Doctoral Thesis

In-Home Fortification with Iron in Africa: Effects on Infant Iron Status and Gut Microbiota

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In-Home Fortification with Iron in Africa:
Effects on Infant Iron Status and Gut Microbiota

A dissertation submitted to
ETH Zurich

For the degree of
Doctor of Sciences

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The most exciting phrase to hear in science, the one that heralds new discoveries,
is not ‘Eureka!’, but ‘That’s funny...’

(Isaac Asimov)
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SUMMARY

Background
Iron deficiency is a major global public health problem, especially in infants and young children with increased iron requirements due to their rapid growth. The consequences of iron deficiency and eventually anemia during infancy are deficits in cognitive, motor, socio-emotional, and neurophysiological development.

Provision of additional iron to populations at risk of iron deficiency anemia has shown to be efficacious. However, iron supplementation of infants has been reported to increased adverse events (diarrhea, malaria, and respiratory tract infections) in malarial areas, and the World Health Organization does no longer recommend untargeted administration of iron supplements. Although the provision of lower doses of iron added to foods (fortification) is generally considered a safer method, recent data suggest adverse events even with this approach.

Episodes of overall, severe and bloody diarrhea and respiratory tract infections have been suggested to be increased by in-home fortification with iron in a recent large but non-blinded trial in Pakistani infants. Furthermore, iron fortification in African school children has been associated with potentially dangerous changes in children’s gut microbiota composition and increased gut inflammation.

The iron-regulatory mechanisms in infants in Sub-Saharan Africa are not well understood. Serum hepcidin is the major iron regulator, but there are little data available of concentrations and predictors in infants; especially from developing countries where competing stimuli such as iron deficiency and infection/inflammation are commonly present.

Objectives
The overall aim of this thesis was to explore efficacious and safe ways to improve the iron status of Sub-Saharan African infants. More specifically we aimed to: (1) investigate the determinants of hepcidin in Kenyan infants with a high burden of both iron deficiency and infection; (2) in a one-year randomized controlled trial (RCT), assess the efficacy of in-home fortification using a low dose iron-containing micronutrient powder (MNP) on iron status and growth in infants; (3) explore the effect of a low and a high dose iron-containing MNPs on infants gut microbiota and metabolites, gut inflammation and morbidity.
SUMMARY

Design

**Manuscript 1:** A cross-sectional analysis of determinants of hepcidin-25 in 5-7 month old infants \((n=339)\) from rural Kenya was performed. Serum hepcidin-25, iron and inflammation markers were assessed, and in a subsample of the infants \((n=148)\) the gut inflammation marker fecal calprotectin was measured. The determinants of serum hepcidin-25 where explored and described using a multiple regression analysis.

**Manuscript 2:** A one-year RCT of in-home fortification of complementary food with an MNP containing 2.5 mg iron as NaFeEDTA was conducted. Six month old Kenyan infants \((n=287)\) were recruited to consume either maize porridge fortified with an MNP including 2.5 mg NaFeEDTA \(+2.5\text{mgFeMNP}\) or maize porridge fortified with the same MNP but without iron \(-2.5\text{mgFeMNP}\), daily. Iron status, inflammation, anthropometrics and morbidity (diarrhea, malaria, and respiratory tract infections (RTI)) were measured at baseline, after 6, and 12 months.

**Manuscript 3:** We performed two RCTs in six month old Kenyan infants \((n=101)\) consuming in-home fortified maize porridge for 4 months. In the first, infants received an MNP containing 2.5 mg iron as NaFeEDTA \(+2.5\text{mgFeMNP}\) or the identical MNP without iron \(-2.5\text{mgFeMNP}\). In the second, they received an MNP containing 12.5 mg iron as ferrous fumarate \(+12.5\text{mgFeMNP}\) or the identical MNP without iron \(-12.5\text{mgFeMNP}\). The primary outcome was the change in the composition of the gut microbiome, analyzed by using 16S rRNA pyrosequencing and by using quantitative PCR (qPCR) for selected taxa, after 3 weeks and 4 months. Secondary outcomes were changes in fecal calprotectin and incidence of treated diarrhea during the study, as well as anemia, iron status and systemic inflammation. We analyzed the two trials separately and pooled the two trials \(+\text{FeMNP}s vs. -\text{FeMNP}s\) to compare the overall effect of iron fortification.

Results

**Manuscript 1:** Prevalence of inflammation, anemia, and iron deficiency was 31%, 71%, 26%, respectively. The overall geometric mean \(\pm SD\) of serum hepcidin-25 was 6.0 \(\pm 3.4\) ng/mL, and was significantly lower in males \((4.9 \pm 3.5 \text{ ng/mL})\) than females \((7.2 \pm 3.3 \text{ ng/mL})\). Inflammation (C-reactive protein and interleukin-6) and iron status (serum ferritin, zinc protoporphyrin and soluble transferrin receptor) were significant predictors of serum hepcidin-25, explaining nearly 60% of its variance. There were
small, but significant differences in serum hepcidin-25 comparing iron deficient anemic infants without inflammation to iron deficient anemic infants with inflammation (1.2 ±4.9 vs. 3.4 ±4.9 ng/mL; P < 0.001). Fecal calprotectin correlated with blood/mucus in the stool but not with hepcidin-25 concentration. Similarly, the gut-linked cytokines IL-12 and IL-17 did not correlate with hepcidin-25 concentration.

*Manuscript 2:* At baseline 71% of the infants were anemic and 22% iron deficient, prevalence of inflammation was high (31% had an elevated CRP). During the one-year RCT of in-home fortification of maize porridge using +2.5mgFeMNP versus -2.5mgFeMNP, infants hemoglobin increased and serum ferritin decreased in both groups, without significant treatment effects on iron status indicators. There were no significant differences in the weekly measures of morbidity. At endpoint, the weight of the infants consuming +2.5mgFeMNP was greater than the one of the infants consuming -2.5mgFeMNP (9.9 kg vs. 9.5 kg; P=0.038).

*Manuscript 3:* Six months old Kenyan infants harbor a *Bifidobacteriaceae* dominated gut microbiome with a high prevalence of pathogenic bacteria, including *Salmonella*, *Clostridium difficile*, *Clostridium perfringens*, and pathogenic *Escherichia coli*. Iron status (SF, sTfR, ZPP, and body iron stores) was significantly increased by +12.5mgFeMNP, but not by +2.5mgFeMNP. Using pyrosequencing, +FeMNPs (pooled +2.5mgFeMNP and +12.5mgFeMNP) increased the enterobacteria, particularly *Escherichia/Shigella* (P=0.048), the enterobacteria to bifidobacteria ratio (P=0.020), and *Clostridium* (P=0.030) compared to -FeMNPs. Most of these effects were confirmed using qPCR; e.g., +FeMNPs increased pathogenic *E. coli* strains (P=0.029). +FeMNPs also increased fecal calprotectin (P=0.002). During the trial, 27.3% of infants in +12.5mgFeMNP required treatment for diarrhea versus 8.3% in -12.5mgFeMNP (P=0.092). There were no study-related serious adverse events in either group.

**Conclusions**
*Manuscript 1:* From the results of the cross-sectional analysis we conclude that hepcidin regulatory pathways, driven by iron status and inflammation, are functional in 6 month-old infants. However, serum hepcidin-25 alone may provide insufficient discriminatory power to distinguish between iron deficient anemic infants with and without infection and further data are needed before hepcidin can be recommended as an exclusive point-of-care marker for targeted iron interventions. Furthermore, we...
propose gender-specific reference values for serum hepcidin-25 in iron-replete infants without inflammation.

Manuscript 2: In the efficacy study, a low dose iron-containing MNP did not improve Kenyan infant’s iron status or reduce anemia prevalence compared to the same MNP without iron, likely because iron absorption was inadequate due to high infection/inflammation prevalence and low iron dose. Concerning its safety, there was no difference in the prevalence of diarrhea, malaria and RTI between the two treatment groups. However, iron fortification seems to have increased infant weight.

Manuscript 3: From the results of the two studies using high and low iron fortification in infants, we conclude that in a rural African setting, iron fortification modified the gut microbiome composition of weaning infants by increasing enterobacteria, especially specific enteropathogens, e.g. pathogenic E. coli, and decreasing beneficial bifidobacteria. Furthermore, iron fortification increased fecal calprotectin levels, indicating intestinal inflammation. These data provide a possible mechanism for the increases in diarrhea seen in recent infant fortification studies. Our findings, together with others suggest that, until safer formulations are available, +FeMNPs should be targeted to infants with iron deficiency anemia, while providing adequate protection from malaria and diarrhea in areas with high infectious disease burden. A priority for future research is to develop +FeMNPs, and other in-home strategies such as crushable tablets or fat-based spreads, which are both effective and safe.
ZUSAMMENFASSUNG

Hintergrund

Die Verabreichung von zusätzlichem Eisen in Risikogruppen hat sich als wirksam erwiesen. Jedoch haben Eisensupplemente bei Kindern zu erhöhten Nebenwirkungen geführt (Durchfall, Malaria und Infektionen der Atemwege). Deshalb empfiehlt die Weltgesundheitsorganisation Eisensupplemente nur noch für eine zielgerichtete Abgabe an Personen mit Eisenmangel. Auch wenn die direkte Lebensmittelanreicherung mit geringeren Eisenmengen (die sogenannte Fortifizierung) generell als sicherer betrachtet wird, wurden kürzlich auch bei Studien mit dieser Methode Nebenwirkungen festgestellt.


Die Mechanismen der Eisenregulierung in afrikanischen Säuglingen sind nicht gut untersucht. Serum Hepcidin ist der Hauptregulator von systemischem Eisen, jedoch existieren in Säuglingen nur wenige Daten über Hepcidin Konzentrationen und Faktoren welche diese beeinflussen; insbesondere aus Entwicklungsländern, in welchen kompetitive Reize wie Eisenmangel und Infektionen/Entzündungen stark verbreitet sind.

Ziele
Das Hauptziel dieser Doktorarbeit war es, wirksame und sichere Methoden zur Verbesserung des Eisenstatus von afrikanischen Säuglingen südlich der Sahara zu erforschen. Der Fokus lag auf: (1) der Erforschung der Faktoren, welche Serum Hepcidin in kenianischen Säuglingen mit einem hohen Risiko von Eisenmangel und Infektionen
beeinflussen; (2) der Untersuchung der Wirksamkeit von Eisenfortifizierung mittels eines Mikronährstoffpulvers (MNP) mit einer geringen Eisendosis auf den Eisenstatus und das Wachstum von Säuglingen während einer einjährigen randomisierten und kontrollierten Studie; (3) der Erforschung der Effekte von tiefen und hohen Eisendosen in MNPs auf die Darmflora und deren Stoffwechselprodukte, sowie Darmentzündung und Morbidität in Säuglingen.

Design

Manuskript 1: Es wurde eine Querschnittsanalyse der bestimmenden Faktoren von Serum Hepcidin-25 in 5-7 Monate alten Säuglingen (n=339) aus einem ländlichen Gebiet Kenias durchgeführt. Serum Hepcidin-25, Eisenstatus- und Entzündungsmarker wurden erfasst und in einer Untergruppe der Kinder (n=148) wurde der Darmentzündungsmarker fäkales Calprotectin gemessen. Die bestimmenden Faktoren wurden mittels multiplen Regressionsanalysen erforscht und beschrieben.

Manuskript 2: Es wurde eine einjährige, randomisierte Studie mit einer mit Eisen angereicherten Ergänzungsnahrung, welche 2.5 mg Eisen in Form von NaFeEDTA enthält, durchgeführt. Kenianische Säuglinge im Alter von sechs Monaten (n=287) wurden eingeschlossen und mussten täglich entweder einen Maisbrei fortifiziert mit Mikronährstoffen inklusive Eisen (+2.5mgFeMNP) oder einen Maisbrei mit den gleichen Mikronährstoffen aber ohne Eisen verzehren (-2.5mgFeMNP). Eisenstatus, Entzündung, anthropometrische Daten und Morbidität (Durchfall, Malaria und Entzündungen der Atemwege) wurden zu Beginn der Studie, nach 6 und 12 Monaten gemessen.

Manuskript 3: Wir haben zwei randomisierte, kontrollierte Studien mit sechs Monate alten Kenianischen Säuglingen durchgeführt, welche während 4 Monaten täglich mit MNP angereicherten Maisbrei verzehrt haben. In der ersten Studie, erhielten die Säuglinge entweder ein MNP welches 2.5 mg Eisen in Form von NaFeEDTA (+2.5mgFeMNP) beinhaltete, oder das identisches MNP ohne Eisen (-2.5mgFeMNP). Im Rahmen der zweiten Studie, erhielten die Säuglinge entweder ein MNP mit 12.5 mg Eisen in Form von Eisenfumarat (+12.5mgFeMNP), oder das identisches MNP ohne Eisen (-12.5mgFeMNP). Der primäre Zielparameter war die Veränderung in der Zusammensetzung der Darmflora, welche mittels 16S rRNS Sequenzierung (pyrosequencing) und für bestimmte Bakterienarten und -gattungen mittels quantitativem PCR (qPCR) zu Beginn der Studie, nach 3 Wochen und nach 4 Monaten
bestimmt wurden. Sekundäre Zielparameter waren die Veränderungen von fäkalem Calprotectin und die Inzidenz von zu behandelnder Durchfall, Malaria und Atemwegsentzündungen während der Studien, sowie Anämie, Eisenstatus und systemische Entzündung. Wir haben die zwei Studien separat analysiert und haben die Daten vereint (+FeMNPs vs. -FeMNPs) um den generellen Effekt der Eisenfortifizierung zu zeigen.

**Resultate**

*Manuskript 1:* Die Prävalenzen von Entzündungen, Anämie und Eisenmangel waren 31%, 71% und 26%. Der geometrische Mittelwert ±SD von Serum Hepcidin-25 war 6.0 ±3.4 ng/mL und war signifikant tiefer in männlichen (4.9 ±3.5 ng/mL) als in weiblichen (7.2 ±3.3 ng/mL) Säuglingen. Entzündung (C-reactives Protein und Interleukin-6) sowie Eisenstatus (Serum Ferritin, Zinkprotoporphyrin und löslicher Transferrinrezeptor) haben Serum Hepcidin-25 signifikant beeinflusst und erklärt beinahe 60% von dessen Varianz. Der Unterschied in Serum Hepcidin-25 Werten von Säuglingen mit Eisenmangelanämie ohne Entzündung und von jenem mit Eisenmangelanämie mit Entzündung war klein aber signifikant (1.2 ±4.9 vs. 3.4 ±4.9 ng/mL; P <0.001). Fäkales Calprotectin korrelierte mit Blut/Schleim im Stuhl jedoch nicht mit der Konzentration von Hepcidin-25. Ähnlich, korrelierten auch die Zytokine IL-12 und IL-17, welche oft mit Darmentzündungen in Verbindung gebracht werden, nicht mit Hepcidin-25.

*Manuskript 2:* Zu Beginn der Studie waren 71% der Säuglinge anämisch und 22% hatten Eisenmangel, die Entzündungsprävalenz war hoch (31% hatten ein erhöhtes CRP). Über den Zeitraum der einjährigen, randomisierten und kontrollierten Studie, in welcher das Fortifizieren von Maisbrei mit +2.5mgFeMNP oder -2.5mgFeMNP verglichen wurden, stieg das Hämoglobin und das Serum Ferritin sank in beiden Gruppen ohne signifikante Behandlungseffekte auf den Eisenstatus. Die Morbidität unterschied sich nicht zwischen den beiden Gruppen. Am Ende der Studie waren Säuglinge welche Maisbrei mit +2.5mgFeMNP verzehnten schwerer als jene welche -2.5mgFeMNP konsumierten (9.9 kg vs. 9.5 kg; P=0.038).

*Manuskript 3:* Sechs Monate alte Kenianische Säuglinge besassen eine Darmflora welche von *Bifidobacteriaceae* dominiert wurde und jedoch eine hohe Prävalenz von Pathogenen wie *Salmonella*, *Clostridium difficile*, *Clostridium perfringens* und pathogene *Escherichia coli* aufwies. Der Eisenstatus (SF, sTfR, ZPP, und der Körpereisenspeicher)
wurde durch +12.5mgFeMNP signifikant erhöht, jedoch nicht durch +2.5mgFeMNP. Mittels pyrosequencing wurde eine Erhöhung der Enterobakterien, hauptsächlich *Escherichia/Shigella* (p=0.048), das Verhältnis von Enterobakterien zu Bifidobakterien (p=0.020) und *Clostridium* (p=0.030) durch +FeMNP festgestellt. Die Mehrheit dieser Effekte wurden durch die qPCR Methode bestätigt, wie etwa die Zunahme von pathogenen *E. coli* Stämme (P=0.029). +FeMNP haben zudem das fäkale Calprotectin erhöht (P=0.002). Während der Studie benötigten 27.3% der Kleinkinder von der +12.5mgFeMNP und 8.3% von -12.5mgFeMNP medizinische Behandlung wegen Durchfall (P=0.092). Es gab in keiner Gruppe schwerwiegende studienbezogene Nebenwirkungen.

**Schlussfolgerungen**


*Manuskript 3:* Aus den Resultaten der beiden Eisenfortifizierungstudien, welche hohe und tiefe Fortifizierungsdosen untersuchten, schliessen wir: In Säuglingen aus einer ländlichen afrikanischen Umgebung modifizierte Eisenfortifizierung die
Darmflorazusammensetzung indem sie Enteropathogene erhöht, im Speziellen pathogene E. coli, so wie gesundheitsfördernde Bifidobakterien reduziert. Darüber hinaus erhöhte die Eisenfortifizierung das fäkale Calprotectin, welches auf Darmentzündung hinweist. Diese Daten liefern einen möglichen Mechanismus für die Häufigkeit von Durchfall in mehreren kürzlich publizierten Fortifizierungstudien. Erkenntnisse von diesen und anderen Studien implizieren, dass +FeMNPs, in Gebieten mit häufig verbreiteten Infektionskrankheiten, nur zielgerichtet an Kinder mit Eisenmangel und Anämie abgegeben werden sollten, während gleichzeitig ein ausreichender Schutz vor Malaria und Durchfall geboten wird. Die Forschung an der Entwicklung von sicheren und wirksamen +FeMNPs, oder anderen geeigneten Fortifizierungstrategien, wie zum Beispiel Tabletten welche zerteilt werden können oder Aufstriche auf Fettbasis, sollte eine Priorität sein.
Iron deficiency is a major public health problem, and infants and young children are a highly vulnerable population (Zimmermann and Hurrell 2007). The consequences of iron deficiency and eventually anemia during infancy are deficits in cognitive, motor, socio-emotional, and neurophysiological development (Lozoff 2007, Georgieff 2011). Healthy infants are born with body iron stores covering their needs during the first 4-6 months of life, afterwards additional iron needs to be provided through complementary feeding (Dallman 1986). During this weaning period, iron requirements relative to energy intake are highest (Dewey 2013). Unfortunately, iron bioavailability from cereal products, often used for weaning foods, is often very low due to high concentrations of the absorption inhibitor phytic acid (Cook et al. 1997).

There are three main strategies to combat iron deficiency in populations: (A) nutritional education and dietary modification or diversification, (B) iron fortification of foods, (C) iron supplementation (Zimmermann and Hurrell 2007, Berger et al. 2011). Iron fortification of complementary foods is considered to be a practical, sustainable, cost-effective long-term approach to increase iron status in children (WHO 2001, Baltussen et al. 2004, WHO 2006). Systematic reviews have shown that iron-fortified micronutrient powders can be efficacious in improving iron status and reducing anemia in infants and young children (De-Regil et al. 2011, Eichler et al. 2012, Gera et al. 2012). Furthermore, reviews of iron-fortified food, in contrary to iron supplements, in young children have not shown an increase in morbidity from malaria or other infections and adverse events in tropical regions (Oppenheimer 2001, Gera and Sachdev 2002). However, in the Pemba trial, iron supplementation in young children increased morbidity and mortality, raising safety concerns for untargeted iron interventions in malarial regions (Sazawal et al. 2006). In 2006, the World Health Organization (WHO) stated that even in-home iron fortification of complementary foods for infants and young children in malaria-endemic areas cannot be recommended until their safety has been demonstrated (Fontaine 2007). Very recently, two large trials in Pakistan and Ghana reported increased incidence of adverse events (severe and bloody diarrhea, and higher hospitalization) in infants and young children consuming iron-containing micronutrient powders (Soofi et al. 2013, Zlotkin et al. 2013).

Nearly all intestinal bacteria require iron for metabolic or signaling functions, and lactobacilli are the only notable exception (Archibald 1983, Weinberg 1997, ...
INTRODUCTION

Wandersman and Delepelaire 2004). Furthermore, virulence of pathogenic bacteria has been shown to increase with sufficient iron acquisition (Genco and Desai 1996, Vasil and Ochsner 1999, Berlutti et al. 2005, Coray et al. 2012, Kortman et al. 2012). Iron fortification of Ivorian children has been associated with a potentially more pathogenic gut microbiota profile, consisting of higher concentrations of enterobacteria and less lactobacilli, as well as increased gut inflammation (Zimmermann et al. 2010). However, there are little data available on the effect of iron fortification on infant gut microbiota.

Systemic iron metabolism regulation in young African children is a further knowledge gap: it is uncertain if the expression of circulating hepcidin, the major iron regulator, is already functional and how it is influenced by concurrent and competing stimuli such as infection/inflammation and iron deficiency, to which African infants are highly vulnerable (Lonnerdal and Kelleher 2007).

The first part of this thesis is a review of the published literature starting with the epidemiology of iron deficiency, iron metabolism and safety of iron interventions in infants and young children living in Sub-Saharan Africa. Further, the human gut and the complex coexistence of microorganisms will be introduced followed by an evaluation of the published studies focusing on the effect of iron on the gut microbiota. The second part of this thesis is presenting original research and contains 3 manuscripts: manuscript 1 is an evaluation of the iron-regulatory mechanisms in 6 month-old Kenyan infants prone to iron deficiency and infections. Manuscript 2 reports the one-year randomized controlled trial in the same population using in-home fortification of maize porridge with a micronutrient powder containing 2.5 mg iron as NaFeEDTA or the same micronutrient powder without iron. Finally, manuscript 3 describes the impact of a low (2.5 mg NaFeEDTA) and a high (12.5 mg ferrous fumarate) iron-containing MNP on the gut microbiota composition, its metabolites and on intestinal inflammation.
1. Epidemiology of iron deficiency in infants and young children

1.1 Prevalence of iron deficiency, anemia and iron deficiency anemia

Iron deficiency (ID) is the most common and widespread nutritional disorder worldwide (WHO 2008). It is defined as a reduction in total body iron to such an extent that body iron stores are fully depleted and also tissue iron deficiency is present (Cook 2005). A long-term negative iron balance can result in iron deficiency anemia (IDA), defined as an insufficient concentration of hemoglobin in the blood, caused by the lack of iron (Stoltzfus 1998). It is estimated that ~50% of the world’s anemia cases may be due to iron deficiency (WHO 2001). Further causes for anemia are deficiencies in other micronutrients (folic acid, Vitamin B12, Vitamin A), infectious and chronic diseases (e.g. malaria, hookworm, HIV), and inherited disorders (i.e. sickle cell disease and thalassemia) (Tolentino and Friedman 2007, Agarwal and Prchal 2009).

Prevalence of ID varies according to hosts age, gender, physiological, pathological, environmental, and socioeconomic conditions (WHO 2001). Africa has the highest estimated anemia prevalence (65-70% in preschool children) (WHO 2008). A recent review of anemia prevalence in children (6-59 months) from 1995 to 2011, including data from 257 surveys, reported a decrease in anemia in central and west Africa (from 80% to 71%) and in east Africa (from 74% to 55%) and an increase in southern Africa (from 30% to 46%) (Stevens et al. 2013). Global data on iron deficiency are limited, but have been estimated based on anemia prevalence (Figure 1): most preschool children in developing countries are considered to be iron deficient (WHO 2001, WHO 2006). However, the use of low hemoglobin and therefore anemia to assess ID has its limitations, as sensitivity and specificity are low. This means an individual has to lose a large portion of its body iron before developing anemia (low sensitivity) and there are various causes of anemia other than ID (low specificity) (Cook 2005). The available iron status indicators for infants will be further discussed in chapter 2.5. Iron deficiency is estimated to account for 841,000 deaths and 35,057,000 disability-adjusted life years (DALYs) lost, the vast majority in developing countries (Stoltzfus 2003). In the recently published Global Burden of Disease Study, IDA is at rank 3 of the global years lived with disability, as it was 1990 (Vos et al. 2012). Consequences of iron deficiency, especially during infancy, will be further elucidated in the next chapter (1.2).
1. Epidemiology of iron deficiency in infants and young children

1.2 Consequences of iron deficiency in infancy

Iron deficiency and anemia in infancy are associated with poorer cognitive, motor, socio-emotional, and neurophysiological outcomes in later life (Lozoff 2007, Georgieff 2011). A review by Lozoff et al. summarizes studies examining differences of IDA and non-IDA children aged 6 to 24 months, and also long-lasting effects of IDA during infancy. The reviewed studies suggest that IDA during infancy decreases overall mental, motor, and socio-emotional functioning and neurocognitive development at preschool, school age and adolescence (Lozoff et al. 2006). A recently published long-term study investigated functional outcomes in young adulthood following chronic ID in infancy and reported poorer adult mental health and education in subjects having a history of ID in infancy (Lozoff et al. 2013).

Studies in rats have shown that iron is essential for proper neurogenesis and differentiation of brain cells and regions (Rao et al. 2003, 2007, Ward et al. 2007). Especially the hippocampus and striatum are morphologically and metabolically altered due to ID. There is a decrease in branching of the dendrites and therefore number and complexity of interneuronal connections. Furthermore, the myelin-producing
oligodendrocytes are affected by ID and can affect the composition and amount of myelin and white matter. Also neurochemistry is suspected to be altered through ID, especially the monoamine biosynthetic pathway, altering the metabolism of dopamine and norepinephrine (Beard 2008).

1.3 Strategies to combat nutritional iron deficiency

Nutritional iron deficiency occurs when physiological requirements cannot be covered by iron absorption from diet. Iron bioavailability is low in populations consuming monotonous plant-based foods with little meat (Zimmermann and Hurrell 2007). The absorption of non-heme iron has recently been suggested to be 1.7-16.9% (10th, 90th percentile) in the average adult consuming a mixed diet (Armah et al. 2013). Meat and ascorbic acid enhance non-heme iron absorption, whereas phytates, polyphenols, and calcium inhibit it (Hurrell 2002, 2006). In meat about 30-60% of the iron is heme iron, of which 15-35% is absorbed (Cook and Monsen 1976, Monsen et al. 1978). Since iron and energy intake are directly related; the risk of ID is highest when iron requirements are greater than energy needs, as during infancy, when rapid growth exhausts iron stores and provided breast milk and unfortified weaning foods are low in iron (Zimmermann and Hurrell 2007).

Three main strategies to combat ID in populations have been advocated: nutritional education and dietary modification and diversification, iron fortification of foods, and iron supplementation (Zimmermann and Hurrell 2007, Berger et al. 2011).

Dietary modification and diversification is the most sustainable approach; however, behavioral changes are difficult to sustain and foods providing highly bioavailable iron (e.g. meat) or enhancing iron absorption (e.g. containing ascorbic acid and organic acids) are expensive and not always accessible (Zimmermann and Hurrell 2007). A study in 12-17 years old IDA Beninese girls has shown a significant reduction in prevalence of anemia and IDA through nutritional education and dietary modification after 22 weeks (Alaofe et al. 2009). A 18 weeks nutrition intervention in 1-6 year old Burkinabe children, during which consumption of heme iron-rich foods (goat meat and liver) and enhancers of non-heme absorption such as oranges was promoted, successfully increased Hb and decreased anemia and ID (Sanou et al. 2010).

Iron supplementation can be quickly implemented and is likely to improve iron stores in populations at risk rapidly, but is only a short-term solution (Pasricha et al. 2013).
Supplementation of children in tropical regions with iron has shown increases in malaria incidence and morbidity from other infections (Oppenheimer 2001), such as diarrheal episodes (Gera and Sachdev 2002). In a trial in preschool children on Pemba, untargeted iron and folic acid supplementation increased mortality (Sazawal et al. 2006). Therefore in 2006, WHO advised against untargeted iron supplementation in children (Fontaine 2007). However, the Pemba trial co-administered iron and folic acid, which was a confounding factor, especially in combination with the use of an antifolate (sulfadoxine-pyrimethamine) as antimalarial therapy (Brittenham 2007). In 2009, the Cochrane review of untargeted iron supplementation in children in malaria-endemic areas concluded from 68 trials, that iron does not increase the risk of clinical malaria or death as long as malaria surveillance and treatment is assured (Ojukwu et al. 2009).

Iron fortification can be split into bio-fortification of plants and food fortification (including mass fortification and in-home fortification). Increasing iron content and bioavailability from plants (bio-fortification) may be achieved using traditional breeding or genetic engineering as reviewed by Lonnerdal et al. (Lonnerdal 2003) and will not be further discussed in this review. Iron fortification of foods, especially mass fortification of staple foods, is considered to be a practical, sustainable, cost-effective long-term approach to increase iron status in populations (WHO 2001, Baltussen et al. 2004, WHO 2006). However, iron fortification of foods is difficult, as there is a trade-off between high bioavailability of the iron fortification compound and the introduction of flavor and color changes in the food to be fortified. A second problem is the presence of absorption inhibitors such as phytic acid or phenolic compounds in the food vehicle (Hurrell 2002). One of the iron compounds that can partly overcome this inhibitory effect is NaFeEDTA, chelating iron and therefore preventing the binding of iron to absorption inhibitors, especially phytic acid (Hurrell et al. 2000, Hurrell 2002). In-home fortification (specific enrichment of meals at home) combines the advantages of supplementation and food fortification and is especially important for infants which may have limited access to staple foods (Davidsson 2003). For untargeted in-home fortification of complementary foods in malarial regions, a low dose highly bioavailable iron compound (NaFeEDTA) has shown promising results (Troesch et al. 2009, 2011). A review of iron-fortified food trials showed improvement in hemoglobin, serum ferritin (SF) and reduced risk of anemia and ID (Gera et al. 2012). Another systematic review and meta-analysis of the impact of multi-micronutrient fortified milk and cereal products
have shown their efficacy in reducing anemia in infants and young children up to 3 years (Eichler et al. 2012). Iron-fortified food in young children (<18 months) has not increased morbidity from infections or other adverse effects (Oppenheimer 2001, Gera and Sachdev 2002). A summary of the currently published iron fortification trials in African infants and young children is given in chapter 1.4.

1.4 Iron fortification trials in young children in Sub-Saharan Africa

A summary of studies assessing the effect of iron-fortified foods in infants and young children (<5 years) in Sub-Saharan Africa is given in Table 1. Fifteen studies were identified, 8 randomized controlled trials (RCTs) (Power et al. 1991, Larrey et al. 1999, Zlotkin et al. 2003, Faber et al. 2005, Adu-Afarwuah et al. 2007, Adu-Afarwuah et al. 2008, The Chilenje Infant Growth 2010, Macharia-Mutie et al. 2012, Suchdev et al. 2012) and 7 intervention studies lacking an iron-free control arm (Zlotkin et al. 2001, Oelofse et al. 2003, Zlotkin et al. 2003, Christofides et al. 2006, Ouedraogo et al. 2008, Seal et al. 2008, Ndemwa et al. 2011). In the studies lacking a control/placebo arm it was considered unethical to deny these anemic and vulnerable study populations the access to iron fortification.

However, in infant studies without a proper control group, it is impossible to rule out age-driven effects. A cross-sectional study in almost 2000 Tanzanian children showed the course of hemoglobin values from birth to 4 years of age. During the first month of life these infants had very high Hb concentrations (135 g/L), which rapidly decreased reaching the lowest values when the child is 6-11 month old (76 g/L), from there the Hb slowly increased with age (Schellenberg et al. 2003). A similar increase of Hb could be seen in the control arms of several RCTs with children >6 months of age at baseline (Larrey et al. 1999, Macharia-Mutie et al. 2012, Suchdev et al. 2012). Most of the studies without iron-free control groups, including children of 6 months and older, showed an increase in Hb over the time of the intervention (Zlotkin et al. 2001, Oelofse et al. 2003, Zlotkin et al. 2003, Christofides et al. 2006, Ouedraogo et al. 2008, Seal et al. 2008), with the exception of the study by Ndemwa using a low dose iron compound (2.5 mg NaFeEDTA) in 6-59 month old Kenyan children for 13 months. The later study found no change in Hb and anemia but a decrease of TfR concentration over the time of the intervention (Ndemwa et al. 2011). Many of these uncontrolled iron fortification studies had the inclusion criterion of low hemoglobin (Zlotkin et al. 2001, Zlotkin et al. 2003, Christofides et al. 2006, Ouedraogo et al. 2008) or were conducted in a refugee camp.
This unfavorable baseline conditions may further complicate the evaluation of the intervention and may limit their generalizability to other populations.

