SPSS Inc., Chicago, IL).

Data distributions were visually assessed. Most variables were normally distributed with the exception of the visual search efficiency score and ferritin concentration. Group imbalances in baseline characteristics were also assessed, separately for children with and without anaemia at baseline. We additionally assessed associations of test scores with baseline factors in pooled data of anaemic and non-anaemic children. Based on these comparisons, we adjusted for sex, age, height-for-age z-score, malaria infection, and inflammation in the final analyses.

Linear regression analysis was used to compare groups that received fortified and unfortified porridge regarding scores for each of the tests. The results of normally distributed test variables are expressed as difference in mean test scores relative to the control. Scores that were not normally distributed were log-transformed before analysis, and results exponentiated to express them in original units. Such results are expressed as percent difference relative to the control.

Table 3.1 Baseline characteristics of children, by anaemia status at baseline

	High-dose NaFeEDTA	Low-dose NaFeEDTA	Electrolytic iron	Control
Children with anaemia at baseline				
n	39	34	39	34
Age, years	6.9 ± 0.8	7.2 ± 0.9	6.8 ± 0.7	6.7 ± 0.8
Male, % (n)	39.0% (15)	50.0% (17)	46.0% (18)	62.0% (21)
Haemoglobin concentration, g/L	102.6 ± 8.9	106.6 ± 5.8	105.0 ± 8.6	106.4 ± 6.8
Plasma ferritin concentration, µg/L[1]	26.0 (14.0;45.0)	29.0 (16.5;37.0)	20.0 (14.0;32.0)	30.0 (16.8;48.8)
Iron deficiency, % (n)	26.0% (10)	21.0% (7)	26.0% (10)	21.0% (7)
Malaria, % (n)	67.0% (26)	65.0% (22)	69.0% (27)	65.0% (22)
Inflammation % (n)	15.0% (6)	9.0% (3)	5.0% (2)	6.0% (2)
Hookworm, % (n)	13.0% (5)	18.0% (6)	13.0% (5)	15.0% (5)
Weight, kg	17.6 ± 2.6	17.9 ± 2.4	18.2 ± 2.5	17.1 ± 1.8
Height, cm	111.1 ± 7.1	111.8 ± 6.0	112.3 ± 6.9	109.1 ± 5.4
Height for age z-score	-1.6 ± 1.4	-1.9 ± 1.1	-1.4 ± 1.1	-2.0 ± 0.8
Weight for height z-score	-0.9 ± 0.8	-0.8 ± 0.8	-0.7 ± 0.7	-0.7 ± 0.6
Weight for age z-score	-1.6 ± 1.1	-1.8 ± 0.9	-1.4 ± 0.9	-1.8 ± 0.6

(Table 3.1 continued)

	High-dose NaFeEDTA	Low-dose NaFeEDTA	Electrolytic iron	Control
Children with no anaemia at baseline				
n	14	13	18	18
Age, years	6.6 ± 1.2	7.0 ± 0.9	6.9 ± 0.8	6.7 ± 0.8
Male, % (n)	57.0% (8)	54.0% (7)	50.0% (9)	44.0% (8)
Haemoglobin concentration, g/L	120.0 ± 5.1	121.5 ± 5.6	123.9 ± 6.1	124.5 ± 6.5
Plasma ferritin concentration, µg/L[1]	27.5 (14.8;43.3)	27.0 (19.0;46.5)	34.5 (25.5;50.8)	30.5 (19.0;62.5)
Iron deficiency, % (n)	21.0% (3)	8.0% (1)	17.0% (3)	11.0% (2)
Malaria, % (n)	21.0% (3)	31.0% (4)	50.0% (9)	39.0% (7)
Inflammation % (n)	0.0% (0)	8.0% (1)	0.0% (0)	6.0% (1)
Hookworm, % (n)	21.0% (3)	8.0% (1)	6.0% (1)	0.0% (0)
Weight, kg	17.5 ± 2.0	19.1 ± 2.0	18.8 ± 2.4	18.0 ± 2.4
Height, cm	112.4 ± 6.0	114.2 ± 4.6	115.4 ± 4.6	112.4 ± 6.6
Height for age z-score	-1.4 ± 1.7	-1.6 ± 1.1	-1.0 ± 1.0	-1.4 ± 1.3
Weight for height z-score	-1.1 ± 0.6	-0.5 ± 0.6	-0.9 ± 0.8	-0.8 ± 0.6
Weight for age z-score	-1.7 ± 0.9	-1.4 ± 0.9	-1.3 ± 1.0	-1.4 ± 0.9

Results are given as mean ± SD, [1]median (25^{th};75^{th} percentiles), or prevalence % (n)

Table 3.2 Baseline factors associated with test scores, by anaemia status at baseline

	Children with anaemia at baseline			Children without anaemia at baseline		
	Self-ordered pointing test (total errors)	Visual search (efficiency)	Bead threading (total beads)	Self-ordered pointing test (total errors)	Visual search (efficiency)	Bead threading (total beads)
Ferritin concentration (log transformed)[1]	2.11 (-1.63;5.85)	0.09 (-0.04;0.21)	0.15 (-2.40;2.70)	-0.55 (-8.49;7.39)	0.09 (-0.22;0.41)	-1.90 (-7.41;3.60)
Haemoglobin concentration, g/L[2]	0.01 (-0.15;0.17)	0.20(-.50;1.21)	0.08 (-0.03;0.18)	-0.33 (-0.62;-0.04)	0.00 (-0.01;0.01)	0.09 (-0.12;0.29)
Age, years	-1.33 (-2.72;0.06)	0.10 (0.05;0.14)	2.6 (1.7;3.4)	-0.81 (-3.07;1.45)	0.05 (-0.04;0.14)	2.01 (0.52;3.49)
Weight for height z score	0.09 (-1.53, 1.71)	-0.00 (-0.06, 0.05)	-0.5 (-1.6, 0.6)	1.37 (-1.14, 3.89)	-0.12 (-0.22, -0.03)	-0.61 (-2.36, 1.15)
Height for age z score	-1.45 (-2.40;-0.50)	0.10 (0.05;0.11)	0.70 (0.02;1.4)	-0.52 (-1.90;0.86)	0.01 (-0.04;0.07)	0.39 (-0.57;1.35)
Sex[3]	1.32 (-0.97;3.62)	-0.01 (-0.09;0.06)	-1.1 (-2.7;0.5)	0.94 (-2.62;4.49)	0.04 (-0.11;0.18)	2.94 (-5.30;-0.59)
Malaria[4]	1.7 (-0.7;4.1)	-0.02 (-0.10;0.06)	1.4 (-0.2;3.1)	1.25 (-2.43;4.94)	-0.01 (-0.15;0.14)	0.58 (-1.99;3.14)

Results are indicated as difference relative to control (95% CI); adjusted for [1] baseline inflammation and malaria, or [2] baseline inflammation, malaria and α-thalassaemia genotype; test score of [3] males (difference of test scores from females test scores) [4] children with malaria (difference in scores from scores of children without malaria)

Results

Tests were administered to 209 of 218 children who were eligible for the study. Eight children who were anaemic at baseline were absent at the testing date; one child, who was not anaemic at baseline, had fever on the testing date and therefore did not participate.

At baseline, treatment groups differed in their haemoglobin concentration, plasma ferritin concentration and prevalence of iron deficiency, both in children with and without anaemia at baseline (**table 3.1**). Children who received electrolytic iron had higher weight-for-age z-scores and height-for-age z-scores than their counterparts in the other treatment groups. In children with anaemia at baseline, increments in age, and height-for-age z-scores were associated with better scores for all tests (**table 3.2**). In their peers without anaemia at baseline, associations with test scores were observed with age and height. In both anaemic and non-anaemic children, girls threaded more beads than boys. No associations were found between iron status indicators and test scores in children with or children without anaemia at baseline when these two subgroups were considered separately (table 3.2). Self-ordered pointing test scores were associated with baseline haemoglobin in the control group (data not shown).

Mean scores for self-ordered pointing tests and bead threading did not differ among the groups, neither was there evidence of an effect of iron treatment on any of the test scores (**table 3.3, figure 3.1**). However, general trends in self-ordered pointing test scores indicated more errors in children with anaemia at baseline than in their peers without anaemia at baseline. There were no evident trends indicating any relationship between treatment and bead threading scores in children with or without anaemia at baseline. Visual search efficiency was better in the control group, even after adjustment of potential confounders, both in children with and without anaemia at baseline.

Table 3.3 Effect of iron interventions on test scores, by anaemia status at baseline

	High-NaFeEDTA	Low-NaFeEDTA	Electrolytic iron	Control
Children with anaemia at baseline				
n	39	34	39	34
Self-ordered pointing test				
Score	19.8 ± 7.0	19.7 ± 7.0	20.7 ± 7.0	21.5 ± 8.0
Intervention effect				
Crude	-1.71 (-4.97;1.56)	-1.82 (-5.20;1.56)	-0.81 (-4.08;2.46)	Reference
Adjusted[1]	-0.50 (-3.78;2.78)	-0.47 (-3.82;2.87)	0.52 (-2.72;3.74)	Reference
Visual search				
Score[2]	0.2 (0.2;0.4)	0.2 (0.2;0.3)	0.3 (0.2;0.4)	0.2 (0.2;0.4)
Intervention effect				
Crude[3]	-6.9 (-27.6;19.4)	-6.0 (-27.6;21.6)	5.9 (-17.4;36.1)	Reference
Adjusted [1,3]	-18.9 (-34.2;0.2)	-21.8 (-36.9;-2.9)	-9.6 (-26.7;11.2)	Reference
Bead threading				
Score	26.9 ± 5.0	29.2 ± 5.0	27.6 ± 5.0	27.3 ± 4.0
Intervention effect				
Crude	-0.40 (-2.61;1.81)	1.85 (-0.43;4.13)	0.32 (-1.89;2.52)	Reference
Adjusted[1]	-1.49 (-3.47;0.50)	0.15 (-1.88;2.18)	-0.76 (-2.72;1.20)	Reference

(table 3.3continued)

Children with no anaemia at baseline				
n	14	13	18	18
Self-ordered pointing test				
Score	19.4 ± 7.5	22.9 ± 6.9	19.6 ± 7.1	17.1 ± 6.1
Intervention effect				
Crude	2.25 (-2.66;7.16)	5.81 (0.80;10.83)	2.44 (-2.15;7.04)	Reference
Adjusted[1]	1.94 (-3.31;7.19)	6.19 (0.96;11.4)	2.69 (-2.01;7.39)	Reference
Visual search				
Score[2]	0.3 (0.2;0.4)	0.2 (0.2;0.5)	0.3 (0.2;0.5)	0.3 (0.2, 0.5)
Intervention effect				
Crude[3]	-7.3 (-41.8;47.6)	-4.1 (-40.4;54.2)	-14.7(-44.7;31.8)	Reference
Adjusted [1,3]	-10.1 (-46.9;52.4)	-9.2 (-46.3;53.1)	-19.3 (-49.6;29.1)	Reference
Bead threading				
Score	27.9 ± 7.3	27.1 ± 3.6	27.9 ± 4.1	27.6 ± 4.4
Intervention effect				
Crude	0.25 (-3.30;3.80)	-0.53 (-4.16;3.09)	0.28 (-3.04;3.60)	Reference
Adjusted[1]	0.71 (-2.82;4.24)	-0.79 (-4.30;2.72)	-0.44 (-3.60;2.72)	Reference

Scores are indicated as mean score ± SD or [2]median score (25^{th}, 75^{th} percentiles)
Effects are indicated as mean difference in scores (95% CI) or [3]percent difference (95% CI) relative to the control
[1]Adjusted for sex and age, and baseline height-for-age z-score, malaria, inflammation, haemoglobin concentration and plasma ferritin concentration

Discussion

We found no evidence that daily intake for five months of iron-fortification foods had an effect on attention, working memory or fine motor control as assessed by visual search efficiency, self-ordered pointing test and bead threading, respectively.

Our findings are surprising because there is evidence from other contexts that iron interventions led to improvements in attention and memory in this age group, [10,31-33]. In our study, the iron status of children who received NaFeEDTA at high and low doses improved [13]. Hence we would have expected to see some differences in group test scores associated with iron treatment.

We selected tests that target specific constructs that are postulated to be associated with iron status [5,6,8,9]. Furthermore the tests were culturally appropriate for this population and for the age group included, and the performance of the testers was carefully assessed before the study. The tests have also been shown to have a high test-retest reliability in this population, are sensitive to the constructs tested [23] and have been applied in other studies in similar populations of African children [28], (Penny Holding, unpublished results). Study participants were selected from children who participated in a double-blind controlled trial to assess the efficacy of iron fortification. We adjusted for group imbalances that arose from the selection of this sub-group. Because tests assessing developmental outcomes are known to be affected by a variety of factors [34,35], we additionally adjusted for baseline imbalances in potential confounders.

The lack of evident effect in our study may have been caused by several factors. First, because we did not measure test scores at baseline, we cannot rule out residual confounding due to group differences in cognitive or motor performance that may have existed at baseline. Second, the dose of iron in our study was low and the duration of our study may have been inadequate to observe effects on developmental outcomes. Whilst supplementation trials have used higher doses of iron, a fortification trial in a similar age group that reported an effect of iron treatment on memory had a longer duration than ours [11].