In the 8 identified RCTs, doses of 2.5-40 mg iron (as NaFeEDTA, ferrous fumarate, or ferrous sulfate) daily were efficacious in increasing Hb (Power et al. 1991, Faber et al. 2005, Adu-Afarwuah et al. 2007, Adu-Afarwuah et al. 2008, The Chilenje Infant Growth 2010, Macharia-Mutie et al. 2012, Suchdev et al. 2012); whereas in Ghanaian children doses of 12.5-40 mg iron (as ferrous fumarate, ferrous sulfate, or microencapsulated ferrous fumarate) daily did not have a significant impact on Hb compared to the control group (Lartey et al. 1999, Zlotkin et al. 2003, Adu-Afarwuah et al. 2008). Further treatment effects on iron status (SF, sTfR, ZPP) could be found in 6 of the 8 RCTs (Power et al. 1991, Lartey et al. 1999, Faber et al. 2005, Adu-Afarwuah et al. 2008, The Chilenje Infant Growth 2010, Macharia-Mutie et al. 2012). These studies indicate that iron fortification in African preschool children can be efficacious.

A nutrition review of the effect of iron on growth, found an association of increased weight through high doses (40-66 mg) of supplemental iron in children aged 6 years or older (Vucic et al. 2013). However, the only study in African children <6 years, using 60 mg iron as ferrous sulfate, did not indicate any effect of iron on growth (Dossa et al. 2001). Similarly, none of the iron fortification studies discussed in this chapter was able to show an impact of iron on growth; with one exception of a subgroup of Zambian infants with HIV-infected mothers, who stopped breastfeeding, showing modest improvement in linear growth (The Chilenje Infant Growth 2010).

Iron supplementation, but not fortification, has indicated to increase malaria incidence and morbidity from other infections in young children living in tropical countries (Oppenheimer 2001, Gera and Sachdev 2002). Also in the presently discussed iron fortification studies in Sub-Saharan African settings, no effect was identified on morbidity, such as diarrhea, malaria, fever or respiratory tract infections (RTI).

A recent study in Ghanaian children (6-35 months) with the primary outcome to assess the safety of an MNP including 12.5 mg ferrous fumarate compared to an MNP without iron, did not observe an increase in malaria in the children consuming the iron-fortified foods. However, there was an increased hospitalization of infants in the iron-fortified group during the study, which was not significant over the entire trial, including a post-
intervention period (Zlotkin et al. 2013). Since efficacy was not the primary outcome of this RCT, this study was not included in the current review.
<table>
<thead>
<tr>
<th>Baseline age</th>
<th>Country</th>
<th>Groups, iron compound and dose (number of subjects)</th>
<th>Duration (months)</th>
<th>Main findings (iron status, growth)</th>
<th>Morbidity</th>
<th>Reference</th>
</tr>
</thead>
</table>
| 12-59 months | Kenya, Mwingi district | A. Unfortified maize porridge, 4.1 mg Fe/d (93)  
B. Maize and amaranth porridge, 23 mg Fe/d (93)  
C. Maize porridge (A) and MNP +2.5 mg NaFeEDTA/d (93) | 4 | Hb and SF increased in all groups, but significantly more in Fe fortified group (C) than control (A)  
Overall (A,B,C) prevalence of anemia, ID and IDA reduced  
Prevalence of underweight decreased in B and C but did not differ from control (A) post intervention  
note: baseline CRP higher in control (A) | No differences in endpoint CRP, or malaria prevalence | (Macharia-Mutie et al. 2012) |
| 6-35 months | Kenya, Nyanza province | A. ~1.6 mg ferrous fumarate/d (estimated average 11.3 mg/week) (515)  
B. Control (502)  
Community based marketing and distribution of MNP | 12 | Fe (A): larger increase in Hb, and decrease ID (SF <12 ng/mL) compared to control (B)  
No difference in anemia, TfR, total body iron stores or ZPP between groups  
Stunting, wasting and underweight did not differ between groups | No differences in malaria prevalence, others not stated | (Suchdev et al. 2012) |
| 6 months | Zambia, Lusaka | A. Basal porridge, fortified with MNP +6.5 mg Fe/kg flour (370)  
B. Porridge fortified with MNP +250 mg ferrous fumarate/kg flour (~7.25 mg/d actually consumed) (373)  
The MNP in porridge B contained higher amounts of Calcium, Vitamin C and others | 12 | Fe (B) increased Hb and maintained iron status (TfR and SF) compared to baseline and control (time and treatment)  
No differences in stunting; in subgroup with HIV-infected mothers who stopped breastfeeding modest improvement in linear growth seen | No differences in morbidity (malaria, diarrhea, pneumonia) | (The Chilenje Infant Growth 2010, Gibson et al. 2011) |
| 6 months | Ghana, Eastern Region | A. Sprinkles, 12.5 mg ferrous fumarate/d (98)  
B. Nutritabs, 9 mg ferrous sulfate/d (102)  
C. Nutributter, 9 mg ferrous sulfate/d (98)  
D. Control (96) | 6 | Higher SF and lower TfR in A, B and C compared to D at endpoint. Hb higher in Nutritabs and Nutributter (B, C) but not in Sprinkles (A) compared to control (D)  
WAZ and HAZ endpoint significantly higher in Nutributter (C) compared to Nutritabs (B) | No differences in morbidity between treatment groups | (Adu-Afarwuah et al. 2007, Adu-Afarwuah et al. 2008) |
<table>
<thead>
<tr>
<th>Study Duration</th>
<th>Location</th>
<th>Inclusion Criteria</th>
<th>Interventions</th>
<th>Outcome</th>
</tr>
</thead>
</table>
| 6-12 months    | South Africa, KwaZulu-Natal province | Hb >80 g/L; bw >2.5 kg | A. Unfortified maize porridge (145)  
B. Maize porridge +11 mg ferrous fumarate and other micronutrients daily (144) | Fe (B) showed an intervention effect on SF and Hb compared to control (A)  
No differences in weight and length between treatment groups  
Fe (B) increased motor development scores compared to control (A) | Not stated (Faber et al. 2005) |
| 8-20 months    | Ghana, Kintampo district | Hb ≥100 g/L | A. Sprinkles, 40 mg microencapsulated ferrous fumarate/d (76)  
B. SP and Fe and vitamin A, 40 mg microencapsulated ferrous fumarate/d (83)  
C. Fe drops, 12.5 mg ferrous sulfate/d (85)  
D. Control (80) | No time nor treatment effect on Hb and SF  
WAZ and HAZ significantly decreased over the trial in all groups and did not differ between groups at endpoint | Not stated (Zlotkin et al. 2003) |
| 6 months       | Ghana, Kintampo district | bw ≥2.5 kg | A. Weanimix: maize, soybean, peanuts, 2.1-4.6 mg Fe/d (53)  
B. Weanimix and MNP, 15.8-20.9 mg electrolytic Fe/d (51)  
C. Weanimix and fish powder (52)  
D. Fermented maize porridge ('Koko') (52) | Fe (B) protected from low SF seen in A,C, and D at end of study; no differences in Hb  
No differences in weight or length | No differences in diarrhea, fever or respiratory illness (Lartey et al. 1999) |
| 3 months       | South Africa | weight (3 months): girls ≥5 kg; boys ≥5.5 kg; Hb >90 g/L | A. Control: infant formula, 8.3 mg ferrous sulfate/100 g (74)  
B. infant formula as A, +40 mg Fe/100 g (75) | Additional Fe (B) increased Hb, ZPP and SF  
No differences in weight or length | No differences in infection prevalence (Power et al. 1991) |
<table>
<thead>
<tr>
<th>Study Duration</th>
<th>Location</th>
<th>Study Design and Population</th>
<th>Inclusion Criterion</th>
<th>Treatment</th>
<th>Outcome</th>
<th>Other Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-59 months</td>
<td>Kenya, Kakuma Refugee Camp</td>
<td>Inclusion criterion: Hb &gt; 80 g/L</td>
<td>A. 2.5 mg NaFeEDTA/d (410)</td>
<td>Not stated</td>
<td>Hb and anemia did not change over the time of the intervention, but TfR concentration decreased between baseline and endpoint</td>
<td>(Ndemwa et al. 2011)</td>
</tr>
<tr>
<td>6-59 months</td>
<td>Zambia, Nangweshi Refugee Camp</td>
<td>Reduction in anemia through intervention (Hb increased)</td>
<td>A. Maize meal +35 mg Fe/kg flour (155)</td>
<td>Malaria did not correlate with Hb</td>
<td></td>
<td>(Seal et al. 2008)</td>
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<tr>
<td>6-23 months</td>
<td>Burkina Faso, Kongoussi district</td>
<td>Inclusion criterion: Hb=70-109 g/L</td>
<td>A. 15 mg ferrous fumarate 5d/w (96)</td>
<td>No differences in weight stated</td>
<td>Hb increased and anemia decreased in all groups, but strongest in the MNP +Fe +Zn (C) group</td>
<td>(Ouedraogo et al. 2008)</td>
</tr>
<tr>
<td>6-18 months</td>
<td>Ghana, Kintampo district</td>
<td>Inclusion criterion: Hb=70-99 g/L</td>
<td>A. Sprinkles (SP), 12.5 mg ferrous fumarate/d (26)</td>
<td>No difference in diarrhea between treatment groups</td>
<td>Hb increased and IDA decreased in all treatment groups (no dose effect)</td>
<td>(Christofides et al. 2006)</td>
</tr>
<tr>
<td>6-18 months</td>
<td>Ghana, Kintampo district</td>
<td>Inclusion criterion: Hb=70-99 g/L</td>
<td>A. 80 mg ferrous fumarate/d (115)</td>
<td>Not stated</td>
<td>Hb and SF increased in both groups; Hb at endpoint was significantly higher in Fe (A) compared to Fe and Zn (B) group</td>
<td>(Zlotkin et al. 2003)</td>
</tr>
<tr>
<td>Study Duration</td>
<td>Location</td>
<td>Inclusion Criteria</td>
<td>Intervention A</td>
<td>Intervention B</td>
<td>Time</td>
<td>Treatment Effect</td>
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</table>
| 6 months       | South Africa, Western Cape, black urban | **Inclusion criterion:** bw ≥ 2.5 kg  
A. Complementary food, 8 mg Fe/meal (25)  
B. Normal diet, 8.8 mg Fe/meal (21)  
Normal diets (B) were complementary foods similar to intervention arm (A)! | 6 | Hb and total iron decreased in both groups  
No effect on weight and height | Not stated | (Oelofse et al. 2003) |
| 6-18 months    | Ghana, Kintampo district | **Inclusion criterion:** Hb=70.0-99.9 g/L  
A. Sprinkles, 80 mg microencapsulated ferrous fumarate/d (246)  
B. Ferrous sulfate drops, 40 mg Fe/d (247) | 2 | Time: Hb and SF increased in both groups  
Treatment: SF significantly higher at endpoint in infants receiving Fe drops (B) compared to infants consuming Sprinkles (A)  
No treatment effect on WAZ or WHZ | No differences in diarrhea between groups | (Zlotkin et al. 2001) |

Doses represent amount of elemental iron. **Abbreviations:** bw: birth weight; Fe: iron; Hb: hemoglobin; SF: serum ferritin; SP: Sprinkles; TfR: transferrin receptor; Zn: zinc; MNP: micronutrient powder.
2. Iron metabolism in infants and young children

There is no natural pathway for the human body to excrete iron. At the same time, iron is an extremely reactive electron donor when freely dissolved in aqueous solution, which may cause the generation of free radicals (Rao and Georgieff 2007). Body iron needs therefore to be bound to a protein (e.g. transferrin, lactoferrin, ferritin, haptoglobin, and hemopexin) in order to be made unreactive, avoid its precipitation in the neutral pH of biological fluids, and remain inaccessible for pathogens (Hallberg and Hulthen 2002, Drakesmith and Prentice 2012). Systemic iron homeostasis must be very tightly controlled to ensure coordinated iron absorption by enterocytes, reutilization in macrophages, and correct iron redistribution to the site of utilization (mainly for erythropoiesis) or storage in hepatocytes (Hentze et al. 2010, Lipinski et al. 2013).

In weaning infants dietary iron needs to cover the requirements during this period of rapid growth, especially for the increase in blood volume and red blood cells (Lipinski et al. 2013). Collard et al. reviewed iron homeostasis in this critical phase of development, concluding with the urgent need for further research on this neglected topic (Collard 2009). The current knowledge of iron metabolism and its regulation, in infants and young children is discussed in the next chapters.

2.1 Iron requirements

Healthy infants are born with body iron stores covering their needs during the first 4-6 months of life, after this period additional iron needs to be provided through complementary feeding (Dallman 1986). Breast milk, although high in energy and nutrients, is low in iron (0.40-0.56 mg/L), but the little iron is highly bioavailable and bound to lactoferrin (Silvestre et al. 2001, Lonnerdal and Kelleher 2007). A study in 151 African and 100 European lactating mothers of 1-18 month old children showed a mean iron content of 0.20 and 0.19 mg/kg milk respectively (Daneel-Otterbech 2000). The iron content in the breast milk from mothers with preterm infants was found to be higher than in the breast milk from mothers with term infants (Ejezie et al. 2011).

Iron requirements are mainly dependent on the needs for growth and coverage of iron losses. In children, iron losses are restricted to basal losses, and estimated to be 0.17 mg/d from 6-12 months, 0.19 mg/d from 12-36 months and 0.27 mg/d from 4-6 years; whereas iron needed for growth is 0.55 mg/d (6-12 months), 0.27 mg/d (12-36 months), and 0.23 mg/d (3-6 years), respectively. This sums up to absolute daily requirements of
0.72 mg/d (6-12 months), 0.46 mg/d (12-36 months), and 0.5 mg/d (3-6 years), respectively (WHO/FAO 2004). The recommended daily allowance depend on the bioavailability of the provided iron and are estimated to be 0.27 mg/d during the first 6 months, 11 mg/d from 6-12 months, 7 mg/d from 12-36 months and 10 mg/d for 4-6 years (Medicine 2001). During the weaning period, iron requirements relative to energy intake are highest during the whole lifespan of man (Dewey 2013).

2.2 Iron absorption

Iron is absorbed in the proximal duodenum; where polarized absorptive cells (enterocytes) are arranged in villi protruding into the intestinal lumen and therefore maximizing the absorptive surface area. Each enterocyte further presents microvilli at its apical side (Andrews 2008). The majority of dietary non-heme iron is present in the ferric form (Fe$^{3+}$) and needs to be reduced to its ferrous form (Fe$^{2+}$) by dietary reducing agents and the membrane ferric reductase duodenal cytochrome b (Dcytb) before absorption (McKie et al. 2001). Iron enters the enterocyte through the divalent metal transporter 1 (DMT1) and is either stored inside the enterocyte by ferritin or transported to the blood stream by ferroportin, where it is oxidized and incorporated into transferrin. However little is known about these mechanisms of absorption in infants (Lönnerdal and Kelleher 2007).

The absorption of iron from human milk was assessed in a supplementation study in 6 month old Swedish infants using ferrous sulfate and estimated to be 16.4%, irrespective of iron intake or status. At nine months iron absorption was 36.7% in unsupplemented infants with generally lower iron stores and 16.9% in supplemented infants with higher iron stores, but no correlation with iron status was found. The authors hypothesized that regulation of non-heme iron absorption is immature at 6 months of age, whereas it may increase the absorption from low-iron diets at 9 months (Domellof et al. 2002). The plausibility of this hypothesis was supported by a study in rat pups, unable to down-regulate iron absorption in early infancy but able to do so in later infancy (Leong et al. 2003). The expression of DMT1 has been shown to be low in early infancy in murine and pig models and an alternative iron absorption pathway trough lactoferrin receptor (LfR) has been suggested (Lopez et al. 2006, Lipinski et al. 2010). Though highly bioavailable, the little iron from breast milk is insufficient to cover iron requirements during infancy and after the age of 6 months additional iron needs to be provided through complementary feeding (WHO 2001).
Enhancers for non-heme iron absorption are meat protein and ascorbic acid, whereas phytates, polyphenols, and calcium inhibit it (Hurrell 2002, 2006). Unfortunately, iron bioavailability from cereal products, often used for weaning foods, is generally very low (<1%) due to the inhibition of high concentrations of phytic acid (Cook et al. 1997). Iron absorption from fortified complementary foods (wheat and soy flour) in 4-6 month-old US-American infants was found to be 3.7% from NaFeEDTA and 4.9% from ferrous sulfate and ascorbic acid (Davidsson et al. 2005). A study in 6 month-old iron sufficient (Hb >100 g/L) British infants showed no enhancing effect of heme concentrate on non-heme iron absorption (Martinez et al. 1998), whereas heme-protein increased non-heme iron absorption in adults (Hallberg et al. 2003, Hurrell et al. 2006). In a 7 day balance study in 6 months infants, iron retention was 28.8% for heme iron and 24.4% for ferrous sulfate (Martinez et al. 1998). However, the values from balance studies might be biased by heme iron from the study subject or iron from shed gut cells (Fomon et al. 2005). Other data on heme iron absorption in infants are lacking. From studies in adults, absorption of non-heme and heme iron is suggested to be 1.0-4.3% and 15-35%, respectively (Monsen et al. 1978, Zimmermann et al. 2005).

2.3 Systemic iron homeostasis in healthy infants
Since neonates iron supply is low and absorption may not be fully developed, hepatic iron stores represent primary source to cover the metabolic demand (Lipinski et al. 2013). A delayed clamping of the umbilical cord for at least 2 minutes, has shown to increase iron status in full-term neonates (Hutton and Hassan 2007), and boost the amount of erythrocytes by up to 50% (Yao et al. 1969, Mercer 2001). Total body iron varies with birth weight and has been estimated to be 75 mg/kg (Widdowson and Spray 1951). The distribution of body iron in the newborn is suggested as follows: 75-80% in erythrocytes (hemoglobin), 10% tissue iron (myoglobin and cytochromes), and 10-15% storage iron (ferritin and hemosiderin) (Rao and Georgieff 2007). At birth, when the neonate enters the oxygen-rich atmosphere, hemoglobin synthesis is stopped and concentration falls from approximately 170 g/L to 120 g/L within the first weeks. The iron from senescent red blood cells (RBCs) is then not reinvested in hemoglobin but in ferritin stores, which is then successively used during the first 6 months (Figure 2) (Kling et al. 1996, Domellof 2007).
Figure 3 illustrates the iron turnover in a healthy 6 month-old child proposed by Domellof et al. (Domellof 2007).

At the basolateral enterocyte membrane ferrous iron is oxidized by a ferroxidase enzyme (ceruloplasmin and its membrane-bound homologue hephaestin) and binds to transferrin (Vulpe et al. 1999, Collard 2009). However, several studies have consistently shown that ceruloplasmin and transferrin are low in newborns (Scott et al. 1975, Fryer et al. 1993, Galinier et al. 2005). Ferrous iron is the most reactive in the generation of free radicals (Halliwell and Gutteridge 1990). Therefore, the antioxidant property of serum albumin has been suggested to be especially important in infants, in order to prevent non-transferrin bound iron (NTBI) (Loban et al. 1997).

In adults, the transferrin-bound iron is taken up into hepatocytes through transferrin receptors and stored in ferritin until needed. However, the exact mechanisms of action are still unknown (Lonnerdal and Kelleher 2007, Muckenthaler et al. 2008).

Figure 2 Changes of total body iron content and its distribution (hemoglobin, tissue and storage), during the first year of life. Adapted from (Domellof 2011).

Figure 3 Estimated iron turnover between the main iron compartments in a healthy 6 months old infant. The width of the arrows is proportional to iron flux and the area of the circles to compartment size. Adapted from (Domellof 2007).
Bracci et al. summarize the current knowledge on oxidative stress in neonatal erythrocytes. Fetal and newborn erythrocytes seem to be predisposed to tissue injury by reactive oxygen species (ROS), being deficient in antioxidant enzymes, and release iron during hypoxia (Bracci et al. 2002); unlike in adults, where circulating erythrocytes are scavenging free radicals and therefore protecting other cells and tissues (Cimen 2008).

Figure 4 Control of body iron fluxes in adults: (1) Non-heme iron absorption by duodenal enterocytes, (2) phagocytosis of senecent red blood cells and iron recycling in the macrophage, (3) iron storage in hepatocytes, and (4) potentially iron excretion by kidney. Red arrows represent the hepcidin regulatory pathways. Abbreviations: CP: carrier protein; DMT: divalent metal transporter; dcytb: duodenal cytochrome b; HFE: hemochromatosis; HO: heme oxygenase; LIP: labile iron pool; RBC: red blood cell; TfR: transferrin receptor (Muckenthaler et al. 2008).

2.4 Hepcidin – the major iron regulator

Hepcidin is a 25-amino acid peptide, synthesized mainly in the liver and released to the blood stream. It is considered to be the major regulator of systemic iron homeostasis (Nemeth and Ganz 2009, Hentze et al. 2010). Hepcidin has been detected in the early 21st century by three independent laboratories (Krause et al. 2000, Park et al. 2001,
Pigeon et al. 2001); the name ‘hepcidin’ derives from its origin in the liver (hep-) and its microbicidal activity (-cidin) (Drakesmith and Prentice 2012). The primarily produced 84-amino acid prohepcidin is posttranslationally processed into the biologically active 25-amino acid form (hepcidin-25), released into plasma and excreted in the urine (Park et al. 2001, Kemna et al. 2005). Hepcidin regulates cellular iron efflux from macrophages and enterocytes through binding to ferroportin and thereby inducing its internalization and degradation (Nemeth et al. 2004, Kemna et al. 2008). It may also reduce intestinal iron absorption through ubiquitin-dependent proteasome degradation of divalent metal transporter 1 (DMT1) on the apical enterocyte membrane (Brasse-Lagnel et al. 2011).

Serum hepcidin transcription is decreased during iron deficiency, hypoxia and by erythropoietic stimuli, while it is increased during infection, inflammation and iron overload (Pigeon et al. 2001, Nemeth et al. 2004, Hentze et al. 2010, Wessling-Resnick 2010). Expression of the hepcidin gene is influenced by three main factors: (A) high iron status upregulates hepcidin expression (mainly through the bone morphogenic protein/hemojuvelin (BMP/HJV) pathway), (B) infection and inflammation increase hepcidin transcription in response to inflammatory mediators such as IL-6 and CRP (primarily through the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway), and (C) erythropoietic activity decreases hepcidin mRNA levels (Wrighting and Andrews 2006, Beutler 2007, Krijt et al. 2010).

The regulation of circulating hepcidin by concurrent and competing stimuli such as infection and iron deficiency has not been extensively studied in humans. Infants, particularly in developing countries, are highly vulnerable to both serious infections and iron deficiency (Lonnerdal and Kelleher 2007). Hepcidin may be a sensitive marker for iron utilization and absorption, but little data are available on hepcidin concentration and its relationship to established markers in population studies. A study in anemic Tanzanian children, aged 2 months to 13 years, showed high concentrations of urinary hepcidin associated with malaria, which could contribute to malarial anemia and an impaired erythropoietic response to iron supplementation (de Mast et al. 2009). In addition, it was shown that asymptomatic malarial parasitemia was associated with increased serum hepcidin concentrations and anemia in Indonesian school children, aged 5-15 years (de Mast et al. 2010). Moreover, a recent study in anemic Gambian children aged 1.5-3 years, showed that serum hepcidin was the major predictor of
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erythrocyte iron incorporation. A low-cost hepcidin assay to improve safety and efficacy of iron supplementation programs for children in developing countries has been suggested (Prentice et al. 2012). However, no published data are yet available on how inflammation and iron status interact to determine serum hepcidin concentrations in infants. As immune responses and hepatic metabolism are not entirely mature in early infancy, hepcidin regulatory pathways may not yet be fully functional. Moreover, although reference values for serum hepcidin have been proposed for adults (Galesloot et al. 2011), data on serum hepcidin concentrations in healthy, iron sufficient, non-anemic infants and the regulation of hepcidin expression in infants in general are lacking.

Several mass spectrometry and immunochemical methods have been developed to quantify hepcidin in plasma and urine (Kroot et al. 2009). The harmonization of the various methodologies for plasma used in different laboratories was aimed by two round robins, where a series of native plasma samples and samples spiked with synthetic hepcidin-25 were analyzed by all involved laboratories (Kroot et al. 2009, Kroot et al. 2012). The synthetic hepcidin standards were not commutable, thus unsuitable for harmonization; but from the native plasma samples algorithms were constructed with which international hepcidin consensus values (HEPCON1) can be calculated and compared (Kroot et al. 2012).

2.5 Iron status indicators

Hemoglobin (Hb)

Hemoglobin is the iron-containing oxygen-binding protein of the RBCs (Riggs 1965). It is the best determinant for assessing the prevalence of anemia. For field purpose the portable HemoCue system using the cyanmethemoglobin method is rapid, cheap and reliable (WHO 2001). However, for the definition of iron status, sensitivity of hemoglobin is poor, since the impact of ID on hemoglobin levels is marginal and large losses of body iron are needed to develop anemia. In addition, specificity is low in developing countries with high prevalence of poverty, malnutrition and infections that contribute to anemia of chronic disease, also called anemia of inflammation (Cook et al. 2003, Cook 2005). Suggestions to adjust of Hb cut-offs for ethnicity have been made (Johnson-Spear and Yip 1994, Beutler and West 2005, Sullivan et al. 2008). However, due to lacking consensus, WHO does not consider ethnicity in their guidelines for cut-off use (WHO 2001, WHO 2008).
Adjustments for altitudes are proposed, -2 g/L at 1000 meters above sea level (MASL) and up to -45 g/L at 4500 MASL (Hurtado et al. 1945, WHO 2011). However, this does not apply to our study population living at the Kenyan coast.

Studies have reported lower hemoglobin concentrations in male compared to female infants (Domellof et al. 2002, Wieringa et al. 2007, Pasricha et al. 2010), which were partly attributed to gender-specific growth rates (Domellof et al. 2002, Wieringa et al. 2007). However, more research is needed to elucidate gender-differences in hemoglobin.

**Serum ferritin (SF)**

Ferritin is an intracellular iron storage protein (main storage sites are liver, spleen and bone marrow) and under steady-state serum ferritin concentration reflects iron stores (Harrison and Arosio 1996). Serum ferritin is a universally available and well-standardized measurement that has been the most useful assessment of iron status (Cook 2005, Fontaine 2007).

Neonates have a mean SF of 125 µg/L, which increases to a maximum of 250 µg/L after 3-4 weeks. From where it decreases through infancy to 10-20 µg/L as iron stores are used for increasing erythrocytes mass (Finch et al. 1986). Cord SF concentrations from American neonates have been suggested to be higher in girls (166 µg/L) than boys (123 µg/L) (Tamura et al. 1999). This gender difference has also been shown in Honduran and Swedish infants aged 4 months (girls: 110 µg/L; boys: 65 µg/L) and 6 months (girls: 64 µg/L; boys: 37 µg/L), independently of weight or country. At 9 months the gender difference was significant in Swedish (girls: 53 µg/L; boys: 29 µg/L) but not Honduran infants (Domellof et al. 2002). The suggested cut-off for SF, below which iron stores are considered depleted in children <5 years are <12 µg/L and in the presence of infection <30 µg/L (WHO 2001). However, Domellof et al. suggested SF cut-off values of <20 µg/L at 4 months, <9 µg/L at 6 months and <5 µg/L at 9 months of age (Domellof et al. 2002).

A limitation of SF is its nature as an acute phase protein and thereby its increase during inflammation, independently of the iron status (Cook 2005). WHO suggest the use of one or more acute phase proteins such as CRP and AGP to estimate the prevailing incidence of infection and inflammation (WHO 2007). Thurnham et al. proposed to correct SF values during subclinical inflammation. The suggested correction factors
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(CFs) are 0.77 during incubation (elevated CRP), 0.53 during early convalescence (elevated CRP and AGP), and 0.75 during late convalescence (elevated AGP) (Thurnham et al. 2010). However, the use of CFs may not be generalizable to different populations and further work is needed to assess their benefits and usefulness for the assessment of nutritional status (Grant et al. 2012).

**Soluble transferrin receptor (sTfR)**

Transferrin receptor (TfR) is the cellular entry point for plasma iron, and reflects overall tissue iron need, but especially in erythrocyte precursors in the bone marrow. The circulating soluble transferrin receptor is a cleaved form of the entire molecule and reflects total body TfR (Skikne 2008). The two main determinants of sTfR are erythropoietic activity and tissue iron deficiency, which increase sTfR (Skikne et al. 1990, Beguin 2003). In iron-replete subjects sTfR is a marker of erythropoiesis and becomes an iron status indicator in subjects with tissue ID, with or without adequate iron stores (Beguin 2003).

The lack of international standardization of the sTfR immunoassays, which all use different standards and reference ranges, is the main limitation of sTfR as an iron status indicator (Speeckaert et al. 2010). The development of a recombinant sTfR WHO reference reagent to use as a common standard could help to solve this problem (Thorpe et al. 2010). Pfeiffer et al. compared the immunoturbidimetric assay by Roche and the Ramco ELISA by Flowers (Flowers et al. 1989), and suggested the equations

\[
\text{Flowers} = 1.5 \times \text{Roche} + 0.35 \text{ or } \text{Roche} = 0.667 \times \text{Flowers} - 0.233
\]

(Pfeiffer et al. 2007).

A study in young children and infants showed higher sTfR concentrations compared to adults. This was explained as a response to physiologically low iron stores and not due to increased erythropoiesis, which was estimated to be higher in adults than infants. Therefore age-specific reference ranges are strongly recommended (Virtanen et al. 1999). In 9-12 month old infants without inflammation (CRP <5 mg/L) and defined ID as SF <10 µg/L, the best cut-off value for sTfR (using Roche method) is suggested >7.4 mg/L (Vendt et al. 2009).

Although sTfR is considered to be unaffected by inflammation (Carriaga et al. 1991), some studies in Sub-Saharan Africa have shown associations between sTfR and inflammation markers (Kasvosve et al. 2006, Grant et al. 2012, Righetti et al. 2013). Specificity of sTfR in African infants and children may be lowered due to the enhancing

**Zinc protoporphyrin (ZPP)**

During phases of insufficient iron availability, zinc (Zn\(^{2+}\)) replaces iron (Fe\(^{2+}\)) in the last step of the heme synthesis leading to ZPP formation (Labbe et al. 1999). ZPP does not bind O\(_2\) and therefore does not undergo the HO-mediated oxidative degradations characteristics of iron-containing heme (Maines 1981).

ZPP has been widely used to screen for lead intoxication and iron deficiency anemia (Lamola and Yamane 1974). The erythrocyte zinc protoporphyrin/heme ratio (ZPP/heme), is a simple and reliable indicator for iron deficient erythropoiesis (Labbe et al. 1999). The quantification of ZPP in red blood cells is done using fluorescence (WHO 2007). Though, it is currently recommended to report ZPP results as the ZPP to heme molar ratio (NCCLS 1996), ZPP can also be expressed as a concentration (free erythrocyte protoporphyrin (EP), EP, or ZPP; and these are not interchangeable) (Zimmermann 2008).

A cohort study in Zambian infants showed their mean ZPP concentration at birth, 4 and 6 months of age to be 96, 127, and 163 \(\mu\text{mol/mol heme}\), respectively (van Rheenen et al. 2008). However, since washing of the RBCs was not mentioned in the methods, this high values may be due to interfering substances which can increase measured ZPP several fold in absence of iron deficiency (Hastka et al. 1992). Labbé et al. suggested to define low ZPP as \(<60 \mu\text{mol/mol heme}\), midrange ZPP as 60-80 \(\mu\text{mol/mol heme}\), and high ZPP, indicating iron deficient erythropoiesis, as \(>80 \mu\text{mol/mol heme}\) (Labbe et al. 1999). WHO sets the thresholds for ZPP between 40-70 \(\mu\text{mol/mol heme}\) depending on whether RBCs are washed or not and suggests further research, especially for cut-offs in infants and young children (WHO 2007).

The specificity of ZPP as an indicator for ID may be limited by the fact that ZPP is elevated in response to lead poisoning, anemia of inflammation or chronic disease, chronic infections and inflammation, hemolytic anemias or hemoglobinopathies (Zimmermann 2008).
Body iron

The concentration of SF is directly proportional to body iron stores in healthy adults and can be estimated as 1 µg/L SF=8-10 mg body iron stores. However, in infants and children body size should be taken into consideration using the formula 1 µg/L SF=120 µg storage iron/kg body weight (Finch et al. 1986).

Cook et al. established an estimation of body iron through a series of repeated phlebotomies in healthy adult subjects (Skikne et al. 1990). The amount of storage iron at baseline was calculated from the amount of heme iron removed to reduce SF concentration to less than 12 µg/L. The amount iron removed at each bleeding was corrected for the absorption of dietary iron and body iron (mg/kg body weight) can be estimated from the ratio of sTfR to SF according to the equation body iron (mg/kg)=[\log_{10} (sTfR * 1000/SF) -2.8229]/0.1207. Positive body iron values depict storage iron, whereas negative values indicate tissue iron deficiency. This method should help to increase sensitivity of iron interventions and the evaluation of iron status in populations in which inflammation is minor (Cook et al. 2003). The use of this ratio improves specificity and sensitivity of these two parameters (Punnonen et al. 1997). The agreement of body iron and the ferritin model (abnormal value of at least 2 of 3 indicators (SF, erythrocyte protoporphyrin, and transferrin saturation)) to define ID in US-American preschool children was fair to good (Cogswell et al. 2009). However, the ratio of SF and sTfR, combines the limitations of these two parameters. Furthermore the estimation of body iron is based on one single phlebotomy study of 14 healthy white adults (Skikne et al. 1990) and the change in iron status was measured several weeks after the bleeding when the establishment of a new equilibrium between sTfR and SF in the plasma was assumed (Cogswell et al. 2009).

Table 2 summarizes the parameters and methods used to assess iron status and anemia in the 6-18 month old Kenyan study population of the original work in this thesis.
Table 2 Iron status and anemia indicators and methods used in Kenyan infant study.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Used to define</th>
<th>Device/ Method</th>
<th>Cut-off</th>
<th>Advantages (+) and limitations (-) (adapted from (Cook 2005))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>Anemia</td>
<td>HemoCue&lt;sup&gt;1&lt;/sup&gt;, HemoControl&lt;sup&gt;2&lt;/sup&gt;, cyanmethemoglobin method</td>
<td>&lt;110 g/L; acute: &lt;70 g/L (WHO 2001)</td>
<td>(+) Low costs, accessible ( -) Low specificity and sensitivity</td>
</tr>
<tr>
<td>Serum ferritin</td>
<td>Iron stores</td>
<td>Cobas Integra&lt;sup&gt;3&lt;/sup&gt;, ECLIA</td>
<td>&lt;12 ng/mL (WHO 2001)</td>
<td>(+) Quantitative, standardized (-) Affected by inflammation</td>
</tr>
<tr>
<td>Soluble transferrin receptor</td>
<td>Tissue iron needs</td>
<td>Cobas Integra&lt;sup&gt;3&lt;/sup&gt;, Tinaquant, immunoturbidimetry; converted to Flowers assay (Flowers = 1.5 * Roche + 0.35)</td>
<td>&gt;7.4 mg/L (Vendt et al. 2009)</td>
<td>(+) Quantitative, unaffected by inflammation (-) Lack of standardization</td>
</tr>
<tr>
<td>Zinc protoporphyrin</td>
<td>Impaired erythropoiesis</td>
<td>AVIV Hematofluorometer&lt;sup&gt;4&lt;/sup&gt;</td>
<td>&gt;80 µmol/mol heme (Labbe et al. 1999)</td>
<td>(+) Low costs, accessible ( -) Automation difficult, affected by lead exposure/inflammation</td>
</tr>
<tr>
<td>Hepcidin</td>
<td>Iron regulation</td>
<td>WCX-TOF MS&lt;sup&gt;5&lt;/sup&gt;</td>
<td>NA</td>
<td>Influenced by competing stimuli i.e. iron status and inflammation</td>
</tr>
<tr>
<td>Body iron stores</td>
<td>Iron stores</td>
<td>BIS = -[log10 (sTfR * 1000/SF) -2.8229)]/0.1207</td>
<td>ID: &lt;0</td>
<td>(+) Increased sensitivity ( -) see SF and sTfR</td>
</tr>
</tbody>
</table>

<sup>1</sup>HemoCue AB, Ängelholm, Sweden; <sup>2</sup>EKF diagnostics Sales GmbH, Barleben/ Magdeburg, Germany; <sup>3</sup>Roche, Basel, Switzerland; <sup>4</sup>AVIV Biomedical, Lakewood, USA; <sup>5</sup>Bruker Daltonics. Abbreviations: BIS: body iron stores; NA: not available; TOF MS: time-of-flight mass spectrometry; WCX: weak cation exchange chromatography.

2.6 Iron homeostasis during inflammation

As discussed in the last chapters, SF, ZPP, hepcidin and under some conditions sTfR are elevated during acute and chronic inflammation (Nemeth et al. 2004, Cook 2005, Zimmermann 2008, Righetti et al. 2013). This chapter gives an overview of the complex interplay of these indicators during inflammatory response.

If the body is affected by an inflammation, iron is redistributed towards storage sites in liver and macrophages, leading to an increase of intracellular ferritin and decrease of serum iron mediated by cytokine-induced hepcidin expression (Feelders et al. 1998, Drakesmith and Prentice 2012). This results in a reduction of easily available iron for pathogens (Weiss 2009). Several iron-binding plasma proteins concentrations are expressed differently during inflammation such as ceruloplasmin (30-60% increase),
transferrin (−30% decrease), lactoferrin (200–500% increase), ferritin (up to 300% increase), haptoglobin (200–500% increase) (Thurnham and Northrop-Clewes 2004). Hepcidin transcription is increased through elevated IL-6 and CRP and inhibits release of iron from macrophages and enterocytes through degradation of ferroportin (see chapter 2.4.) (Wrighting and Andrews 2006). Beside hepatic hepcidin, which is the main source of circulating hepcidin, there are also small amounts of hepcidin produced by mammalian monocytes and macrophages (Weiss 2009). A recent review of monocyte/macrophage hepcidin, mainly regulated by inflammatory stimuli, highlights its importance for local modulation of tissue and organ inflammation in an autocrine and paracrine way (Zhang and Rovin 2013). Furthermore, the stimulation of NTBI uptake via DMT1 in monocytes and its retention by down-regulation of ferroportin has been reported in response to pro-inflammatory cytokines. On the other side the anti-inflammatory cytokine IL-10 induced TfR-mediated iron uptake into activated monocytes (Ludwiczek et al. 2003).

The time course of an acute phase reaction after the administration of cytokines (recombinant TNFα and IFNγ) in cancer patients during limp perfusion is illustrated in Figure 5. The experiment started with a pretreatment of rIFNγ for 2 days, to prime activated macrophages, and rTNFα at day 0. CRP and SF increase within few hours as early acute phase responders and CRP decreases at day 2, whereas SF concentration decreased slowly but was still elevated at day 7. AGP is reacting less promptly and remains elevated until day 7. The concentration of sTfR fell during rIFNγ administration and further decreased after TNFα treatment until day 2 paralleling the maximum concentration of CRP and SF (Feelders et al. 1998). The response of sTfR could be either reflecting impaired erythroid growth or the expression of sTfR was

![Figure 5](image-url)
inhibited by the cytokines (Northrop-Clewes 2008).

This hypoferremia of inflammation can result in anemia of inflammation or chronic disease (Wessling-Resnick 2010). A study in South African preschool children attempted to differentiate between study participants with IDA and those with anemia of inflammation or chronic disease, using a log ratio of sTfR and SF. However, the ratio was not able to distinguish IDA in the presence of inflammation, but a high ratio (>2.55) was clearly indicating ID and not AI (Malope et al. 2001).

Malaria infection has several counteractive effects on hepcidin expression (Figure 6) and therefore iron homeostasis (de Mast et al. 2009, 2010). First, malaria-induced hemolysis and clearance of infected RBCs by macrophages result in anemia, leading to increased erythropoiesis (Mockenhaupt et al. 1999, Lamikanra et al. 2007) and therefore a decrease in hepcidin concentration. Second, inflammation directly, as well as through the effect of the acute phase cytokines on the bone marrow resulting in decreased erythropoiesis, may be associated with increased hepcidin production (de Mast et al. 2009). Third, parasite metabolites, especially the disposal product hemozoin, may have an inhibitory effect on erythropoiesis (Lamikanra et al. 2007, Thawani et al. 2013) and therefore increase hepcidin.

**Figure 6** Impact of febrile *Plasmodium falciparum* malaria on hepcidin expression and therefore iron absorption and release from macrophages (de Mast et al. 2009).
3. Safety of oral iron interventions in Sub-Saharan Africa

This chapter will review potential safety concerns of untargeted oral iron administration in malaria-endemic areas and the challenges during the diagnosis of febrile episodes in Sub-Saharan Africa. The specific effects of iron on the gut microbiota composition will be elucidated in chapter 5, after an introduction into the ecosystem of the human gastrointestinal tract and its inhabitants (chapter 4).

3.1 Iron interventions and malaria incidence

Untargeted daily supplementation of iron and folic acid in preschool children (1-36 months) has shown to increase morbidity and mortality in iron-replete children in Pemba, Zanzibar (Sazawal et al. 2006). A parallel trial in Nepal reported no adverse events in preschool children receiving iron and folic acid supplementation (Tielsch et al. 2006), thus implicating malaria as the major safety issue (Hurrell 2011). However, the co-administration of folic acid with iron is a confounding factor, especially since the standard antimalarial sulphadoxine-pyrimethamine (SP) used during the trial was an antifolate drug (Brittenham 2007). Folic acid can increase the risk of plasmodial infection as well as induce treatment failure of SP (Gregson and Plowe 2005).

Reviews and meta-analyses of the safety of iron in malarial areas are discordant. In 2009, a Cochrane review concluded that iron supplements are safe for children living in malarial areas, when surveillance and treatment are available (Ojukwu et al. 2009). This would however, not be the case in many developing countries (Hurrell 2011). A link between iron, mainly in high doses, and increased risk of clinical malaria has been shown repeatedly (Oppenheimer 2001, Prentice et al. 2007). Further evidence that iron could have an enhancing effect on malaria comes from studies suggesting a low host iron status to be protective against malaria risk in young Sub-Saharan African children (Gwamaka et al. 2012, Jonker et al. 2012). In both studies ID was defined using SF concentration and even though the authors tried to account for infection (using elevated CRP), the results could be biased by the effect of infection on SF. Further confounding factors might be differences in hemoglobinopathies, associated with both hemoglobin and protection from malaria (Prentice 2008).

A review of safety of iron fortification of complementary foods, found no evidence that low dose provisions of iron increase malaria risk. However, there are not enough studies with adequate sample sizes conducted in this area and the authors express the need for further research and the implementation of iron intervention together with malaria
control strategies according to WHO guidelines (Dewey and Baldiviez 2012). A very recent publication by Zlotkin et al. was powered to assess malaria incidence in children aged 6-35 months during a large RCT in a high malaria-burden area in Ghana. The children received in-home fortified porridge including an MNP with 12.5 mg iron or a control MNP without iron, daily for 5 months. The study did not show an increase in malaria incidence through the iron fortification, but higher rates of hospitalization in the iron group, which differed significantly during the intervention period, but not in the entire trial including a postintervention period. Furthermore, a subgroup analysis showed that baseline anemia was associated with a lower risk of malaria (Zlotkin et al. 2013). A comment by Prentice et al. highlights the demur that the MNP might not have been efficacious in reducing anemia to such an extent that the protective effect of ID against plasmodial infection is lost (Prentice et al. 2013).

There are four possible ways by which iron could influence susceptibility to malaria infection and severity. (A) Through an increase in NTBI, formed when the iron influx in the plasma exceeds the rate of iron binding to transferrin (Hurrell 2011). It is suggested that plasmodia cannot use the abundant heme iron and depend on this restricted source of labile iron (Scholl et al. 2005). NTBI is taken up by hepatocytes and stimulate merozoites during the liver stage of *Plasmodium falciparum*. Furthermore it seems likely that NTBI promotes sequestration of malaria-infected erythrocytes to capillaries leading to more cerebral malaria (Hurrell 2010). (B) Iron might enhance malaria susceptibility through stimulated erythropoiesis, since plasmodia favor reticulocytes. However, this only applies to *P. vivax* (Prentice 2008). (C) Iron supplementation decreases ZPP concentration, which may have antimalarial properties (Iyer et al. 2003). (D) Iron excess can trigger immunological responses (Prentice 2008).

The knowledge in this field is imperfect and applicable approaches for strategies to prevent ID and malaria need to be implemented. Currently there are two complementary strategies suggested, namely to reduce iron doses and to increase malaria control efforts (Stoltzfus 2012).

3.2 Challenges in diagnosis of fever in Sub-Saharan Africa

The clinical signs of severe malaria and other infections as of invasive bacteria are often hard to distinguish (Evans et al. 2004). Ghanaian children presenting with symptoms of severe malaria (WHO 2000) but with a negative blood smear, have shown a high risk
for bacterial sepsis and death (Evans et al. 2004). In Ugandan children 30% of cases, using integrated management of childhood illness (IMCI) guidelines, qualified for the diagnosis of both malaria and pneumonia (Kallander et al. 2004). However, overlap in symptoms does not mean that both diseases are present, as seen in Gambian children where the seasonal malaria could not be clinically distinguished from the perennial pneumonia incidence, although maximal 14% of the children had confirmed coinfections (O’Dempsey et al. 1993).

Verbal autopsies often attribute children having died from a short illness associated with fever as malarial deaths (Mulholland and Adegbola 2005). However, in Kenyan children less than 5 years of age, similar numbers of hospital deaths were attributed to bacteremia as to malaria using clinical and laboratory measures (Marsh et al. 1995, Berkley et al. 2005). Furthermore, even in children infected with P. falciparum parasites, severe illness might be due to other causes, since in holoendemic areas up to 80% of children are parasitemic (Bodker et al. 2006). Thus, in Sub-Saharan Africa, clinical signs of severe malaria can overlap with other infections such as bacterial sepsis and the risk of over-diagnosis of malaria and neglect of other infections, especially from invasive bacteria, is given (Gwer et al. 2007, Crump et al. 2011).

Not only are the symptoms similar; often children in malarial regions suffer from double burden of infections. In Mozambican children with severe malaria 5.4% had concomitant bacteremia, mainly Streptococcus pneumonia, gram-negative bacteria, Staphylococcus aureus and non-typhoidal Salmonella (NTS), increasing the risk of death significantly (Bassat et al. 2009). An autopsy study in Malawian children, fulfilling WHO criteria for cerebral malaria, revealed that 23% of deaths were from other causes, many of bacterial sepsis (Taylor et al. 2004). In an earlier study in Kenya 8-12% of children with severe malaria had bacteremia, and this was associated with a 3-fold increase in mortality (Berkley et al. 1999). Another study in Kenyan children aged 1-3 years, identified 11.7% bacteremia, mostly Salmonella typhimurium (belonging to NTS) and S. aureus, in children infected with P. falciparum. The mortality rate was increased 8.5-fold with bacteremia (Were et al. 2011). Of febrile Tanzanian children admitted to hospital, 10% carried pathogenic bacteria in their blood (Mtove et al. 2010). And in febrile Nigerian infants 46.1% harbored malaria parasites and 38.2% had bacteremia (Ayoola et al. 2005). In children presenting with bacteremia and malaria, the presence of malarial parasites on admission may delay diagnosis of bacteremia and worsen outcomes.
(Graham et al. 2000). Hence, all these studies show that, due to the high possibility of coinfection, febrile children should be tested for bacterial infections even if there are positive for malaria (Ayoola et al. 2005).

The major causes for childhood mortality in Africa are estimated to be pneumonia (17%), malaria (15%), prematurity (12%), diarrhea (11%) and neonatal sepsis (5%) (WHO 2013). Studies on hospital admissions of children from Sub-Saharan Africa have shown that 3-11% of severe pneumonia is of bacterial origin (O’Callaghan-Gordo et al. 2011, Hammitt et al. 2012, Feikin et al. 2013). Furthermore, enterotoxigenic Escherichia coli and Shigella spp. have been identified to be the major bacterial contributors to moderate-to-severe infant diarrhea in developing countries (Kotloff et al. 2013).

Malaria has been identified as an important risk factor for gram-negative bacteremia in a study in Kenyan infants, where admissions because of bacteremia decreased in parallel with malaria (Scott et al. 2011). A similar trend has been seen between 1979 and 2005 in Gambia (Mackenzie et al. 2010). However, the mechanisms by which malaria predisposes to acute gram-negative bacterial infections are unclear (Obaro and Greenwood 2011).

A systematic review and meta-analysis of bloodstream infections in Africa showed that 8.2% of febrile children had bloodstream infections. Unlike adults, children were more likely to carry gram-positive bacteria like S. pneumonia, S. aureus, and streptococci, but also gram-negative enterobacteria (Reddy et al. 2010). On Pemba, in an area of low malaria transmission, the leading causes of sepsis in febrile children were Salmonella enterica serovar Typhi and S. pneumonia (Thriemer et al. 2012). Blood cultures from Ghanaian children under 5 (n=1,196) living in a malarial area, revealed the four most prevalent pathogens were non-typhoidal Salmonella (53%), S. aureus (13%), S. pneumonia (9%), and Salmonella Typhi (7%). Systemic bacterial infections have been found in nearly 20% of hospitalized Ghanaian children (Nielsen et al. 2012). Published studies on invasive Salmonella infection in children in Sub-Saharan Africa, identified pathogenic bacterial isolates in 1 to 46%, the fraction of Salmonella Typhi ranged from 0-42% and of NTS from 9-84% (Mtove et al. 2010). These and other studies indicate that especially invasive NTS is associated with P. falciparum infections (Mackenzie et al. 2010). A recent publication hypothesizes that this association of NTS and malaria might involve iron as a cofactor. Enhanced erythropagocytosis and inflammation during
malaria infection increases macrophageal iron content and thereby promotes growth and replication of the intracellular NTS (van Santen et al. 2013).
4. Gut microbiota - an organ in itself

4.1 Introduction to the human gastrointestinal tract

The human gastrointestinal (GI) tract is organized in sections, separating digestion and nutrient absorption, in the proximal GI tract, largely from the gut microbiota (Walter and Ley 2011). We can divide the GI tract into five major areas (Figure 7): esophagus, stomach, small intestine, cecum, and large intestine (Savage 1977).

![Human gastrointestinal tract](http://en.wikibooks.org/wiki/Human_Physiology/The_gastrointestinal_system);

During mastication, digestive enzymes are secreted from the salivary glands. The food is retained and acidified in the stomach for the proteases to be active. Degradable food
components are hydrolyzed into simple sugars, amino acids, and fatty acids by the digestive enzymes and absorbed in the small intestine (Grabitske and Slavin 2008, Walter and Ley 2011). Other components, such as fibers, resistant starch, and some peptides and lipids, are passed via the ileocecal valve to the large intestine (Walter and Ley 2011).

The luminal surface of the intestine consists of a single layer of columnar epithelium cells. The surface is folded into deep cavities also called crypts of Lieberkühn, and in the small intestine finger-like villi protrude from the epithelial lining of the intestinal wall (Wright and Alison 1984, Potten et al. 1997). In the crypts of Lieberkühn, stem cells continuously divide and their progenies move towards the luminal surface, progressively differentiating, reaching maturation and being exposed to the gut content until they are sloughed from the tip of the villus after 2 to 7 days (Figure 8, A) (Wright and Alison 1984, Crosnier et al. 2006). Four distinct cell types can be distinguished in the gut epithelium (Figure 8, B), the absorptive enterocyte, the secretory goblet cell, the enteroendocrine cell, and the Paneth cell (Cheng and Leblond 1974, Crosnier et al. 2006). Cells programmed to become columnar enterocytes change their function and structure during migration to the tip of villus (Smith 1985).

Figure 8 Epithelial cell types in small intestine: (A) stem cells dividing into progeny in the crypt and moving as differentiated cells towards the villus tips, and (B) differentiated cell types deriving from the stem cell (Crosnier et al. 2006).
The maturation of the enterocyte involves an increase and elongation of the microvilli at the apical surface (Merrill et al. 1967). Enterocytes synthesize the glycoprotein enzymes needed for terminal digestion and absorption, and transport substances from the intestinal lumen to the circulatory system. However, they also have an active role in shaping the intestinal immune defense (Miron and Cristea 2012). Goblet cells secrete a mucus layer covering the epithelium and thus providing the first line of defense against physical and chemical injury from food, microbes or microbial products (Kim and Ho 2010). There are many different subtypes of enteroendocrine cells (Moran-Ramos et al. 2012), which produce more than 20 different hormones regulating food intake, energy expenditure, glucose homeostasis, and several metabolic functions in response to food ingestion (Murphy and Bloom 2006, Murphy et al. 2006). The Paneth cells are situated in the crypts and secrete granules rich in microbicidal peptides when exposed to bacteria or their antigens, further they produce factors that help sustain and modulate the epithelial stem and progenitor cells (Clevers and Bevins 2013).

The human gut has an estimated surface area of 200-300 m² (Gebbers and Laissue 1989) and therefore represents a major surface for microbial colonization and interaction (Sekirov et al. 2010), which will be discussed in the next chapters.

### 4.2 Overview of human gut microbiota

The adult human gut harbors up to 100 trillion (10^{12}) microbes, which is equivalent to 10^{11}–12 cells/g colonic content with an estimated biomass of >1 kg (Whitman et al. 1998, O’Hara and Shanahan 2006). All major microbial groups are present in the gut, predominantly bacteria, but also a variety of protozoans (Clarke 1977, Mackie et al. 1999). Within the GI tract, the microbial biomass is increasing with pH and with a decelerated flow rate (Figure 9, A) from around 10^{2} cells/g in the stomach (pH 1.5-5) to about 10^{12} cells/g in the colon (pH 5-7) (Mackie et al. 1999, Sekirov et al. 2010, Walter and Ley 2011). Over 99% of the bacteria in the human gut are strict anaerobes, dominating the facultative anaerobes and aerobes (Savage 1970, Savage 1977). From the 50 phyla described to date (Schloss and Handelsman 2004), Bacteroidetes and Firmicutes prevail the human gut microbiota, while Proteobacteria, Actinobacteria, Verrucomicrobia, Fusobacteria, and Cyanobacteria are present in minor concentrations (Eckburg et al. 2005). It is estimated that 500-1,000 different species are present in the human gut (Xu and Gordon 2003). There are regionally different niches provided by the intestinal ecosystem (Figure 9, A and B), occupied by autochthonous (indigenous)
4. GUT MICROBIOTA – AN ORGAN IN ITSELF

Microbiota; whereas allochthonous (transient) microbiota is usually not colonizing particular habitats (Savage 1977, Berg 1996, Xu and Gordon 2003). The transient microbes found at any point of the intestine, may be dislodged indigenous members of more proximal niches, or ingested by food and water (Xu and Gordon 2003).

Figure 9 Spatial and temporal aspects of human intestinal microbiota composition: (A) increasing concentration and diversity of bacteria across the length of the gastrointestinal tract, (B) longitudinal variations in microbial composition in the intestine, and (C) temporal pattern and parameters influencing colonization of the human GI tract (Sekirov et al. 2010).

The intestinal microbiota shows heterogeneity in the longitudinal and latitudinal axis of the GI tract (Sekirov et al. 2010). The majority of bacteria found in the stomach and proximal small intestine (duodenum and jejunum) are acid-tolerant members of the genera Lactobacillus or Streptococcus, which are considered transients, able to survive the gastric acid, bile and pancreatic secretions (Berg 1996, O’Hara and Shanahan 2006). Helicobacter pylori, though rapidly disappearing from human population (Blaser 1993), is the single dominant bacterial species in the human stomach (Bik et al. 2006). Bacterial density increases in the distal small intestine (ileum), and rises to $10^{11-12}$ cells/g colonic content, representing 60% of the feces (Eckburg et al. 2005). At the latitudinal axis, the composition of the microbiota in the intestinal lumen differs significantly from the composition in the close proximity of the epithelium attached and embedded in the mucus layer (Figure 9, B) (Eckburg et al. 2005, Sekirov et al. 2010). For instance the ratio of anaerobes to aerobes is lower at the mucosal surfaces than in the lumen (O’Hara and Shanahan 2006). In a rat study, Swidsinski et al. showed that many bacterial species present in the intestinal lumen do not access the mucus layer and...
epithelial crypts. *Bacteroides, Bifidobacterium, Streptococcus*, members of *Enterobacteriaceae, Enterococcus, Clostridium, Lactobacillus*, and *Ruminococcus* were all found in feces, whereas only *Clostridium, Lactobacillus*, and *Enterococcus* were detected in the mucus layer and epithelial crypts of the small intestine (Swidsinski et al. 2005).

### 4.3 Colonization of the infant gut

The adult gastrointestinal tract has several mechanisms to control bacterial growth and composition, such as the low pH in stomach (Kanno et al. 2009), the production of bacteriocins produced by the commensal gut microbiota (Simon and Gorbach 1986), and the immune response (Mestecky et al. 1999). However, apart from the low pH in the stomach, which is established in the first month of life (Kaye 2011); most of these intrinsic control mechanisms are not yet fully functional in infants (Kirjavainen and Gibson 1999). Infants’ gut microbial composition therefore depends on extrinsic factors, as discussed in this chapter.

At birth, the sterile fetus is inoculated with its mother’s vaginal, gastrointestinal, and skin microbiota; as well as the microbes of the hospital staff and environment (Mandar and Mikelsaar 1996, Bezirtzoglou 1997). Because the gastrointestinal tract of neonates shows a positive redox potential, the pioneers to colonize the GI tract are facultative anaerobes such as *Staphylococcus, Enterobacteriaceae* and *Streptococcus* (Yoshioka et al. 1991, Bezirtzoglou 1997). These facultative anaerobes lower the redox potential, permitting the subsequent growth of strict anaerobes such as *Bifidobacterium spp.*, clostridial species (Rotimi and Duerden 1981, Stark and Lee 1982) and *Bacteroides* (Jost et al. 2012) within the first few days of life. A rapid rise of the overall bacterial count is seen and the level of $10^9$ CFU/g of feces is reached after one week (Bezirtzoglou 1997). The mode of delivery determines the later gut microbiota; infants born vaginally showed higher numbers of bifidobacteria, *Bacteroides* and total bacteria at 1 months of age compared with infants born by Caesarian section, who are more often colonized with *Clostridium difficile* (Penders et al. 2006, Huurre et al. 2008); a potential pathogen (Khoruts and Sadowsky 2011). Eggesbo et al. describe the development of gut microbiota during the first 4 months in infants not exposed to disruptive effects and medical interventions such as preterm birth, Caesarian section, antibiotics or lack of breast-feeding. The study highlights the dominant role of *Bifidobacterium* and *Enterobacteriaceae*, as well as common colonization with *Bacteroides* and
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*Lachnospiraceae* in infants; and shows strong associations of early and late infant gut microbiota (Eggesbo et al. 2011).

The feeding practices have a major impact on the infant gut microbiota. Breast-fed infants show higher concentrations of bifidobacteria, whereas formula-fed infants host a more diversified gut microbiota with *Bacteroides* and members of the *C. coccoides* and *Lactobacillus* groups (Fallani et al. 2010). Recently, a review investigated the potential prebiotic effects of human milk oligosaccharides in promoting the growth of probiotic bacteria such as bifidobacteria (Barile and Rastall 2013). Another review reported the bacterial composition of human milk being predominantly staphylococci, streptococci, lactic acid bacteria, especially bifidobacteria, and suggests anti-infectious, immunomodulatory and metabolic bacterial properties. It was furthermore hypothesized that certain bacteria from the maternal gut, using mononuclear immune cells, can colonize the mammary gland and therefore also the breast milk (Fernandez et al. 2013).

Weaning introduces the next big change in infants gut microbiota. Introduction of solid foods causes disturbances in the microbial ecology, especially in exclusively breast-fed infants (Stark and Lee 1982). Although the impact is not seen in total bacterial numbers or groups; introduction of solid food changes the composition of species and strains within this groups, which results in changes in metabolic activity (Mackie et al. 1999). Through complementary food, the microbiota profile of breast-fed infants changes towards that of formula-fed infants; this implies an increase in enterococci and enterobacteria, and the appearance of *Bacteroides*, clostridia, and other anaerobic streptococci (Stark and Lee 1982, Mackie et al. 1999, Guaraldi and Salvatori 2012).

At the age of 12-36 months, an adult-like microbiota profile has typically established (Figure 9, C) (Mandar and Mikelsaar 1996, Mackie et al. 1999, Sekirov et al. 2010, Fouhy et al. 2012, Yatsunenko 2012). This is characterized by the dominance of *Bacteroides* and Firmicutes, a common occurrence of Verrucomicrobia, and a low abundance of Proteobacteria and aerobic gram-negative bacteria in general (Palmer et al. 2007).

### 4.4 Bacterial iron homeostasis

Nearly all intestinal bacteria, lactobacilli being the only notable exception (Archibald 1983, Weinberg 1997), require iron for metabolic or signaling functions (Wandersman and Delepelaire 2004). Lactobacilli employ unique co-factors in various enzymes such
as cobalt-cofactored ribonucleotide reductase, a manganese-cofactored DNA-dependent RNA polymerase, and a non-heme mangano-catalase (Weinberg 1997). The virulence of pathogenic bacteria has been shown to increase with sufficient iron acquisition (Genco and Desai 1996, Vasil and Ochsner 1999, Berlutti et al. 2005, Coray et al. 2012, Kortman et al. 2012). The water soluble ferrous form (Fe^{2+}) can be taken up by ubiquitous divalent metal transporters. However, in most human microbial habitats, Fe^{2+} is oxidized to the poorly soluble ferric form (Fe^{3+}) by spontaneous oxidation or enzymatically during assimilation and circulation in the host (Miethke and Marahiel 2007). Ferrous iron can be extremely toxic, reacting with oxygen and reduced oxygen species to form reactive oxygen species (ROS) via the Fenton reaction (Touati 2000). Therefore, iron uptake and storage is strictly controlled in bacteria, as in all organisms requiring iron for their metabolism (Cornelis et al. 2011).

4.4.1 Bacterial iron acquisition
As all living forms, also the human body protects itself rigorously against free iron and assimilates iron by tightly binding it to proteins, such as transferrin, hemoglobin, lactoferrin and ferritin (Wandersman and Delepelaire 2004, Miethke and Marahiel 2007). Faced with this iron restriction by their hosts; most bacteria have mechanisms to acquire iron from several sources.

Various iron compounds, such as lactoferrin, transferrin, ferritin, heme, and hemoproteins can be taken-up by the bacteria directly (Wandersman and Delepelaire 2004). The disadvantage of the direct uptake is the requirement of specific receptors for each iron source (Miethke and Marahiel 2007).

Indirect iron acquisition strategies involve small-molecular weight compounds called siderophores, acting as high-affinity ferric iron (Fe^{3+}) chelators and competing with host iron-binding proteins (Andrews et al. 2003, Miethke and Marahiel 2007, Saha et al. 2012). Siderophores can be divided into three groups depending on the oxygen ligand donor for Fe^{3+}-coordination: (A) catecholates (and phenolates), (B) hydroxamates (or carboxylates), and (C) mixed types (Miethke and Marahiel 2007). Siderophores have six donor atoms and form a 1:1 complex with Fe^{3+} (Carrano and Raymond 1978), which is binding to a receptor with a coupled ABC-type permease, and then internalized to the cytoplasm (Cornelis et al. 2011, Saha et al. 2012). Gram-negative bacteria, having two membranes, require a receptor in the outer membrane and an intermediate
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ferrisiderophore-binding protein in the periplasm as well as an ABC-type permease (Koster 2001, Hannauer et al. 2010, Cornelis et al. 2011).

The ferrous (Fe$^{2+}$) iron transporter ‘Feo’ is particularly important in an environment low in oxygen, in which Fe$^{2+}$ is stable and predominates Fe$^{3+}$ (Andrews et al. 2003). The importance of this mechanism has been shown in feoB mutants of E. coli and Salmonella being unable to colonize the anaerobic mouse gut (Stojiljkovic et al. 1993, Tsolis et al. 1996).

Heme-chelating molecules, commonly called hemophores, are produced by gram-negative bacteria to chelate and escort heme to specific membrane receptors (Cope et al. 1994, Letoffe et al. 1994). Since heme in its free form is toxic and hydrophobic, it is only found in complex with hemoproteins such as hemoglobin or hemopexin and needs to be extracted from the protein before uptake (Wyckoff et al. 2005). The second pathway for heme uptake is the direct uptake via a specific receptor which binds hemoproteins and extracts heme (Wandersman and Stojiljkovic 2000, Wandersman and Delepelaire 2004).

4.4.2 Intracellular iron storage

Many bacteria build intracellular iron stores in order to sustain the metabolism when external iron sources are restricted (Andrews 1998). Three types of iron-storage proteins have been discovered: (A) ferritins also present in eukaryotes, (B) heme-containing bacterioferritins, and (C) smaller DNA-binding proteins from starved cells (Dps) present in prokaryotes only (Romao et al. 2000, Andrews et al. 2003, Chiancone et al. 2004). Interestingly, we can find all three storage types in the same bacterium and several ferritin or bacterioferritin genes are common. They are constructed of 24 (ferritin and bacterioferritin) or 12 (Dps) subunits folded in a four-α-helix bundle. This allows to accommodate at least 2,000-3,000 iron atoms per 24-dimer and about 500 iron atoms per 12-dimer. The storage proteins take up iron in the ferrous form (Fe$^{2+}$), but iron is deposited in the oxidized ferric form (Fe$^{3+}$) (Andrews et al. 2003).

Redox stress resistance systems

The intracellular storage of iron in protein-complexes not only serves as a constant iron supply, but at the same time protects the cell from free iron interacting with reactive oxygen species (ROS) generating highly reactive and extremely damaging hydroxyl radicals (Andrews et al. 2003, Cornelis et al. 2011). Especially Dps has been shown to
have an important role in protecting DNA from damage by interaction of iron with ROS (Zeth 2012).