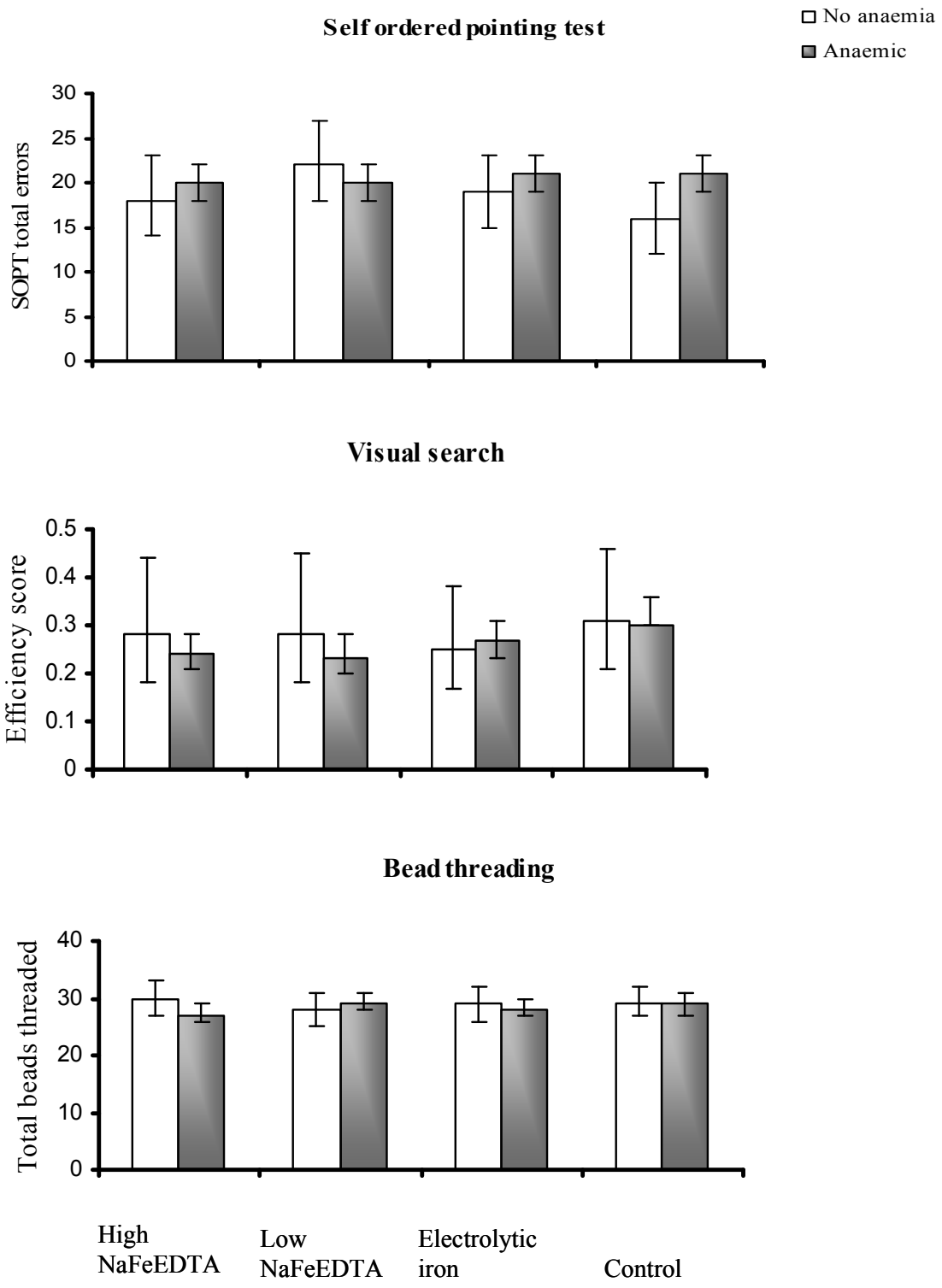

Figure 3.1

Adjusted test scores of children who were anaemic or non-anaemic at baseline indicated as scores of female children aged 7 years (average age) with average baseline concentrations of haemoglobin and ferritin and with average height for age and without malaria.

Third, we selected anaemic children in this study because we expected a substantial proportion of such children to be iron deficient. Improvements in cognition are more evident in individuals with iron deficiency anaemia [1]. Because anaemia in our study was largely due to malaria, however, and the prevalence of iron deficiency anaemia was smaller than expected, we may not have been able to detect effects. By contrast, in India, iron treatment using supplements improved in children with low haemoglobin at baseline;[31] although ferritin concentrations were not measured, anaemia in that study may have been largely due to iron deficiency.

Fourth, the absence of evidence in our trial may be because iron deficiency co-existed with other nutritional deficiencies that were not redressed by our intervention, and that are limiting for cognitive and motor development. We found that stunting and wasting were highly prevalent at baseline, and that these anthropometric indices were strongly associated with higher test scores after the intervention. Others have observed that nutritionally disadvantaged children are likely to have poorer developmental trajectories than children with better nutritional status [36,37]. Whereas there may be improvements in cognitive or motor function as a result of nutritional interventions that increase energy and iron intake in such children, they may continue to lag behind their better nourished counterparts [37,38]. Most studies have assessed the effects of iron treatment on cognitive or motor outcomes in anaemic but otherwise either less stunted or healthier children than those in our study sample [10,32,33]. The presence of stunting could also imply energy deficits or other nutritional deficiencies that may additionally affect developmental outcomes.

We found no evidence that iron fortification of whole maize flour with either NaFeEDTA or electrolytic iron, at the fortification levels used in our study, improved indicators of attention, spatial working memory or fine motor dexterity and control over a 5-month period. Evidence remains scarce that iron provides benefits beyond improved haematological indicators of iron status [7]and should be obtained in suitably designed randomised controlled trials.

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Chapter 4

The effect of iron fortification using NaFeEDTA on potential iron overload in Kenyan school children with α^{+}-thalassaemia

Andang'o PaulineEA, Osendarp SJM, Mbugi E
Kok FJ, Mwaniki DL, Ayah R, Kraaijenhagen R
Clive E West[†]
Verhoef H

Submitted for publication

[†]Deceased

Abstract

Suggestions that iron absorption may be increased in individuals with α^+-thalassaemia have raised concerns that iron interventions in such individuals can produce iron overload.

This study aims to assess to what extent α^+-thalassaemia genotype determines the magnitude of the effect on iron status of daily consumption for 5 months of maize flour fortified with iron.

490 children aged 3-8 years were randomly assigned to consume maize porridge that was unfortified or fortified with either high-dose NaFeEDTA (56 mg iron/kg flour), low-dose NaFeEDTA (28 mg/kg) or electrolytic iron (56 mg/kg). Primary outcomes were plasma concentrations of ferritin and transferrin receptor (TfR). Plasma ferritin concentrations, adjusted for the effect of inflammation and malaria, were considered to indicate possible iron overload when >90 µg. Modification by α^+-thalassaemia genotype of intervention effects was assessed by stratified and multiple regression analyses.

The prevalence of normal, heterozygote and homozygote genotypes were 31%, 53% and 16% respectively. Only 5 children (1%) had adjusted ferritin concentrations >90 µg/L. There was no evidence that α^+-thalassaemia influenced the magnitude of the fortification effect on plasma concentrations of ferritin or TfR. There was no evidence of interaction between α^+-thalassaemia genotype and treatment. Interaction estimate in the high NaFeEDTA relative to normal controls (% difference, 95% CI) was 0.19 (-0.08, 0.46) and –0.02 (0.04, 0.35)µg/L in heterozygotes and homozygotes, respectively.

Daily consumption for 5 months of maize flour fortified with NaFeEDTA improved iron status. We found no evidence that it resulted in iron overload, or that α^+-thalassaemia genotype influenced the response to iron fortification.

Introduction

Haemoglobin disorders such as the thalassaemias are common in the tropics. α^+-thalassaemia is particularly common in sub-Saharan Africa;[1,2] the Middle East, the Indian subcontinent, parts of Southeast Asia, Melanesia and in African Americans.[3] In heterozygous α^+-thalassaemia, one of the linked pair of α-globin genes in the haploid genome is deleted (α-/αα), whilst in the homozygous state, there are two deleted genes (α-/α-).[3]

In α-thalassaemia major, one of the more severe forms of thalassaemia, ineffective erythropoiesis is accompanied by increased iron absorption, which may result in iron overload.[3 4] Two studies have shown that transferrin receptor concentrations are raised in α^+-thalassaemia, indicating some degree of ineffective erythropoiesis.[5 6] In one of these studies, there was additional evidence of higher ferritin concentrations in thalassaemics compared to individuals with normal genotype.[6] The authors suggested that the raised concentrations of ferritin and transferrin receptor may indicate increased iron absorption in individuals with α^+-thalassaemia. Thus individuals with α^+-thalassaemia may benefit more from the interventions. On the other hand, increased iron absorption over a prolonged period might lead to iron overload. It is not clear, however, whether sustained iron intakes at levels used in fortification programs (30-60 mg iron/kg flour depending on type of iron and the nature of the food vehicle [7]) can be harmful, especially in individuals who are likely to absorb more iron than normal individuals.

We conducted a randomised controlled iron fortification trial with NaFeEDTA in children with a high prevalence of α^+-thalassaemia.[8] Because NaFeEDTA is highly bioavailable, in the present study we aimed to assess to what extent α^+-thalassaemia modified the effect of the intervention, and to what extent it led to risk of iron overload.

Subjects and methods

Subjects and study design

The current study was conducted in Malindi district, near the Kenyan coast. As elsewhere in sub-Saharan Africa, α^+-thalassaemia is predominantly due to a 3.7-kb deletion ($-\alpha^{3.7}$). Children aged 3-8 years were drawn from 4 schools in Marafa location of Malindi district, and individually randomised to daily consumption of porridge (*uji*) made from whole maize

flour, which had been either unfortified, or fortified with NaFeEDTA at 56 mg iron or 28 mg iron per kg flour. A third group received flour fortified with electrolytic iron (56 mg/kg flour), but results are not reported because, contrary to, NaFeEDTA we found no effect on iron status. Consumption of *uji* was supervised, with a target daily intake of 700 mL *uji* (containing 100 g flour) for children aged 3–5 years and 1000 mL *uji* (containing 150 g flour) for children aged 6–8 years. The intervention lasted 5 months. All children were treated for malaria 2 weeks before the end of the intervention to eliminate infection-induced inflammation on plasma ferritin concentration that are unrelated to iron stores. Details of the study design are provided elsewhere.[8]

Laboratory analyses

Haemoglobin was analysed in a haematology analyzer (KX-21, Sysmex Corporation, Japan). Plasma concentrations of C-reactive protein, ferritin and soluble transferrin receptor (sTfR) were measured in The Netherlands (Meander Medical Centre, Amersfoort) on a nephelometer (model BN-Prospec, Dade-Behring, Marburg, Germany), using Behring kits and calibration and assay procedures according to the manufacturer's instructions. Plasma concentration of C-reactive protein was determined as a marker of current inflammation, to take into account that infection-induced inflammation is known to increase ferritin concentrations independent of iron status.[9] Genotyping for α^+-thalassaemia was done by PCR.[10] Current or recent infection with *Plasmodium falciparum* was assessed by dipstick test (Paracheck; Orchid Biomedical, Goa, India). This test has a reported sensitivity and specificity of more than 90% relative to microscopy.[11-12]

Data analysis

We included 490 children of 516 in the analysis, and excluded 25 children for whom genotype could not be determined because of missing samples, mislabelling, or technical problems with the PCR tests. We also excluded one child because of abnormal iron status indicators and a reported history of attending a special clinic for sickle cell anaemia.

Iron overload causes high plasma concentrations of ferritin; concentrations within the normal range provide good evidence against iron overload.[13] Plasma ferritin concentrations of 90 µg/L are the upper value of the normal range[14] observed in apparently healthy American children aged 4-8 years.[15] Thus we considered plasma ferritin concentrations >90 µg/L as an indication for possible iron overload. Anaemia was defined as haemoglobin concentrations

<110 g/L in children <5 years and <115 μg/L in children ≥5 years.[16] Normal ferritin concentrations were defined as <12 μg/L in children <5 years and <15 μg/L in children ≥5 years.[16] Inflammation was defined as C-reactive protein concentrations >10 mg/L.

Data was analysed using SPSS (version 12·0 for Windows, SPSS Inc., Chicago, IL). We inspected the distribution of plasma ferritin concentration at baseline and at the end of the intervention to detect possible iron overload as defined above. Because malaria and inflammation affect plasma ferritin concentrations independent of iron status we assessed baseline ferritin using two methods: restricting the assessment to children who did not have malaria infection or inflammation; or using ferritin concentrations that were corrected for malaria and inflammation. To obtain such adjusted ferritin concentrations we used linear regression to determine the effect of malaria and inflammation on log ferritin. The effect estimates obtained were used to correct log ferritin concentrations of individual children. The corrected log-ferritin concentrations were exponentiated to obtain adjusted ferritin concentrations. Because malaria but not inflammation has been reported to be associated with sTfR;[17-19] we also adjusted sTfR concentrations for malaria, using similar methods as described above for ferritin concentrations.

We used multiple linear regression analysis to assess the associations between α^+-thalassaemia and iron status, both before and after the intervention. In the latter case, we adjusted for imbalances at baseline in iron status indicators, inflammation and malaria. We used stratified and direct multivariate analysis to assess the influence of α^+-thalassaemia on the magnitude of the effect of the intervention on iron status. In both analyses, we adjusted for baseline differences in plasma concentrations of ferritin and sTfR; inflammation and malaria. Because any adverse effects would most likely be identified in the group that received high-NaFeEDTA, we only report the results for this intervention. For all regression analyses, outcome variables that were not normally distributed (concentrations of ferritin and sTfR) were log-transformed and regression coefficients exponentiated to obtain geometric mean concentrations. Such results are reported as percent difference in concentration relative to that in children with normal genotype.

Table 4.1 Indicators of iron status, malaria and inflammation at baseline, by α^+-thalassaemia genotype

	α^+-thalassaemia genotype		
	Normal ($\alpha\alpha/\alpha\alpha$)	**Heterozygous ($-\alpha/\alpha\alpha$)**	**Homozygous ($-\alpha/-\alpha$)**
All children, n	150	260	80
Children without malaria and without inflammation (n)	51.3% (77)	50.0% (130)	40.0% (32)
Malaria (n)	46.7% (70)	48.8% (127)	57.5% (46)
Inflammation[1] (n)	7.3% (11)	5.0% (13)	10.0% (8)
Ferritin concentration, µg/L			
All children[2]	30.5 (3.0, 441.0)	26.5 (4.0, 289.0)	32.5 (5.0, 144.0)
Restricted to children without malaria or inflammation	25.0 (3.0, 91.0)	24.0 (4.0, 81.0)	26.0 (5.0, 126.0)
Adjusted for malaria and inflammation	24.6 (3.0, 87.5)	23.0 (3.2, 181.2)	26.0 (4.0, 117.0)
All children[3]	29.2 (25.8, 33.0)	26.7 (24.4, 29.2)	32.1 (27.4, 37.5)
Ferritin concentration >90 µg/L (n)			
All children	4.7% (7)	3.5% (9)	7.5% (6)
Restricted to children without malaria or inflammation	1.3% (1)	0.0% (0)	6.3% (2)
Adjusted for malaria and inflammation	0.0% (0)	0.8% (2)	3.8% (3)
Soluble transferrin receptor concentration, mg/L[3]			
All children	2.6 (2.5, 2.8)	2.8 (2.7, 2.9)	2.8 (2.7, 3.0)
Adjusted for malaria	2.5 (2.3, 2.6)	2.6 (2.5, 2.7)	2.6 (2.5, 2.8)

[1] Plasma C-reactive protein concentration > 10 mg/L;
[2]Median (minimum value, maximum value) or [3]geometric mean (95% CI)

Table 4.2 Associations between α^+-thalassaemia genotype and plasma indicators of iron status (concentrations of ferritin and soluble transferrin receptor), at baseline and at the end of interventions[1]

	Baseline, all children	End of intervention[1]		
		High-dose NaFeEDTA	Low-dose NaFeEDTA	Control (unfortified flour)
Ferritin %				
Normal genotype	Reference	Reference	Reference	Reference
Heterozygotes	-5.2 (-17.3, 8.6)	6.9 (-9.8, 26.7)	-5.6 (-21.3, 13.2)	-9.3 (-25.6, 10.4)
Homozygotes	11.9 (-6.9, 34.6)	2.1 (-22.2, 34.0)	2.6 (-20.6, 32.5)	-7.0 (-19.4, 42.0)
Soluble transferrin receptor %				
Normal genotype	Reference	Reference	Reference	Reference
Heterozygotes	4.6 (-2.3, 12.0)	2.7 (-4.8, 10.8)	5.0 (-1.6, 12.1)	3.4 (-2.4, 9.6)
Homozygotes	7.7 (-1.8 18.1)	7.0 (-3.7, 18.9)	13.6 (3.9, 24.1)	3.5 (-4.6, 12.3)

[1] Effect estimates are expressed as % difference in geometric mean concentrations (95% CI) relative to children with an normal genotype, obtained by multiple linear regression

[2] Estimate adjusted for pre-selected baseline indicators (malaria, inflammation, plasma concentrations of ferritin and soluble transferrin receptor)

Results

At baseline, iron status was slightly better in the control group than in the groups that received NaFeEDTA our report published elsewehere[8]. About half the children were infected with malaria, and only a small proportion of children had raised C-reactive protein concentrations (**table 4.1**). Plasma concentrations of ferritin and sTfR were similar across genotypes (table 4.1). When the analysis was restricted to those without malaria or inflammation, only 3 children (1.3%) had ferritin concentrations above 90µg/L. By comparison, when using ferritin concentrations that had been adjusted for malaria and inflammation, only 5 children (1.0%) were detected with ferritin concentrations >90 µg/L. The highest ferritin concentrations observed were 126 µg/L (restricted analysis) and 181 µg/L (adjusted for malaria and inflammation). These cases concerned children with homozygous and heterozygous genotypes, respectively. At the end of the intervention, the highest ferritin value observed was 108 µg/L (a child with heterozygous genotype; **figure 4.1**). Results of analyses to assess whether ferritin and sTfR concentrations depended on alpha thalassaemia genotype at baseline or at the end of the intervention are presented in **table 4.2**. There was no evidence that α^+-thalassaemia genotype was associated with plasma ferritin concentration, or that it was associated with plasma sTfR concentration. However, plasma sTfR concentrations were slightly but consistently higher in children with α^+-thalassaemia than their peers with normal genotype.

Response to the iron intervention

Figure 4.2 shows the effect of high-dose NaFeEDTA on ferritin concentration, stratified by α^+-thalassaemia genotype. Ferritin concentration at the end of the intervention was similar across α^+-thalassaemia genotypes. In children with normal, heterozygous or homozygous genotypes, high-dose NaFeEDTA led to increases in ferritin concentration by 54% (95% CI: 28% to 86%), 84% (24% to 121%) and 43% (1% to 101%), respectively. There was no evidence of differences in the effect of the intervention between genotypes, in the stratified analysis. Also when assessed by direct multivariate analysis, there was no evidence that α^+-thalassaemia genotype modified the effect of high-dose NaFeEDTA. Percent difference of effects of high-dose NaFeEDTA in heterozygotes and homozygotes, relative to children with a normal genotype were 0.19 (- 0.08, 0.46) and -0.02 (-0.04, 0.35) g/L, respectively.

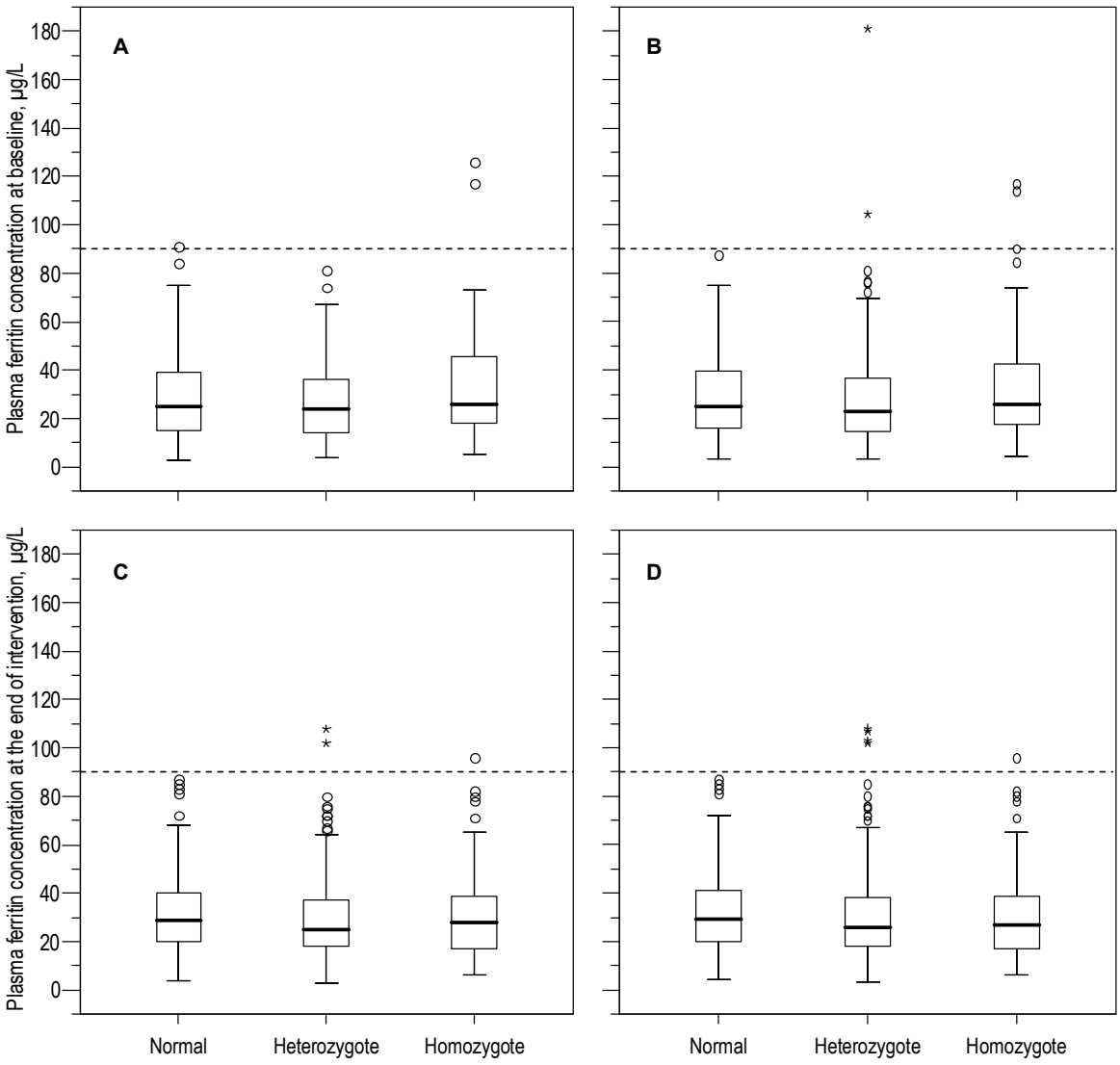

Figure 4.1

Distribution of ferritin concentration in children at baseline (upper panels) and at the end of the intervention (lower panels). Panel A: Restricted to children with no malaria or inflammation; B: ferritin adjusted for malaria and inflammation; C: restricted to children without malaria or inflammation in the group that received high NaFeEDTA; D: all children in the group that received high NaFeEDTA.

Discussion

We found no evidence that α^+-thalassaemia genotype was associated with elevated ferritin concentrations, either before or after the iron intervention. In addition, we found no evidence that children with α^+-thalassaemia responded to the iron intervention differently from children with normal genotype.

Plasma ferritin concentrations indicate the level of body iron stores, but its interpretation is difficult in the presence of inflammation. Plasma sTfR concentrations have been used to validly indicate the level of the iron deficit, even in the presence of inflammatory conditions[20]. Plasma sTfR concentrations are also influenced, however, by the level of erythropoiesis. Malaria may increase erythropoiesis because it necessarily leads to haemolysis, which stimulates the production of erythropoietin. On the other hand, malaria may lead to a decreased erythropoiesis through immune suppression[21]. Thus there is no way to accurately determine iron status in individuals with malaria.

We used three approaches to deal with this problem. First, when assessing iron status by ferritin concentrations status at baseline, we restricted the analysis to children without inflammation and without malaria. Because this method leads to a reduced sample size, it has the disadvantage that the precision of effect estimates is reduced. In addition, it may cause bias in estimating the iron status of populations if iron status is truly associated with infection or malaria. Second, when analysing baseline data, we adjusted ferritin concentrations of individual children for the effects of both malaria and inflammation. This method can result in residual error because it assumes, incorrectly, that the effects of malaria and inflammation in individual children are estimable from the group. In our study population, however, the results of the two methods were comparable, although the absolute number of children identified as having high ferritin concentrations >90g/L was slightly higher when using the adjustment method. Third, we treated children for malaria two weeks before collecting blood samples at the end of the intervention. As a consequence, the prevalence of inflammation and malaria was very low, so that ferritin concentrations measured at the end of the intervention validly reflected iron status.

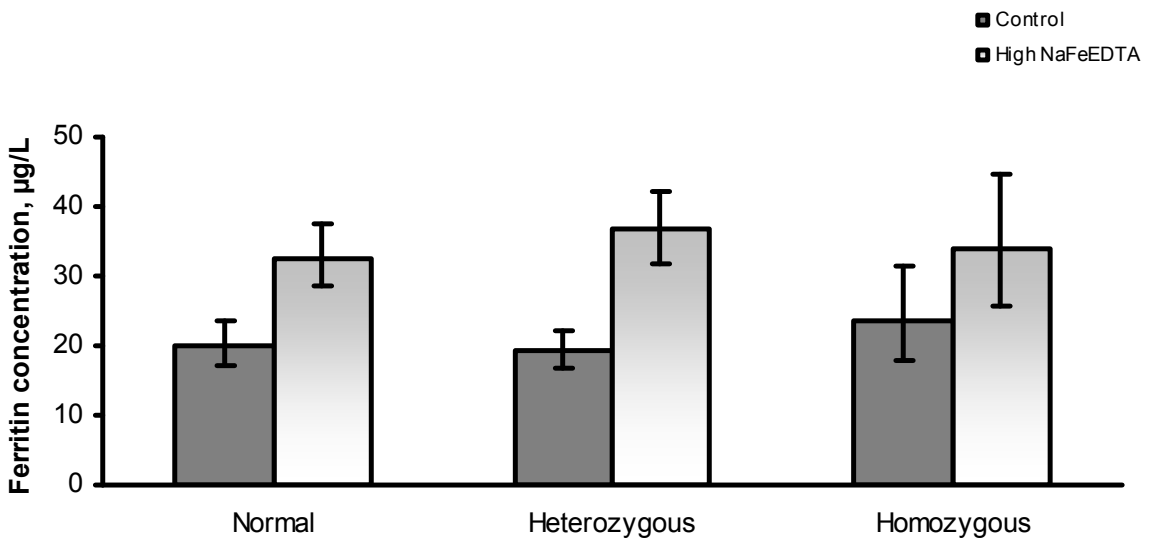

Figure 4.2

Ferritin concentrations (geometric mean) in children who received high NaFeEDTA stratified by α^+-thalassaemia genotype and adjusted for baseline malaria, mean haemoglobin, and median concentrations of ferritin and C-reactive protein. The figure represents end of intervention ferritin concentrations for children with no malaria at baseline, with baseline mean or median iron status indicator concentrations.

Our findings do not support the hypothesis that our iron intervention led to an increased response in ferritin concentration by children with α^+-thalassaemia compared to children with normal genotype. We found no strong evidence of an association between ferritin concentrations and α^+-thalassaemia genotype, even if ferritin concentrations at baseline appeared to be slightly higher in homozygotes than in children with normal genotype. Although a few children had ferritin concentrations above 90µg/L, there also was no clear indication that these high ferritin concentrations were associated with α^+-thalassaemia genotype at baseline or with α^+-thalassaemia genotype and iron treatment at the end of the intervention.

In populations such as the one we studied the safety of iron interventions may depend on several factors that were not addressed in the current study. First, in severely malnourished children, transferrin levels are low and intake of extra iron may result in raised levels of non-transferrin bound iron. Raised ferritin concentrations and the presence of chelatable iron in plasma, in malnourished children, were reported in early studies.[22-24] In a more recent study, raised concentrations of ferritin and non-transferrin bound iron and high transferrin saturation in malnourished children was reported.[25] Because non-transferrin bound iron is possibly a

source of nutrition for pathogens, iron interventions may increase the risk of malaria and other systemic infections[26] in severely malnourished children. In agreement with earlier studies that have found epidemiological evidence for such a link, current guidelines from the World Health Organization specify that iron should not be given initially to severely malnourished children until they have a good appetite and start gaining weight[27]. Similar guidelines may be extended to the use of iron-fortified food in emergency conditions.

Second, supplementation with ferrous sulfate and folic acid in malaria endemic areas has been shown to increase the risk of hospitalization and death.[28] WHO recommends that iron supplementation in malaria-endemic areas should target individuals who are anaemic and at risk of iron deficiency.[29] This recommendation specifically excludes iron fortification because the absorption and metabolism of fortificant iron may be different. Supplementation iron is usually provided as ferrous salts, which may produce non-transferrin bound iron because the supply of iron by duodenal enterocytes to plasma may transiently exceed the availability of transferrin [30]. By contrast, fortificant iron is ingested in lower doses than supplementation iron so that the production of non-transferrin bound iron from fortification is less than from supplementation. In addition, contrary to supplemental iron, NaFeEDTA provides iron in its ferric form, which must be reduced in the intestinal lumen on the enterocyte membrane by duodenal cytochrome B before it can be taken up by the enterocyte. Because the expression of this ferric reductase is reduced in iron sufficiency, this may provide an additional mechanism to prevent iron overload from ferric iron. Further safety assessments of NaFeEDTA should establish its effect on the pool of non-transferrin bound iron. However, because our intervention period was 5 months only, we cannot preclude the theoretical possibility that intake of foods with fortificant iron as NaFeEDTA may lead to iron overload. This should be monitored in fortification programmes.