4.4.3 Regulation of iron homeostasis
Iron metabolism in bacteria is usually regulated in response to iron availability (Escolar et al. 1999). The ferric-uptake regulator (Fur) acts as a positive repressor, blocking the transcription of genes involved in iron uptake by binding at their promoters, if sufficient Fe$^{2+}$ is available (Andrews et al. 2003). Fur has been discovered in the model organism *E. coli*, but many similar regulators have been found in gram-negative and gram-positive bacteria (Crosa 1997, Hantke 2001). Several other iron-dependent regulators have been discovered especially in potential pathogens like *E. coli* and *Salmonella* (McLennan et al. 1981), *Corynebacterium diphtheriae* (DtxR, diphtheria toxin regulator) (Boyd et al. 1990), mycobacteria (IdeR, iron-dependent regulator) (Dussurget et al. 1996), and staphylococci (SirR, staphylococcal iron regulator repressor) (Hill et al. 1998).

Iron-containing prosthetic groups (non-protein components of an enzyme molecule)
Bacteria synthesize heme, an iron-containing prosthetic group, belonging to the family of tetrapyrrroles including also chlorophylls (Panek and O’Brien 2002). In bacteria, heme is involved in energy conserving electron transport chains and a cofactor of various enzymes (Schobert and Jahn 2002).

A second prosthetic group in bacteria are the iron-sulfur clusters (Fe-S), essential as a cofactor of many distinct cell functions (Saini et al. 2012), electron-transfer, maintenance of protein structure, enzyme activity, metabolic regulation, gene expression regulation, and protein folding (Py et al. 2011, Xu and Moller 2011).

4.5 Gut bacteria-host metabolic interplay
Coevolution of host and gut microbiota has been suggested (Ley et al. 2008, Muegge et al. 2011). In reciprocal transplantation experiments with germ-free zebrafish and mice, the recipient animals altered the induced gut microbiota towards their species specific composition (Rawls et al. 2006). Other studies have shown that the gut microbiota species adapt to the host habitat and to other microbial species present (Samuel et al. 2007, Xu et al. 2007). Moreover, complex mechanisms allow the host to control its resident microbiota (chapter 4.5.2) (Macpherson and Uhr 2004).
Probiotics are defined as life microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO 2002). In 1995, Gibson and Roberfroid introduced the concept of prebiotics as ‘non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempting to improve host health’ (Gibson and Roberfroid 1995).

In the recent years, the importance of the commensal gut microbiota in the modulation of host’s immune system, and its influence in host development and physiology has become evident (Bischoff 2011, Sommer and Backhed 2013). These host-microbiota interactions will be elucidated in the following chapters.

4.5.1 Immunomodulation

The crucial role of the gut microbiota in the development of mucosal immunity is not surprising, considering that the intestinal mucosa represents the largest body surface area in contact with external antigens. The majority of these antigens derives from gut microbes covering the mucosa in a dense carpet and is presented to the intestinal immune cells (Rakoff-Nahoum and Medzhitov 2008, Sekirov et al. 2010). Evidence for the impact of gut microbiota on the development of the intestinal mucosal and systemic immune systems, can be found in studies with germ-free (GF) animals, possessing abnormal numbers of several immune cells, immune cell products, and lymphoid structures (Macpherson and Harris 2004, Bouskra et al. 2008).

Several studies have shown the importance of the gut microbiota in the maturation and development of its host’s mucosal and systemic immunity. For instance, bacterial polysaccharide A (PSA) of Bacteroides fragilis has been demonstrated to be essential for the activation of CD4+ T cells by intestinal cells to trigger appropriate cytokine production (Mazmanian et al. 2005). Furthermore, different species of Lactobacillus have been identified to influence cytokine expression and the Th1/Th2/Th3 ratio (Christensen et al. 2002, Liu et al. 2010, van Hemert et al. 2010), as well as the activation of natural killer (NK) cells (Fink et al. 2007). Moreover, peptidoglycan of gram-negative bacteria has been shown to induce the formation of isolated lymphoid follicles (ILF), which upon recognition of gut microbiota through toll-like receptors (TLR) mature into B cell clusters (Bouskra et al. 2008). Also, segmented filamentous bacteria (SFB) have been demonstrated to induce CD4+ T helper cells (Th17 cells) that produce IL-17 and IL-
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22 (Ivanov et al. 2009), which play a role in innate defense mechanisms at mucosal surfaces (Rubino et al. 2012). However, not only the presence of a single bacterial species but also the interplay of a particular combination of commensal microbes can be necessary to modulate host immune system. A study by Ivanov et al. has shown the need of members of the Cytophaga-Flavobacter-Bacteroidetes phylum for the differentiation of Th17 cells (Ivanov et al. 2008).

The intact mucosal immune system has two important functions: (1) tolerate the commensal gut microbiota, and yet (2) control gut microbiota to prevent overgrowth and invasion of systemic sites (Sekirov et al. 2010).

Prevention of excessive immune response and therefore coexistence of host and the vast amount of gut bacteria is sustained through several mechanisms. First, there is a physical separation of epithelial cells and bacteria through mucus. It has been shown that the mucus consists of two layers, of which the inner one is densely packed and devoid of bacteria (Johansson et al. 2008). Second, the modification of the antigenic properties of the bacteria reduces the host immune response. Lipopolysaccharide (LPS) is the major component of the outer membrane of gram-negative bacteria; and systemic reaction towards it can lead to a septic shock (Beutler and Rietschel 2003). Therefore, the host minimizes the toxic potential of the LPS endotoxin by dephosphorylation (Bates et al. 2007) or by down-regulation of the enzyme Interleukin-1 receptor-associated kinase 1 (IRAK-1), which is needed for the endotoxin signaling (Lotz et al. 2006). The third mechanism is the modulation of the local host’s immune response to be more tolerant towards commensal bacteria. Dendritic cells of the intestine have been shown to promote a more tolerogenic state of the GI tract (Sekirov et al. 2010), depending on microbiota-induced secretion of thymic stromal lymphopoietin (TSLP) and transforming growth factor (TGF)-β by epithelial cells (Zeuthen et al. 2008). Another anti-inflammatory mechanism is employed by Bacteroides thetaiotaomicron, preventing the activation of the proinflammatory transcription factor NFκB (Kelly et al. 2004). An alternative way of host tolerance to resident bacteria has been proposed by Lee et al., suggesting that TLR9 in the basolateral region of the intestinal epithelial cells promotes NFκB activation and therefore protects the host from invading microbial agents, whereas apical TLR6 stimulation by commensal bacteria does not (Lee et al. 2006). Furthermore, Clostridium spp. belonging to the clusters IV and XIVa have been shown to strongly promote the
accumulation of CD4+ T regulatory cells, which play an important role in the host’s immune homeostasis (Atarashi et al. 2011).

Although commensal gut microbiota is essential for human health, its overgrowth can be detrimental. There are several strategies for the control of the gut microbiota (Figure 10) (Macpherson and Harris 2004). Intestinal dendritic cells (DC) can retain live commensals for several days and induce immunoglobulin A (IgA), which protects against invading commensals and opportunistic pathogens (Macpherson and Uhr 2004, Tsuji et al. 2008). In order to avoid the induction of a systemic response, the migration of the bacteria-loaded DCs is restricted to the mesenteric lymph node and thus keeps the immune response confined to the mucosa (Macpherson and Harris 2004). The presence of bacteria is essential for IgA production, as it has been shown that IgA is drastically reduced in GF mice and absent in neonates (Macpherson and Harris 2004), whereas LPS can restore its production in DC cultures from GF mice (Massacand et al. 2008). Gram-negative Bacteroides have been shown to induce higher IgA levels than gram-positive Lactobacillus by Peyer’s patches lymphocytes (Yanagibashi et al. 2009). Nevertheless, a Lactobacillus strain has been shown to significantly increase the nonspecific humoral response, especially an elevation in IgA, during an acute phase of a rotavirus infection (Kaila et al. 1992). A study in immunodeficient mice showed that in the absence of IgA, a symbiotic B. thetaiotaomicron strain elicits a more robust oxidative response in the host and therefore suggests IgA promotion of a non-inflammatory relationship between symbiotic microorganisms and the host (Peterson et al. 2007). Another mechanism by which the host controls its gut microbiota is through antimicrobial peptides (AMPs) and will be discussed in the next chapter 4.5.2.
4.5.2 Protection from pathogens

The commensal gut microbiota forms a physical barrier against pathogens by occupying attachment sites, reduces nutrient availability, and produces antimicrobials. Moreover, the gut microbiota can stimulate its host to produce AMPs (Sekirov et al. 2010). These AMPs are generated by various intestinal cells, including enterocytes and Paneth cells; the latter are located at the base of the small intestinal crypts (Ayabe et al. 2000, Hooper 2009). The two major classes of AMPs are defensins and cathelicidins (Muniz et al. 2012). The AMPs are able to lethally damage bacterial cell membranes (Lehrer et al. 1989, Ganz 2003), and thus regulate numbers and/or composition of the gut microbiota (Salzman et al. 2007). A recent review suggests the potential therapeutic use of cathelicidins in targeting inflammatory and cancerous diseases in the GI tract (Chow et al. 2013). Furthermore, studies on probiotics highlight the physical presence of these gut microbes in order to reduce the attachment sites for pathogens (Resta-Lenert and Barrett 2003), upregulate mucin production (Mack et al. 1999, Kim et
4. Gut microbiota – an organ in itself

al. 2008), enhance tight junction integrity (Anderson et al. 2010), or reduce iron availability (Deriu et al. 2013). A study on the function of toll-like receptors (TLRs) showed, that commensal bacteria play a crucial role in their activation and therefore in the maintenance of the host defense against pathogens; as seen in chapter 4.5.1 (Rakoff-Nahoum et al. 2004).

4.5.3 Influence on structure and function of the GI tract
The GI tract of newborns is immature, and the gut microbiota is important for its structural and functional maturation (Berg 1996, Hooper and Gordon 2001, Shanahan 2002). One example is the disturbed vascular development of GI villi in GF mice, with potential implication for nutrient absorption (Stappenbeck et al. 2002). Similarly, the intestinal surface area is reduced in GF animals (Gordon and Bruckner-Kardoss 1961). Furthermore, the villi are thinner due to decreased cell regeneration (Banasaz et al. 2002), or increased cell cycle time and reduced proliferative activity (Alam et al. 1994). Moreover, the gut microbiota increases the peristaltic motility (Husebye et al. 1994). Finally, as discussed in the previous chapter (4.5.2); the commensal gut microbiota assists its host in maintaining barrier functions through mucin production (Mack et al. 1999, Kim et al. 2008), strengthening of tight junctions (Anderson et al. 2010), and activation of TLRs (Rakoff-Nahoum et al. 2004) and thus increases protection towards pathogens.

4.5.4 Fecal calprotectin
Calprotectin is a calcium-binding polypeptide found in the cytosol of neutrophils, monocytes and activated macrophages (Gisbert and McNicholl 2009). Fecal calprotectin levels reflect migration of primarily neutrophils into the intestinal mucosa, and levels are elevated in children with gastroenteritis (Berni Canani et al. 2004). Endoscopy with biopsies is the current gold standard for the assessment of intestinal inflammation, but fecal calprotectin has proven a valuable, rapid and non-invasive alternative (Paduchova and Durackova 2009).

A review by Konikoff et al. suggests a cutoff value for fecal calprotectin concentration of 50 µg/g feces in healthy adults and children. However, in healthy infants below 12 months up to 10-fold higher values were typically identified (Konikoff and Denson 2006). Similarly, high median fecal calprotectin concentrations were reported in Ugandan infants: 345 (0-3 months), 278 (3-6 months), and 183 (6-12 months),
respectively. The median concentrations in Ugandan children aged 1 to 4 and 4 to 12 years were 75 and 28 µg/g, respectively and are comparable to children from high-income countries (Hestvik et al. 2011). Fecal calprotectin concentration were suggested to be lower in infants fed breast milk than those fed formula, potentially due to the anti-inflammatory properties of breast milk or the association of a more rapid decrease in gut permeability compared to formula feeding (Golden et al. 2002).

4.5.5 Effect of various diets on gut microbiota
The human gut microbiota composition is dependent on the dietary pattern of the host, especially on the carbohydrate intake (Walker et al. 2010, Wu et al. 2011). A recent study compared the fecal gut microbial composition of children from Burkina Faso and Italy and reported that the African diet, rich in starch, fiber, and plant polysaccharides, favors Bacteroidetes (particularly Prevotella spp.) and higher short-chain fatty acid (SCFA) concentration, while European children are colonized more frequently by Firmicutes (De Filippo et al. 2010). Moschen et al. reviewed the effect of various diets on the composition of the gut microbiota (Table 3) (Moschen et al. 2012). Inter-individual differences in the gut microbiota have been classified in three discrete enterotypes in healthy humans, namely the Bacteroides, Prevotella and Ruminococcus enterotypes (Arumugam et al. 2011). However, there is a debate if the grouping of the microbiota of individuals into compositional categories or enterotypes, based on the dominance of specific genera may oversimplify this complex situation (Jeffery et al. 2012).

Table 3 Diets and their effect on the gut microbiota. Adapted from (Moschen et al. 2012)

<table>
<thead>
<tr>
<th>Diet/dietary intervention</th>
<th>Change in gut microbiota</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-fat diet</td>
<td>Bacteroidetes ↓, Firmicutes ↑, Proteobacteria ↑</td>
<td>(Hildebrandt et al. 2009)</td>
</tr>
<tr>
<td>High-fat/High-sugar diet</td>
<td>Bacteroidetes ↓, Firmicutes ↑</td>
<td>(Turnbaugh et al. 2009)</td>
</tr>
<tr>
<td>Protein-rich/Saturated fats</td>
<td>‘Bacteroides’ enterotype</td>
<td>(Wu et al. 2011)</td>
</tr>
<tr>
<td>Carbohydrate-enriched diet</td>
<td>‘Prevotella’ enterotype</td>
<td>(De Filippo et al. 2010, Wu et al. 2011)</td>
</tr>
<tr>
<td>Self-reported vegetarians</td>
<td>‘Prevotella’ enterotype</td>
<td>(Wu et al. 2011)</td>
</tr>
<tr>
<td>High fiber content</td>
<td>Bacteroidetes ↑, Actinobacteria ↑, Firmicutes ↓, Proteobacteria ↓</td>
<td>(De Filippo et al. 2010, Wu et al. 2011)</td>
</tr>
<tr>
<td>High animal protein and fat (Western diet)</td>
<td>Firmicutes dominated</td>
<td>(De Filippo et al. 2010)</td>
</tr>
<tr>
<td>Vegetarian lifestyle</td>
<td>Bacteroides spp., Bifidobacterium spp., Enterobacteriaceae spp., and E. coli ↓</td>
<td>(Zimmer et al. 2012)</td>
</tr>
</tbody>
</table>
4. GUT MICROBIOTA – AN ORGAN IN ITSELF

4.5.6 Gut microbial metabolites

The ability of the gut microbiota to ferment non-digestible dietary carbohydrates and host-derived glycans into SCFAs is important for human health (Gibson and Roberfroid 1995, Flint et al. 2012). Furthermore, SCFAs can be a relevant energy source, contributing up to 10% to the daily caloric requirement of humans (McNeil 1984).

During the fermentation in the colon, polysaccharides and oligosaccharides are hydrolyzed to their constituent sugars, which are further fermented (Savage 1986, Topping and Clifton 2001). The primary end-products are SCFAs (acetate, propionate and butyrate), other organic acids (e.g. formate), alcohols (e.g. methanol and ethanol), various gases (CO₂, CH₄, and H₂) and energy, which bacteria require for growth and cell function maintenance (McNeil 1984, Cummings et al. 1987). Acetate, propionate, and butyrate account for 85% of the luminal SCFA production at a suggested molar ratio of 60:25:15. The majority of SCFAs are absorbed in the lumen and less than 5% is excreted in feces (Topping and Clifton 2001). However, some of the metabolites such as ammonia, phenolic and indolic compounds have been shown to exert toxic effects on the lumen (Nyangale et al. 2012). Acetate is rapidly absorbed by the colonocyte and transported through the liver to peripheral tissues, where it serves as main energy source for muscles (Bleiberg et al. 1992). Acetate is also used as a substrate for liver cholesterol and fatty acid synthesis (Nishina and Freedland 1990, Lin et al. 1995), and increases colonic blood flow and oxygen uptake (Schepach 1994). A review by Hosseini et al. suggests that absorbed propionate is thought to lower lipogenesis, serum cholesterol levels, and carcinogenesis, and increases leptin and therefore regulates satiety (Hosseini et al. 2011). Butyrate can be considered to be the most important SCFA, as it is the preferred energy source for colonocytes and appears to promote a normal phenotype in these cells (Topping and Clifton 2001, Louis and Flint 2009). For instance, butyrate production showed a protective effect against colon cancer in a rat model (McIntyre et al. 1993), and decreased cell proliferation and apoptosis of human colon carcinoma cell lines (Heerdt et al. 1994).

Not all bacteria in the human gut are able to degrade polysaccharides (Chassard et al. 2007), a simplified schematic illustrating the relationship between primary degraders, polysaccharide utilizers and bacteria using intermediate fermentation products (e.g. H₂) can be found in Figure 11 (Flint et al. 2008).
A study on fecal SCFA detection in Swedish infants during the first two years of life, has reported a rapid appearance of acetate after birth, followed by propionate in the first 3 months, and subsequently of butyrate (Midtvedt and Midtvedt 1992). The type of feeding influences the development of gut microbiota and therefore SCFA production. In breastfed infants, acetate and lactate are the most abundant SCFAs while propionate is low and butyrate is virtually absent (Siigur et al. 1993, Edwards et al. 1994).

A summary of the most common bacteria in the human GI tract, as well as their fermentation substrates and products can be found in Table 4. The quantitatively most important gram-negative Bacteroides are capable of degrading a wide range of polysaccharides including starch and host-derived glycan (Zocco et al. 2007), producing acetate, propionate and succinate (Salminen et al. 1998). Members of the gram-positive Firmicutes (including Clostridium, Faecalibacterium, Roseburia, Lactobacillus and Eubacterium) are main contributers to colonic butyrate production (Louis and Flint 2009). Lactic acid bacteria (LAB) are a diverse phenotypic group of bacteria producing lactate as their primary fermentation product and include Lactobacillus, Streptococcus, Enterococcus, Lactococcus, Bifidobacterium and Leuconostoc (Masood et al. 2011). Lactate is an important substrate for secondary metabolite fermenters such as Bifidobacterium adolescentis and Eubacterium hallii Anaerostipes caceae (Belenguer et al. 2006), in
addition LAB-derived acetate can be used by Roseburia intestinalis and A. caccae (Falyon et al. 2006). Furthermore, their ability to ferment fructo-oligosaccharides and inulin makes LAB favored prebiotic targets and probiotic organisms (Gibson and Roberfroid 1995, Howard et al. 1995, Bouhnik et al. 1999).

Hydrogen disposal during fermentation is essential for normal colon function and performed through different pathways (Gibson et al. 1993). In developed countries, 30-50% of individuals harbor methanogenic bacteria in their colon (Bond et al. 1971), metabolizing carbon dioxide (CO₂) and hydrogen (H₂) to methane (CH₄) and water (H₂O) (Thauer et al. 1977). In people not excreting methane, other route for H₂ disposal exist, one of them being the reduction of sulphate by the phenotypic group of sulfate-reducing bacteria (SRBs) (Gibson et al. 1990). However, unlike methanogenesis the end-product is not harmless, but the highly toxic metabolic end-product hydrogen sulfide (Gibson et al. 1993). A third way of H₂ disposal is performed by acetogenic bacteria producing acetate (Thauer et al. 1977).

Table 4 Major commensal gut microbiota, their fermentation substrates and products in the human GI tract, adapted from (Salminen et al. 1998, Dethlefsen et al. 2006, Payne 2012)

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Substrate</th>
<th>Major fermentation end-product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroides</td>
<td>Saccharolytic (poly- and oligosaccharides, starch)</td>
<td>Acetate, propionate, succinate</td>
</tr>
<tr>
<td>Clostridia</td>
<td>Saccharolytic and proteolytic</td>
<td>Acetate, propionate, butyrate, lactate, ethanol, hydrogen</td>
</tr>
<tr>
<td>Eubacteria</td>
<td>Saccharolytic and proteolytic</td>
<td>Lactate, acetate, butyrate</td>
</tr>
<tr>
<td>Roseburia</td>
<td>Saccharolytic (starch and inulin)</td>
<td>Butyrate</td>
</tr>
<tr>
<td>Faecalibacteria</td>
<td>Saccharolytic and proteolytic</td>
<td>Lactate, acetate, butyrate</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>Saccharolytic (polysaccharide, fructose)</td>
<td>Lactate</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>Saccharolytic (polysaccharide, fructose)</td>
<td>Acetate, lactate, formate, ethanol</td>
</tr>
<tr>
<td>Ruminococi</td>
<td>Saccharolytic</td>
<td>Acetate</td>
</tr>
<tr>
<td>Streptococci</td>
<td>Saccharolytic and proteolytic</td>
<td>Lactate, acetate</td>
</tr>
<tr>
<td>Desulfovibrios</td>
<td>Sulfate; H₂ and lactate scavenging</td>
<td>Hydrogen sulfide, Acetate</td>
</tr>
<tr>
<td>Methanobrevibacter</td>
<td>H₂ and CO₂</td>
<td>Methane</td>
</tr>
</tbody>
</table>
4.5.7 Effect of gut microbiota on processes outside of the GI tract

The gut microbiota has been suggested to have an impact on various other organ systems of the human body in health and disease. A review of the role of gut microbiota in human obesity summarizes findings from important studies in this field (Tagliabue and Elli 2013). An increased energy harvest through higher SCFA production (as seen in the previous chapter 4.5.5) is one possible way, by which gut microbiota can contribute to obesity. SCFA concentration (especially propionate and butyrate) have been reported to be higher in obese than lean individuals (Schwiertz et al. 2010, Payne et al. 2011). Furthermore, obesity in humans has been associated with reduced bacterial diversity and altered composition (Ley et al. 2006, Armougom et al. 2009, Turnbaugh et al. 2009, Million et al. 2012). However, it is unclear if these observed differences in gut microbiota composition in obese subjects are the cause or effect of obesity (Tagliabue and Elli 2013). Moreover, in animals, high-fat diet decreased Bacteroidetes and increased Firmicutes and Proteobacteria independently of the mice being obese or not (wildtype vs. RELMβ KO), indicating that the diet and not the obese state was responsible for the changes in gut microbiota composition (Hildebrandt et al. 2009).

A review by Howitt and Garrett summarizes current knowledge on the potential link between gut microbiota and cardiovascular disease (CVD) (Howitt and Garrett 2012). A study in atherosclerosis patients and healthy controls identified compositional and functional differences in the gut microbiota that may be related to the disease (Karlsson et al. 2012). Wang et al. suggested the contribution of gut microbiota dependent metabolism of the dietary phosphatidylcholine to the pathogenesis of CVD (Wang et al. 2011).

Recent evidence on the gut-brain axis and the involvement of the gut microbiota in the bidirectional gut-brain communication, brain function and even behavior has been reviewed by Cryan et al. and are summarized in the Figure 12 (Cryan and Dinan 2012).

Furthermore, the gut microbiota composition and alteration of the healthy gut microbiota in early infancy, has been suggested to be involved in the etiology of atopic disease (Penders et al. 2007).
4. Effect of antibiotics on the gut microbial ecosystem

Antibiotics are an important weapon in the battle against infectious bacterial diseases. However, they can have a devastating effect on the host commensal gut microbiota (Willing et al. 2011).

Antibiotics administration is ideally meant to target a specific pathogenic population, but many have a broad-spectrum activity in order to treat many diseases (Nathan 2004). Furthermore, the gut microbiota is a complex ecosystem, in which members depend on each other, based on differential metabolite production and utilization pathways (chapter 4.5.5). For this reason targeting one population in this system can have an indirect effect on others, as illustrated in Figure 13 (Willing et al. 2011). This has been shown with vancomycin, an antibiotic active against gram-positive bacteria, having a big impact on gram-negative bacteria, decreasing Bacteroidetes and increasing Proteobacteria (Robinson and Young 2010).
Many human studies have reported short- and long-term effects of different antibiotics on the commensal gut microbiota (Lofmark et al. 2006, Jernberg et al. 2007, De La Cochetiere et al. 2008, Dethlefsen et al. 2008, Tanaka et al. 2009, Jernberg et al. 2010, Mangin et al. 2010, Perez-Cobas et al. 2012). The disturbance of the commensal gut microbial homeostasis disrupts the protective barrier function and can favor opportunistic pathogens, such as *C. difficile*, through the provision of new niches (De La Cochetiere et al. 2008). Furthermore, exposure to antibiotics increases the number of antibiotics-resistance genes (Lofmark et al. 2006, Jernberg et al. 2007).

*Figure 13* Effect of antibiotics on the gut microbial ecosystem. (A) Mutualistic interactions between different members of the gut microbiota and the host without antibiotic interference. (B) Impact of antibiotics on primary targets, but indirectly also on bacteria that are involved in mutualistic interactions with them, such as secondary metabolite utilizers (Willing et al. 2011).

### 4.7 Techniques to assess gut microbial diversity

The human intestine harbors a complex assembly of $10^{13}$ to $10^{14}$ microorganisms; 10 times more than human cells. Their cumulative genome, called ‘*microbiome*’, contains at least 100 times as many genes as the entire human genome (Gill et al. 2006). First identification of gut microbiota was performed by isolating live bacteria using specified media (Sekirov et al. 2010). However, up to 85% of the gut microbiota is not culturable and therefore undetectable using these techniques (Langendijk et al. 1995). Culture-independent molecular techniques further elucidated the gut microbial ecosystem (Maccaferri et al. 2011). Due to ethical and practical reasons, the vast majority of studies describing the human gut microbiota are based on the analysis of fecal samples.
However, Eckburg et al. showed variation between fecal and surface-adherent mucosal bacteria obtained by colonoscopy, suggesting that fecal microbiota represents a mixture of luminal and shed mucosal bacteria (Eckburg et al. 2005). This chapter describes the available techniques to investigate the gut microbiota and summarizes their advantages and disadvantages in Table 5.

4.7.1 Cultivation
Research on microbiota started with culture-dependent analyses using differential media to select bacterial population based on their metabolic requirements (Sekirov et al. 2010). In an early study, the cultivated fecal microbiota of 20 healthy adults was found to be dominated by the genera Bacteroides, Eubacterium, Ruminococcus, Clostridium, and Bifidobacterium (Moore and Holdeman 1974). Most of these gut microbial species are strict anaerobes (Eckburg et al. 2005) and thus require to be handled in the absence of oxygen using anaerobic chambers or Hungate tubes in order to prevent loss of diversity (Finegold and Rolfe 1983, Tannock et al. 2000). Nevertheless, only about 15-35% of the total gut microbiota is considered culturable (Langendijk et al. 1995). Despite various limitations, the isolation of live species allows further phenotypic characterization and functional studies to be performed (Dethlefsen et al. 2006).

4.7.2 16S rRNA methods
Molecular methods to assess microbial diversity rely on the gene encoding the structural RNA of the small ribosomal subunit, called 16S rRNA (Bik 2009). The 16S rRNA gene is highly suitable for phylogenetic analyses, as it is found in all prokaryotes and contains both conserved and variable regions (Olsen et al. 1986). Its use has been established by Woese and Fox in the 1970s (Woese and Fox 1977). The 16S rRNA gene copy numbers vary among bacterial genomes, which complicate extrapolation to cell numbers (Zoetendal et al. 2004). Although rRNA-based methods cannot discriminate between live and dead bacteria, these techniques helped to discover many microbial groups and have increased the knowledge of phylogenetic relationships between different taxa (Hugenholtz 2002, Rappe and Giovannoni 2003).

Oligonucleotide hybridization
Fluorescent in situ hybridization (FISH) uses labeled oligonucleotide probes crossing the bacterial membrane and hybridizing with the 16S rRNA. Direct quantification of individual bacteria is achieved using epifluorescent light microscopy, confocal laser
microscopy, or flow cytometry (Zoetendal et al. 2004). However, due to the high detection limit of $10^6$ cells/g feces, its use is restricted to abundant members of the gut microbiota (Vaughan et al. 2000). Microarray identification is based on the same probe hybridization principle, but allows rapid and high-throughput screening. This makes it useful for the analyses of microbial community structure, function and dynamics (Zhou 2003). A phylogenetic microarray to study the human gut microbiota, called human intestinal tract chip (HITChip), has been developed (Rajilic-Stojanovic et al. 2009).

**Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE)**

DGGE and TGGE are separation methods for PCR-amplified DNA fragments of equal length but different base-pair (guanine (G) and cytosine (C)) composition or sequence (Muyzer and Smalla 1998). These fingerprinting techniques, using the 16S rRNA gene, are semi-quantitative methods ideal to monitor community shifts (Zoetendal et al. 2004). However, these techniques are only able to detect bacteria that constitute up to 1% of the total community (Muyzer et al. 1993, Zoetendal et al. 1998), and thus only the most dominant bacteria will be assessed (Zoetendal et al. 2004).

**Quantitative real-time PCR (qPCR)**

The quantitative real-time PCR measures the emitted fluorescence by the PCR products in each cycle and need no post-PCR laboratory analysis. Quantitative PCR is a high throughput analysis with short turnaround time and high detection sensitivity (Carey et al. 2007). Using qPCR, as with FISH, primers can be designed to be as specific or general as needed (Kerckhoffs et al. 2009). However, similar to FISH and microarrays, qPCR is limited to already identified bacterial species/strains, since specific primers are required. Moreover a standard curve needs to be generated for accurate quantification, which can be difficult without suitable culturable strain (Sekirov et al. 2010).

**Pyrosequencing**

Pyrosequencing is a real-time DNA sequencing method, based on the detection of DNA polymerase activity by an enzymatic luminometric inorganic pyrophosphate (PPI) detection assay (Nyren 1987, Ronaghi et al. 1996). The single-stranded DNA together with a short annealed primer, DNA polymerase, ATP sulfonylase, luciferase, and apyrase are mixed. Nucleotide bases are then added in a specific order, form a complex with the DNA or are removed by apyrase (Ronaghi et al. 1996). When a base-DNA-complex is
formed, pyrophosphate is released by DNA polymerase and converted to ATP by ATP sulfurylase, subsequently used by luciferase to emit light which is detected (Ronaghi et al. 1998). Pyrosequencing is currently the fastest sequencing method for both confirmatory and de novo sequencing. The major limitations of pyrosequencing are the restricted read length, which makes it difficult to specify certain bacteria to a species level, and the extensive bioinformatics analysis required (Ronaghi 2001, Ahmadian et al. 2006).

**Table 5** Techniques for the analysis of microbial communities (adapted from (Zoetendal et al. 2004, Dethlefsen et al. 2006, Sekirov et al. 2010, Fouhy et al. 2012)).

<table>
<thead>
<tr>
<th>Method</th>
<th>Main use</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivation</td>
<td>Identification and quantification of taxa</td>
<td>Provides isolates for further phenotypic characterization; can focus on recovering strains with desired traits</td>
<td>Incomplete and biased community representation, slow and laborious</td>
</tr>
<tr>
<td>Oligonucleotide hybridization:</td>
<td>Detection and quantification of known phylogenetic groups</td>
<td>Can be high-throughput; can reveal spatial relationships; phylogenetic identification of visible cells</td>
<td>Detects only taxa that hybridize to chosen probes</td>
</tr>
<tr>
<td>FISH and Microarray</td>
<td>Monitor community/population shifts</td>
<td>Rapid; high-throughput</td>
<td>Detects only taxa that hybridize to chosen probes; semi-quantitative; identification requires clone library</td>
</tr>
<tr>
<td>Fingerprinting methods:</td>
<td>Comparison of communities</td>
<td>Rapid, inexpensive assessment of abundant 16S rRNA sequence variants</td>
<td>Broad-range PCR bias; reference strains required for validation; laborious</td>
</tr>
<tr>
<td>DGGE and TGGE</td>
<td>Phylogenetic identification, generates data for other 16S rRNA-based methods</td>
<td>Identification to strain level; can detect novel taxa; analysis possible at multiple phylogenetic levels</td>
<td>Broad-range PCR bias; expensive; short read length; extensive bioinformatics analysis required</td>
</tr>
</tbody>
</table>

1Broad-range PCR bias: Possibility of not including all taxa or accurately representing their relative abundance; however this issue is reduced when compared to the cultivation approach.

*Abbreviations:* FISH: Fluorescent in situ hybridization; DGGE: Denaturing gradient gel electrophoresis; TGGE: Temperature gradient gel electrophoresis; PCR: Polymerase chain reaction.
4.8 Models to study the human gut microbial ecosystem

4.8.1 In vitro

In vitro models range from simple closed systems with pure or defined mixed bacterial populations or fecal material, to more complex multi-stage continuous cultures able to simulate different niches and conditions in the colon (Macfarlane and Macfarlane 2007).

Human intestinal epithelial cell models are widely accepted for the in vitro assessment of drug or nutrient absorption and transport, as well as the study of interactions of probiotics and pathogens with enterocytes (Meunier et al. 1995, Mennigen and Bruewer 2009, Zihler et al. 2011). These models consist of polarized, differentiated monolayers of single or co-culture cell lines, distinguished upon their physiological properties such as absorption (e.g. Caco-2) or production of mucus (e.g. HT29-MTX) (Mathias et al. 2010, Vieira et al. 2010).