We found no evidence that iron fortification with NaFeEDTA at 28 or 56mg iron/kg flour resulted in an increased risk of iron overload.

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Chapter 5

Safety of NaFeEDTA as an iron fortificant: effects of fortification using NaFeEDTA on status of zinc and other nutritionally important mineral elements in Kenyan school children

Pauline EA Andang'o, Hans Verhoef, Frans J Kok
David L Mwaniki, Rosemary Ayah, Clive E West[†]
Saskia JM Osendarp

Submitted for publication

[†]Deceased

Abstract

NaFeEDTA is a promising iron fortificant in populations consuming high-phytate diets. There have been concerns about its safety, however, particularly about its potential deleterious effects on the absorption and status of zinc and other mineral elements.

We assessed the effect of NaFeEDTA on plasma levels of nutritionally important mineral elements (zinc, copper, calcium, magnesium and manganese).

In a 5-month iron fortification trial among children aged 3-8 years, we measured plasma concentrations of mineral elements in samples collected at baseline and at the end of intervention. The children were randomized to consume porridge from whole maize flour that was either unfortified (control) or fortified with 28 iron/kg flour or 56 mg iron/kg from NaFeEDTA or 56 mg electrolytic iron/kg flour. Effects were measured by multiple regression analysis as group differences in means or percent difference, relative to control, of post-intervention mineral element concentrations.

At baseline, 42% of the children had zinc deficiency, whilst 66% and 19% had low calcium and magnesium concentrations, respectively. At the end of the intervention, the plasma concentrations of these mineral elements had decreased in all groups. There was no evidence, however, that NaFeEDTA affected these concentrations.

We found no evidence that daily consumption of flour fortified with NaFeEDTA for a 5-month period at mean levels of EDTA intake of 2.35 mg/kg body weight affected plasma concentrations of the mineral elements investigated.

Introduction

The potential of NaFeEDTA as an iron fortificant has been successfully demonstrated in several absorption studies and field trials with condiments [1-5] and it is now recommended for the fortification of high-phytate flours [6]. We recently confirmed its efficacy in a study that supports this recommendation [7]. The enhanced iron bioavailability from low-bioavailability diets by NaFeEDTA is attributed to its EDTA moiety, a strong chelator that is capable of binding most metal elements (**figure 5.1**). Tight binding of iron to EDTA is favored at low pH such as encountered in the stomach. Thus iron bound to EDTA is unavailable to other ligands such as phytate that would otherwise bind to it and enhance its excretion in stool. At higher pH, as found in the duodenum, the iron dissociates from EDTA, thus promoting its absorption [8]. Most of the EDTA is excreted in the stool. A small proportion of EDTA (5%) is absorbed, but is rapidly excreted in urine [9]. Iron and EDTA are absorbed by separate and independent mechanisms when NaFeEDTA is added to a meal [10]. Thus a risk assessment for excess intake of NaFeEDTA should separately address the risks for excess intake of iron and EDTA.

Acceptance of NaFeEDTA as a fortificant has been delayed because of concerns that EDTA potentially affects the metabolism of other mineral elements. Safety concerns about the use of EDTA involve two issues: its direct toxicity and its potential interaction with other metal elements. There is no evidence of possible toxicity at proposed fortification levels supplying up to 5-10 mg iron from NaFeEDTA a day [8]. Of concern, however, are its potential effects on the absorption and excretion of nutritionally important mineral elements such as zinc, copper, and, probably to lesser degree, calcium and magnesium[8]; and on the absorption of potentially toxic elements such as lead, cadmium, mercury, aluminium and manganese. Once dissociated from iron, EDTA is capable of forming complexes with other cations whose binding to EDTA is optimal at higher pH; potentially increasing their excretion and reducing their absorption. On the other hand it has the potential of solubilizing and increasing the absorption of potentially harmful elements. Alternatively, absorbed EDTA may potentially bind and enhance urinary excretion of absorbed elements [3]. Evidence so far from both animal and human studies do not support potential adverse effects from NaFeEDTA consumption [1-3,5,10,11]. There is need, however, to establish the safety of prolonged intakes of NaFeEDTA regarding its effect on mineral element status, especially in children.

In a field trial to assess the efficacy of NaFeEDTA on iron status of children, we provided this fortificant at fortification levels supplying 28 mg/kg flour and 56 mg/kg flour, and 56 mg electrolytic iron/kg, for five months. In the current study we assessed the effect of consuming NaFeEDTA at these levels on the status of five nutritionally important minerals: zinc, calcium, copper, magnesium and manganese.

Subjects and Methods

Study sample and interventions

Data for the current study were obtained from children who participated in an iron intervention trial conducted near Malindi, on the Kenyan coast. Details of the study design are provided elsewhere [7]. In brief, the participants were children aged between 3-8 years from 4 schools in Marafa location of Malindi district. A total of 516 children were individually randomised to receive whole maize flour porridge (*uji*) that was either unfortified or fortified with NaFeEDTA at 56 mg iron/kg flour or 28 mg/kg, or with 56 mg electrolytic iron/kg flour. The target daily intake was 700 mL *uji* (containing 100 g flour) for children aged 3–5 years and 1000 mL *uji* (containing 150 g fl our) for children aged 6–8 years. This target amount was provided under supervision daily to every child in two equally divided portions. We estimated that addition of iron at a low dose of 28 mg/kg flour and a high dose of 56 mg/kg flour would provide 20% and 40%, respectively, of the daily iron requirements for children aged 3–5 years and 18% and 36%, respectively, of the requirements for children aged 6–8 years. In this calculation, we assumed that 5% of the added iron would be absorbed. The intervention lasted 5 months. All children were treated for malaria 2 weeks before the end of the intervention to avoid inflammation-induced effects on indicators of mineral element status.

Blood collection

Non-fasting venous blood was collected into containers for mineral element analysis, with Na-heparin (Becton-Dickinson; Temse, Belgium). Aliquots of whole blood, transferred into EDTA bottles, were used for haematological analysis and to detect falciparum malaria antigenemia by dipstick test. Heparinised blood was separated in the field within one hour of collection; plasma was kept and transported in a cool box (4-8 ºC) for 2-6 hours whilst in the

field and subsequently stored in liquid nitrogen (-196ºC) and below -70ºC. At 5 months after the start of the intervention we repeated baseline measurements.

Laboratory analyses

Haemoglobin concentrations were measured in a haematology analyzer (KX-21, Sysmex Corporation, Japan). Plasma concentrations of C-reactive protein (CRP), ferritin and soluble transferrin receptor (sTfR) were measured in The Netherlands (Meander Medical Centre, Amersfoort) on a Behring nephelometer (model BN-Prospec, Dade-Behring, Marburg, Germany), using Behring kits and calibration and assay procedures according to the manufacturer's instructions. C-reactive protein concentration was determined as a marker of current inflammation, to take into account that infection-induced inflammation is known to increase ferritin concentrations independent of iron status [12]. Plasma samples were diluted 20 times in milliQ [13] and subsequently analyzed for their concentrations of zinc, copper, calcium, magnesium and manganese by ICP-AES (Vista Axial, Varian, Australia) at NIZO Food Research (Ede, The Netherlands). This method has the advantage over Atomic Absorption Spectrometry (AAS) of simultaneously analyzing several elements in one run, in a small volume of specimen. Plasma samples collected at baseline and at the end of the intervention were analyzed in the same run. To determine variability in outcomes, measurements were replicated five times. With mean values set at 100%, measurements varied between 98% and 103% for calcium, 97% and 102% for copper, 99% to 102% for magnesium, and 97% to 102% for zinc respectively.

Infection by *Plasmodium falciparum* malaria was assessed by conventional microscopical examination of thick and thin blood film, and by dipstick test (Parachek; Orchid Biomedical, Goa, India). This test has a reported sensitivity and specificity of more than 90% [14-16].

Mineral element biomarkers and cut-off values for deficiency or low concentrations

Plasma zinc concentration is currently the most widely used and accepted biomarker of population zinc status although it has poor sensitivity and specificity when applied to detect deficiency in individuals [17]. We defined zinc deficiency and low zinc status as plasma zinc concentrations <9.9 µmol/L and <10.7 µmol/L, respectively [18].

Because of lack of defined cutoff values to indicate deficiency for the remaining mineral elements, we considered plasma concentrations below the lower limit of 'normal' plasma values to indicate low status of specific mineral elements. Biomarkers are lacking that permit reliable determination and interpretation of individual copper status [19]; however, plasma copper concentration is frequently used for this purpose, and is effectively maintained within a relatively narrow range. Responses to depletion are variable, except in cases of severe depletion [20,21]. Therefore, whilst very low levels may relatively safely be interpreted as indicating deficiency, the interpretation of marginally low levels is difficult [22]. More severe dietary restriction depresses copper levels [17], and plasma copper concentrations <7.1 μmol/L lend strong support to the diagnosis of deficiency [23]. Plasma copper is elevated in the acute phase response. We considered 0.80–1.75 mg/L (12.6–27.5 μmol/L) [24] as the normal range for this element.

Plasma manganese concentrations reflect dietary intake [17]. We considered concentrations of 4–12 μg/L (0.007–0.022 μmol/L) to be within the normal range [25]. Plasma concentrations of magnesium are useful in assessing acute changes in magnesium status but may fail to give the true picture of total body magnesium status [26]. However, effects of magnesium deficiency are mainly restricted to extracellular functions of magnesium because intracellular concentrations are under tight regulation [27]. We considered plasma concentrations < 0.745 mmol/L as indicating low magnesium status [28,29].

As with copper, plasma calcium concentration is maintained within a narrow range, but is depressed with habitually low intakes as shown in studies among South African [30,31] and Nigerian [32-34] children. We considered values <2.22 mmol/L to indicate low calcium status [35].

Data analysis

Data was missing for one child at baseline for whom no laboratory results were obtained, and for 20 children (4%) at the end of the intervention. Of these 20 children, 12 were absent at the time of specimen collection and no laboratory results were obtained for 8 children. We imputed these data using the mean values within each treatment group. Because less than 10% of data was missing, this method of imputation should yield similar results to better but more sophisticated methods. Because of the small loss to follow-up however, we do not expect that any differences arising from this method would be substantial. We also conducted a sensitivity analysis by doing a complete-case analysis.

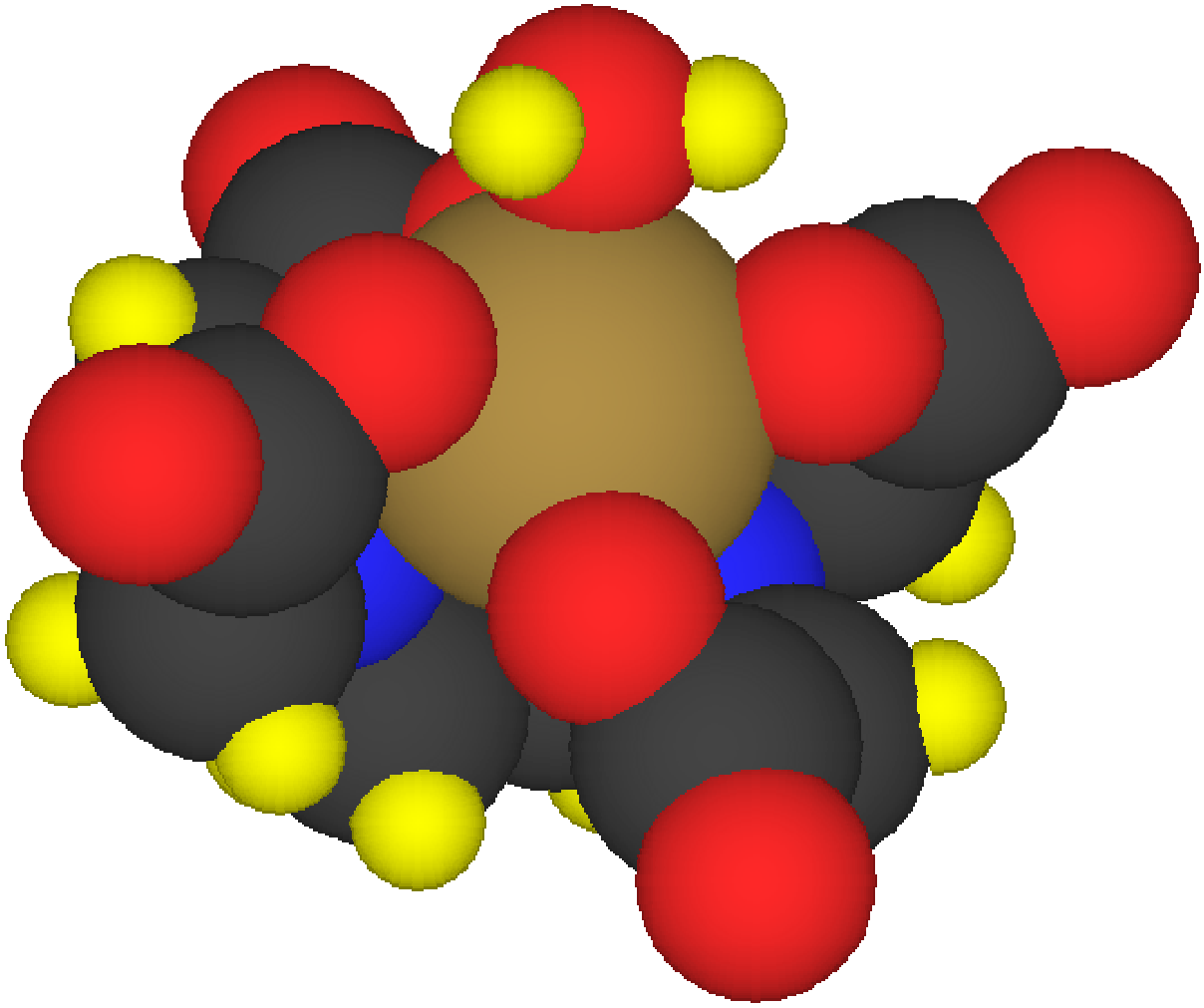

Figure 5.1

Iron (brown ball) in NaFeEDTA.3H_2O is shielded from binding to phytates that hamper absorption of the iron. Dissolved in water, one of the three water molecules remains attached to the central iron-ion. Black: carbon; red: oxygen; blue: nitrogen; yellow: hydrogen. Sodium is not part of the 'jacket' and has therefore not been shown in the diagram. [Colours are displayed only in the electronic version of this thesis].

We calculated EDTA intakes based on actual body weights. Although there is no Acceptable Daily Intake (ADI) available for EDTA, this can be derived from the ADI for $CaNa_2$-EDTA, which was established as 2.5 mg/kg body weight[36]. Thus, assuming the absorption of EDTA moiety from NaFeEDTA to be identical to that from $CaNa_2$-EDTA [10], we determined the ADI for EDTA to be 1.93 mg/kg body weight.

We assessed baseline and end-of-intervention concentrations of mineral elements within treatment groups to evaluate the children's mineral element status at the two time points.

Visual inspection of distribution plots of each element was used to determine normality. We conducted multiple linear regression analyses to assess treatment effects on each of the mineral elements, with the group that received unfortified flour as the control. In these analyses, standardised scores of the end-of-intervention concentration of each mineral element was the dependent variable, with and without adjustment for malaria, age and the respective baseline concentration of the mineral element that was considered as the dependent variable. Standardised scores of non-normally distributed outcomes were obtained from log-transformed values. Results are reported as mean difference of standardised score relative to the control for normally distributed outcomes, and as percent difference relative to the control, for non-normally distributed outcomes.

In a sub-group analysis we assessed whether children who were iron deficient at baseline were more at risk of developing mineral element deficiency than their iron sufficient counterparts, following the iron intervention. To do this, we assessed the treatment effects on the mineral elements in strata of children who were iron deficient or sufficient at baseline.

Results

EDTA intake in children receiving NaFeEDTA-fortified flour

The mean (SD) daily intake of EDTA was 1.15 (0.21) mg/kg body weight and 2.35 (0.40) mg/kg body weight in children in the low-dose and high-dose NaFeEDTA groups, respectively (**figure 5.2**). None of 139 children in the low-dose NaFeEDTA group exceeded the ADI for EDTA. By contrast, 84% (101/121) of their peers in the high-dose NaFeEDTA group exceeded the ADI.

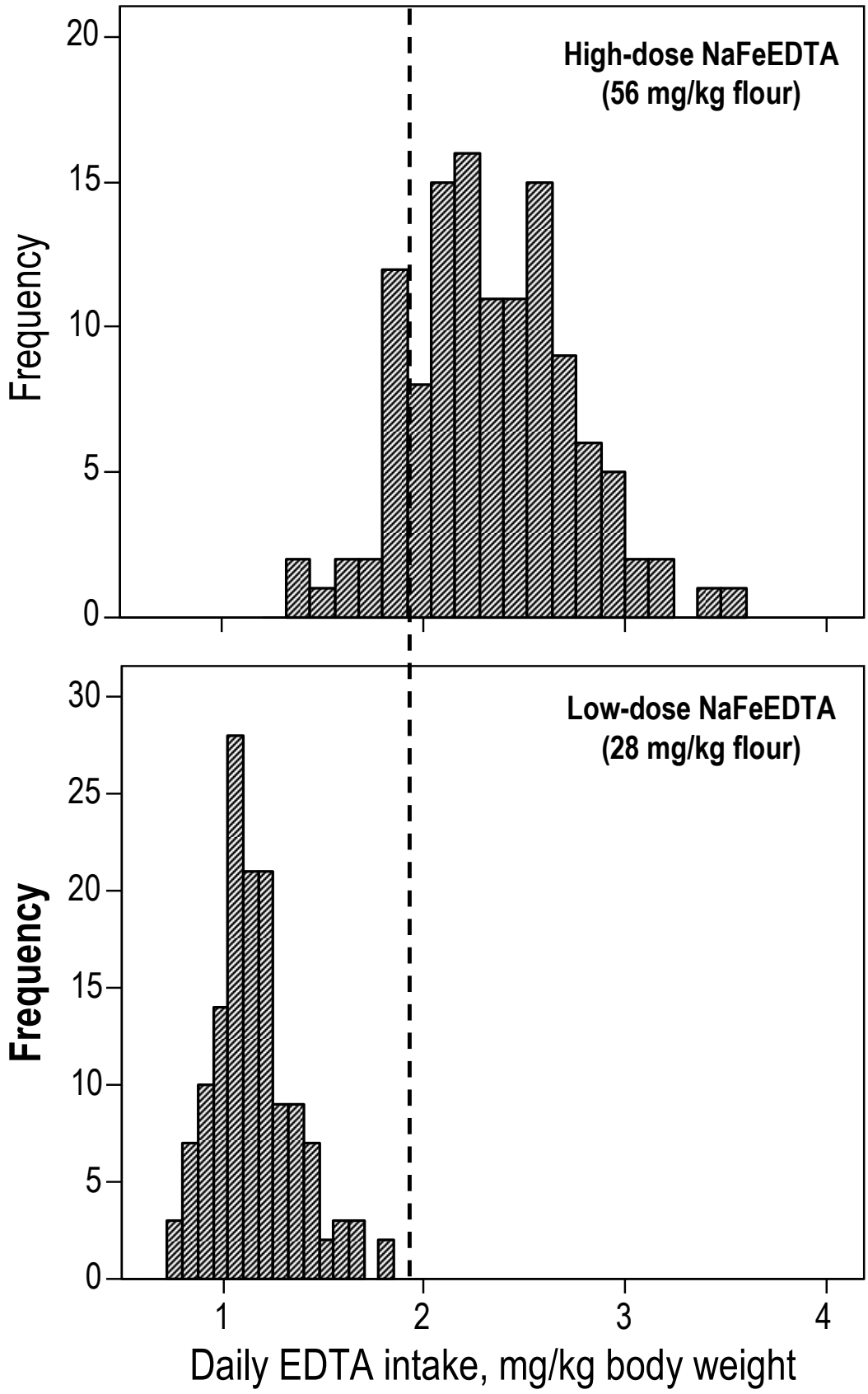

Figure 5.2

EDTA intake (mg/kg body weight) in the high-dose NaFeEDTA group (upper panel) and low-dose NaFeEDTA group (lower panel); the dotted line indicates the ADI for EDTA (1.93 mg/ kg body weight).

Baseline mineral element status

Plasma concentrations of calcium, copper, potassium and magnesium were normally distributed, whilst concentrations of zinc and manganese were normally distributed after log-transformation. At baseline, the following prevalence values were measured: zinc deficiency: 42.0%; low status for zinc, calcium and magnesium: 59.0%, 66.0% and 19.0%, respectively. Plasma concentrations of mineral elements and prevalence of low status at baseline were similar across treatment groups (**tables 5.1-5.2**), with the possible exception of zinc deficiency, which appeared to occur more frequently in the control group. Zinc concentrations were lower and copper values higher in children with malaria and inflammation as compared to their peers without both malaria and inflammation. We found no evidence of associations between malaria and plasma concentrations at baseline or at the end of intervention of magnesium, manganese and calcium. Similarly, we found no evidence of associations between inflammation and these mineral status indicators.

Effect of the iron intervention on the mineral element concentrations

For zinc, calcium and magnesium, the prevalence of low status was higher at the end of the intervention than at baseline. The distributions of these mineral elements were similar across intervention groups (tables 5.1-5.2), with the exception of magnesium and manganese. Plasma concentrations of magnesium seemed reduced – and prevalence of low magnesium status increased – in the high NaFeEDTA group relative to the control group. For plasma manganese concentrations, the data were compatible with reductions in both groups receiving NaFeEDTA relative to that measured in the control group (**table 5.3**).

Lastly, there was no evidence that the iron interventions were associated with changes in zinc status, even when children were stratified by iron status at baseline.

Table 5.1 Mineral element status at baseline and after 5 months of intervention

		High NaFeEDTA	Low NaFeEDTA	Electrolytic iron	Control
n	Baseline	121	140	126	128
	Month 5	120	138	127	128
Zinc concentration (µmol/L)	Baseline	10.4 ± 3.7	10.6 ± 2.8	10.4 ± 2.0	10.4 ± 3.7
	Month 5	9.4 ± 1.8	9.3 ± 1.5	9.4 ± 1.7	9.3 ± 1.5
Calcium concentration (mmol/L)	Baseline	2.19 ± 0.2	2.21 ± 0.3	2.18 ± 0.2	2.15 ± 0.1
	Month 5	2.08 ± 0.1	2.09 ± 0.1	2.09 ± 0.1	2.08 ± 0.1
Copper concentration (µmol/L)	Baseline	22.3 ± 4.2	22.7 ± 5.6	22.5 ± 3.9	21.8 ± 4.3
	Month 5	19.7 ± 3.4	19.4 ± 3.7	19.7 ± 3.5	18.7 ± 3.2
Magnesium concentration (mmol/L)	Baseline	0.81 ± 0.1	0.82 ± 0.1	0.82 ± 0.1	0.80 ± 0.1
	Month 5	0.75 ± 0.1	0.78 ± 0.1	0.78 ± 0.1	0.76 ± 0.1
Manganese concentration (µmol/L) [1]	Baseline	0.03 (0.03, 0.05)	0.03 (0.02, 0.04)	0.03 (0.02, 0.04)	0.03 (0.02, 0.04)
	Month 5	0.03 (0.02, 0.03)	0.026 (0.02, 0.03)	0.03 (0.03, 0.04)	0.030 (0.028, 0.032)

All values are expressed as mean ± SD unless specified

[1]Median (25^{th}, 75^{th} percentiles)

Table 5.2 Proportion of children with low mineral element concentrations at baseline, by intervention group

	High NaFeEDTA	Low NaFeEDTA	Electrolytic Iron	Control
n	121	139	126	128
Baseline				
At 5 months	121	139	127	128
Zinc (low)[1]				
Baseline	51.2% (62)	61.4% (86)	57.0% (72)	67.2% (86)
At 5 months	80.2% (97)	83.6% (117)	78.7% (100)	81.3% (104)
Zinc deficiency [2]				
Baseline	36.4% (44)	42.1% (59)	39.7% (50)	50.8% (65)
At 5 months	67.8% (82)	75.5% (105)	64.6% (82)	68.0% (87)
Low calcium status [3]				
Baseline	62.8% (76)	66.4% (93)	63.5% (80)	69.5% (89)
At 5 months	82.6% (100)	87.8% (122)	85.0% (108)	85.9% (110)
Low copper status [4]				
Baseline	0% (0)	0.7% (1)	0% (0)	0.8% (1)
At 5 months	0% (0)	0.7% (1)	1.6% (2)	2.3% (3)
Low magnesium status [5]				
Baseline	20.7% (0)	15.8% (0)	14.3% (0)	23.4% (1)
At 5 months	43.8% (53)	32.4% (45)	25.2% (32)	37.5% (48)
Low manganese status [7]				
Baseline	2.5% (3)	2.9% (4)	7.9% (10)	2.3% (3)
At 5 months	3.3% (4)	6.5% (9)	2.4% (3)	2.3% (3)

Values are indicated as % (n)

Low status is defined as below the lower value of the reference range

[1]< 10.7 µmol/L; [2]< 9.9 µmol/L; [3]< 2.22 mmol/L; [4]< 12.6 µmol/L; [5]< 0.740 mmol/L for children ≤ 5 years and < 0.745 mmol/L for children > 5 years; [6]< 3.3 mmol/L; [7]< 0.007 µmol/L

Discussion

We found no evidence that daily intake over a 5-month period of flour fortified with NaFeEDTA, even at intake levels above the ADI, affected plasma concentrations of zinc, calcium, or copper. Our data suggested that intake of flour fortified with high-dose NaFeEDTA may be associated with marginally reduced plasma magnesium concentrations, and a possible association between intake of flour fortified with NaFeEDTA with small reductions of plasma manganese concentrations.

Plasma concentrations of zinc, calcium and magnesium at the end of the intervention had declined substantially relative to values measured at baseline. This may have occurred because all children received porridge, which may have led to a growth spurt and a concomitant dilution of these mineral elements. This finding does not invalidate our results, however, because treatment effects are indicated by comparing values at the end of the interventions, and not within intervention groups.

Of the minerals assessed, the potential effect of EDTA on zinc has been of most concern, because adverse effects observed in early animal studies arose from EDTA-induced zinc deficiency [37]. Recent studies have however found no adverse effect of EDTA on zinc under a variety of conditions; by contrast, they indicate that progressively high intakes of EDTA can result in increased zinc excretion and reduced retention. For example, dietary intake of EDTA resulted in increased zinc absorption and increased urinary excretion in rats; because the proportional increase in absorption exceeded the proportional increase in excretion, however, net zinc retention was increased [11]. An earlier study showed that, when NaFeEDTA was added to normal Guatemalan meals, the NaFeEDTA dose “equivalent to that consumed in a day by rural Guatemalans” (40mg) did not influence the zinc absorption of adults. In a more recent study, consumption of a test meal containing NaFeEDTA increased zinc absorption and urinary excretion in apparently healthy adult women relative to consumption of a test meal containing ferrous sulfate. However, there was no evidence that zinc retention differed between groups receiving the two types of meals [4]. In healthy infants, no increase in zinc absorption was observed when NaFeEDTA was compared to ferrous sulfate plus ascorbic acid [3]. Differences between studies in the effects of zinc may be attributable to different molar ratios of zinc: EDTA [11].

Table 5.3 Effects of fortification with NaFeEDTA and electrolytic iron on post-intervention mineral element status

	High-dose NaFeEDTA	Low-dose NaFeEDTA	Electrolytic iron
Zinc	0.03 (-0.24, 0.24)	-0.06 (-0.29, 0.18)	0.03 (0.21, 0.27)
Calcium	0.01 (-0.23, 0.26)	0.04 (-0.20, 0.28)	0.07 (-0.18, 0.31)
Copper	0.25 (0.02, 0.48)	0.11(-0.12, 0.33)	0.23 (-0.00, 0.45)
Magnesium	-0.07 (-0.31, 0.18)	0.22 (-0.02, 0.45)	0.22 (-0.03, 0.46)
Manganese[1]	-0.15 (-0.40, 0.10)	-0.30 (-0.53, -0.06)	0.14 (-0.10, 0.39)

Results are indicated as the difference in sds (95% CI), adjusted for baseline concentrations of the specified nutrient element, age and malaria.

[1]Log-transformed

Our data suggested an association between high-dose NaFeEDTA and marginally reduced plasma magnesium concentrations. On a molar basis, the dietary intakes of magnesium and calcium are much higher than of EDTA, so that it seems unlikely that ingested EDTA has an effect on the absorption of these elements. Thus, although we cannot preclude that high-dose NaFeEDTA leads to reduced magnesium status, our observation is likely to be a spurious finding.

Incremental dietary EDTA intake in rats slightly increased urinary calcium concentration but had no measurable effect on calcium retention [11]. In agreement with our findings, however, studies with stable isotopes showed that NaFeEDTA had no effect on calcium absorption in women [4] nor on the absorption of either calcium or magnesium in healthy infants [3].