In order to study more complex interactions of several bacteria, fermentation systems are used. The simplest and frequently used static batch fermentation is a valuable initial screening tool, but is completely lacking the physiological dynamics of the colon (Venema and van den Abbeele 2013).

A three-stage continuous culture system, mimicking the human proximal, transverse and distal colon was introduced by Gibson and Macfarlane (Gibson et al. 1988, Macfarlane et al. 1989, Macfarlane et al. 1989). The SHIME (Simulator of the Human Intestinal Microbial Ecosystem) is an enhanced model consisting of five connected reactors including the upper and lower digestive tract, from stomach to distal colon (Van den Abbeele et al. 2010). More complex simulation systems of the proximal colon have been introduced with the TIM-2 model including peristaltic movements mixing the chime and with the simulation of microbial metabolite uptake via a dialysis membrane (Minekus et al. 1999, Venema and van den Abbeele 2013). In order increase bacterial concentration and diversity as well as long-term stability, reproducing the situation in the human colon, immobilization of infant fecal microbiota using gel beads in continuous fermentation was introduced by the group of Christophe Lacroix (Cinquin et al. 2004, Cinquin et al. 2006). Nevertheless, in vitro models do not provide host immune or neuroendocrine system functionality and other host attributes like absorption, secretion and defense processes (Macfarlane and Macfarlane 2007).
The combination of intestinal cell models with fermentation models creates an advanced *in vitro* model system where samples obtained from fermenters simulating a gut microbiota ecosystem can be directly applied to monolayer cell models for host-function assessment (Deat et al. 2009, Zihler 2010, Bahrami et al. 2011).

A recent publication summarizes the current possibilities of *ex vivo* models, using human or animal cells, tissues or organs in an artificial environment and under controlled conditions, to study host-microbiota interactions. The authors conclude that these models combine the advantage of high resemblance to the *in vivo* experiment situation and the control and reproducibility of *in vitro* models (Roeselers et al. 2013).

### 4.8.2 *In vivo*

Animal models provide important model systems to investigate metabolic processes mediated by gut microbiota, since diet can be controlled and direct access to intestinal contents as well as tissues or organs at autopsy are possible. However, animal models are rather expensive compared to *in vitro* approaches, and animal digestive physiology may differ from humans (Macfarlane and Macfarlane 2007). Similarities to the human GI tract have been suggested for the following animal models: pigs (colon morphology), dogs (stomach morphology and emptying characteristics), and rabbits (gut microbiota composition) (Kararli 1995).

Germ-free (GF) animal models show how host functions are affected by colonization with commensal bacteria or their absence (Sekirov et al. 2010). Gnotobiology uses living model systems in which the identities of all organisms are known; usually by using GF animals colonized by a defined microbiota. These models have been shown to shed light on the interactions of the microbiota and the host mucosal immune system (Wagner 2008). However, GF animal models do have limitations: since the gut microbiota is essential for host development (as seen in chapter 4.5), responses of GF animals might not be the same as in the naturally raised host (Falk et al. 1998). Human flora-associated animals (HFA) offer the most applicable system for studying the ecology and metabolism of the human intestinal flora (Sekirov et al. 2010). Infant HFA models have tried to mimic infants gut microbiota and colonization and metabolism (Edwards et al. 2003, Zeng et al. 2013).
4.8.3 Human microbiome project

The human microbiome project (HMP) is an interdisciplinary effort of multiple projects, aiming to understand the contribution of the microbiota, of which the vast majority is located in the GI tract, to human physiology (Turnbaugh et al. 2007). It is part of the International Human Microbiome Consortium (IHMC) (Lepage et al. 2013). The genomes of all microbial symbionts (microbiome), which outnumber human cells by a factor of ten, may provide traits that humans did not need to evolve (Gill et al. 2006). Some major goals of HMP are the suggestion of new diagnostic biomarkers of health, development of new probiotics, or industrial applications of the microbial enzymes, and a deeper understanding of the nutritional requirements of humans. To achieve these aims, HMP is using in vivo human and animal studies, as well as in vitro models (Turnbaugh et al. 2007).
5. Effect of oral iron on the gut microbiota

A summary of *in vitro* and *in vivo* studies evaluating the effects of oral iron on commensal and pathogenic gut microbiota is given in Table 6. Thirteen studies were identified to report effects of iron on a consortium of gut bacteria. Two of them are *in vitro* studies using either fermentations (Dostal et al. 2013) or cell models (Kortman et al. 2012). Eleven *in vivo* studies have been found, 5 in mice and rats (Benoni et al. 1993, Tompkins et al. 2001, Werner et al. 2011, Dostal et al. 2012, Ettreiki et al. 2012), one in piglets (Lee et al. 2008), one in school children (Zimmermann et al. 2010) and 4 in infants (Mevissen-Verhage et al. 1985,b,c, Balmer et al. 1989,b, Krebs et al. 2013, Soofi et al. 2013).

5.1 Iron and the commensal gut microbiota

*In vivo* studies showed various significant effects of the provided iron on the gut microbiota composition.

The most common inhibitory effects of iron was a decrease in bifidobacteria found in one animal and 3 infant studies (Mevissen-Verhage et al. 1985,b,c, Balmer et al. 1989,b, Werner et al. 2011, Krebs et al. 2013) and lactobacilli in 2 animal and 2 human studies (Tompkins et al. 2001, Zimmermann et al. 2010, Dostal et al. 2012, Krebs et al. 2013). Iron has been found to enhance enterobacteria in 3 human and a rat study (Mevissen-Verhage et al. 1985,c, Balmer et al. 1989,b, Benoni et al. 1993, Zimmermann et al. 2010), *Bacteroides* in 3 human and 2 animal studies (Mevissen-Verhage et al. 1985,c, Benoni et al. 1993, Werner et al. 2011, Dostal et al. 2012, Krebs et al. 2013), and clostridia in infants fed with fortified formula and complementary food containing heme iron, but not electrolytic iron and temporary (after 2 weeks) in iron supplemented rats (Mevissen-Verhage et al. 1985,c, Benoni et al. 1993, Krebs et al. 2013).

In contrast to this, iron supplementation has been shown to decrease enterobacteria in a rat study (Dostal et al. 2012), clostridia in a mouse study (Werner et al. 2011), and *Bacteroides* in long-term (4 weeks) supplementation of rats (Benoni et al. 1993); whereas lactobacilli increased two weeks after start of Fe supplementation in a rat study (Benoni et al. 1993).

Furthermore, iron has introduced changes in the dominant gut bacterial species in African children (Zimmermann et al. 2010), and altered the gut microbiota of formula-
fed infants away from patterns found in breasts-fed infants (Mevissen-Verhage et al. 1985,c).

In the study by Ettreiki et al., 150 mg/kg/d ferric or ferrous iron did not have any effect on the assessed total bacteria, Firmicutes, Bacteroidetes and enterobacteria in weaning mice (Ettreiki et al. 2012).

5.2 Iron and pathogenic intestinal bacteria

Oral iron supplementation has been associated with increased incidence of malaria and other infectious diseases (Oppenheimer 2001). In chapter 3 the safety of iron in Sub-Saharan Africa was discussed. The focus in this chapter will be on changes in pathogenic and potentially pathogenic bacteria through iron in the above identified studies (Table 6).

*In vitro* studies are an important tool to study introduced infections of pathogens in a controlled ecosystem. Kortman et al. reported an increase in *S. typhimurium* as well as in the opportunistic pathogens *C. freundii*, *E. coli*, and to a certain extent *E. faecalis* through iron administration. In addition, the adhesion of *S. typhimurium, C. freundii* and *E. coli* on enterocytes was enhanced with increasing iron concentration (Kortman et al. 2012). This was in contrast to the fermentation model, where *S. typhimurium* grew slower when it was introduced during a high Fe phase than during normal Fe phase (Dostal et al. 2013).

In mice, iron-fortified diet increased *Desulfovibrio* spp. (Werner et al. 2011), which produce toxic hydrogen sulfides suggested to contribute to pathogenesis in inflammatory bowel disease (Carbonero et al. 2012). Furthermore, luminal but not parental iron increased pathogenesis of chronic ileitis in the genetically susceptible host (Werner et al. 2011). In rats, iron supplementation lead to the presence of the potential pathogenic clostridium species *C. limosum* and *C. sporogenes*, and a single dose of 200 mg FeSO₄ increased *C. perfringens* enterotoxin (Benoni et al. 1993).

In an early human study, most *E. coli* strains in breast-fed and formula-fed infants without iron supplement were non-hemagglutinating, while the majority of strains found in iron-fortified formula-fed infants showed mannose-resistant hemagglutination (Mevissen-Verhage et al. 1985). Zimmermann et al. suggested a general shift towards a more pathogenic microbiota through iron administration in
African school children, with a higher ratio of enterobacteria to lactobacilli and bifidobacteria. Moreover, a significant increase in fecal calprotectin was reported at the end of the trial in the children consuming iron-fortified biscuits. Furthermore, there were more children positive for *Salmonella* infection in the iron-fortified group, however this was not significant (Zimmermann et al. 2010). In a recent study by Soofi et al. iron fortification as part of an MNP compared to a control group receiving no MNP in Pakistani infants has increase overall morbidity, especially from bloody diarrhea and acute respiratory signs. An increased number of *Aeromonas* spp. was reported in groups receiving MNP with iron (Soofi et al. 2013).

5.3 Iron status and anemia as determinant of gut microbiota

Three studies describe differences in gut microbiota composition according to the host’s anemia status (Tompkins et al. 2001, Zimmermann et al. 2010, Dostal et al. 2012). Anemic African children carried an unfavorable, high ratio of enterobacteria to bifidobacteria and lactobacilli, which was further increased by iron fortification (Zimmermann et al. 2010). Similarly, anemic rats showed high numbers of enterobacteria and low numbers of commensal *Roseburia* spp./ *E. rectale* (butyrate-producing *Clostridium* Cluster XIVa member) and *Bacteroides* spp.. However, in this study iron did not have an impact on enterobacteria, but 20 mg of FeSO₄ increased Hb and *Bacteroides* spp. in former anemic rats and restored the metabolic activity of the gut microbiota (Dostal et al. 2012). Another rat study suggests an overall increase of bacterial population in the colon of animals receiving iron-deprived compared to iron-fortified diets (Tompkins et al. 2001).

A systematic review showed the association of *Helicobacter pylori* with low iron stores, though the mechanisms of action are unknown. All identified studies on *H. pylori* focused on the impact of infection on iron status and the difficulties of successful iron interventions during *H. pylori* infection, but none looked on the effect of administered iron on new or enhanced *H. pylori* infection (Muhsen and Cohen 2008). A study in mice showed increased resistance of iron deficient individuals to invasive *Salmonella* infection (Puschmann and Ganzoni 1977).

5.4 Limitations of published studies on dietary iron and gut microbiota

There are various limitations in the above identified studies. First, the translation of the results from *in vitro* or animal studies to the human physiology and gut microbiota is
intricate (Macfarlane and Macfarlane 2007). Furthermore, differences between cecal and fecal gut microbiota have been found, as surface-adherent mucosal bacteria will be underrepresented in feces compared to luminal bacteria (Eckburg et al. 2005). Also, differences in the methods used to assess microbial composition may have influenced study outcomes. The two studies in newborns were conducted almost 30 years ago using culture techniques (Mevissen-Verhage et al. 1985,c, Balmer et al. 1989,b) as did 3 of the animal studies (Benoni et al. 1993, Tompkins et al. 2001, Lee et al. 2008). It is known that only about 15-35% of the total gut microbiota is culturable and cumbersome anaerobic handling is crucial for the successful identification of strictly anaerobes in cultures (Langendijk et al. 1995).

Most studies include small group and sample sizes, due to the elaborate and costly laboratory work involved in the determination of the gut microbiota and the individual character associated with these data. However, small samples size can lead to baseline differences, especially since inter-individual differences in gut microbiota are common and reported in the study in African school children (Zimmermann et al. 2010), breast-fed infants (Krebs et al. 2013) and rats (Dostal et al. 2012). Infant studies are especially vulnerable to these inter-individual differences, since the gut microbiota is not yet fully established and affected by to external influences before and during the study (Sekirov et al. 2010).

The infant study by Krebs et al. compared electrolytic with heme iron without having an iron-free control arm and reports to be underpowered (4-6 subjects per group) to detect changes in low abundant groups like E. coli that account for 1% of all bacteria (Krebs et al. 2013). The study in Pakistani infants presented data from diarrheal samples, which may not represent the situation in the healthy subjects. Moreover, the control group did not receive any of the micronutrients from the MNPs (Soofi et al. 2013). Furthermore, the study in newborns by Balmer et al. had a duration of only 14 days and is therefore limited to identify short term effects (Balmer et al. 1989,b).
Table 6 Studies on the effect of iron interventions on gut microbial analyses, *in vitro* and *in vivo*.

<table>
<thead>
<tr>
<th>Subjects, age</th>
<th>Groups, iron compound and dose (number of subjects)</th>
<th>Duration</th>
<th>Microbial sample/ method</th>
<th>Main effects on gut microbiota</th>
<th>Other effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em></td>
<td>Fermentation of feces from 6-10 year old children</td>
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<tr>
<td></td>
<td>A. Very low Fe, 0.9 mg Fe/L</td>
<td>7 phases, 10 days each</td>
<td>Fermentation of fecal samples/ qPCR, pyrosequencing, HPLC</td>
<td>Very low Fe (A): <em>Roseburia</em> spp./ <em>Eubacterium rectale</em>, <em>Clostridium</em> Cluster IV and <em>Bacteroides</em> spp.; ↓ <em>Lactobacillus</em> spp. and <em>Enterobacteriaceae</em> ↑ <em>Salmonella</em>: grew slower when introduced during high (C) than normal (D) Fe phase</td>
<td>Very low Fe (A): propionate and butyrate ↓ Low Fe (B): acetate ↓ and subsequent high Fe (C): acetate ↑</td>
<td>(Dostal et al. 2013)</td>
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<td></td>
<td>B. Low Fe, 1.56 mg Fe/L</td>
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<td></td>
<td>C. High Fe, 26.5 mg Fe/L</td>
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<td></td>
<td>D. Normal Fe, 5.0 mg/L</td>
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<td></td>
<td>All doses were used alternatingly</td>
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<td></td>
<td>Caco-2 and mucus producing E12 cells</td>
<td>Fe³⁺ citrate 0-50 µmol/L</td>
<td>Cell-monolayer/ culture</td>
<td>Fe increased growth of pathogen <em>Salmonella typhimurium</em> and opportunistic pathogens <em>Citrobacter freundii</em> and <em>Escherichia coli</em>; and had small effects on <em>Enterococcus faecalis</em>. Whereas it did not influence commensal <em>Lactobacillus plantarum</em>. Adhesion of <em>S. typhimurium</em>, <em>C. freundii</em> and <em>E. coli</em> increased with increasing Fe</td>
<td></td>
<td>(Kortman et al. 2012)</td>
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<tr>
<td><em>Rats</em></td>
<td>Rats, 3 weeks</td>
<td>I. Fe depletion of all except A (37)</td>
<td>24 days</td>
<td>Fecal/ TGGE, qPCR, HPLC</td>
<td>ID (F): <em>Bacteroides</em> spp. and <em>Roseburia</em> spp./ <em>E. rectale</em> ↓; <em>Enterobacteriaceae</em> and in some groups (B,C) <em>Lactobacillus</em>/ <em>Leuconostoc</em>/ <em>Pediococcus</em> spp. ↑</td>
<td>ID: butyrate and propionate ↓</td>
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<tr>
<td></td>
<td>A. Control diet, 35 mg Fe/kg (3)</td>
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<td></td>
<td>B. 10 mg FeSO₄/kg diet (8)</td>
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<td></td>
<td>C. 20 mg FeSO₄/kg diet (7)</td>
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<td></td>
<td>D. 10 mg electrolytic Fe/kg diet (7)</td>
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<td></td>
<td>E. 20 mg electrolytic Fe/kg diet (7)</td>
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<td></td>
<td>F. Fe-deficient diet (8)</td>
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<td></td>
<td>II. Fe repletion of B, C, D and E (29)</td>
<td>13 days</td>
<td>Fecal and cecal water(SCFA)/ TGGE, qPCR, HPLC</td>
<td>Fe (B-E): <em>Enterobacteriaceae</em> and <em>Lactobacillus</em>/ <em>Leuconostoc</em>/ <em>Pediococcus</em> spp. ↓ (stronger effects in C, than E; but lactobacilli were very high in B and C before introduction to Fe) 20 mg FeSO₄ (C): <em>Bacteroides</em> spp. ↑</td>
<td></td>
<td>FeSO₄ (B,C) restored metabolic activity</td>
</tr>
<tr>
<td>Rats</td>
<td>Mice and Rats</td>
<td>Mice, juvenile (weanling)</td>
<td>Mice (WT), 7 weeks</td>
<td>Mice, 12-13 weeks</td>
<td>Piglets, 21 days (weanling)</td>
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</table>
| A. Control, water (10)  
B. 10 mg/kg/d Fe in 50 FeSO₄ (10)  
C. 30 mg/kg/d Fe in 150 FeSO₄ (10)  
> Administered via stomach tube | A. Fe²⁺, 150 mg/kg/d (16)  
B. Fe⁺⁺ Lipofer®, 75 mg/kg/d (16)  
C. Fe⁺⁺ Lipofer®, 150 mg/kg/d (16)  
D. Control, water (16) | A. FeSO₄, 180 mg/kg diet, ±28 µg/day (5)  
B. Fe-free control diet, <10 mg Fe/kg diet (5) | A. Low, <2 mg Fe/kg diet + NaCl (8)  
B. Intermediate, 121 mg FeCl₃/kg diet + NaCl (8)  
C. High, 1590 mg FeCl₃/kg diet (8) | A. Basal diet (BD), 70 mg Fe/kg (36)  
B. BD + 50 mg Fe²⁺ sulfate/kg diet (36)  
C. BD + 100 mg Fe²⁺ sulfate/kg diet (36)  
D. BD + 250 mg Fe²⁺ sulfate/kg diet (36) | |
| 4 weeks | 6 weeks | 11 weeks | 12 weeks | 4 weeks | |
| Fecal/ culture | Cecal/ qPCR | Cecal/ pyrosequencing | Upon sacrifice sample collection from jejunum, ileum and transverse colon/ culture | Fecal samples/ culture | |
| 2 weeks: E. coli, lactobacilli and clostridia ↑ (B, C); low Fe (B): Bacteroides ↑; enterococci ↓; high Fe (C): peptostreptococci ↑  
4 weeks high Fe (C): enterococci and bacteroides ↓, clostridia ↑, presence of potential pathogens C. limosum and C. sporogenes | No alteration of the microbiota profile (total bacteria, Firmicutes, Bacteroidetes and enterobacteria) observed | Fe (A): Bifidobacterium, Succinovibrio, Turicibacter and Clostridium ↓; and Desulfovibrio and Bacteroides ↑ | Fe (B, C): decreases anaerobes, microaerophiles, lactobacilli and enterococci in transverse colon, coliforms in jejunum and lactobacilli in ileum (only C), compared to ID mice (A) | Higher coliform bacteria counts after 14 days in Fe groups (B-D)  
No effect of Fe on total anerobic bacteria, Bifidobacterium spp., Lactobacillus spp. and Clostridium spp. | Fe (B-D): linear increase of diarrhea with Fe concentration  
Diarrheal samples: lower Bifidobacterium and Lactobacillus spp.; higher coliforms compared to normal feces |
| A single dose study with 200 mg ferrous sulphate /kg: tissue necrosis (stomach and duodenum) and increase in C. perfringens enterotoxin | no inflammation or alteration of the gut mucosa through Fe; Fe⁺⁺ decreased TNBS-induced colitis in adulthood | Luminal but not systemic Fe increased chronic ileitis in susceptible host (TNFΔARE/WT, not WT) | Intermediate Fe (B) increased body weight significantly, but not high Fe (C) compared to mice receiving low Fe diet (A) | | (Benoni et al. 1993) | (Ettreiki et al. 2012) | (Werner et al. 2011) | (Tompkins et al. 2001) | (Lee et al. 2008) |
### Children

<table>
<thead>
<tr>
<th>Country</th>
<th>Age</th>
<th>Intervention A</th>
<th>Fe (mg)</th>
<th>Time</th>
<th>Method</th>
<th>Findings</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ivorian, 6-14 years</td>
<td>6 months</td>
<td>A. Biscuits +20 mg electrolytic Fe/d, 4 times/week (30)</td>
<td>-20</td>
<td>6 months</td>
<td>Fecal/ TGGE, qPCR</td>
<td>Fe (A) increased profile dissimilarity, enterobacteria ↑, lactobacilli ↓ (baseline differences in lactobacilli)</td>
<td>Fe (A) increased fecal calprotectin, but no effect on reported morbidity (Zimmermann et al. 2010)</td>
</tr>
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<td></td>
<td></td>
<td>B. Biscuits +no Fe/d, 4 times/week (30)</td>
<td>0</td>
<td></td>
<td></td>
<td>No effect of Fe on <em>Bacteroides</em>, <em>Bifidobacterium</em>, <em>Salmonella</em>, pathogenic <em>E. coli</em> and <em>Shigella</em></td>
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</table>

### American, 5 months (breast-fed)

<table>
<thead>
<tr>
<th>Country</th>
<th>Age</th>
<th>Intervention A</th>
<th>Fe (mg)</th>
<th>Time</th>
<th>Method</th>
<th>Findings</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 months</td>
<td>A. Cereals +7.8-15.6 mg electrolytic Fe +1.2-2.4 mg Zn/d (6)</td>
<td>-7.8-15.6</td>
<td>4 months</td>
<td>Fecal/ pyrosequencing</td>
<td>Fe (B): Actinobacteria (especially <em>Bifidobacterium</em> and <em>Rothia</em>), and Lactobacillales ↓; whereas Bacteroidales ↑, compared to A and C</td>
<td>(Krebs et al. 2013)</td>
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<td></td>
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<td>B. Cereals +6.2-12.4 mg electrolytic Fe/d (4)</td>
<td>-6.2-12.4</td>
<td></td>
<td></td>
<td>Heme Fe (C): <em>Clostridium</em> Cluster XIVa ↑</td>
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<td>C. Meat, 1.0-2.0 mg heme Fe/d (4)</td>
<td>-1.0-2.0</td>
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### Pakistani, 6 months

<table>
<thead>
<tr>
<th>Country</th>
<th>Age</th>
<th>Intervention A</th>
<th>Fe (mg)</th>
<th>Time</th>
<th>Method</th>
<th>Findings</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 months</td>
<td>A. Control (947)</td>
<td>-947</td>
<td>12 months</td>
<td>Diarrheal samples (~600 available samples of totally 3000 episodes per group)/ PCR and culture</td>
<td>Increased morbidity, especially bloody diarrhea and acute respiratory signs (Soofi et al. 2013)</td>
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<td></td>
<td></td>
<td>B. MNP +12.5 mg Fe²⁺ fumarate (910)</td>
<td>-12.5</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>C. MNP +12.5 mg Fe²⁺ fumarate +10 mg Zn (889)</td>
<td>-10</td>
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### British, newborn

<table>
<thead>
<tr>
<th>Country</th>
<th>Age</th>
<th>Intervention A</th>
<th>Fe (mg)</th>
<th>Time</th>
<th>Method</th>
<th>Findings</th>
<th>Notes</th>
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<tbody>
<tr>
<td></td>
<td>14 days</td>
<td>A. Basal formula (BF) (20)</td>
<td>-20</td>
<td>14 days</td>
<td>Fecal/ culture</td>
<td>Fe (C) day 4: <em>E. coli</em> ↑ and staphylococci ↓</td>
<td>(Balmer et al. 1989,b)</td>
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<tr>
<td></td>
<td></td>
<td>B. BF +2.8 mg lactoferrin/L (18)</td>
<td>-2.8</td>
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<tr>
<td></td>
<td></td>
<td>C. BF +2.8 mg lactoferrin/L +9.2 mg Fe/L (20)</td>
<td>-9.2</td>
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### Dutch, newborn

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<thead>
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<th>Country</th>
<th>Age</th>
<th>Intervention A</th>
<th>Fe (mg)</th>
<th>Time</th>
<th>Method</th>
<th>Findings</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 weeks</td>
<td>A. Breast milk (10)</td>
<td>-10</td>
<td>12 weeks</td>
<td>Fecal/ culture</td>
<td>Fe (C vs. A, B): isolation frequency and counts of bifidobacteria ↓; isolation frequency of <em>Bacteroides</em> spp., clostridia and <em>E. coli</em> ↑</td>
<td>Breast-fed infants had a low fecal pH (Mevissen -Verhage et al. 1985,b,c)</td>
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<td></td>
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<td>B. Cow milk product (7)</td>
<td>-7</td>
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<tr>
<td></td>
<td></td>
<td>C. Cow milk product +5 mg /L Fe (6)</td>
<td>-5</td>
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</table>

*Abbreviations*: BD: Basal diet; BF: Basal formula; Fe: Iron; FeCl₃: Ferric chloride; ID: iron deficient; MNP: Micronutrient powder; NaCl: Sodium chloride; TNBS: Trinitrobenzene sulfonic acid, TNF: Tumor necrosis factor, WT: Wild type.
6. References


Anderson, R. C., A. L. Cookson, et al. (2010b). Lactobacillus plantarum MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. BMC Microbiol 10: 316.


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REFERENCES


MANUSCRIPT 1: Predictors of hepcidin in Kenyan infants

Iron Status and Systemic Inflammation, but not Gut Inflammation, Strongly Predict Gender-Specific Concentrations of Serum Hepcidin in Infants in Rural Kenya

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Abstract

Hepcidin regulation by competing stimuli such as infection and iron deficiency has not been studied in infants and it’s yet unknown whether hepcidin regulatory pathways are fully functional in infants. In this cross-sectional study including 339 Kenyan infants aged 6.0±1.1 months (mean±SD), we assessed serum hepcidin-25, biomarkers of iron status and inflammation, and fecal calprotectin. Prevalence of inflammation, anemia, and iron deficiency was 31%, 71%, 26%, respectively. Geometric mean (±SD) serum hepcidin was 6.0 (±3.4) ng/mL, and was significantly lower in males than females. Inflammation (C-reactive protein and interleukin-6) and iron status (serum ferritin, zinc protoporphyrin and soluble transferrin receptor) were significant predictors of serum hepcidin, explaining nearly 60% of its variance. There were small, but significant differences in serum hepcidin comparing iron deficient anemic (IDA) infants without inflammation to iron-deficient anemic infants with inflammation (1.2 (±4.9) vs. 3.4 (±4.9) ng/mL; P <0.001). Fecal calprotectin correlated with blood/mucus in the stool but not with hepcidin. Similarly, the gut-linked cytokines IL-12 and IL-17 did not correlate with hepcidin. We conclude that hepcidin regulatory pathways are already functional in infancy, but serum hepcidin alone may not clearly discriminate between iron-deficient anemic infants with and without infection. We propose gender-specific reference values for serum hepcidin in iron-replete infants without inflammation.
Introduction

Hepcidin, a 25-amino acid peptide produced and secreted mainly by hepatocytes, is a major regulator of systemic iron homeostasis (Nemeth and Ganz 2009; Hentze et al. 2010). Hepcidin regulates iron efflux from macrophages and enterocytes through its binding to ferroportin and subsequent internalization of the receptor-ligand complex (Nemeth et al. 2004; Kemna et al. 2008). It may also reduce intestinal iron absorption through ubiquitin-dependent proteasome degradation of divalent metal transporter 1 (DMT1) on the luminal enterocyte membrane (Brasse-Lagnel et al. 2011).

Serum hepcidin transcription is decreased in iron deficiency, hypoxia and by erythropoietic stimuli, and it is increased in infection, inflammation and iron overload (Pigeon et al. 2001; Nemeth et al. 2004; Hentze et al. 2010; Wessling-Resnick 2010). Iron status regulates hepcidin expression primarily through the bone morphogenic protein/hemojuvelin (BMP/HJV) pathway, while infection and inflammatory cytokines such as interleukin-6 (IL-6) increase hepcidin transcription mainly through the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (Beutler 2007; Krijt et al. 2010).

The regulation of circulating hepcidin by concurrent and competing stimuli such as infection and iron deficiency has not been extensively studied in humans. Infants, particularly in low-income countries, are highly vulnerable to both, serious infections and iron deficiency (Lonnerdal and Kelleher 2007). Hepcidin may be a sensitive marker for iron utilization and absorption, but little data is available on hepcidin concentration and its relationship to established markers in population studies. A previous study in anemic Tanzanian children, aged 2 months to 13 years, showed high concentrations of urine hepcidin were associated with malaria, which could contribute to malarial anemia and an impaired erythropoietic response to iron supplementation (de Mast et al. 2009). In addition it was shown, that asymptomatic malarial parasitemia was associated with increased serum hepcidin concentrations and anemia in Indonesian schoolchildren, aged 5-15 years (de Mast et al. 2010). Moreover, a recent study in anemic Gambian children, aged 1.5-3 years, showed, that serum hepcidin was the major predictor of erythrocyte iron incorporation (Prentice et al. 2012). Together, these studies advocate a low-cost hepcidin assay to improve safety and efficacy of iron supplementation programs for children in developing countries. However, no published data are yet available on how inflammation and iron status interact to determine
serum hepcidin concentrations in very young infants at the age of 6 month. As immune responses and hepatic metabolism are not fully mature in early infancy, hepcidin regulatory pathways may not yet be fully functional. Moreover, although reference values for serum hepcidin have been proposed for adults (Galesloot et al. 2011), there are no published data on serum hepcidin concentrations in healthy, iron sufficient, non-anemic infants.

Therefore, the aims of this study were to (a) investigate how iron status and inflammation interact to predict serum hepcidin concentrations in African infants, aged 6 month, in a low-income rural setting; and (b) propose a reference values for serum hepcidin in healthy, term, iron sufficient African infants.

**Methods**

**Ethics Statement**

Ethical approval was given by committees at the Kenyatta National Hospital/ University of Nairobi (KNH-ERC/A/337), the University of KwaZulu-Natal (BF121/08), and the Swiss Federal Institute of Technology Zurich (EK 2009-N-53). Written informed consent was obtained from the care-givers. The trial was registered at clinicaltrials.gov (NCT01111864).

**Study Population and Design**

We conducted a cross-sectional study as part of an iron intervention trial in Kenyan infants. From February 2010 to August 2011, we recruited 5-7 months old infants (n=339) in the rural Msambweni district of southern coastal Kenya. The study period included two long rainy seasons from April to July (2010 and 2011) and a short rainy season from October to November 2010. Most families in this area live from subsistence farming with maize as the staple crop.

Malaria, fever, and diarrhea morbidity over the previous 3 months was assessed by questionnaire from mother’s recall, with a focus on the previous 1 week and on the day of sample collection (confirmed by health personnel). Feeding history (breastfeeding and complementary feeding) was assessed via mother’s recall. The antenatal card (n=148) or mothers’ recall was used to record birth history and included mode of delivery, birth date and birth weight. Preterm birth was defined as registered birth before the 37th week of gestation. Infant weight was measured to the nearest 100 g using a hanging scale (Salter model number 235-6S, 25 kg x 100 g; Salter Brecknell, UK)
and height to the nearest 0.1 cm using a measurement board (Shorr Production, LLC., Olney, MD). A whole blood sample was taken by venipuncture in the infants and a finger prick was done to measure the hemoglobin in their mothers. In a subgroup of infants (n=148) we collected a stool sample.

**Laboratory Methods**

Hemoglobin (Hb) was measured by using a HemoCue (HemoCue AB, Ängelholm, Sweden) or a HemoControl device (EKF diagnostics Sales GmbH, Barleben/Magdeburg, Germany) from a venous blood sample (3 mL) in the infants or a finger prick in their mothers. Serum was separated by centrifugation on collection day. The remaining erythrocytes were washed 3 times with normal saline and the zinc protoporphyrin to heme ratio (ZPP) was measured by using a calibrated AVIV hematofluorometer (AVIV Biomedical, Lakewood, USA). Serum ferritin (SF), soluble transferrin receptor (sTfR) and C-reactive protein (CRP) were measured at Lancet Laboratories Nairobi by using the Cobas Integra (Roche, Basel, Switzerland). The lower limit of detection (LLOD) for CRP was 0.9 mg/L. The following cut-offs were used: (a) anemia: Hb <110 g/L for infants and <120 g/L for adult women (WHO 2001), (b) iron deficiency: SF <12 ng/mL (WHO 2001) or sTfR ≥7.4 mg/L (Vendt et al. 2009), and (c) elevated CRP: ≥4.1 mg/L (manufacturer’s reference range).

Frozen serum was transported to the Netherlands (Radboud University Medical Centre, Nijmegen) and serum hepcidin measurements were performed by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF MS) (Kroot et al. 2010). An internal standard (synthetic hepcidin-24; Peptide International Inc.) was used for quantification (Swinkels et al. 2008). Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionization TOF MS platform (Bruker Daltonics). Serum hepcidin-25 concentrations were expressed as nM and calculated to ng/mL (1 nM=2.789 ng/mL). The LLOD of this method was 0.5 nM or 1.4 ng/mL, respectively. For comparison of WCX-TOF MS generated data with a previously used enzyme-linked immune-sorbent assay (ELISA) (Galesloot et al. 2011), hepcidin values were recalculated using the equation (ELISA-1.00)/1.52=WXC-TOF MS determined by Kroot et al. (Kroot et al. 2010).