In rats, it was found that NaFeEDTA can improve the absorption of copper especially from low-zinc diets but otherwise it did not influence absorption, excretion or retention of copper. In infants, no effect of NaFeEDTA on copper absorption was observed as compared to ferrous sulfate given at doses that supplied similar amounts of iron [3]. As judged by plasma copper concentrations, children in our study appeared to have adequate copper status. Because plasma copper concentrations within the normal range do not reflect the dietary

supply of copper, however, we cannot preclude effects of NaFeEDTA on copper status that were not manifested in plasma copper concentrations.

We observed apparently lower manganese concentrations in the groups receiving NaFeEDTA relative to the control, and an increase relative to the control in the group receiving electrolytic iron; however, these changes were small and our estimates were imprecise. In addition, the prevalence of children with low manganese status was low. Davidsson et al [3] found no influence of NaFeEDTA on manganese absorption or excretion in adult women. Thus we cannot preclude an effect of NaFeEDTA from our data, but there is no evidence that this should be a concern.

Contrary to our expectations, the EDTA intake in a substantial fraction of children in the group receiving high-dose NaFeEDTA exceeded the ADI. The reasons were three-fold: Firstly, when designing our study, we calculated the intake of EDTA based on weights of US children because we had no data on weight distribution for our target population of Kenyan children. In fact, however, the Kenyan children in our study weighed less than their US counterparts. Second, we based our calculations on average weight of US children instead of the weight of children from an arbitrarily selected upper percentile. Third, based on earlier reports [38], [39], [8], we assumed that the ADI for EDTA was 2.5 mg/kg body weight. This value, however, was set by JECFA [36] as the ADI for $CaNa_2EDTA$, whereas recalculation indicated an ADI for EDTA of 1.93 mg/kg (see our Subjects and Methods section).

There are two important arguments, however, why the ADI that we derived for EDTA should not be applied too rigidly, particularly with short-term exposure as in our study. First, it was derived from the ADI set for $CaNa_2$-EDTA, which is an additive without any nutritional value that is used as a shelf life-enhancing agent. The ADI value for $CaNa_2$-EDTA was based on the no-observed-effect level (NOEL) of 250 mg/kg body weight reported for rats and dogs, with the application of a safety factor of 100 to provide a conservative margin of safety on account of the inherent uncertainties in extrapolating animal toxicity data to potential effects in the human being, and to take into account variation within the human species[36]. By contrast, NaFeEDTA is added solely to improve health and to satisfy dietary needs for iron, which may warrant the use of a lower safety factor when setting an ADI for EDTA. Because iron may be toxic at intake levels less than 10 times those for optimal nutrition, a safety factor

of 100 as applied for food additives should not be considered immutable [40], and a lower factor may be more appropriate.

Second, the ADI is an estimate by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) of the amount of a food additive, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk [40]. Thus short-term exposure to intake levels exceeding the ADI, as occurred in our trial for EDTA, should not necessarily be viewed as a health concern.

We found no evidence that daily consumption of flour fortified with NaFeEDTA for a 5-month period at mean levels of EDTA intake of 2.35 mg/kg body weight affected the status of zinc, calcium, magnesium, copper or manganese as indicated by the plasma concentrations of these mineral elements.

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Chapter 6

General discussion

This thesis aimed to assess: the benefits of consuming whole maize flour fortified with NaFeEDTA or electrolytic iron on: 1) the iron status of school-aged children, and 2) cognitive function and fine motor skills; and to assess two aspects of the safety of the use of NaFeEDTA; 3) the safety of NaFeEDTA in children with α-thalassaemia; and 4) the safety of the interventions on the status of nutritionally important mineral elements.

MAIN FINDINGS

The main findings of this thesis are summarised in **figure 6.1**. The study that assessed the potential benefits of NaFeEDTA and electrolytic iron, when added as iron fortificants to whole maize flour, showed that NaFeEDTA improved iron status, but electrolytic iron did not (chapter 2). In the groups receiving high-dose NaFeEDTA and low-dose NaFeEDTA, respectively, iron deficiency anaemia decreased by 89% and 48%; iron deficiency decreased by 91% and 70%. The prevalence of anaemia decreased only in the high-dose NaFeEDTA group (by 36%). Electrolytic iron was given at a dose of iron equivalent to that contained in high-dose NaFeEDTA, but did not improve iron status.

Contrary to expectations, there was no evidence that the improved iron status observed in children who received NaFeEDTA resulted in better scores of tasks assessing cognitive and motor function (chapter 3).

The next two chapters address the safety of NaFeEDTA as an iron fortificant. The study in chapter 4 assessed whether α^+-thalassaemia modified the effect of the intervention. In doing so, the study aimed to determine if individuals with α^+-thalassaemia responded better to the intervention than their peers with normal genotype; and if such a response might even put them at risk of developing iron overload. There was no such evidence. This conclusion is limited, however, by the 5-month intervention period used in this trial and does not preclude the theoretical possibility that intake of foods fortified with iron as NaFeEDTA may lead to iron overload. In the final study (chapter 5), the safety of NaFeEDTA was further addressed by assessing its potential to adversely affect the status of other mineral elements. There was no evidence that NaFeEDTA affects zinc, copper, calcium, magnesium and manganese status. Because small changes in magnesium and manganese could reflect a large effect on their

status, the effects of prolonged intake of NaFeEDTA on these nutrients should be monitored in fortification programmes.

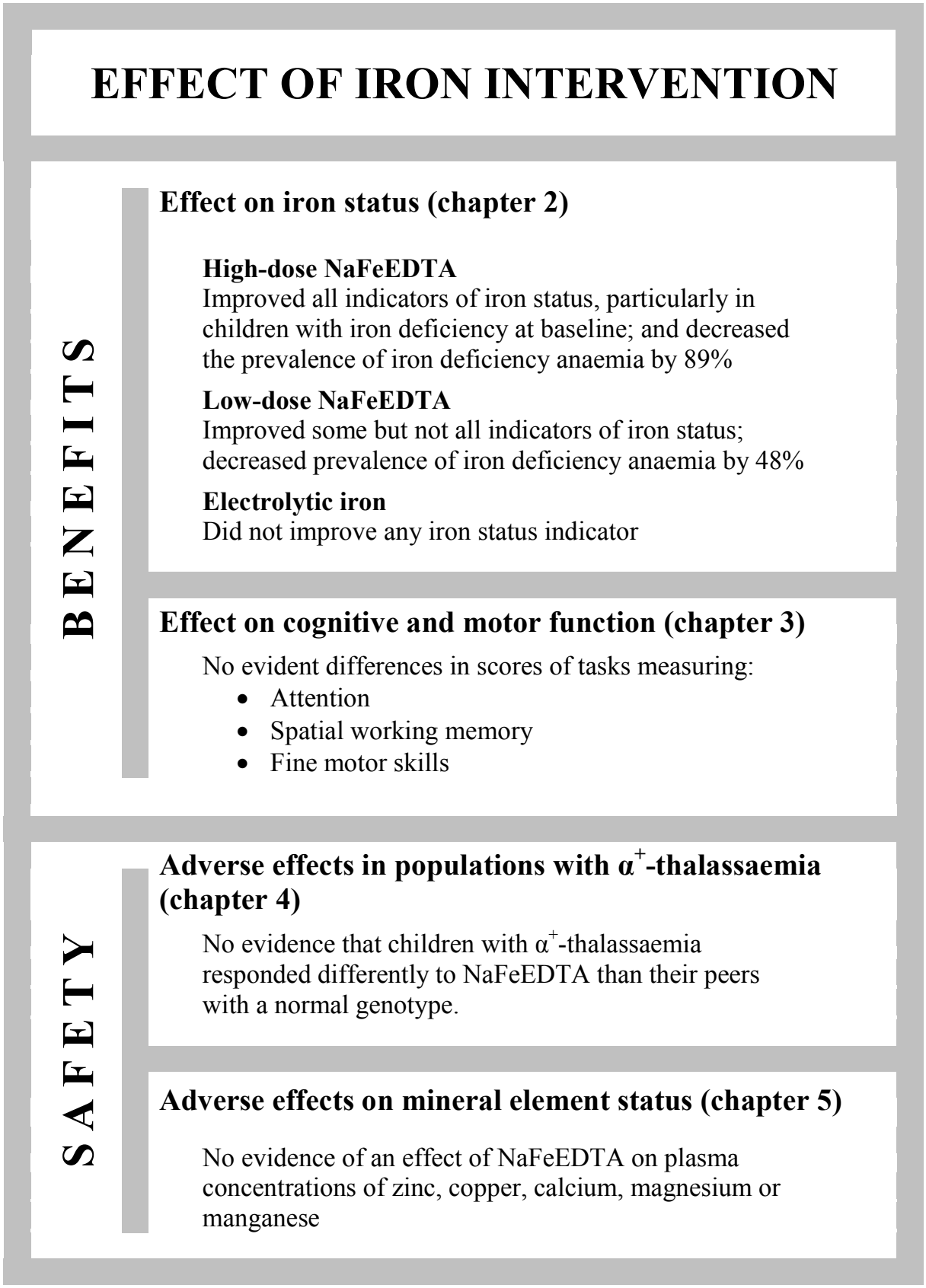

Figure 6.1 Overview of the main findings.

METHODOLOGICAL ISSUES

The findings discussed within this thesis are based on a randomised double-blind placebo-controlled trial. Participants were individually randomised to treatments, giving each an equal probability of being in a given treatment group. Objective measures (biomarkers of iron status) determined by laboratory analysis were used to determine the outcome. Similarly, graduated mugs were used to determine *uji* intake (chapter 2). Researchers, staff and participants, and laboratory personnel were unaware of the identity of the treatments; and there was small loss-to follow-up. The above factors strengthened the findings of the thesis.

The following sections address the potential influence of three issues that are important for the validity of the findings in this thesis, namely the selection of the study population, potential misclassification of outcomes, and confounding.

Selection of study population

A relatively large sample size is necessary to detect small to moderate treatment differences. Of importance to the validity of a trial, however are the compliance to treatment, and the total number of endpoints experienced. In the trial described in this thesis, compliance to treatments was high and was similar between groups (chapter 2). On the other hand, the proportion of children likely to achieve the desired endpoint (improved iron status) was smaller than expected. The trial was designed primarily to assess the effect of the iron interventions on iron deficiency anaemia; accordingly, this outcome was used to determine the sample size required for the trial. The study was conducted in a site with confirmed high prevalence of anaemia; data on the prevalence of iron deficiency were not available at the time of planning the trial. The prevalence of anaemia in the study population was 56%. It has been assumed that, at a global level, approximately half the number of anaemia cases are due to iron deficiency, with the remaining half being due to malaria, other infections or other nutrient deficiencies.[1] Thus a substantial proportion of the study population in this thesis was theoretically expected to be iron deficient. However, although the prevalence of anaemia was very high in this study population, the prevalence of iron deficiency was much less (15%) than expected. This may have reduced the precision of the study assessing the efficacy of NaFeEDTA and electrolytic iron (chapter 2), but does not affect the validity of the findings. The study shows compelling evidence that NaFeEDTA improves iron status. Larger benefits can be expected when NaFeEDTA is used in populations with a larger burden of iron

deficiency. The low prevalence of iron deficiency is also unlikely to have affected the findings in the studies described in chapters 4 (modification of the intervention by α^+-thalassaemia) and 5 (the effect of the intervention on other mineral elements); but may have contributed to the inability to observe an effect of the iron intervention on cognitive and motor function in the study described in chapter 3. As indicated in that chapter, an effect of improved iron status on cognitive or motor function is more likely to be observed in individuals with iron deficiency anaemia [2].

Misclassification

As explained in the introduction of this thesis (chapter 1), malaria, infection and α^+-thalassaemia complicate the determination of iron status. Malaria and inflammation can increase plasma ferritin concentrations independent of iron status, whilst malaria and α^+-thalassaemia can also increase plasma concentrations of soluble transferrin receptor (sTfR) independent of iron status. Because of these considerations, body iron stores based on the ratio of sTfR to ferritin were not calculated [3]. Others have addressed this complication by using two or more abnormal indicators of body iron status to define iron deficiency. Rather than using such composite measures to determine iron deficiency, treatment effects were assessed on three separate measures of iron status: haemoglobin, ferritin and transferrin receptor concentrations. The definition of iron deficiency used here was based solely on ferritin concentrations and may have led to an underestimation of the prevalence of iron deficiency: some iron deficient children whose ferritin concentrations were raised by underlying infections may have been classified as iron sufficient. Such potential misclassification would reduce the contrast between the groups that were classified as iron sufficient and iron deficient, and thus underestimate any differences that may exist between the groups. In chapter 4, a method is described to adjust for the effect of malaria and inflammation on ferritin concentrations. The possibility of misclassification of iron deficiency was assessed by comparing the prevalence of iron deficiency obtained from adjusted ferritin values to the prevalence reported in chapter 2. Prevalence estimates obtained from the two methods were similar, although, as pointed out in chapter 4, residual confounding cannot be ruled out in the adjusted ferritin estimates.

Confounding

Because this thesis is based on a randomised trial, confounding is an unlikely explanation of the study that assessed the efficacies of NaFeEDTA and electrolytic iron (chapter 2); or the

two studies that addressed the safety of NaFeEDTA (chapters 4 and 5). The findings in these studies include the whole randomised sample hence give confidence that the baseline comparability of the groups was retained. By chance, the iron status of children in the control group was better at baseline than that of their peers in the treatment groups. Although the difference was small, baseline iron status is strongly associated with the outcome, end of intervention iron status. Even small differences in the distribution of factors that are strongly associated with the outcome can lead to substantial confounding in the estimate of the treatment effect. This problem was redressed because it was decided *a priori* to give more credence to estimates of treatment effect that were adjusted for baseline iron status.

In the study described in chapter 3, no evidence was found that iron interventions improved cognitive or motor function. In this study, both the small sample size and confounding may have contributed to this lack of evidence. Because the study was based on a subgroup of the original randomised sample, the balance in baseline factors was lost, and the study effectively became an observational study. Confounding occurs if such factors are associated with both treatment group and outcome, in this case scores of tasks assessing cognitive and motor function. Although this was remedied by adjusting for several baseline factors, confounding by baseline factors that were not measured cannot be precluded. In particular, cognitive and motor function were not measured at baseline, which, if they had been performed, would most likely have been strongly associated with results of similar tests at the end of the intervention. The disadvantage of administering such tests repeatedly, however, is that children are likely to learn from the first test, and thus to score better at the second test. This would have resulted in improvement in scores across all treatment groups. The result of such a 'Hawthorne' effect is that effect estimates tend towards zero.

INTERPRETATION OF FINDINGS

The efficacy of NaFeEDTA

One of the greatest challenges to the application of iron fortification is to identify a fortificant that can provide iron in a bioavailable form to populations mainly consuming cereal-based diets. In chapter 1, the difficulties are described in obtaining adequate iron from such diets. The bioavailability of ingested iron in the form of ferrous sulfate and ferrous fumarate is relatively high, but is substantially reduced in the presence of phytates and other iron

absorption inhibitors that occur in cereal-based diets. The findings of this thesis are in agreement with previous reports from absorption studies using stable isotopes that iron from NaFeEDTA has higher bioavailability than other forms of iron, particularly when given in high-phytate diets [4-6]. They add to previous work in which its efficacy was demonstrated in condiments such as soy and fish sauce in Asia,[7,8] curry powder in South Africa,[9] and in sugar in Central America [10].

Furthermore, the findings described in chapter 2 show that NaFeEDTA improves iron status even in malaria-endemic areas. In populations not exposed to malaria, the beneficial effects of NaFeEDTA can be even higher than those observed in this thesis. This is because infections may reduce the utilisation of iron for haemoglobin synthesis.[11] The higher improvements in individuals with iron deficiency and the much smaller response in individuals who were iron sufficient corroborates evidence that absorption of iron from NaFeEDTA is regulated according to the body's iron needs [9]. Thus, consumption of this product fortified with NaFeEDTA by iron sufficient individuals results in a down-regulation of iron absorption.

The efficacy of electrolytic iron in improving iron status has only been assessed in low-phytate vehicles [12,13]. In one of these studies,[12] no effect of electrolytic iron on iron status was found; this has been attributed to low fortification levels.[14] In the second study, electrolytic iron added to wheat flour improved the iron status of Thai women.[13] As described in chapter 2, no evidence was found that electrolytic iron added to whole maize flour improved the iron status of young children. This finding shows that electrolytic iron, at least in the form and at the dose described, is not suitable for fortification of a high-phytate food vehicle such as maize flour.

The effect of the intervention on cognitive and motor function

The findings of the study described in chapter 3 are contrary to those reported by others. In controlled trials, iron interventions either through supplementation or fortification have been shown to improve cognitive and motor function in this young children.[15-17] However, compelling evidence of a causal link between iron and cognitive and motor functions is yet to be established [18]. The study reported here is limited by possible confounding, and because iron deficiency anaemia was much less prevalent than anticipated.

Effect modification of the iron intervention by α^+-thalassaemia

There have been no published reports on the effect of increasing iron intake over a prolonged period in individuals with α^+-thalassemia. The findings of this thesis suggest that in the short term there is no indication that individuals with α^+-thalassemia genotypes respond any differently to an iron fortification intervention from their peers with a normal genotype.

The effect of NaFeEDTA on nutritionally important mineral elements

The Acceptable daily intake (ADI) is expressed on a body weight basis and is an estimate of the amount of a food additive that can be ingested daily "over a lifetime without appreciable health risk."[19]. Previous reports of the ADI for EDTA are confusing. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) specifically defined the ADI for $CaNa_2EDTA$ as a food additive, with a maximum daily intake of $CaNa_2EDTA$ from all sources of 2.5 mg/kg body weight [20]. Other reports [5,21,22] erroneously quoted this value as the ADI for EDTA. Based on the ADI for $CaNa_2EDTA$, an equivalent ADI for EDTA of 1.93 mg/kg body weight for EDTA is calculated in this thesis (chapter 5). In 1999, JECFA allowed NaFeEDTA for use as a source of iron for food fortification [23]. Based on the earlier ADI for $CaNa_2EDTA$, JECFA in its latest report established an ADI for EDTA of 1.9 mg/kg body weight for [24], which is in agreement with the calculated value reported in this thesis (chapter 5).

The key documented safety issue for NaFeEDTA concerns the potential adverse effect of EDTA on zinc and other mineral elements [20,21]. Despite earlier concerns, however, evidence from isotope studies in both animals and humans indicate that NaFeEDTA consumed at doses applicable to food fortification does not affect the metabolism of trace elements and other important mineral elements. The findings have included studies on zinc, magnesium, copper, calcium and manganese [25-28]. In this thesis, also no evidence was found that at intakes of EDTA above the ADI for an extended period affected the status of such mineral elements. Because the intervention period of the trial described in this thesis was 5 months only, it is recommended that the effect of prolonged consumption of NaFeEDTA on these mineral elements is monitored in programmes where it is used as a fortificant.

In the trial reported here, children were carefully supervised to ensure that they actually consumed as much as they could of the target amount of flour. The target intake of flour in the trial was probably higher than the intake of flour by children under real-life conditions.

Thus, when flour is fortified at levels of the high-dose NaFeEDTA, both the benefits on iron status and the actual EDTA intake would probably be less than reported in this thesis. This leads to two conclusions. First, for reasons outlined in chapter 5, JECFA may consider revising the ADI for EDTA on the basis of a safety factor below the value of 100 that has been used to establish the ADI for $CaNa_2EDTA$. Second, when establishing fortificant levels of NaFeEDTA in flour fortification programmes, it should be considered to use levels equivalent or close to the high-dose NaFeEDTA in this trial (56 mg iron as NaFeEDTA/kg flour).

BIOLOGICAL MECHANISMS

Efficacy of NaFeEDTA and electrolytic iron

Mechanisms underlying the higher bioavailability of iron from NaFeEDTA involve its property as a metal chelator. The mechanism by which NaFeEDTA promotes the absorption of fortificant and intrinsic iron is the subject of the study in chapter 5. EDTA forms complexes with iron protecting it from other potential chelators. Iron remains tightly bound to EDTA in the acid medium of the stomach but less so at a lower pH. As a consequence, iron becomes available for absorption in the duodenum.[22,29] By contrast, electrolytic iron easily binds to phytates and other chelating substances in the diet, so that it becomes unavailable for absorption. It may additionally easily be converted to ferric iron and precipitated as insoluble iron hydroxides.

Regulation of iron absorption.

Current evidence indicates that non-haem iron is absorbed by duodenal enterocytes exclusively in the ferrous form. As described in the first chapter of this thesis, supplemental iron is usually provided in the form of ferrous salts, which can be taken up directly via divalent metal transporter-1 (DMT1). By contrast, iron in NaFeEDTA occurs in the ferric form. Ferric iron must first be reduced to the ferrous form before it can be absorbed [30]. The absorption of ferric iron may be regulated by the expression of duodenal cytochrome b (Dcytb), thus reducing the risk of excess uptake of ferric iron from NaFeEDTA as compared to identical doses of ferrous iron from ferrous sulfate.

The finding that α^+-thalassaemia did not modify the effect of the intervention described within this thesis suggests that iron regulation in this condition is comparable to that in individuals with a normal genotype.

Iron status and cognitive function

Adverse effects on cognitive and motor development of iron deficiency are plausible and may be irreversible [31,32]. As is indicated in chapter 3, such relationships can be mediated by reduced oxygen resulting from iron deficiency-induced anaemia [33,34]. Alternatively, iron deprivation may directly affect the functioning of specific brain areas [32,35]. Associations between iron deficiency and impaired cognitive function have been observed mostly in animal studies and observational studies in humans. On the other hand, there is as yet no compelling evidence of a causal link between iron and either of these functions. Observational studies are liable to bias: children with iron deficiency are likely to be also deficient in other nutritional deficiencies that may explain the relationship observed.

FUTURE RESEARCH

1. Fortification is easiest to achieve, and has mostly been studied, when applied to industrially-processed foods. Most industrially-processed flour, however, has a low phytate content. Thus another trial may be needed to compare the cost-benefit of NaFeEDTA versus other forms of iron in low-phytate flour.

2. As reported in chapter 5, a marked proportion of children had low status of micronutrients other than iron. This thesis shows the efficacy of using NaFeEDTA as an iron fortificant in a high-phytate food vehicle. When using fortification mixes with multiple micronutrients, several vitamins are readily broken down by ferrous iron salts that are added in the mix. Because iron in NaFeEDTA may be less oxidative than ferrous iron, it should be investigated whether these vitamins are better retained when iron is added as NaFeEDTA.

3. Because maize flour in rural Africa is almost exclusively obtained from small-scale mills, research is needed to further develop methodologies for such settings.

4. Research is desirable to compare NaFeEDTA to ferrous iron salts regarding their safety in populations with high incidence of malaria and other infections. This however should not delay implementation of programmes to fortify flour with NaFeEDTA;

POLICY IMPLICATIONS

Technically, iron has generally been considered the most challenging micronutrient to add to foods.[1] This is particularly the case when iron is added to flours with high phytate content. With the findings presented in this thesis, the technology is now available to fortify flour even in the most demanding conditions. In the last decade, technical guidelines for flour fortification have become available,[36-38] and several organisations have been established to provide technical and financial assistance to start national fortification programmes. Fortification of the diet with iron is the most cost-effective strategy to prevent iron deficiency.[5] In short, it is time for governments to act and implement such programmes.

The evidence presented in this thesis support the recommendation that NaFeEDTA is the preferred iron fortificant where high phytate levels substantially reduce iron absorption [38]. This includes flours from sorghum and millet, for which the production is increasingly becoming centralised in countries such as Kenya.

Acceptance of NaFeEDTA in fortification programmes has been slow, mainly because of reservations about its cost and safety. The findings presented in this thesis, and work from other groups [22,25-28,39-41] indicate that safety concerns should no longer delay the use of NaFeEDTA in flour fortification programmes. Electrolytic iron can theoretically improve iron status when used in high-phytate flours at higher doses or other forms than used in this thesis; it should be noted, however, there is at present no empirical evidence to support this. Thus technical guidelines should be reviewed with a view to recommend NaFeEDTA as the only iron fortificant with proven efficacy in high-phytate flours.

CONCLUSIONS

NaFeEDTA improves iron status when added as an iron fortificant to whole maize flour and is the preferred fortificant for iron fortification of whole maize flour, a high-phytate food vehicle. Iron deficient individuals benefit more from consuming NaFeEDTA-fortified whole maize flour than their iron-sufficient peers. On the other hand, electrolytic iron, when given in the form and fortification levels as reported in this thesis, does not improve iron status when added as an iron fortificant to whole maize flour.

The data in this thesis suggests that individuals with α^{+}-thalassaemia are unlikely to be at risk of iron overload as a result of consuming whole maize flour fortified with NaFeEDTA at fortification levels of 56mg iron/kg flour over a 5-month period. In such individuals, iron status should be monitored in fortification programmes to confirm that this also applies following longer periods of consumption of NaFeEDTA-fortified flour.

No evidence was found within this thesis that daily EDTA consumption of up to 2.3mg/kg body weight adversely affected the status of nutritionally important mineral elements. This too should be monitored in fortification programmes.

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Summary

Summary

Food fortification is recognized to be an effective strategy for reaching substantial proportions of populations with micronutrient deficiencies. This strategy has the potential to play a key role in reducing the prevalence of iron deficiency, which remains currently the most common nutrient deficiency worldwide.

A large proportion of the world's population consumes flour, making it one of the potentially most effective food vehicles for fortification. Iron fortification of flour is difficult, however, because food grains contain natural substances such as phytates that inhibit iron absorption. Electrolytic iron has relatively low bioavailability but is favoured for fortification in many countries because it is low-cost, stable and it does not affect the organoleptic properties of food vehicles. Ferrous sulfate, which is highly bioavailable and commonly used for fortification and supplementation, is not suitable in hot climates because it causes rancidity and can only be used in flours that are stored for short periods. Ferrous fumarate is also highly bioavailable under normal circumstances and causes fewer sensory problems than ferrous sulfate. The bioavailability of all three fortificants is substantially reduced, however, in the presence of iron absorption inhibitors, limiting their effective use in flour especially from whole grains or in high-phytate diets.

Findings from stable isotope studies indicate that the bioavailability of fortificant iron from NaFeEDTA is substantially less affected by absorption inhibitors than that of electrolytic iron, or iron from inorganic ferrous salts. In addition to its satisfactory bioavailability in high-phytate diets, NaFeEDTA is light in colour, relatively stable and is unaffected by cooking or prolonged storage; making it an attractive iron fortificant for regions where high temperatures and likely prolonged storage may limit the use of other forms of iron. The efficacy of NaFeEDTA had also been shown in field trials with condiments as fortification vehicles. It was also proposed by the International Nutritional Anaemia Consultative Group (INACG) for the fortification of high-phytate flours, but its potential in a high-phytate food vehicle had not been assessed, in human efficacy studies. The work described in this thesis comprised a randomised placebo-controlled iron intervention trial among 3-8 year old Kenyan school children (n = 516) between May and November 2005. It aimed to assess the efficacy and benefits of consuming whole maize flour fortified with NaFeEDTA or electrolytic iron, and to explore the safety of fortification with NaFeEDTA. In addition to measuring the effect of NaFeEDTA on iron status, its benefits were assessed on cognitive and motor function.

The use of NaFeEDTA in fortification programmes has been delayed by concerns about its safety. Evidence from animal studies assessing the toxicity of EDTA complexes shows that the most important potential consequence of the prolonged administration of large quantities of an EDTA complex is deficiency of zinc and other nutritionally important mineral elements, especially divalent cations. Other workers used isotope studies to assess potentially adverse effects of NaFeEDTA on other mineral elements, especially zinc. Findings from such studies have shown, however, that the intake of NaFeEDTA at levels present in the diet as a result of food fortification, are unlikely to affect the status of these mineral elements. In addition to assessing the efficacy and further benefits of NaFeEDTA, this thesis examined the safety of NaFeEDTA by assessing its effect on the status of five mineral elements after five months of daily consumption of the fortificant. The setting of the field trial in this thesis was a malaria-endemic area with a high prevalence of the red cell disorder, α^+-thalassaemia. It has been suggested that iron absorption may be higher in individuals with this condition than in individuals with a normal genotype. Because the bioavailability of iron from NaFeEDTA is relatively high, the thesis further addressed its safety by assessing potential effect modification of the iron intervention by α^+-thalassaemia. The objective of this assessment was to determine if individuals with α^+-thalassaemia may potentially load more iron than their counterparts with a normal genotype.

The findings of this thesis show that, even when used in a high-phytate food vehicle, NaFeEDTA is efficacious in improving iron status, whereas electrolytic iron is not (**chapter 2**). Two levels of NaFeEDTA fortification were used. Fortification with NaFeEDTA at high levels (56 mg iron per kg flour), or low levels (28 mg/kg) reduced the prevalence of iron deficiency anaemia by 89% (95% CI 49% to 97%) and 48% (-20% to 77%), respectively. Similarly, NaFeEDTA consumption reduced the prevalence of anaemia and iron deficiency. High-dose fortification with NaFeEDTA led to larger gains in iron status than low-dose level, and fortification with NaFeEDTA was more efficacious in children with poor iron status at baseline. Although other studies have shown that supplying iron to children of a similar age group improves cognitive function, this thesis found no evidence of differences in scores of cognitive or motor development tests between children who consumed iron fortified whole maize flour and those who consumed unfortified whole maize flour (**chapter 3**).