Serum levels of human IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFNγ, TNFα, and GM-CSF were determined in 156 infants by using a human cytokine multiplex kit (Cytokine 10-plex
panel, Invitrogen, Breda, Netherlands) according to the manufacturer’s instructions. The LLOD of these cytokine assays was 15 pg/mL, 10 pg/mL, 5 pg/mL, 3 pg/mL, 3 pg/mL, 10 pg/mL, 5 pg/mL, 15 pg/mL, 5 pg/mL, 10 pg/mL, respectively. Serum levels of human IL-12 (p40/p70) and IL-17 were determined in 39 samples by using Singleplex bead kits (Invitrogen), also according to the manufacturer’s instructions. The LLODs were 15 pg/mL and 20 pg/mL, respectively.

Stool samples were stored at -20 ºC until analysis. Fecal calprotectin, a marker for intestinal inflammation, was measured in 148 stool samples using an ELISA (Calprest, Eurospital, Trieste, Italy).

**Statistical Analysis**

All data were analyzed using IBM SPSS Statistics 20.0.0 (SPSS Inc., Chicago, IL) and Microsoft Office EXCEL 2010 (Microsoft, Redmond, WA). Values for samples that were below the LLOD for serum hepcidin, CRP and the cytokines were imputed using randomly generated numbers between 0 and the specific LLODs. Weight-for-age (WAZ), height-for-age (HAZ), weight-for-height (WHZ), and BMI-for-age (BAZ) z-scores were calculated using the WHO Anthro software (version 3.2.2) and standards. Definition for stunting was a HAZ < -2 and for wasting a WHZ < -2 (WHO 2007).

The distribution of the data was checked for normality and log transformation was performed, if needed, on non-normally distributed data and for the calculation of the geometric means (GM). Spearman correlation coefficients (rho) were determined to assess the relationship of serum hepcidin with iron status, inflammation and morbidities. Independent samples t-test and the Mann-Whitney U were used to compare serum hepcidin, iron status indices, and CRP values among groups. To explore associations between hepcidin, iron status, and inflammation, linear regression models were fitted with hepcidin, Hb, SF, sTfR, ZPP as the dependent variables. In iron replete (SF ≥12 ng/mL and sTfR < 7.4 mg/L), non-anemic (Hb ≥110 g/L), full-term infants without inflammation (elevated CRP), reference values for serum hepcidin were calculated and presented as geometric means (GM) and the 2.5 and 97.5 percentiles (P2.5 and P97.5).
Results

Study Population
The sample included 339 mother-infant pairs. Mean (±SD) maternal age was 26 (±6.4) years, body mass index (BMI) was 21.3 (±3.1) kg/m², and Hb was 116.9 (±11.7) g/L; nearly half (49.2%) of the women were anemic. Among the mothers, 95.9% were breastfeeding (n=325) and 86.4% (n=293) were giving complementary foods, predominantly sweetened maize gruel (‘uji’), starting from four months post-delivery. Of the infants, 61.9% were born at home, 33.9% in hospital; 3.5% were preterm and 1.8% were delivered by Caesarian section. Mean (±SD) age of the infants at assessment was 6.0 (±1.1) months, and mean weight-for-age and height-for-age z-scores were -0.4 and -0.8, respectively; 4.1% of infants were wasted and 14.7% were stunted. The prevalence of anemia, iron deficiency and IDA was high: 70.5%, 25.4% and 22.4%, respectively (Table 1).

Infectious Diseases
There was a high burden of infectious disease among the infants. Systemic inflammation (elevated CRP) was present in nearly one-third (30.7%) of infants, and 119 (35.1%) had a CRP below the LLOD of 0.9 mg/L. Based on maternal reports, 6.2% of infants were currently passing watery or loose stools, 1.5% were passing more than 4 stools a day, and 17.1% reported blood or mucus in the stools. One infant had malaria on the day of the venipuncture (assessed by a Giemsa stained blood smear), and 7.4% of infants had had malaria in the previous 3 months (reported by caretaker). Mothers reported 8% of infants had received antibiotics, 2.9% supplements for diarrhea, and 2.7% treatment for intestinal parasites (reported as ‘worms’) within the previous week.
Table 1 Characteristics of the study population.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male</th>
<th>Female</th>
<th>Both</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>173 (51.0%)</td>
<td>166 (49.0%)</td>
<td>339</td>
</tr>
<tr>
<td>Age, months*</td>
<td>6.0 (1.1)</td>
<td>6.1 (1.1)</td>
<td>6.0 (1.1)</td>
</tr>
<tr>
<td>Hemoglobin, g/L*</td>
<td>102.2 (11.8)</td>
<td>103.6 (11.1)</td>
<td>102.9 (10.1)</td>
</tr>
<tr>
<td>ZPP, µmol/mol heme*</td>
<td>99.6 (1.8)</td>
<td>84.6 (1.8)</td>
<td>91.9 (1.8)</td>
</tr>
<tr>
<td>Serum ferritin, ng/mL*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All children</td>
<td>24.1 (2.5)</td>
<td>32.2 (2.7)</td>
<td>27.8 (2.6)</td>
</tr>
<tr>
<td>Children without inflammation†</td>
<td>21.5 (2.5)</td>
<td>29.5 (2.6)</td>
<td>24.9 (2.6)</td>
</tr>
<tr>
<td>Soluble transferrin receptor, mg/L*</td>
<td>5.8 (1.4)</td>
<td>5.4 (1.4)</td>
<td>5.6 (1.4)</td>
</tr>
<tr>
<td>C-reactive protein, mg/L*</td>
<td>1.7 (4.8)</td>
<td>2.3 (4.4)</td>
<td>2.0 (4.6)</td>
</tr>
<tr>
<td>Hepcidin, ng/mL‡</td>
<td>4.9 (3.5)</td>
<td>7.2 (3.3)</td>
<td>6.0 (3.4)</td>
</tr>
<tr>
<td>IL-6, pg/mL*</td>
<td>11.9 (1.9)</td>
<td>11.7 (1.9)</td>
<td>11.8 (1.9)</td>
</tr>
<tr>
<td>Fecal calprotectin, mg/kg*</td>
<td>160.7 (2.1)</td>
<td>175.5 (2.2)</td>
<td>167.1 (2.1)</td>
</tr>
<tr>
<td>Inflammation†</td>
<td>50 (28.9%)</td>
<td>57 (34.3%)</td>
<td>103 (30.7%)</td>
</tr>
<tr>
<td>Anemia§</td>
<td>132 (76.3%)</td>
<td>107 (64.5%)</td>
<td>239 (70.5%)</td>
</tr>
<tr>
<td>Iron deficiency (ID)¶</td>
<td>51 (29.8%)</td>
<td>35 (21.3%)</td>
<td>86 (25.7%)</td>
</tr>
<tr>
<td>SF &lt;12 ng/mL</td>
<td>31 (18.3%)</td>
<td>22 (13.6%)</td>
<td>53 (15.6%)</td>
</tr>
<tr>
<td>sTfR ≥7.4 mg/L</td>
<td>36 (20.5%)</td>
<td>23 (14.0%)</td>
<td>59 (17.4%)</td>
</tr>
<tr>
<td>Iron deficiency anemia (IDA)‡</td>
<td>50 (29.2%)</td>
<td>29 (17.7%)</td>
<td>79 (23.6%)</td>
</tr>
<tr>
<td>Birth weight, kg</td>
<td>3.2 (1.2)</td>
<td>3.1 (1.2)</td>
<td>3.1 (1.2)</td>
</tr>
<tr>
<td>Height-for-age (z-score)</td>
<td>-0.9 (1.2)</td>
<td>-0.7 (1.2)</td>
<td>-0.8 (1.2)</td>
</tr>
<tr>
<td>Weight-for-age (z-score)</td>
<td>-0.4 (1.2)</td>
<td>-0.4 (1.2)</td>
<td>-0.4 (1.2)</td>
</tr>
<tr>
<td>BMI-for-age (z-score)</td>
<td>0.1 (1.2)</td>
<td>-0.03 (1.3)</td>
<td>0.06 (1.3)</td>
</tr>
<tr>
<td>Maternal hemoglobin, g/L*</td>
<td>117.4 (11.5)</td>
<td>116.3 (12.0)</td>
<td>116.9 (11.7)</td>
</tr>
</tbody>
</table>

Estimates are mean (±SD) or number (%) unless indicated otherwise
*geometric mean (±SD); †CRP >4.1 mg/L; ‡conversion factor: 1 ng/mL=0.358 nM; §Hb <110 g/L; ¶SF <12 ng/mL or sTfR ≥7.4 mg/L; ††Concurrent ID and Anemia

Serum Hepcidin and its Interaction with Biochemical, Anthropometric and Health Markers

Among infants, geometric mean (GM, (±SD)) serum hepcidin was 6.0 (±3.4) ng/mL (Table 1), and was below the lower limit of detection (LLOD) in 51 (15.0%) infants. Serum hepcidin was significantly lower in males than in females (GM (±SD) 4.9 (±3.5) ng/mL vs. 7.2 (±3.3) ng/mL; P <0.05; Figure 1, (i) total population). Infants born preterm had significantly lower serum hepcidin (0.7 (±7.0) ng/mL (58% below LLOD) vs. 7.1 (±3.9) ng/mL (13% below LLOD); P <0.05) and higher ZPP values (143.7 (±1.9) mol/mol heme vs.
83.8 (±1.8) mol/mol heme; P <0.05) compared to full-term infants. There were no statistically significant differences in Hb, SF, TfR and CRP concentrations comparing preterm to term infants, but these comparisons were limited as only 3.5% of infants were known to be born preterm.

Serum hepcidin was significantly correlated with Hb, ZPP, SF, sTfR, CRP, IL-6, weight, height, gender and premature birth (Table 2). There were no significant correlations of serum hepcidin with maternal Hb, place of delivery (hospital or home) or infectious disease history. In the multiple linear regression analyses including gender and weight-for-age z-score (WAZ); CRP (β=.273), IL-6 (β=.221), ZPP (β=-.337), and SF (β=.274) were significant predictors of serum hepcidin, resulting in an adjusted R² of .595 (P <0.001; Table 3). CRP and IL-6 together explained 22.8% of the variance in serum hepcidin (adjusted R²=.228). Omitting CRP and IL-6 from the model, the remaining iron biomarkers explained 36.7% of the variance in serum hepcidin (adjusted R²=.367). Replacing ZPP with sTfR (β=-.202, P <0.001) did not change the predictive power of the model. ZPP and sTfR correlated strongly (Spearman coefficient rho=.628, P <0.001; Table 2), and including one of these predictors in the regression model explained nearly the same variance in serum hepcidin as both together. IL-6 was the only cytokine with a significant correlation with hepcidin (rho=.358; P <0.01, Table 2); moreover IL-6 correlated with SF (rho=.210; P <0.01) and CRP (rho=.554; P <0.01). IL-5 correlated with Hb (rho=-.176; P <0.05), and IL-12 with SF (rho=-.364; P <0.05). Most interleukins correlated with CRP, but not with serum hepcidin (Table 4).

The gender-specific regression models had a R² of .553 and .586 in male and female infants respectively (Table 3). Serum ferritin was a stronger predictor of hepcidin in females (β=.297, P <0.001) than in males (β=.265, P <0.05), while CRP was a slightly stronger predictor of hepcidin in males (β=.288, P <0.001) compared to females (β=.257, P <0.001). WAZ was a significant predictor (P <0.05) for hepcidin in the total population and in female infants, but not in male infants.
Figure 1 Median levels, lower and upper quartile plus standard error, of serum hepcidin (ng/mL) by sex, measured in different subgroups. Criteria for subgroups were as follows: (1) total population; (2) no iron deficiency (ID), no anemia, and no elevated CRP concentration; (3) ID and anemia (IDA), but no elevated CRP concentration; (4) no ID and anemia, but elevated CRP concentration; (5) IDA and elevated CRP concentration. Cut-offs were as follows: anemia: Hb <110 g/L; ID: SF <12 ng/mL or sTfR ≥7.4 mg/L; IDA: concurrent anemia and ID; no ID: SF ≥12 ng/mL and sTfR <7.4 mg/L; elevated CRP: CRP ≥4.1 mg/L. Note: all individuals with ID and no elevated CRP were anemic. Letters (a, b, c, d, e, and f) indicate significant differences at P <0.05.

Table 2 Spearman correlation coefficients (rho) from iron and inflammation markers and anthropometric data of the study infants.

<table>
<thead>
<tr>
<th></th>
<th>ZPP</th>
<th>SF</th>
<th>sTfR</th>
<th>CRP</th>
<th>Hepcidin</th>
<th>IL-6</th>
<th>Gender</th>
<th>BW</th>
<th>Weight</th>
<th>Height</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb, g/L</td>
<td>.498*</td>
<td>.242*</td>
<td>.431*</td>
<td>.144*</td>
<td>.186*</td>
<td>.132*</td>
<td>.107</td>
<td>.107</td>
<td>.135*</td>
<td>.009</td>
</tr>
<tr>
<td>ZPP, µmol/mol heme</td>
<td>- .439*</td>
<td>.628*</td>
<td>.002</td>
<td>.437*</td>
<td>.001</td>
<td>.205*</td>
<td>.170*</td>
<td>.205*</td>
<td>.010</td>
<td></td>
</tr>
<tr>
<td>SF, ng/mL</td>
<td>-</td>
<td>-</td>
<td>.417*</td>
<td>.251*</td>
<td>.609*</td>
<td>.210*</td>
<td>.144*</td>
<td>.201*</td>
<td>.195*</td>
<td>.134*</td>
</tr>
<tr>
<td>sTfR, mg/L</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>.017</td>
<td>.371*</td>
<td>.065</td>
<td>.158*</td>
<td>.161</td>
<td>.130*</td>
<td>.056</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>.426*</td>
<td>.554*</td>
<td>.122*</td>
<td>.014</td>
<td>.037</td>
<td>.056</td>
<td></td>
</tr>
<tr>
<td>Hepcidin, ng/mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>.358*</td>
<td>.206</td>
<td>.131</td>
<td>.232*</td>
<td>.152*</td>
<td></td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>.011</td>
<td>.062</td>
<td>.116</td>
<td>.034</td>
<td></td>
</tr>
<tr>
<td>Gender, (m/f)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-.113</td>
<td>-.258*</td>
<td>-.243*</td>
<td></td>
</tr>
<tr>
<td>BW, kg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>.213*</td>
<td>.135</td>
<td>.632*</td>
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<tr>
<td>Weight, kg</td>
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<td>-</td>
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</tbody>
</table>

*P <0.01; †P <0.05; BW=birth weight
Table 3 Results of linear regression analyses for serum hepcidin (ng/mL) in the total population and by gender separately.

<table>
<thead>
<tr>
<th></th>
<th>Total population (n=141)</th>
<th>Male (n=65)</th>
<th>Female (n=76)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>SE B</td>
<td>B</td>
</tr>
<tr>
<td>Constant</td>
<td>1.264</td>
<td>.274</td>
<td>.987</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>.168</td>
<td>.039</td>
<td>.162</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>.306</td>
<td>.088</td>
<td>.287</td>
</tr>
<tr>
<td>ZPP, µmol/mol</td>
<td>-.574</td>
<td>.125</td>
<td>-.390</td>
</tr>
<tr>
<td>sTfR, mg/L</td>
<td>-.101</td>
<td>.157</td>
<td>-.126</td>
</tr>
<tr>
<td>SF, ng/mL</td>
<td>.246</td>
<td>.056</td>
<td>.267</td>
</tr>
<tr>
<td>WAZ</td>
<td>-.052</td>
<td>.02</td>
<td>-.056</td>
</tr>
<tr>
<td>Gender</td>
<td>.076</td>
<td>.043</td>
<td>.097</td>
</tr>
</tbody>
</table>

Note: Adjusted R² Total=.595; Adjusted R² Male=.553; Adjusted R² Female=.586; *P < .001; †P < .05.

The dependent variable hepcidin and the independent variables were log-transformed before inclusion in the models. Interpretation for these betas is as follows: 1% change in the independent variable corresponds to a beta% change in the dependent variable.

Table 4 Mean levels of cytokines and fecal calprotectin and their correlation with hepcidin and CRP.

<table>
<thead>
<tr>
<th></th>
<th>Geometric mean (± SD)</th>
<th>Correlation (rho) with</th>
<th>Correlation (rho) with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hepcidin</td>
<td>CRP</td>
</tr>
<tr>
<td>IL-1, pg/mL</td>
<td>18.5 (2.5)</td>
<td>.023</td>
<td>.257†</td>
</tr>
<tr>
<td>IL-2, pg/mL</td>
<td>24.0 (1.6)</td>
<td>.084</td>
<td>.261†</td>
</tr>
<tr>
<td>IL-4, pg/mL</td>
<td>12.0 (7.5)</td>
<td>.086</td>
<td>.252†</td>
</tr>
<tr>
<td>IL-5, pg/mL</td>
<td>4.5 (1.7)</td>
<td>.021</td>
<td>.159</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>11.8 (1.9)</td>
<td>.405*</td>
<td>.538*</td>
</tr>
<tr>
<td>IL-8, pg/mL</td>
<td>42.5 (2.4)</td>
<td>.190</td>
<td>.291*</td>
</tr>
<tr>
<td>IL-10, pg/mL</td>
<td>28.4 (1.5)</td>
<td>.093</td>
<td>.265†</td>
</tr>
<tr>
<td>IFN-γ, pg/mL</td>
<td>3.0 (3.7)</td>
<td>-.034</td>
<td>-.052</td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>9.5 (2.7)</td>
<td>-.040</td>
<td>.088</td>
</tr>
<tr>
<td>GM-CSF, pg/mL</td>
<td>8.2 (3.1)</td>
<td>-.002</td>
<td>.194</td>
</tr>
<tr>
<td>IL-12, pg/mL</td>
<td>237.3 (2.0)</td>
<td>.011</td>
<td>.179</td>
</tr>
<tr>
<td>IL-17, pg/mL</td>
<td>38.7 (2.1)</td>
<td>.216</td>
<td>.383</td>
</tr>
<tr>
<td>Calprotectin, mg/kg</td>
<td>167.1 (2.1)</td>
<td>-.087</td>
<td>-.088</td>
</tr>
</tbody>
</table>

*P < .01; †P < .05
CRP correlated significantly with SF (rho=.251; P <0.01), Hb (rho=-.144; P <0.01), and hepcidin (rho=.426; P <0.01), but not with sTfR and ZPP. Figure 2 shows scatterplots of hepcidin with inflammation and iron markers. Separate linear regression analyses with the iron markers (Hb, SF, sTfR, ZPP and hepcidin) as dependent variables and inflammation markers (CRP and cytokines) as independent variables, identified CRP as a significant predictor for SF (β=.254; P <0.001), Hb (β=-.185; P =0.001) and hepcidin (β=.394; P <0.001), but not for sTfR and ZPP. CRP and the cytokines together resulted in an R² of .345 (P <0.05) for the prediction of SF concentration, whereas sTfR and ZPP were not predicted by CRP and cytokines.

Fecal Calprotectin
Geometric mean (±SD) of fecal calprotectin (FC) was 167 (±1.0) mg/kg. Calprotectin did not correlate with any other inflammation or iron marker, but it correlated significantly with weight (rho=-.258; P <0.01) and the report of the mothers of blood or mucus in stool (rho=-.256; P <0.01). Fecal calprotectin values did not correlate with any other morbidity indices or the duration of breastfeeding. Only four mothers reported current diarrhea (reported as 4 or more loose stools a day) in their children, in 3 of them FC values are available. There was a trend for a significant correlation between diarrhea cases and FC (rho=-.158; P =0.06). Girls had slightly higher fecal calprotectin values (GM (± SD)) than boys, of 175.5 (±1.0) mg/kg and 160.7 (±1.0) mg/kg, respectively. Fecal calprotectin did not correlate with the gut-linked cytokines IL-12 and IL-17 measured in serum, and these cytokines did not correlate with hepcidin.

Subgroup Analysis of Serum Hepcidin According to Iron and Inflammation Status
Figure 1 shows gender-stratified serum hepcidin concentration in different subgroups based on inflammation (measured by CRP) and iron status and anemia. Twenty-nine male and thirty-nine female infants were iron sufficient, non-anemic and had no elevated CRP. The reference values for serum hepcidin from these were 4.1 ng/mL in male and 9.0 ng/mL in female infants (Table 5). Serum hepcidin (GM ±SD) in iron deficient infants without elevated CRP were low (1.0 (±4.2) ng/mL and 1.5 (±3.8) ng/mL for males and females respectively); 23 out of the 36 males (63.9%) and 9 out of the 18 females (50.0%) had serum hepcidin concentration below the detection limit of 1.4 ng/mL. We found small, though significant differences in mean hepcidin concentration comparing iron deficient anemic infants without inflammation (3 in Figure 1) to iron
deficient anemic infants with inflammation (5 in Figure 1; 1.2 (±4.9) ng/mL vs. 3.4 (±4.9) ng/mL; P < 0.001).

Figure 2 Scatter plots and regression equations derived from the correlation of inflammation markers (A: CRP and B: IL-6) and iron markers (C: SF, D: sTfR, and E: ZPP) with serum hepcidin.
Table 5 Reference values for serum hepcidin (nM and ng/mL) from non-anemic, iron replete, Kenyan infants without elevated CRP (CRP <4.1 mg/L AND SF ≥12 ng/mL AND sTfR <7.4 mg/L AND Hb ≥110 g/L).

<table>
<thead>
<tr>
<th>Reference values for serum hepcidin</th>
<th>Geometric Mean</th>
<th>P2.5</th>
<th>P97.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (n=29)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nM</td>
<td>1.5</td>
<td>0.0</td>
<td>5.3</td>
</tr>
<tr>
<td>ng/mL</td>
<td>4.1</td>
<td>0.1</td>
<td>14.8</td>
</tr>
<tr>
<td>Female (n=39)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nM</td>
<td>3.2</td>
<td>0.1</td>
<td>18.2</td>
</tr>
<tr>
<td>ng/mL</td>
<td>9.0</td>
<td>0.3</td>
<td>50.1</td>
</tr>
<tr>
<td>All (n=68)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nM</td>
<td>2.3</td>
<td>0.1</td>
<td>18.1</td>
</tr>
<tr>
<td>ng/mL</td>
<td>6.4</td>
<td>0.2</td>
<td>50.6</td>
</tr>
</tbody>
</table>

Discussion

We are not aware of previous reports of serum hepcidin (hepcidin-25) concentrations in young full-term infants. Previous data on serum hepcidin in infancy is limited to measurements in preterm infants, low birth weight newborns in cord blood and children over the age of 18 months (de Mast et al. 2010; Galesloot et al. 2011; Prentice et al. 2012). Pro-hepcidin concentrations in healthy non-anemic Turkish infants were reported to be 118 ±102.2 ng/mL (mean ±SD) (Ulukol et al. 2007), although it is questionable whether these reflect any physiological relevance (Kemna et al. 2008; Kroot et al. 2011). In our Kenyan male infants, hepcidin concentrations were much lower than those previously found in 18-24 years old Dutch men and adapted for the differences in methods (4.1 ng/mL vs. 14.9 ng/mL) (Galesloot et al. 2011). In female Kenyan infants, however, we found higher values (9.0 ng/mL vs. 2.9 ng/mL) than in 18-24 years old women from the previously mentioned Dutch population. Therefore, in contrast to serum hepcidin concentrations in adults where males have generally higher values than females (Ganz et al. 2008; Galesloot et al. 2011), serum hepcidin in our study was found to be around 50% higher in female infants than in males, after controlling for weight and height. In our regression model including iron status (sTfR, SF and ZPP) and inflammation markers (CRP and IL-6), gender is not a significant predictor (Table 3). This gender difference in hepcidin concentration can mainly be explained by the lower overall iron status in males, and the higher CRP concentration in females. Several studies have reported higher rates of anemia and lower iron status in male compared
to female infants (Domellof et al. 2002; Soh et al. 2004; Wieringa et al. 2007; Schneider et al. 2008; Pasricha et al. 2010), which were attributed to gender-specific growth rates (Wieringa et al. 2007) or hormone-mediated differences in iron metabolism (Domellof et al. 2002). However, more research is needed to elucidate gender-differences in iron status in infants. The percentage of exclusively breastfed infants was slightly higher in males (6.6%) compared to females (3.1%). Even if not statistically significant, the higher proportion of breastfed boys may contribute to the lower prevalence of infection in boys found in this population group; as breast milk has shown to be protective against infectious diseases (WHO 2000).

This study demonstrates that circulating hepcidin-25 concentrations are strongly predicted by both infection/inflammation markers (CRP and IL-6) and iron status markers (SF, ZPP and sTfR) in infants in rural sub-Saharan Africa. These data suggest that the known adult hepcidin regulatory pathways are functioning well as early as 6 months of age. Inflammation and iron status explained nearly 60% of the variance in serum hepcidin, with a roughly equal contribution from each in this setting: CRP and IL-6 explained 23% of the variance in serum hepcidin, SF alone (a reflection of both iron status and, as an acute-phase protein, inflammation) explained 32% of the variance, while ZPP and sTfR together explained 21% (ZPP alone 20% and sTfR 12%, respectively).

In a previous study, in cord blood from low birth weight newborns and preterm infants, circulating hepcidin concentration was also correlated with iron status (Berglund et al. 2011; Muller et al. 2012). Also in Dutch adults, the strongest predictor of serum hepcidin was SF ($R^2$=.58 and $R^2$=.62 in adult men and women, respectively), followed by CRP (Galesloot et al. 2011), but infection/inflammation is much more common in our sample of Kenyan infants. Notably, hepcidin levels in iron deficient and anemic (IDA) children with elevated CRP were significantly lower than those in non-anemic children with elevated CRP. Although IDA children with inflammation tended to have higher hepcidin levels compared to IDA children without inflammation, this difference was significant in male infants only (Figure 1). These findings suggest that the inflammation mediated stimuli (through IL-6) are to a large extent overruled by iron demand and erythropoiesis stimuli down regulating hepcidin synthesis.

Defining iron status in Sub-Saharan Africa and other settings with a high burden of infectious disease is difficult (Zimmermann et al. 2005). The use of SF is limited by its confounding by inflammation, and ZPP and sTfR have been proposed as preferable
indices in these settings, although ZPP may also be confounded by inflammation and sTfR by the rate of erythropoiesis. In our infant population, CRP correlated significantly with SF but not with sTfR and ZPP. In a multiple regression model, CRP and the cytokines together explained SF with an $R^2$ of .345 ($P < 0.005$), whereas sTfR and ZPP were not predicted by CRP and the cytokines. Thus, these data suggest sTfR and ZPP are suitable iron biomarkers in this age group and setting, but standardized cut-offs for infants are needed (Kung’u et al. 2009; Grant et al. 2012a,b).

CRP, a downstream biomarker of inflammation, was a major predictor of serum hepcidin in our infant population. IL-6 induces hepcidin expression (Kemna et al. 2005; Wrighting and Andrews 2006) and it is thought that the increase in circulating hepcidin during infection/inflammation is primarily mediated by IL-6 (Kroot et al. 2011). The inclusion of IL-6 in our regression analysis significantly increased the variance in serum hepcidin explained by the model ($R^2 = 0.587$). CRP and IL-6 together showed an adjusted $R^2$ of .228, while the adjusted $R^2$ of CRP and IL-6 alone was .199 and .162, respectively. These data suggest other factors, along with IL-6, may increase CRP during inflammation; however, IL-6 was the only cytokine with a significant correlation with serum hepcidin in our models.

Fecal calprotectin (FC), a biomarker of localized gut inflammation, was correlated with the report of recent blood or mucus in the infant stool. Similarly, there was a borderline significant trend for a correlation of FC with the few cases of diarrhea reported in this population at the time of assessment. However, FC was not correlated with systemic inflammation, as measured by CRP or the cytokines. Especially, IL-12 and IL-17, which have been linked to gut inflammation (Siegemund et al. 2009; Sarra et al. 2010), did not show any correlation with FC. Furthermore, FC, IL-12 and IL-17 were not correlated with serum hepcidin, suggesting that gut inflammation is not a predictor for hepcidin in this population. It should however be noted that, except for IL-6, none of the other measured cytokines were correlated with hepcidin. Gut inflammation has been associated with an IL-6-mediated hepcidin increase in a mouse model for intestinal colitis, but results in humans with inflammatory bowel disease (IBD) are equivocal (Cherayil et al. 2011). The young age of the Kenyan infants, which is associated with a developing gut microbiota and immature intestinal immune system (Hooper et al. 2012), might play a role in the absence of hepcidin induction in the infants with increased FC levels. Higher values of FC have been reported in breastfed compared to
formula fed Italian infants (Savino et al. 2010), however, in our population duration of breastfeeding did not predict FC. The younger Italian infants (2-13 weeks) showed median FC concentration of 555 mg/kg, thrice as high than in our Kenyan infant population (167.1 mg/kg) (Savino et al. 2010). However, healthy Ugandan infants aged 0-1 years had a median FC of 249 mg/kg, similar to our study population (Hestvik et al. 2011).

The present study provides important data on serum hepcidin concentration in iron replete, iron deficient, and iron deficient anemic infants in rural sub-Saharan Africa. We also propose a gender-specific reference values for serum hepcidin measured by WCX-TOF MS in healthy, full-term, iron replete, non-anemic African infants. The association of hepcidin with iron status and infection markers in infancy indicates the potential relevance of hepcidin as iron status marker in this population group. Our data shows significant differences in hepcidin concentration in IDA infants with and without inflammation. However, a separate analysis of this subgroup by sex resulted in significant differences in boys, but not in girls. Furthermore the size of the differences and the relatively large standard deviations also suggest that serum hepcidin alone may not provide sufficient discriminatory power between anemic infants with and without inflammation (Figure 1). Thus, because provision of iron to infants with infections may be dangerous in certain settings, more research is clearly needed on the potential utility of hepcidin as a point-of-care marker to increase the safety of iron supplementation in infants and children in the developing world, as has been proposed (de Mast et al. 2009; de Mast et al. 2010). In addition, our data suggest serum hepcidin is not correlated with gut inflammation during infancy; thus, the potential local injurious effect of dietary iron supplements on infectious or inflammatory intestinal disease (Werner et al. 2011; Kortman et al. 2012) may not be predicted by serum hepcidin levels. In conclusion, although promising, our study underscores that the potential clinical advantage of serum hepcidin to guide safe iron supplementation in infancy needs further clarification.

Funding
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References


MANUSCRIPT 2: Low dose iron fortification in Kenyan infants

In-home fortification with 2.5 mg iron as NaFeEDTA does not reduce anemia but increases weight gain: a randomized controlled trial in Kenyan infants

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Maternal & Child Nutrition; under revision
Abstract

In-home fortification of infants with MNPs containing 12.5mg iron may increase morbidity from infections; therefore, an efficacious low dose iron-containing MNP might be advantageous. Effects of iron-containing MNPs on infant growth are unclear. We assessed the efficacy of a low-iron MNP on iron status and growth and monitored safety in a randomized, controlled, double-blind, 1-year trial in 6-month-old infants (n=287) consuming daily a maize porridge fortified with either an MNP including 2.5mg iron as NaFeEDTA (MNP+Fe) or the same MNP without iron (MNP-Fe). At baseline, after 6 and 12 months, we determined hemoglobin (Hb), iron status (serum ferritin (SF), soluble transferrin receptor (sTfR), and zinc protoporphyrin (ZPP)), inflammation (C-reactive protein (CRP)) and anthropometrics. We investigated safety using weekly morbidity questionnaires asking for diarrhea, cough, flu, bloody or mucus-containing stool, and dyspnea and recorded any other illness. Also feeding history and compliance were assessed weekly. At baseline 71% of the infants were anemic and 22% iron deficient, prevalence of inflammation was high (31% had an elevated CRP). Over the 1-year, hemoglobin increased and serum ferritin decreased in both groups, without significant treatment effects of the iron fortification. At endpoint, the weight of infants consuming MNP+Fe was greater than in the MNP-Fe group (9.9 vs. 9.5kg, P=0.038). There were no significant differences in the weekly measures of morbidity. In this study, low dose iron-containing MNP did not improve infant’s iron status or reduce anemia prevalence, likely because absorption was inadequate due to the high prevalence of infections and the low iron dose.
Introduction

Iron deficiency (ID) is the most common micronutrient deficiency worldwide, particularly in infants (Stoltzfus 1998). Iron deficiency anemia (IDA) can lead to poor cognitive and motor development (Stoltzfus 1998; Sachdev et al. 2005). Infants’ iron stores at birth are generally sufficient to cover their needs over the first 4-6 months, but during the subsequent weaning period complementary foods rich in iron are needed to meet the increased requirements for growth (Zimmermann and Hurrell 2007). Thus, iron fortification of complementary foods is recommended to prevent ID during infancy and early childhood (Fontaine 2007; Dube et al. 2010).

Untargeted iron supplementation at high doses (0.8-1.2 mg Fe/kg body weight) increased morbidity in preschool children in Tanzania (Sazawal et al. 2006). An earlier systematic review of randomized controlled trials suggested iron supplementation may increase risk of diarrhea (Gera and Sachdev 2002) and some experts suggest food fortification with low doses of iron as the safest iron intervention (Oppenheimer 2001; Gera and Sachdev 2002). Micronutrient powders for in-home fortification containing high doses of iron are efficacious in improving iron status in infants and school children (Zlotkin et al. 2003; Adu-Afarwuah et al. 2008). However, their safety in malarial areas has been recently questioned. A large intervention study in Pakistani infants reported that an MNPs containing high doses of iron (12.5 mg/d) reduced anemia and iron deficiency, but increased diarrhea and respiratory morbidities (Soofi et al. 2013). The same dose of iron has been reported to increase hospitalization, possibly due to diarrhea, in Ghanaian infants (Zlotkin et al. 2013).

Iron from NaFeEDTA is highly bioavailable, even in phytate-rich whole flours often used in complementary porridges, and may allow the use of lower, safer iron dosages for infants and children (Andang'o et al. 2007; Troesch et al. 2009). However, its efficacy in complementary foods has not yet been assessed in infants.

Iron is essential for growth, but meta-analyses have shown limited or no effect of iron intervention and/or MNPs on child growth (Ramakrishnan et al. 2004; Sachdev et al. 2006). Furthermore, studies in breast-fed and iron-replete infants have reported negative effects of iron on growth (Dewey et al. 2002) and on weight-for-age z-scores (WAZ) (Lind et al. 2008). The intervention in Pakistani infants reported a small positive effect of high dose iron fortification (12.5 mg/d) on linear growth (Soofi et al. 2013).
We aimed to assess the efficacy of a low-iron MNP (containing 2.5 mg iron as NaFeEDTA) in 6 month old Kenyan infants. Co-primary outcomes were anemia, iron status and growth. A secondary outcome was the safety of the low-iron MNP based on recording weekly morbidity.

**Subjects and Methods**

**Study site**

The study was conducted between February 2010 and August 2012 in the capture area of the Kikoneni health center (Msambweni County), in southern coastal Kenya. Since this rural area is sparsely populated, recruitment was done continuously and completed by August 2011. The intervention period included three long rainy seasons from April to July (2010-2012) and two short rainy seasons from October to November (2010 and 2011). The main economic activity in the area is subsistence farming with maize as the staple food crop. The typical local weaning food is a liquid maize porridge ‘uji’; a regular portion consists of about 8-10 g maize flour boiled in 100-150 mL water and sweetened with sugar.

**Study design and participants**

This efficacy study was embedded in a randomized, double-blind, controlled trial of a low dose iron-containing MNP, with cognitive and motor development as primary outcome (Kvalsvig unpublished). Inclusion criteria were: (a) age of 6 months (±3 weeks); (b) hemoglobin ≥ 70 g/L; and (c) no acute or chronic illness. The study was explained by the study nurse to one of the caretakers and written informed consent was obtained. Random assignment was performed using a computer-generated list and eligible subjects were allocated to one of the four color-labeled groups (two containing MNP+Fe and two containing MNP-Fe). After a baseline assessment, 379 eligible infants were enrolled into the cognition study. For this efficacy trial, the primary outcome was hemoglobin. We estimated that 80 infants in each group were required to achieve a statistical power of 95% at an alpha level of 0.05, and to detect difference in hemoglobin concentration of 4 g/L between groups, assuming a standard deviation of 11 g/L.
The intervention was a daily consumed maize porridge fortified with the allocated MNP. The mothers were given standard instructions on how to prepare and fortify the porridge. The micronutrient powder (MixMe, DSM Nutritional Products Europe Ltd, Basel, Switzerland) was packed in four different colored sachets, two for each treatment group (MNP with 2.5 mg NaFeEDTA (MNP+Fe) and MNP without iron (MNP-Fe)). The composition of the MNPs is shown in Table 1. Before the intervention started, we tested the acceptability of the MNPs and potential sensory changes, using a triangle test (Meilgaard et al. 2007). The MNPs (MNP+Fe and MNP-Fe) were indistinguishable and acceptability was high among the local community (data not shown). Samples of the maize flour were analyzed in triplicate at the Laboratory of Human Nutrition at the ETH Zurich for their iron and phytate content; these were 1.15±0.06 mg and 310±20 mg per 100 g, respectively.

Weekly, the mothers collected 2-3 kg maize flour (Dola, Kitui Flour Mills Ltd, Mombasa, Kenya) and seven micronutrient sachets at the nearest of six distribution points. Compliance was assessed by collecting and recording empty and full MNP sachets from the previous week. Feeding history and the child’s overall health status were assessed using a weekly recall covering the previous seven days. If the mother reported any current illness, the child was examined by the study nurse at the health center of Kikoneni or referred to the district hospital in Msambweni. In addition, the study nurse probed for prior and current episodes of malaria, diarrhea and respiratory tract infections at baseline, after 6 and 12 months. Infant weight was measured to the nearest 100 g using a hanging scale (Salter 235-6S, 25 kg x 100 g; Salter Brecknell, UK) and recumbent length to the nearest 0.1 cm using a measurement board (Shorr Production, LLC., Olney, MD).

Table 1 Composition of micronutrient powder used in the intervention study.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Amount per 1g sachet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>100 µg</td>
</tr>
<tr>
<td>Iron</td>
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</tr>
<tr>
<td>Vitamin D</td>
<td>5 µg</td>
</tr>
<tr>
<td>Copper</td>
<td>0.34 mg</td>
</tr>
<tr>
<td>Tocopherol Equivalent</td>
<td>5 mg</td>
</tr>
<tr>
<td>Iodine</td>
<td>30 µg</td>
</tr>
<tr>
<td>Vitamin K1</td>
<td>30 µg</td>
</tr>
<tr>
<td>Selenium</td>
<td>17 µg</td>
</tr>
<tr>
<td>Thiamine</td>
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</tr>
<tr>
<td>Zinc</td>
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</tr>
<tr>
<td>Riboflavin</td>
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</tr>
<tr>
<td>Pyridoxine</td>
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</tr>
<tr>
<td>Folic Acid Anhydrous</td>
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</tr>
<tr>
<td>Niacinamide</td>
<td>6 mg</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>0.9 µg</td>
</tr>
<tr>
<td>Vitamin C</td>
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</tr>
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</table>
There was a labeling error from the first to the second production batch of the MNP, where two of the four colors (1 MNP+Fe and 1 MNP-Fe) were interchanged. This was only discovered during unblinding after the study was completed. Data from all subjects affected by this labeling error were excluded (MNP+Fe: n=45; MNP-Fe: n=46) and 287 infants were included in the final analysis (Figure 1, trial profile).

![Trial profile](image)

**Figure 1** Trial profile and group allocation illustrating permanent and temporary losses in the two trial arms over the 12 months intervention. MNP+Fe: Micronutrient powder with iron, MNP-Fe: Micronutrient powder without iron. Others: missed drugs, official withdrawal, 1 death in MNP+Fe before midpoint that was not associated with the treatment (included in 'others').

**Biochemical indicators**

At baseline, after 6, and 12 months venous blood samples (3 mL) were drawn by the study nurse using heparin vacutainers and butterfly needles. Hemoglobin (Hb) was measured using a HemoCue (HemoCue AB, Ängelholm, Sweden) or a HemoControl device (EKF diagnostics Sales GmbH, Barleben/ Magdeburg, Germany). Serum was separated by centrifugation on collection day. The remaining erythrocytes were washed 3 times with normal saline, and the zinc protoporphyrin to heme ratio (ZPP) was
measured using a calibrated AVIV hematofluorometer (AVIV Biomedical, Lakewood, USA). Serum ferritin (SF), soluble transferrin receptor (sTfR) and C-reactive protein (CRP) were measured at Lancet Laboratories in Nairobi by using the Cobas Integra (Roche, Basel, Switzerland). We converted the Roche sTfR concentration to the Flowers assay (Flowers et al. 1989) using the regression equation by Pfeiffer (Flowers=1.5 * Roche +0.35) (Pfeiffer et al. 2007).

Body iron stores (mg/kg body weight) were calculated from the ratio of sTfR to SF according to the equation by Cook et al. (body iron (mg/kg)=[log10 (sTfR * 1000/SF) - 2.8229] /0.1207) (Cook et al. 2003), and total body iron stores (mg) by multiplying with the body weight. The following cut-offs were used: (a) anemia: Hb <110 g/L (WHO 2001), (b) ID: body iron stores <0 mg/kg; and (c) inflammation: CRP ≥4.1 mg/L (manufacturer’s reference range).

Ethics
This study was approved by the ethics and research committees of the Kenyatta National Hospital/ University of Nairobi (P167/6/2009), the University of KwaZulu-Natal (BF121/08), and the ETH Zurich (EK 2009-N-53). Caregivers signed an informed consent. This study is registered at clinicaltrials.gov as NCT01111864.

Statistical analysis
Data were analyzed using IBM SPSS Statistics 20.0.0 (SPSS Inc., Chicago, IL) and Microsoft Office EXCEL 2010 (Microsoft, Redmond, WA). Data were double entered and its distribution checked for normality (Kolmogorov-Smirnov test); not normally distributed data were log transformed. We obtained geometric means and corresponding standard deviations (SD) or standard errors of the mean (SEM) for absolute concentrations by taking the antilog of these values. Weight-for-age (WAZ), height-for-age (HAZ) and weight-for-height (WHZ) were calculated by using the WHO Anthro software (version 3.2.2) and standards. The definition for stunting was a HAZ < -2, for underweight WAZ < -2, and for wasting a WHZ < -2 (WHO 2007).

Baseline, mid-, and endpoint differences between the treatment groups were assessed using independent samples t-test for continuous variables and Pearson’s chi-square test for ID and IDA prevalence. Estimated intervention effects were assessed using general linear models (GLM), baseline values were used as covariates and P values <0.05 was considered significant. A subgroup analysis according to baseline iron stores was
done to assess the intervention effects in iron deficient (body iron stores <0) and iron replete (body iron stores ≥0) infants separately.

The effect of the treatment on the total days with diarrhea, cough, flu, bloody or mucus-containing stool, and dyspnea were compared using Mann-Whitney U test. The effect of the treatment on diarrhea and malaria incidence was assessed using Pearson’s chi-square test.

**Results**

**Study participants**

At baseline, the overall prevalence of anemia was 70.7%; 21.8% of infants were iron deficient and 31.0% had an elevated CRP (**Table 2**). The prevalence of underweight was 7.8%, while 13.5% of infants were stunted. Mothers indicated that 62.1% of the infants were born at home and 26.2% in hospital; 9 infants were born preterm (3.2%) and 4 were delivered by caesarian section (1.4%). Nearly all infants were still being breastfed (98.2%, n=277) but 90.1% (n=254) had already been introduced to complementary foods, predominantly sweetened maize gruel (‘ujii’), starting on average at four months of age.

Although randomization was checked by comparing baseline hemoglobin, weight and length, which did not differ between groups, there were significant differences in sTfR (P=0.004), ZPP (P=0.005, **Table 2**), body iron stores (P=0.004), and the prevalence of ID (P=0.0003) and IDA (P=0.002), between the two treatment groups at baseline.

In total, 170 (MNP+Fe=88, MNP-Fe=82) children completed the intervention (Figure 1). The main reasons for discontinuing the study were refusal and relocation. The overall compliance to the MNP of all participants in the study was 99%. The most frequent reason for a child not to consume the sachet was absence/ travel or sickness.
Table 2 Baseline characteristics by intervention group of per protocol analysis

<table>
<thead>
<tr>
<th></th>
<th>MNP+Fe</th>
<th>MNP-Fe</th>
<th>Both</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>144</td>
<td>143</td>
<td>287</td>
</tr>
<tr>
<td>Age (months)</td>
<td>6.0 ± 1.1</td>
<td>6.0 ± 1.1</td>
<td>6.0 ± 1.1</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>74 (51.4)</td>
<td>72 (50.3%)</td>
<td>146 (50.9%)</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>104.1 ±10.5</td>
<td>102.1 ±10.6</td>
<td>103.0 ±10.5</td>
</tr>
<tr>
<td>Zinc protoporphyrin (µmol/mol heme)</td>
<td>85.1 ±1.6*</td>
<td>103.8 ±1.8</td>
<td>94.1 ±1.7</td>
</tr>
<tr>
<td>Serum ferritin (ng/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All children</td>
<td>34.1 ±1.8</td>
<td>27.8 ±2.0</td>
<td>30.9 ±1.9</td>
</tr>
<tr>
<td>Children without inflammation’</td>
<td>30.5 ±1.7</td>
<td>25.2 ±2.1</td>
<td>27.8 ±1.9</td>
</tr>
<tr>
<td>Soluble transferrin receptor (mg/L)</td>
<td>8.6 ±1.2*</td>
<td>9.6 ±1.2</td>
<td>9.1 ±1.2</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>4.9 ±1.7</td>
<td>3.7 ±1.4</td>
<td>4.3 ±1.6</td>
</tr>
<tr>
<td>Inflammation’</td>
<td>45 (31.2%)</td>
<td>44 (30.8%)</td>
<td>89 (31.0%)</td>
</tr>
<tr>
<td>Anemia’</td>
<td>99 (68.8%)</td>
<td>104 (72.7%)</td>
<td>203 (70.7%)</td>
</tr>
<tr>
<td>Iron deficiency (ID)’</td>
<td>18 (12.8)**</td>
<td>43 (30.9%)</td>
<td>61 (21.8%)</td>
</tr>
<tr>
<td>Iron deficiency anemia (IDA)’</td>
<td>15 (10.6%)**</td>
<td>38 (27.3%)</td>
<td>53 (18.9%)</td>
</tr>
<tr>
<td>Weight baseline (kg)</td>
<td>7.3 ±1.1</td>
<td>7.3 ±1.1</td>
<td>7.3 ±1.1</td>
</tr>
<tr>
<td>Length baseline (cm)</td>
<td>64.8 ±1.0</td>
<td>64.1 ±1.1</td>
<td>64.4 ±1.1</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>3.2 ±1.0</td>
<td>3.1 ±1.0</td>
<td>3.2 ±1.0</td>
</tr>
<tr>
<td>Body iron stores (mg/kg)</td>
<td>3.26 ±1.0*</td>
<td>1.86 ±1.0</td>
<td>2.57 ±1.0</td>
</tr>
</tbody>
</table>

Data are geometric means ±SD or number (%) unless indicated otherwise. 1CRP ≥ 4.1 mg/L; 2Hb <110 g/L; 3body iron stores (mg/kg) < 0; 4Concurrent ID and anemia; *different from control (MNP-Fe), P <0.05 using t-test; **different from control (MNP-Fe), P <0.05 using Pearson chi-square test; MNP+Fe: Micronutrient powder with iron, MNP-Fe: Micronutrient powder without iron.

Iron status and inflammation

There was no effect of iron on any of the iron status or inflammation markers adjusted for baseline differences, except for a decrease of SF (P=0.0001) and CRP (P=0.012) in the MNP+Fe group at midpoint (Table 3). Anemia prevalence decreased during the intervention in both treatment groups (MNP+Fe and MNP-Fe), from 68.8% and 72.7% at baseline, to 67.4% and 61.9% after 6 months, and 55.8% and 41.4% after 12 months, respectively. The prevalence of IDA (negative body iron stores and Hb <110 g/L) differed significantly at baseline 10.4% in the MNP+Fe and 26.6% in the MNP-Fe (P=0.001); and was not different at midpoint 32.1% and 18.5% (P=0.130), and differed again at endpoint...
32.9% and 12.9% (P=0.035), in MNP+Fe and MNP-Fe respectively, using Pearson’s chi-square tests.

In the gender-specific analysis, boys iron status and inflammation did not differ between groups (SF, P=0.068 and CRP, P=0.089), while girls of the MNP+Fe had lower SF along with a tendency for lower CRP compared to those in the MNP-Fe group (SF, P=0.0001 and CRP, P=0.068). Furthermore, we identified in girls an effect of the iron on ZPP (midpoint: P= 0.033, endpoint: P=0.022), sTfR (midpoint: P= 0.002, endpoint: P=0.048), and Hb (midpoint: P= 0.001, endpoint: P=0.003, Table 3).

**Table 3** Changes in iron status and inflammation and weight during the 12 months intervention.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Time point (month)</th>
<th>Hb (g/L)</th>
<th>ZPP (µmol/mol heme)</th>
<th>sTfR (mg/L)</th>
<th>SF (µg/L)</th>
<th>CRP (mg/L)</th>
<th>Weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>baseline (0)</td>
<td>104.0 ±10.5</td>
<td>92.7 ±1.6**</td>
<td>9.0 ±1.2**</td>
<td>29.8 ±1.8</td>
<td>45 ±1.8</td>
<td>7.6 ±1.1</td>
</tr>
<tr>
<td>(n=74)</td>
<td>midpoint (6)</td>
<td>106.0 ±10.5</td>
<td>106.3 ±1.7</td>
<td>10.0 ±1.1</td>
<td>18.8 ±1.6</td>
<td>2.4 ±1.3</td>
<td>9.0 ±1.1</td>
</tr>
<tr>
<td></td>
<td>endpoint (12)</td>
<td>108.9 ±10.5</td>
<td>81.2 ±1.8</td>
<td>8.9 ±1.2</td>
<td>23.0 ±1.9</td>
<td>2.9 ±1.6</td>
<td>10.0 ±1.1</td>
</tr>
<tr>
<td>Female</td>
<td>baseline (0)</td>
<td>104.2 ±10.5</td>
<td>77.8 ±1.5</td>
<td>8.3 ±1.1</td>
<td>39.2 ±1.8</td>
<td>5.3 ±1.7</td>
<td>7.1 ±1.1</td>
</tr>
<tr>
<td>(n=70)</td>
<td>midpoint (6)</td>
<td>103.0 ±10.5</td>
<td>97.7 ±1.7*</td>
<td>10.1 ±1.3**</td>
<td>16.1 ±1.5*</td>
<td>3.4 ±1.7</td>
<td>8.8 ±1.1</td>
</tr>
<tr>
<td></td>
<td>endpoint (12)</td>
<td>104.8 ±10.8</td>
<td>90.0 ±1.6**</td>
<td>9.1 ±1.2*</td>
<td>16.1 ±1.5**</td>
<td>2.9 ±1.4</td>
<td>9.9 ±1.1</td>
</tr>
<tr>
<td>Both</td>
<td>baseline (0)</td>
<td>104.1 ±10.5</td>
<td>85.1 ±1.6**</td>
<td>8.6 ±1.2**</td>
<td>34.1 ±1.8</td>
<td>4.9 ±1.7</td>
<td>7.3 ±1.1</td>
</tr>
<tr>
<td>(n=144)</td>
<td>midpoint (6)</td>
<td>104.6 ±10.5</td>
<td>102.2 ±1.7</td>
<td>10.0 ±1.2</td>
<td>17.5 ±1.6**</td>
<td>2.9 ±1.5*</td>
<td>8.9 ±1.1</td>
</tr>
<tr>
<td></td>
<td>endpoint (12)</td>
<td>107.1 ±10.6</td>
<td>85.0 ±1.7</td>
<td>9.0 ±1.2</td>
<td>19.7 ±1.7</td>
<td>2.9 ±1.5</td>
<td>9.9 ±1.1**</td>
</tr>
<tr>
<td>Male</td>
<td>baseline (0)</td>
<td>101.2 ±10.6</td>
<td>118.7 ±1.8</td>
<td>10.6 ±1.2</td>
<td>22.3 ±1.8</td>
<td>3.9 ±1.5</td>
<td>7.7 ±1.1</td>
</tr>
<tr>
<td>(n=72)</td>
<td>midpoint (6)</td>
<td>103.3 ±10.6</td>
<td>120.1 ±1.7</td>
<td>10.4 ±1.2</td>
<td>21.9 ±1.7</td>
<td>3.5 ±1.4</td>
<td>9.3 ±1.1</td>
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<tr>
<td></td>
<td>endpoint (12)</td>
<td>105.7 ±10.8</td>
<td>88.2 ±1.8</td>
<td>10.5 ±1.3</td>
<td>21.2 ±1.7</td>
<td>2.3 ±1.3</td>
<td>9.6 ±1.1</td>
</tr>
<tr>
<td>Female</td>
<td>baseline (0)</td>
<td>102.9 ±10.6</td>
<td>90.5 ±1.8</td>
<td>8.7 ±1.2</td>
<td>34.8 ±2.2</td>
<td>3.6 ±1.4</td>
<td>7.0 ±1.1</td>
</tr>
<tr>
<td>(n=71)</td>
<td>midpoint (6)</td>
<td>109.6 ±10.5</td>
<td>86.3 ±1.7</td>
<td>8.2 ±1.1</td>
<td>26.7 ±1.6</td>
<td>5.4 ±1.7</td>
<td>8.5 ±1.1</td>
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<tr>
<td></td>
<td>endpoint (12)</td>
<td>113.8 ±10.5</td>
<td>96.8 ±1.6</td>
<td>8.2 ±1.1</td>
<td>26.1 ±1.5</td>
<td>2.8 ±1.4</td>
<td>9.3 ±1.1</td>
</tr>
<tr>
<td>Both</td>
<td>baseline (0)</td>
<td>102.1 ±10.6</td>
<td>103.8 ±1.8</td>
<td>9.6 ±1.2</td>
<td>27.8 ±2.0</td>
<td>3.7 ±1.4</td>
<td>7.3 ±1.1</td>
</tr>
<tr>
<td>(n=143)</td>
<td>midpoint (6)</td>
<td>106.8 ±10.6</td>
<td>99.9 ±1.7</td>
<td>9.1 ±1.2</td>
<td>24.5 ±1.6</td>
<td>4.5 ±1.5</td>
<td>8.8 ±1.1</td>
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<td></td>
<td>endpoint (12)</td>
<td>110.3 ±10.7</td>
<td>77.2 ±1.7</td>
<td>9.2 ±1.2</td>
<td>24.0 ±1.6</td>
<td>2.6 ±1.3</td>
<td>9.5 ±1.1</td>
</tr>
</tbody>
</table>

Data are geometric means ± SD; *different from control (MNP-Fe), P <0.05, using univariate GLM with baseline variable as covariate; **different from control (MNP-Fe), P <0.05 using t-test; MNP+Fe: Micronutrient powder with iron, MNP-Fe: Micronutrient powder without iron.
Baseline body iron stores as a subgroup analysis criterion

Infants with negative iron stores at baseline increased them (Figure 2 A) irrespective of the treatment; whereas infants with positive iron stores at baseline appeared to stabilize them at about 1-1.6 mg Fe/kg body weight (Figure 2 B). Figure 2 C shows the changes in body iron stores in all infants.

Figure 2 Body iron stores ±SD (mg/kg, geometric means) over the 12 months intervention in (A) in infants with a negative iron stores at baseline (MNP+Fe (n=18) and MNP-Fe (n=43); (B) in infants with a positive iron store at baseline (MNP+Fe (n=123) and MNP-Fe (n=96); and (C) all infants (MNP+Fe (n=141) and MNP-Fe (n=139). The differences between the groups from baseline to midpoint was significant in the graphs B (P=0.000034) and C (P=0.0001) using GLM, but not from baseline to endpoint. MNP+Fe: Micronutrient powder with iron, MNP-Fe: Micronutrient powder without iron.
Growth

There was no significant effect of iron on weight using general linear model statistics (p=0.185). However, after the 1-year intervention infants in the MNP+Fe group were significantly heavier (9.9 kg vs. 9.5 kg P=0.038, t-test, Table 3). The gender-specific analysis showed: girls in the MNP+Fe were borderline significant heavier than in the MNP-Fe (9.9 kg vs. 9.3 kg, P=0.067), but not boys (10.0 kg vs. 9.6 kg, P=0.324). The prevalence of underweight did not differ between groups and was 9.2% and 6.4% at baseline, 6.2% and 7.8% at midpoint, and 14.9% and 21.2% at endpoint in the MNP+Fe and MNP-Fe, respectively. Also, changes in WHZ and HAZ were not different between the two groups. The height did not differ between the treatment groups.

Morbidity

During the 1 year intervention, the days spent with diarrhea, cough, flu, bloody or mucus-containing stool, dyspnea and any other illness did not differ between the two treatment groups. The mothers reported on average 2 days with diarrhea, 7 days cough, 8 days flu, 1 day bloody or mucus-containing stool, and 2 days dyspnea. Further, the incidence of treated malaria and diarrhea did also not differ between the treatment groups. The incidence of treated malaria from baseline to midpoint was 5.4% and 7.0% in the MNP+Fe and the MNP-Fe respectively, and increased from midpoint to endpoint to 13.8% (MNP+Fe) and 8.9% (MNP-Fe). The incidence of diarrhea from baseline to midpoint was 13.5% and 14.1% in the MNP+Fe and the MNP-Fe respectively and 12.5% (MNP+Fe) and 15.2% (MNP-Fe) from midpoint to endpoint.

Discussion

In this study, in-home fortification of maize porridge with micronutrients and 2.5 mg iron as NaFeEDTA daily, for one year, did not improve infant’s iron status or reduce their anemia prevalence compared to fortification with micronutrients only. Previous fortification studies using higher iron doses (11-12.5 mg/d) as ferrous fumarate have reduced ID in infants in Sub-Saharan Africa (Faber et al. 2005; Adu-Afarwuah et al. 2008). In older children (1-5 years of age), 2.5 mg NaFeEDTA daily for 4 months, reduced prevalence of iron deficiency and anemia (Macharia-Mutie et al. 2012). There are several possible explanations why the lower dose of iron used in this study did not improve infants’ iron status.
First, although iron bioavailability from NaFeEDTA in inhibitory meals is high, the iron dose may have been too low. The phytic acid to iron molar ratio of the plain porridge was 22.4:1; fortification reduced this ratio to 0.9:1. Former absorption studies using similar meals in adults estimated an absorption of NaFeEDTA of 4.4% (Troesch et al. 2009) and 5.2% (Hurrell et al. 2000), for a SF of 40 and 39 ng/mL; extrapolating this to our mean SF of 31 ng/mL at baseline (Cook et al. 1991), we anticipated an absorption of ≈5-6%. This means 0.14-0.16 mg of the provided iron were expected to be absorbed, covering ≈20% and ≈30% of the daily requirement of 6-12 (0.72 mg) and 12-18 (0.46 mg) month old children, respectively (WHO 2001). Recently, in a study by Macharia-Mutie et al. the same MNP (2.5 mg iron as NaFeEDTA) daily for 4 months reduced IDA in Kenyan 1-5 year old children (Macharia-Mutie et al. 2012). These contradicting results may be due to the overall higher food intake in older children, which, combined with low dose fortification, may allow to reach sufficient bioavailable iron intake at similar estimated daily requirements (WHO 2001).

The regulations of JECFA (Joint FAO/WHO Expert Committee on Food Additives) limit the use of EDTA to 1.9 mg per kg bodyweight. The rationale for this restriction is the estimated absorption of 5% of the EDTA which can negatively affect the metabolism of minerals by increased urinary excretion (Candela et al. 1984; Hurrell et al. 1994). Therefore, in our study population with an average baseline bodyweight of 7.3 kg we could not add more than 2.5 mg iron as NaFeEDTA (containing 13 mg EDTA). In young Beninese children iron absorption from a lipid-based complementary food mixed with millet porridge and fortified with a mixture of NaFeEDTA and FeSO4 was significantly lower than from meals fortified with FeSO4 only (Cercamondi et al. 2013). The authors explained this rather surprising outcome by the possibility that the ascorbic acid provided enhanced absorption of FeSO4 but not NaFeEDTA. Furthermore, EDTA has been shown to reduce the absorption of native iron in the diet (Cook and Monsen 1976). However, since the complementary food in this study did not provide a considerable amount of iron this effect may be negligible.

Second, inflammation is a major determinant for hepcidin expression and therefore iron absorption (Nemeth and Ganz 2009). In our study population, malaria and other infectious diseases were common and one third of the infants had an elevated CRP at baseline. In contrast to this, in Macharia-Mutie’s study, the prevalence of inflammation was ≈10 times lower in the iron group (3.4%) and significantly elevated in
the control group (16.5%) at baseline (Macharia-Mutie et al. 2012). A cross-sectional analysis of our infant population indicated that inflammation increases hepcidin concentration even in ID subjects (Jaeggi et al. 2013). However, a subgroup analysis in infants without elevated CRP did not show an effect of iron treatment on iron status (data not shown).

Third, despite randomization and equivalent Hb and anthropometrics at baseline, prevalence of ID and IDA were lower in the iron fortified group than in the control group. Although we attempted to correct for these effects using baseline values as a covariate, these may have resulted in smaller treatment effects, as iron status is a major determinant of dietary iron absorption.

A recent study in Pakistani infants reported a small but significant positive effect of iron-containing MNP on length gain (Soofi et al. 2013). Former meta-analyses have shown an effect of micronutrients (Ramakrishnan et al. 2004), but not of iron alone on child growth (Ramakrishnan et al. 2004; Sachdev et al. 2006). However, in one of these meta-analyses, a trend was suggested for an increase in weight through iron supplementation in children from malarial endemic areas (Sachdev et al. 2006). In a study in South African school children, maize porridge fortified with micronutrients including phytase and 2.5 mg NaFeEDTA, daily for 5 months, showed a small treatment effect on WAZ scores along with an increase in body iron stores compared to unfortified porridge (Troesch et al. 2011). In the current study infants consuming iron-fortified maize porridge weighed significantly more at the end of the one year intervention (0.4 kg). There are several potential explanations for this higher weight in the MNP+Fe group at endpoint. Differences in infant feeding practices between the two groups could have had an effect; however, the trial was randomized and we have no evidence that there were feeding differences between groups. Another possibility might be that EDTA from the NaFeEDTA increased the bioavailability of the zinc from the MNP (as zinc oxide) and/or from the maize (Hettiarachchi et al. 2004), improving zinc status, and contributing to increased weight gain (Brown et al. 2009). However, we did not measure serum zinc concentration in this study. Furthermore, iron has shown to increase the population of short-chain fatty acids (SCFA) producing bacteria in rats (Dostal et al. 2012). And these SCFAs can be a significant energy source for the host, contributing up to 10% of the daily caloric requirements of humans (McNeil 1984).
We conclude that in-home fortification with a MNP containing 2.5 mg iron as NaFeEDTA did not improve iron status in infants in an area with high burden of infectious disease, likely because of the combination of low dose and high prevalence of inflammation. However, infants consuming maize porridge fortified with MNP+Fe had a higher weight after the one year trial compared to infants consuming maize porridge fortified with MNP-Fe. Future large-scale studies are needed to confirm these results in infants from rural African settings.

**Key Messages**

- In-home fortification of complementary feeding in Kenyan infants was well accepted and compliance was high.

- Low dose iron MNP did not improve iron status or hemoglobin in this rural African infant population with high rates of infection and inflammation.

- Morbidity was not increased in infants receiving the iron-fortified porridge.

- After the one year intervention, infants consuming iron-fortified maize porridge had greater weight gain compared to controls.

- Future large-scale studies are needed to evaluate safe and effective iron fortification strategies in infants.

**Source of funding**

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Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants

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Gut; in press
Abstract

Background In-home iron fortification for infants in developing countries is recommended for control of anemia but low absorption typically results in >80% of the iron passing into the colon. Iron is essential for growth and virulence of many pathogenic enterobacteria. We determined the effect of high and low dose in-home iron fortification on the infant gut microbiome and intestinal inflammation.

Methods We performed two double-blind randomized controlled trials in 6-month-old Kenyan infants (n=115) consuming home-fortified maize porridge daily for 4 months. In the first, infants received a micronutrient powder (MNP) containing 2.5 mg iron as NaFeEDTA or the MNP without iron. In the second, they received a different MNP containing 12.5 mg iron as ferrous fumarate or control. The primary outcome was gut microbiome composition analyzed by 16S pyrosequencing and targeted real-time PCR (qPCR). Secondary outcomes included fecal calprotectin (marker of intestinal inflammation) and incidence of diarrhea. We analyzed the trials separately and combined. The study is registered with ClinicalTrials.gov, number NCT01111864.

Results At baseline, 63% of the total microbial 16S rRNA could be assigned to Bifidobacteriaceae but there were high prevalences of pathogens, including Salmonella, Clostridium difficile, Clostridium perfringens, and pathogenic Escherichia coli. Using pyrosequencing, +FeMNPs increased enterobacteria, particularly Escherichia/Shigella (p=0.048), the enterobacteria/bifidobacteria ratio (p=0.020), and Clostridium (p=0.030). Most of these effects were confirmed using qPCR; for example, +FeMNPs increased pathogenic E. coli strains (p=0.029). +FeMNPs also increased fecal calprotectin (p=0.002). During the trial, 27.3% of infants in +12.5mgFeMNP required treatment for diarrhea versus 8.3% in -12.5mgFeMNP (p=0.092). There were no study-related serious adverse events in either group.

Conclusions In this setting, provision of iron-containing MNPs to weaning infants adversely affects the gut microbiome, increasing pathogen abundance and causing intestinal inflammation.
Significance of this study

What is already known on this subject?