Findings of the safety assessments showed no evidence that individuals with α^+-thalassaemia responded differently to the iron intervention than their counterparts with a normal genotype

(**chapter 4**). There was also no evidence that NaFeEDTA adversely affected the status of zinc, calcium, copper, magnesium or manganese, even at levels of NaFeEDTA intake exceeding the Acceptable Daily Intake (ADI) of EDTA (**Chapter 5**). The ADI is an estimate of the amount of a food additive, expressed on a body weight basis, which can be ingested daily over a lifetime without appreciable health risk. The ADI for EDTA was derived in this thesis from the ADI set for $CaNa_2$-EDTA, which is an additive without any nutritional value that is used as an agent to enhance shelf life. By contrast, NaFeEDTA is added solely to improve health and to satisfy dietary needs for iron, which may warrant the use of a lower safety factor when setting an ADI for EDTA. Because iron may be toxic at intake levels less than 10 times those for optimal nutrition, a safety factor of 100 as applied for food additives should not be considered immutable, and a lower factor may be more appropriate.

This thesis concludes that NaFeEDTA is the preferred iron fortificant for high-phytate flours and that electrolytic iron should not be used in these flours until there is adequate evidence from human studies that it is efficacious at higher fortification levels or in other forms than used in our trial. The thesis found no evidence that the benefit of improved iron status extends to improved cognitive or motor function. There was no evidence that NaFeEDTA, when consumed at the levels in our trial, poses a risk of potential iron overload in individuals with α^+-thalassaemia, or that it adversely affects the status of zinc, calcium, copper, magnesium of manganese. We recommend monitoring the effect of prolonged use of NaFeEDTA, both in individuals with α^+-thalassaemia and on the status of other mineral elements. This should however not delay the use of NaFeEDTA in fortification programmes. There is no evidence that NaFeEDTA at levels used for fortification is unsafe, whereas it has the potential to substantially contribute to the reduction of iron deficiency.

A substantial proportion of the whole maize flour consumed in African countries, particularly in rural areas, is produced in local hammer mills. Future research should assess the potential of fortifying whole maize flour with NaFeEDTA at this small scale level, and develop appropriate methodologies for the application of such fortification. Because deficiencies of several micronutrients usually co-exist, multiple micronutrient fortification may be more effective than single nutrient fortification in improving the health status of populations. Some vitamins are readily broken down by inorganic ferrous salts that are added in the fortification mix. Because iron in NaFeEDTA may be less oxidative than ferrous iron, it should be investigated whether these vitamins are better retained when iron is added as NaFeEDTA.

Samenvatting

Samenvatting

Voedselverrijking wordt algemeen gezien als een effectieve manier om populaties met gebreksziekten te bereiken. Deze strategie kan mogelijk een sleutelrol spelen in het bestrijden van ijzergebrek, nog altijd de meest vóórkomende gebreksziekte ter wereld.

Meel is één van de meest geschikte soorten voedsel om te verrijken, omdat een groot deel van de wereldbevolking dit als basisvoedsel heeft. Het is echter moeilijk om meel te verrijken met ijzer, omdat granen van nature stoffen bevatten die de ijzeropname remmen, zoals fytaten. In veel landen wordt elektrolytisch ijzer gebruikt, omdat het relatief goedkoop en stabiel is en omdat het de smaak en geur van voedsel niet verandert. Het heeft echter een relatief lage biobeschikbaarheid. IJzersulfaat heeft een hoge biobeschikbaarheid en wordt vaak gebruikt voor verrijking en suppletie. Het is echter niet geschikt voor gebruik in warme klimaten, omdat het ranzigheid kan veroorzaken en alleen toepasbaar is in meel dat voor korte perioden wordt opgeslagen. IJzerfumaraat kan ook goed worden opgenomen en beïnvloedt de smaak- en geurwaarneming minder dan ijzersulfaat. De biobeschikbaarheid van alle drie de ijzerzouten wordt echter aanzienlijk verlaagd in de aanwezigheid van stoffen die de ijzeropname remmen. Hun toepasbaarheid in meel is daarom beperkt, vooral als het volkorenmeel betreft, of als het voedingspatroon rijk is aan fytaten.

Vergeleken met elektrolytisch ijzer of ijzer in de vorm van anorganische zouten, wordt de beschikbaarheid van toegevoegd ijzer in de vorm van NaFeEDTA aanzienlijk minder beïnvloed door opnameremmers. Dit blijkt uit resultaten van studies met stabiele isotopen. De werkzaamheid van NaFeEDTA is ook aangetoond in studies waarin het was toegevoegd aan specerijen en kruidensauzen, maar nog niet in humane studies waarbij het toegevoegd is aan voedsel dat hoge fytaatgehaltes bevat. In dit proefschrift wordt een interventie-studie bij 3- tot 8-jarige Keniaanse schoolkinderen beschreven, waarin werd gekeken naar de effectiviteit van meel uit hele maïskorrels, dat verrijkt was met NaFeEDTA of elektrolytisch ijzer. Verder is de veiligheid van deze NaFeEDTA-verrijking onderzocht. Naast het effect van NaFeEDTA op ijzerstatus, is ook de invloed op de cognitieve en motorische ontwikkeling van de kinderen gemeten.

Tot nu toe wordt ijzer als NaFeEDTA nauwelijks gebruikt in voedselverrijkingsprogramma's vanwege zorgen over de veiligheid. Verscheidene studies hebben aangetoond dat langdurige toediening van grote hoeveelheden van het EDTA-complex mogelijk leidt tot een gebrek aan

zink en andere mineralen die vanuit voedingskundig oogpunt belangrijk zijn, met name tweewaardige kationen. In de studie beschreven in dit proefschrift, werd naast de werkzaamheid ook de veiligheid van NaFeEDTA onderzocht. Dit werd gedaan door na vijf maanden dagelijks toedienen van NaFeEDTA de status van vijf andere mineralen te meten. Deze studie werd uitgevoerd in een gebied waarin malaria endemisch is en waarin α^+-thalassemie, een afwijking in de rode bloedcellen, veel voorkomt. Opname van ijzer zou in individuen met deze aandoening hoger zijn in vergelijking tot degene met een normaal genotype. Omdat de biobeschikbaarheid van NaFeEDTA relatief hoog is, werd in deze studie apart gekeken naar de veiligheid in deze groep. Het doel hiervan was om te bepalen of kinderen met α^+-thalassemie meer ijzerstapeling hebben dan degenen met een normaal genotype.

De resultaten van dit proefschrift tonen aan dat verrijking met NaFeEDTA de ijzerstatus verbetert, zelfs wanneer het gebruikt wordt in voedsel met hoge fytaatgehaltes. In tegenstelling tot NaFeEDTA bleek elektrolytisch ijzer niet effectief te zijn. NaFeEDTA werd toegediend in twee verschillende doseringen: met een hoog gehalte van verrijking (56 mg ijzer per kg meel) en met een laag gehalte van verrijking (28 mg/kg). Hoge en lage NaFeEDTA-verrijking leidde tot een verlaging van de prevalentie van ijzergebreks-bloedarmoede, met respectievelijk 89% (95% BI: 49% tot 97%) en 48% (-20% tot 77%). Inname van NaFeEDTA verlaagde eveneens de prevalentie van bloedarmoede en ijzergebrek. Verrijking met hoge gehaltes NaFeEDTA leidde tot een sterkere verbetering in ijzerstatus dan lage gehaltes. Verder was verrijking met NaFeEDTA effectiever in kinderen die bij aanvang van de interventie een lage ijzerstatus hadden.

Er waren geen aanwijzingen dat individuen met α^+-thalassemie anders op de ijzerinterventie reageerden dan kinderen met een normaal genotype. Bovendien waren er geen indicaties die erop duidden dat NaFeEDTA een negatief effect had op de bloedwaarden voor zink, calcium, koper, magnesium of mangaan, zelfs met inname-niveaus van NaFeEDTA waarbij de 'Acceptable Daily Intake' (ADI) voor EDTA werd overschreden. De ADI is een schatting van de hoeveelheid van een aan voedsel toegevoegde stof, uitgedrukt per kilogram lichaamsgewicht, die levenslang dagelijks kan worden ingenomen zonder relevante gezondheidsrisico's. De ADI van EDTA werd in dit proefschrift afgeleid van de ADI die was bepaald voor $CaNa_2$-EDTA, een stof zonder voedingswaarde die wordt toegevoegd om de houdbaarheid van voedsel te verhogen. Omdat NaFeEDTA, in tegensteling tot $CaNa_2$-EDTA,

uitsluitend wordt gebruikt om de gezondheid te verbeteren, zou bij het berekenen van de ADI voor EDTA mogelijk een lagere veiligheidsfactor gebruikt moeten worden dan bij $CaNa_2$-EDTA. Omdat ijzer toxisch is bij innameniveaus die minder dan 10 keer lager zijn dan nodig voor een optimale voeding, moet een veiligheidsfactor van 100, zoals toegepast bij de berekening van de ADI voor toegevoegde stoffen, niet gezien worden als onherroepelijk; een lagere factor is mogelijk geschikter.

In dit proefschrift wordt o.a. geconcludeerd dat meel met hoge gehaltes aan fytaten het beste verrijkt kan worden met NaFeEDTA. Het verdient aanbeveling om elektrolytisch ijzer niet te gebruiken in volkoren-meel, totdat de mogelijke werkzaamheid voldoende wordt aangetoond bij hogere niveaus van verrijking of bij andere vormen dan gebruikt in deze interventie-studie. In dit proefschrift werden geen aanwijzingen gevonden die erop duiden dat een verbeterde ijzerstatus gepaard gaat met een verbeterde cognitieve of motorische ontwikkeling. Er zijn ook geen aanwijzingen gevonden dat NaFeEDTA, met de gebruikte innameniveaus, een risico van ijzerstapeling vormt in kinderen met α^+-thalassemie, of dat het een negatief effect had op de status voor zink, calcium, koper, magnesium of mangaan. Het verdient wel aanbeveling om het effect van langdurige inname van NaFeEDTA in individuen met α^+-thalassemie op de bloedwaarden van andere mineralen te volgen. Omdat NaFeEDTA kan bijdragen aan een aanzienlijke verlaging van ijzergebreksbloedarmoede en er geen aanwijzingen zijn dat NaFeEDTA onveilig is bij de hoeveelheden die gebruikt worden voor verrijking, zou dit niet moeten leiden tot uitstel van het gebruik van NaFeEDTA in verrijkingsprogramma's.

In Afrika wordt een aanzienlijk deel van het volkoren meel geproduceerd in kleinschalige fabriekjes. Toekomstig onderzoek zou gericht moeten zijn op het ontwikkelen en toepassen van methoden om maïsmeel op kleine schaal te verrijken. Omdat gebreksziekten van verscheidene micronutriënten vaak tegelijkertijd vóórkomen, kan verrijking met meerdere micronutriënten effectiever zijn in het verbeteren van de gezondheidstoestand van populaties dan wanneer met één voedingsstof wordt verrijkt. Sommige vitaminen worden snel afgebroken door anorganische ijzerzouten wanneer deze worden verwerkt in een micronutriënten-mix die voor verrijking wordt gebruikt. Omdat ijzer in NaFeEDTA minder oxidatief is dan in anorganische zouten, zijn studies nodig om te onderzoeken of deze vitaminen beter behouden blijven als ijzer wordt gebruikt in de vorm van NaFeEDTA.

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Curriculum Vitae

The Author

Pauline Emma Aoko Andang'o was born in Kisumu, Kenya on the 15th January 1968, and grew up in Njoro, near Nakuru, Kenya. In 1990 she graduated from Kenyatta University, Nairobi, Kenya, with a Bachelor of Education in Home Economics after which she was posted to teach Home Science at Koru Girls' secondary school, where she served for two and a half years.

In 1992, having received the John Crawford scholarship, Pauline joined Flinders University of South Australia, where she was finally able to pursue studies in Dietetics, in which she developed an interest in her first year of high school. In 1995 she obtained a Masters degree in Nutrition and Dietetics after which she joined Maseno University as a tutorial fellow and eventually became a lecturer in the same institution. In 2003 Pauline joined the Division of Human Nutrition, Wageningen University, as a PhD sandwich student, on a collaborative research project involving Unilever Food and Health Research Institute, R&D, Vlaardingen; the Centre for Public Health Research, Kenya Medical Research Institute and the Division of Human Nutrition, Wageningen University. During her time in the Netherlands she attended several PhD courses at Wageningen University and the Erasmus Summer course offered by NIHES, Rotterdam, and also attended several conferences and discipline-specific meetings.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

Courses

Introduction to Epidemiology and Statistics, 2003

Principles of Research in Medicine, Erasmus Summer Programme, Rotterdam, 2005

Clinical Trials, Erasmus Summer Programme, Rotterdam, 2005

Topics in Evidence-based Medicine, Erasmus Summer Programme, Rotterdam, 2005

Introduction to Data-analysis, Erasmus Summer Programme, Rotterdam, 2005

Regression Analysis, Erasmus Summer Programme, Rotterdam, 2005

History of Epidemiologic Ideas, Erasmus Summer Programme, Rotterdam, 2005

Master class Dietary Influences on Blood Pressure VLAG, 2007

Philosophy and Ethics of Food Science and Technology VLAG, 2007

Meetings and Conferences

18th International Nutrition Congress, Durban, South Africa (Poster presentation)

Symposium on Iron, Unilever

Bioavailability 2006, Chiang Mai, Thailand. (oral presentation, workshop).

Biofortification workshop, HarvestPlus, Mombasa, Kenya, April 2006

African Nutrition Leadership Programme (ANLP), November 2006

Micronutrient Forum 2007, Istanbul (Poster presentation; participant: Young investigators' Workshop).

General courses

How to write and read a medical paper, Erasmus Summer Programme, Rotterdam, 2005

Writing and presenting a scientific paper, Wageningen Graduate Schools (WGS), 2006

Project and Time Management WGS, 2006

Advanced statistics WGS, 2007

Optional

Preparation PhD research proposal, 2003

HNE-38802 Concepts and Methods in Epidemiology in 2006/2007

Staff seminars, Wageningen University, 2003-2007

Journal club 2006/2007, Tanzania 2006 and Wageningen, Oldsmobiles 2007

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Cover

A child in the compound of one of the study schools; donkey cart used to ferry porridge to the neighbouring nursery school (back cover). Photo by Saskia Osendarp.

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