- The composition of the infant gut microbiota may vary depending on dietary iron supply, but most data are from culture methods, or have come from animal experiments.
- In-home iron fortification for infants in developing countries is recommended to control anemia, but low absorption typically results in >80% of the iron passing into the colon.
- Two recent iron fortification trials in infants in developing countries have raised safety concerns: in Ghana, there was an increased rate of hospitalizations possibly due to diarrhea, and in Pakistan, a small but significant increase in overall diarrhea prevalence.
- There is little known about the composition of the African infant gut microbiota during the weaning period or the effects of iron fortification at this age.

What are the new findings?

- This is the first controlled intervention trial to examine the effect of iron fortification on the African infant gut microbiome
- Iron fortification modifies the gut microbiome in weaning African infants, increasing enterobacteria and decreasing bifidobacteria, and increases abundances of specific enteropathogens, for example pathogenic *Escherichia coli*.
- Iron fortification in weaning African infants increases fecal calprotectin levels, indicating intestinal inflammation.
- These data provide a probable mechanism for the increases in diarrhea seen in recent infant fortification studies.

How might it impact on clinical practice in the foreseeable future?

Our findings suggest that, until safer formulations are available, iron fortification should not be given to all infants, but should be targeted only to infants with clear iron deficiency anemia, while providing adequate protection from diarrhea.
**Introduction**

While infants have the highest rates of iron deficiency anemia (IDA), they are also the group less well covered by universal fortification programs. Micronutrient powders (MNPs), added directly to complementary foods after cooking (in-home fortification), can be an effective approach for providing additional dietary iron and reducing anemia rates (Adu-Afarwuah et al. 2008). Currently, in-home fortification programs are in place or planned in 36 countries including 10 in Sub-Saharan Africa (UNICEF-CDC 2013).

However, the safety of iron-containing MNPs (+FeMNPs) is uncertain. A 2007 WHO consultation, on the results of the Pemba trial where iron and folic acid supplementation increased child mortality (Sazawal et al. 2006), did not recommend the use of +FeMNPs in malaria-endemic areas because of concerns about potential increases in infection (Fontaine 2007). Two recent fortification trials in infants with an MNP containing 12.5 mg ferrous fumarate have raised safety concerns: in Ghana, there was an increased rate of hospitalizations possibly due to diarrhea (Zlotkin et al. 2013), and in Pakistan a small but significant increase in overall diarrhea prevalence, bloody diarrhea, and respiratory illness (Soofi et al. 2013). An earlier systematic review concluded iron supplementation, but not fortification, may increase risk for diarrhea (Gera and Sachdev 2002). If +FeMNPs increase risk for infection and diarrheal disease, this would be an important adverse effect, as diarrhea contributes to the death of ≈1 in 9 under 5-year-old children in Sub-Saharan Africa (WHO 2013).

The food matrix of most cereal-based and legume-based complementary foods is rich in phytic acid, a potent inhibitor of iron absorption; therefore, usually less than 20% of iron added to these foods is absorbed (Tondeur et al. 2004, Zimmermann and Hurrell 2007). In rural African populations with high levels of inflammation and infection, absorption is likely to be even lower, as inflammation increases circulating hepcidin the major iron regulator, which reduces dietary iron absorption through binding and degradation of the iron efflux protein, ferroportin, at the basolateral membrane of the enterocytes (Nemeth et al. 2004). Thus, providing +FeMNPs to individuals with concurrent inflammation results in most of the iron passing unabsorbed into the colon. Once absorbed, iron in the body is bound to proteins limiting iron access to potential pathogens, and during infection, iron supply is further reduced in the extracellular compartment and remains in macrophages and enterocytes (Cassat and Skaar 2013). There is no comparable system for sequestration of dietary iron known in the gut.
lumen, although neutral pH and the presence of defense molecules, such as lipocalin 2 (Raffatellu et al. 2009), may reduce iron solubility and availability to gut microbes.

Iron is an essential, growth-limiting nutrient for many gut bacteria, competing for unabsorbed dietary iron (Andrews et al. 2003). For most enteric gram-negative bacteria (eg Salmonella, Shigella or pathogenic Escherichia coli), iron acquisition plays an essential role in virulence and colonization (Naikare et al. 2006). By contrast, lactobacilli, a major group of beneficial ‘barrier’ bacteria improving gut integrity and reducing colonization by enteric pathogens (Anderson et al. 2010), do not require iron, but instead rely on manganese (Weinberg 1997). Therefore, an increase in unabsorbed dietary iron through fortification or supplementation could modify the colonic microbiota equilibrium and favor growth of pathogenic strains over ‘barrier’ strains.

Colonization of the human gastrointestinal tract begins at birth and depends on the mode of delivery, hygiene and prematurity (Penders et al. 2006). The iron-binding protein, lactoferrin, in breast milk limits iron availability to the gut microbiota (Yen et al. 2011), and may have a protective effect in breastfed infants. The relatively simple gut microbiota of breast-fed infants further diversifies with the introduction of complementary feeding (Vael and Desager 2009). In a study comparing the gut microbiome among infants from the USA, Venezuela and Malawi, there were significant differences between sites, but a common pattern was the dominance of bifidobacteria throughout the first year of life, thereafter, bifidobacteria diminish steadily leading to the establishment of an adult-like gut microbiome at about 3 years of age (Yatsunenko 2012).

In a controlled trial of iron fortification in schoolchildren in Côte d’Ivoire, iron increased enterobacteria and intestinal inflammation, and decreased lactobacilli (Zimmermann et al. 2010). In older studies in European infants using culture methods, iron induced an increase in E. coli and a decrease in bifidobacteria (Balmer et al. 1989), along with higher counts of Bacteroides (Mevissen-Verhage et al. 1985). A recent small study in US infants (n=14) receiving complementary feeding regimens providing iron from fortified cereals or meat reported an increase in Bacteroidales and decreases in bifidobacteria, Lactobacillales and Rothia (Krebs et al. 2013). In infants in Pakistan, +FeMNPs caused an increase in Aeromonas species compared to the non-supplemented control group (Soofi et al. 2013).
Our study aim was to determine the effects of two widely used high and low dose +FeMNPs on the gut microbiome, using barcoded 16S rRNA pyrosequencing and targeted real-time PCR (quantitative PCR, qPCR), and intestinal inflammation in Kenyan infants prone to diarrheal disease. Our hypotheses were that iron fortification would: (1) increase fecal enterobacteria and its ratio to bifidobacteria and/or lactobacilli; (2) favor colonization by potential pathogens and (3) increase intestinal inflammation.

**Materials and Methods**

**Study site and participants**

The study was conducted in Msambweni County, in southern coastal Kenya. This is a malaria-endemic area, where 40% of the pediatric hospital admissions in 2006 were due to plasmodial infections (Okiro et al. 2007). The region experiences a long rainy season from April to July, and short rains from October to November. Farming is the main economic activity and maize the staple food crop in this sparsely populated area. The typical local weaning food is the liquid maize porridge, *uji*; a regular portion consists of about 8-10 g maize flour boiled in 100-150 mL water and sweetened with sugar.

In the catchment area of the Kikoneni health center, we continuously recruited infants aged 5.5 months. We recruited 80 infants from March 2010 until September 2011 and randomly assigned them to receive either an MNP with or without 2.5 mg iron as sodium iron EDTA (NaFeEDTA, ±2.5mgFeMNP, MixMe, DSM Nutritional Products Europe, Basel, Switzerland). We recruited 80 more infants from September 2011 to May 2012 and randomly assigned them to receive either an MNP with or without 12.5 mg iron as ferrous fumarate (±12.5mgFeMNP, Sprinkles, Hexagon Nutrition, Mumbai). The composition of the MNPs is shown in Table 1. Inclusion criteria were an infant age of 5.5 months (±3 weeks), mother at least ≥15 years of age, infant hemoglobin (Hb) ≥70 g/L, and no maternal or infant chronic diseases. The MNPs were packed in group-coded sachets (containing one daily dose).
Table 1 Composition of the two micronutrient powders, with and without iron*

<table>
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<th></th>
<th>+2.5mgFeMNP</th>
<th>-2.5mgFeMNP</th>
<th>+12.5mgFeMNP</th>
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*Amounts per 1g powder

Study design

Prior to intervention, we conducted triangle sensory tests (Meilgaard et al. 2007) in local adults (n=25 per MNP). In these tests, the +FeMNPs, were indistinguishable from the corresponding -FeMNPs and their acceptability was high (data not shown).

Trained field workers instructed the mothers in cooking the uji and its fortification with the MNPs. Further, the participating mothers were trained on the home collection of infant stool samples. Before starting the intervention with MNPs, we conducted a 2-week run-in period to familiarize the families with the stool collection method and the introduction of the maize porridge.

Then, weekly for 4 months, we dispensed 7 MNP sachets and 2 kg of maize flour (Dola, Kitui Flour Mills, Mombasa, Kenya) directly to the participating mothers from six distribution points. We analyzed triplicate samples of the maize flour for iron concentration by using atomic absorption spectroscopy, and for phytic acid (Makower 1970, Vanveldhoven and Mannaerts 1987) at the ETH Zurich. During the weekly distribution, field workers collected the previous week’s used and unused MNP sachets.
to assess compliance and recorded the infants’ feeding history and the health status by using a multiple-choice questionnaire. If a mother reported any illness, the dedicated study nurse examined the child and recorded treated episodes of malaria, diarrhea and respiratory tract infections (RTI). The study nurse used a forced-choice questionnaire to record treated cases of malaria, diarrhea and RTI at baseline (covering the last 3 months) and after 4 months (covering the entire intervention period).

Gut microbiome, short-chain fatty acids (SCFA) and fecal calprotectin
Stool samples were collected at baseline, 3 weeks and 4 months. The mothers were provided with plastic diapers, containers, spatulas, Anaerocult sachets (Merck KGaA, Darmstadt, Germany) to generate an anaerobic environment, a falcon tube for the measurement of 8 mL water to wet the Anaerocult and an illustrated pamphlet to reinforce the sampling instructions. The stool samples were collected in the morning, kept anaerobic, and aliquots were frozen at -20°C the same day.

Full descriptions of the materials and methods used for the following are available in the online Supplementary Materials and Methods: DNA extraction, gut microbiome analysis using 16S rRNA pyrosequencing, targeted qPCR, and short-chain fatty acid (SCFA) measurements.

Biochemical indicators
At baseline, after 4 months (±12.5mgFeMNP) and 6 months (±2.5mgFeMNP), venous blood samples were drawn using heparin vacutainers and butterfly needles. Serum was separated and frozen on collection day.

The following parameters were determined, of which the full description is available in the online Supplementary Materials and Methods: hemoglobin (Hb), zinc protoporphyrin to haem ratio (ZPP), serum ferritin (SF), soluble transferrin receptor (sTfR) and C-reactive protein (CRP), serum hepcidin-25, body iron stores, serum IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFNγ, TNFα, GM-colony-stimulating factor (CSF), IL-12 (p40/p70) and IL-17. The following cut-offs were used: (1) anemia: Hb <110 g/L (WHO 2001), (2) ID: body iron stores <0 mg/kg (Cook et al. 2003) and (3) inflammation: CRP ≥4.1 mg/L (manufacturer’s reference range).
Anthropometric indicators
At baseline and after 4 months, infant weight was recorded using a hanging scale (Salter 235-6S, 25 kgx100 g; Salter Brecknell, UK) and length using a measurement board (Shorr Production, Olney, Maryland, USA). We calculated weight-for-age (WAZ), height-for-age (HAZ), weight-for-height (WHZ) and BMI-for-age (Body Mass Index; BAZ) Z-scores using the WHO Anthro software (WHO 2007).

Statistical analysis
Based on previous studies on gut microbiota conducted by our group in schoolchildren (Zimmermann et al. 2010) and young adults (Petry et al. 2012), we estimated a sample size of 25-30 subjects in each group to detect a relevant difference of about 0.85 log number of copies/g feces in enterobacteria, lactobacilli and bifidobacteria, considering a SD of 1.09-1.37 for the three microbial groups, with \( \alpha=0.05 \) and \( \beta=0.8 \).

Longitudinal effects of the iron on the gut microbiome (assessed by pyrosequencing) were assessed by comparing change over time, which was calculated by dividing the relative abundance of a taxon at 4 months or 3 weeks by the relative abundance of a taxon at baseline and a subsequent Mann-Whitney U test (MWU) was done. Cross-sectional differences were compared by MWU. Estimated intervention effects of iron on gut microbiota (assessed by qPCR), iron status and anthropometrics were evaluated using univariate general linear models (GLM) with baseline values as covariates. Full descriptions of statistical methods and software used can be found in the online Supplementary Materials and Methods.

Ethical aspects
This study was approved by the ethics and research committees of the Kenyatta National Hospital/ University of Nairobi (P167/6/2009 and P168/05/2011), ETH Zurich (EK 2009-N-53). Caregivers of the infants gave written informed consent. A data safety monitoring board, including a pediatrician and a nutritionist, performed safety monitoring for the study.
Results

Our estimated sample size was 100-120 infants, and we enrolled 115 infants into the study (Figure 1, trial profile). We originally recruited 160 infants, but during the 2-week run-in period, 21 infants were excluded because they had received antibiotics in the previous 2 months, and 24 infants did not provide an adequate baseline stool sample. During the study, a further 11 infants were excluded because they received antibiotics, or were unable to provide adequate stool samples. Use of antibiotics was unexpectedly high in this setting; this was the local standard of care. Due to a labelling error at the factory in a second batch of the ±2.5mgFeMNP sachets, three infants received mixed treatments and were excluded. Therefore, the final analysis was completed on 101 infants: 28 from +2.5mgFeMNP, 21 from -2.5mgFeMNP, 26 from +12.5mgFeMNP, and 26 from -12.5mgFeMNP.

Baseline prevalence of anemia, iron deficiency (negative body iron stores) and systemic inflammation were 67.3%, 25.5% and 29.7%, respectively. Nearly all infants were still being breastfed (99.0%), but 80.2% had already been introduced to complementary foods, predominantly uji starting on average, at 4 months of age. Compliance with the sachets was 99.4% for ±2.5mgFeMNP and 96.2% for ±12.5mgFeMNP. The native iron and phytic acid concentrations in the maize flour were 1.15±0.06 mg and 310±20 mg per 100 g, respectively.

Figure 1 Trial profile
Baseline infant gut microbiome and its development over time

Totally, 934,853 bacterial 16S rRNA sequences were analyzed by pyrosequencing. At baseline, the infant gut microbiome consisted of the phyla Actinobacteria (64.3% of reads), Firmicutes (22.4%; including 4.7% Lactobacillus), Bacteroidetes (8.9%; including 3.6% Bacteroides and 4.3% Prevotella), and Proteobacteria (4.1%; including 3.3% Escherichia/Shigella); and was highly dominated by the family of Bifidobacteriaceae, contributing 63.0% of the total 16S rRNA (Figure 2). There was no significant effect of baseline anemia status on phylogenetic diversity among the infants (data not shown). However, an exploratory analysis revealed significant differences in taxa between anemic and non-anemic infants at baseline: non-anemic infants harbored lower abundances of Prevotella (2.0% vs 4.5%, p=0.014), whereas, they showed higher abundances of Actinomycetales (0.14 % vs 0.09%, p=0.004) and Streptococcus (6.3% vs 3.9%, p=0.023) (Figure 3).

The phylogenetic diversity in all stool samples increased after 3 weeks (p=0.004) and further increased to 4 months (p=0.005, see online Supplementary Figure S1). Redundancy analysis throughout the study found: (1) a strong interindividual variation of the gut microbial composition (p=0.001); (2) a significant difference in the gut microbiome of anemic and non-anemic infants (p=0.031) and (3) a significant difference in the gut microbiome of infants in the two MNP trials (±2.5mgFeMNP and ±12.5mgFeMNP, p=0.015). Furthermore, strong time-specific signatures were found (p=0.001). Gender, season in which the intervention started, and the starting date of complementary feeding did not significantly affect the gut microbiome.

Significant changes over time in the infants gut microbiome composition of the -FeMNP group from baseline (6 months old) to endpoint (10 months old) are illustrated in online Supplementary Figure S2. These changes involved an increase in Faecalibacterium and Prevotella, and a decrease in Enterobacteriaceae (p=0.010, p=0.006, p=0.002, respectively).
Figure 2 Baseline gut microbiome of the 6 month-old Kenyan infants enumerated by 16S rRNA pyrosequencing (n=101). The fraction of 16S rRNA reads (in %) attributed to specific taxonomic level is given below the taxon name.
Figure 3 Differences in baseline gut microbiome composition in anaemic versus non-anaemic 6 month-old Kenyan infants. Nodes represent taxa; edges link the different taxonomic levels. The fold increase is calculated as the 2log of the ratio of the relative abundance in anaemic and non-anaemic (0=no difference between anaemia status, 1=twice as abundant in non-anaemic). The significance is expressed as the p value of a Mann-Whitney U test of the anaemic and non-anaemic infant samples. The node-size corresponds to the relative abundance. Taxa (ie, nodes) were included in this visualization if they met the following criteria: all samples together have an average relative abundance of >0.1% for the taxon and the study groups have a fold difference of at least 0.1 with a significance of p<0.05 or the taxon has a child (ie, more specific taxonomic classification) meeting the criteria.

Composition of the infant gut microbiome during iron fortification

The phylogenetic diversity of the gut microbiome was not significantly modified by +FeMNP versus -FeMNP (see online Supplementary Figure S1). Differences in the gut microbiome at endpoint for the combined ±FeMNP groups and the separate analysis of the ±2.5mgFeMNP and ±12.5mgFeMNP are shown in Figure 4A-C. The changes over time in the relative abundance of a taxon between the ±FeMNP groups and the ±2.5mgFeMNP and ±12.5mgFeMNP are shown in Figure 5I-VI. Comparing +FeMNP versus -FeMNP, there were significantly higher abundances of the genera Clostridium, and Escherichia/Shigella (p=0.033, p=0.010, respectively), and a trend towards lower abundances of the genus Bifidobacterium (p=0.085) in the +FeMNP group at endpoint (Figure 4A). Additionally, the change over time in relative abundances (4 months vs baseline) was significantly different in +FeMNP versus -FeMNP for Firmicutes, Escherichia/Shigella, and Clostridium (p=0.034, p=0.030, p=0.048, respectively; Figure 5I-III). Comparing +2.5mgFeMNP with -2.5mgFeMNP, there were significantly higher abundances of the genera Escherichia/Shigella (p=0.044) and a trend towards higher in Roseburia (p=0.083) in +2.5mgFeMNP versus -2.5mgFeMNP at endpoint (Figure 4B). Additionally, the change over time in relative abundances (4 months vs baseline) was significantly different in +2.5mgFeMNP compared with -2.5mgFeMNP for Escherichia/Shigella (p=0.034; Figure 5IV). Comparing +12.5mgFeMNP with -12.5mgFeMNP, there were significantly higher abundances of Firmicutes (p=0.018),
Bacteroides \((p=0.045)\), a trend towards higher abundances of Clostridium species \((p=0.052)\) and Escherichia/Shigella \((p=0.067)\), and significantly lower abundance of Bifidobacterium \((p=0.047)\) in +12.5mgFeMNP versus -12.5mgFeMNP at endpoint (Figure 4C). Additionally, the change over time in relative abundances (4 months vs baseline) was significantly higher in +12.5mgFeMNP compared with -12.5mgFeMNP for Firmicutes \((p=0.046; \text{Figure 5V})\) and showed a significantly larger decrease for Bifidobacterium \((p=0.049, \text{Figure 5VI})\).

Figure 4 Differences in gut microbial composition after 4 months in Kenyan infants receiving iron containing micronutrient powders (+FeMNP) versus no-iron micronutrient powders (-FeMNP). (A) +FeMNP vs -FeMNP; (B) +2.5mgFeMNP vs -2.5mgFeMNP; (C) +12.5mgFeMNP vs -12.5mgFeMNP. Nodes represent taxa; edges link the different taxonomic levels. The fold increase is calculated as the 2log of the ratio of the relative abundance in +FeMNP and -FeMNP \((0=\text{no difference between group}, 1=\text{twice as abundant in +FeMNP, etc.})\). The significance is expressed as the \(p\) value of a Mann-Whitney U test. The node-size corresponds to the relative abundance (in %). Taxa displayed were selected based on the list of targets of our primary interest.
Figure 5 Change from baseline to 4 months in taxa that differed significantly between +FeMNP and -FeMNP infants at 4 months. The boxplots (I-VI) report on significant changes over time from baseline to endpoint (I: p=0.034, II: p=0.030, III: p=0.048, IV: p=0.034, V: p=0.046, VI: p=0.049) of taxa being different in groups at endpoint (corresponding roman numerals and colours in Figure 4). Boxplots of 2log ratios (0=no difference for the two time points, 1=twice as abundant at 4 months, etc.) are displayed with the 10-90th percentiles.

Comparing +FeMNP versus -FeMNP by qPCR analysis, revealed a borderline significant effect of iron on enterobacteria (p=0.061), with higher concentrations in +FeMNP (8.9±0.3 log number of gene copies/g feces) versus -FeMNP (8.0±0.4) at endpoint. Furthermore, there was a significant treatment effect on Roseburia species/Eubacterium rectale (p=0.020), with lower concentrations in +FeMNP (4.4±0.4) versus -FeMNP (5.8±0.4). In the separate analyses of the two MNPs, a significant treatment effect was found on Roseburia species/E. rectale in ±12.5mgFeMNP (p<0.0001), with lower concentrations in +12.5mgFeMNP (3.2±0.4) versus -12.5mgFeMNP (6.2±0.5) at endpoint; while a significant treatment effect was seen for Eubacterium hallii in ±2.5mgFeMNP (p=0.009), with higher concentrations in +2.5mgFeMNP (8.4±0.6) versus -2.5mgFeMNP (7.1±0.5) at endpoint.

Examining the effect of iron on pathogenic gut microbiota in all analyzed stool samples, we detected Bacillus cereus in 39.5% (mean in detected samples: 4.6 log copies/g feces (range: 3.3-7.2)), Staphylococcus aureus in 65.4% (6.6 (3.0-10.0)), Clostridium difficile in 56.5% (7.2 (3.4-10.4)), members of the Clostridium perfringens group in 89.7% (7.7 (3.1-10.4)), Salmonella in 22.4% (5.7 (4.4-7.9)) and Vibrio cholera in 0%.
Further, we detected enteropathogenic \textit{E. coli} (EPEC) in 65.0\% (mean in detected samples: 6.0 log copies/g feces (range: 3.2-9.3)), enterotoxigenic \textit{E. coli} producing heat-labile toxin (ETEC LT) in 49.2\% (6.0 (3.0-10.5)), ETEC producing heat-stable toxin (ETEC ST) in 7.0\% (5.7 (3.5-8.7)), enterohemorrhagic \textit{E. coli} producing shiga-like toxin 1 (EHEC stx1) in 9.6\% (4.7 (3.4-7.4)), and EHEC stx2 in 8.5\% (4.6 (3.6-6.3)). There was a significant treatment effect on the sum of the pathogenic \textit{E. coli} at endpoint (p=0.029), with higher concentration in the +FeMNP (6.0±0.5 log numbers of copies/g feces) versus -FeMNP (4.5±0.5). In the separate analyses of the two MNPs, there was a significant treatment effect on the sum of the pathogenic \textit{E. coli} in ±2.5mgFeMNP at midpoint (p=0.012), and at endpoint (p=0.043), with transient lower concentrations in the +2.5mgFeMNP (4.9±0.7) versus -2.5mgFeMNP (7.0±0.5) at midpoint and higher concentrations in +2.5mgFeMNP (6.5±0.6) versus -2.5mgFeMNP (4.1±0.8) at endpoint. No effect of iron on pathogenic \textit{E. coli} could be detected in the ±12.5mgFeMNP. The increase in the sum of pathogenic \textit{E. coli} through iron fortification was significant in infants with baseline iron deficiency (p=0.012), but not in infants who were iron sufficient (p=0.327).

\textbf{Figure 6A-D} shows the ratio of abundances of enterobacteria to bifidobacteria and lactobacilli analyzed by pyrosequencing and qPCR. Using pyrosequencing, the ratio of the relative abundances of enterobacteria to bifidobacteria changed significantly over time from baseline to endpoint in the +FeMNP versus -FeMNP (p=0.020; Figure 6A). At endpoint, the enterobacteria to bifidobacteria ratio was significantly higher in the +FeMNP versus -FeMNP (p=0.004); similarly, in the separate analysis of the MNPs, the enterobacteria to bifidobacteria ratio was significantly higher in +2.5mgFeMNP (p=0.030) and in +12.5mgFeMNP (p=0.049), compared to the respective control groups at endpoint. The qPCR analysis confirmed this, showing a significant effect on the ratio of enterobacteria to bifidobacteria in +FeMNP compared with -FeMNP group at endpoint (p=0.008, Figure 6B); with a trend towards a significant effect in the separate analysis of ±2.5mgFeMNP (p=0.053) and ±12.5mgFeMNP (p=0.098). Using pyrosequencing, the ratio of the relative abundance of enterobacteria to lactobacilli in +FeMNP did not change over time from baseline to endpoint, after a temporary decrease at 3 weeks (p=0.018) in +FeMNP versus -FeMNP; however, at endpoint, the ratio of enterobacteria to lactobacilli was significantly higher in +FeMNP (p=0.023; Figure 6C). Using qPCR, a similar trend towards a higher ratio of enterobacteria to
lactobacilli was detected at endpoint in +FeMNP compared to -FeMNP (p=0.062, Figure 6D). Furthermore, pyrosequencing analysis of ±2.5mgFeMNP and ±12.5mgFeMNP separately showed a borderline significantly higher ratio of enterobacteria to lactobacilli in +12.5mgFeMNP versus -12.5mgFeMNP (p=0.055) at endpoint, with a temporary decrease after 3 weeks (p=0.021); but did not differ at any time point in +2.5mgFeMNP versus -2.5mgFeMNP.

Figure 6 Ratio of infant enterobacteria to bifidobacteria at baseline, 3 weeks and 4 months comparing +FeMNP and -FeMNP assessed by: (A) pyrosequencing (log2 ratio of the relative abundance); and (B) qPCR (ratio of log numbers of copies/g faeces). Ratio of infant enterobacteria to lactobacilli assessed by: (C) pyrosequencing; and (D) qPCR. Boxplots are displayed with the 10-90th percentiles. Values differed significantly between groups (+FeMNP and -FeMNP): (A) the change over time from baseline to endpoint in ratios was significant different between groups (p=0.020); at endpoint the ratios were significantly higher in +FeMNP versus -FeMNP (p=0.004). (B) the ratios were significantly different between groups at 4 months using general linear models (GLM) and adjusted for baseline differences (p=0.008). (C) the change over time from baseline to 3 weeks in ratios was significantly different (p=0.018), but the change over time from baseline to endpoint was not different between +FeMNP versus -FeMNP; at endpoint the ratios were significantly higher in +FeMNP versus -FeMNP at endpoint (p=0.023). (D) the ratios were borderline significantly different between groups at 4 months using GLM and adjusted for baseline differences (p=0.062).
Fecal calprotectin, SCFAs, iron status and systemic inflammation

Intestinal inflammation, assessed by fecal calprotectin, was significantly higher in infants receiving +FeMNP (229.2±1.9 µg/g) versus -FeMNP (123.3±2.1 µg/g, p=0.002). In the separate analyses of the MNPs, fecal calprotectin values were significantly elevated in +12.5mgFeMNP (248.9±2.2 µg/g) versus -12.5mgFeMNP (102.5±2.2 µg/g, p=0.008), but were not significantly higher in +2.5mgFeMNP (215.6±1.6 µg/g) versus -2.5mgFeMNP (165.6±1.8 µg/g, p=0.164, Figure 7). The increase in fecal calprotectin through iron fortification was significant in infants who were iron sufficient at baseline (p=0.0002) but not in infants with iron deficiency (p=0.912). There were no significant correlations of fecal calprotectin with the overall gut microbiome composition assessed by pyrosequencing. The qPCR data revealed intrasample correlations of fecal calprotectin with none of the commensal bacteria, but with the sum of pathogenic E. coli (p=0.011, t=0.177), and enterobacteria (p=0.008, t=0.201), and members of the C. perfringens group (p=0.007, t=-0.206) at 3 weeks, and EPEC (p=0.024, t=0.190) at 4 months. There were no significant differences in fecal acetate, propionate, or butyrate concentrations between +FeMNPs and -FeMNPs during the intervention (see online Supplementary Table S2).

Figure 7 Infant faecal calprotectin concentrations at baseline and 4 months in the pooled data from the -FeMNP versus +FeMNP groups, in the -2.5mgFeMNP versus +2.5mgFeMNP, and in the -12.5mgFeMNP versus +12.5mgFeMNP. Values differed significantly over the trial between the ±12.5mgFeMNP groups (p=0.008) and the pooled MNP groups (±FeMNP, p=0.002), using general linear models and baseline variables as covariates. Boxplots are displayed with the 10-90th percentiles.
There was a significant treatment effect of +12.5mgFeMNP versus -12.5mgFeMNP on body iron (p=0.001), SF (p=0.004), sTfR (p=0.008), ZPP (p=0.039) and a trend towards an effect on hepcidin-25 (p=0.052, see online Supplementary Table S3). By contrast, there was no significant treatment effect of +2.5mgFeMNP versus -2.5mgFeMNP on any iron status indicator or hepcidin-25. There was no treatment effect of either +FeMNP on serum CRP (see online Supplementary Table S3) or serum IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17, IFN, TNFα or GM-CSF (data not shown). There was a significant treatment effect of +2.5mgFeMNP versus -2.5mgFeMNP on IL-12, which was higher in +2.5mgFeMNP (523.6±1.5 µg/g vs 431.9±1.4 µg/g, p=0.028).

**Growth**

There was no significant treatment effect of iron on weight gain. However, we identified a significant increase of linear growth in +FeMNPs (70.2±1.1 cm vs 68.5±1.1 cm at endpoint, p=0.017, see online Supplementary Table S3). This effect was significant in the infants of ±12.5mgFeMNP (70.2±1.1 cm vs 68.1±1.1 cm at endpoint, p=0.011), but not in ±2.5mgFeMNP. Linear growth was not correlated to gut microbiota or fecal calprotectin.

**Morbidity**

On enrolment, 19.2% of mothers reported a treated episode of diarrhea in their infant during the previous 3 months, 29.7% a treated RTI, and 3.8% a treated malaria episode. During the intervention, incidences of treated RTI and malaria did not significantly differ between +FeMNP versus -FeMNP. However, there was a trend towards a greater incidence of treated episodes of diarrhea in +12.5mgFeMNP versus -12.5mgFeMNP: 27.3% (n=6/22) versus 8.3% (n=2/24, p=0.092).

**Discussion**

This study shows that in breastfed, 6-months-old infants from rural Africa, the gut microbiome is dominated by *Bifidobacteriaceae*, but harbors many gram negative and gram positive pathogens. Our findings indicate that the iron in MNPs favors growth of several of these potentially pathogenic subgroups. At the same time, iron decreases abundances of bifidobacteria, shifting gut microbial balance away from beneficial ‘barrier’ strains towards a potentially more pathogenic profile. This is accompanied by an increase in intestinal inflammation. Our findings in Kenyan infants are comparable with those of a recent controlled trial in school-aged children in Côte d’Ivoire receiving
iron-fortified biscuits containing 20 mg iron/day as electrolytic iron for 6 months (Zimmermann et al. 2010). The expected absorption of electrolytic iron in that setting was <5%, and there was no decrease in anemia or iron deficiency in the iron-fortified group. Although the study was a secondary analysis, only qPCR methods were used, and the authors did not report increases in specific pathogens; it did demonstrate that iron fortification can extensively modify the gut microbiota, increasing enterobacteria and decreasing lactobacilli, along with increased intestinal inflammation measured by fecal calprotectin. The differences between these two studies are likely due to agerelated differences in the gut microbiota between infants and older children, but may also reflect differences in methods used to characterize the gut microbiome, differences in geographic setting and, possibly, differences in the iron compound and/or dose given. A recent high-dose iron supplementation trial in low to middle income South African schoolchildren residing in an area with an improved water supply and a lower risk of contaminated food (Dostal et al. 2014), supports the suggestion that environmental variables modulate the effects of iron on intestinal inflammation and gut microbiota. Despite the high iron dose, there were no measurable effects on intestinal inflammation measured by fecal calprotectin, nor on gut microbiota evaluated by qPCR. Overall, these data suggest that the effects of supplemental or fortification iron on the gut microbiota and intestinal inflammation in children are more pronounced in settings where hygiene standards are low and the microbiome is likely to be populated by opportunistic enteropathogens.

Iron fortification in the present study, and in the Ivorian study (Zimmermann et al. 2010) favored growth of enterobacteria over bifidobacteria and/or lactobacilli, and this could be due to their different iron requirements and metabolism. Many pathogenic enterobacteria require iron acquisition for bacterial virulence (Bullen et al. 2000, Boyer et al. 2002) and/or gastrointestinal tract colonization (Tsolis et al. 1996). Only few bacteria do not require iron, of which lactobacilli is the major group (Weinberg 1997). Lactobacilli do not produce siderophores, and their growth is similar in media with and without iron (Pandey et al. 1994). *Bifidobacterium breve*, an important bifidobacteria species in breastfed infants, can sequester luminal iron using a divalent metal permease (Bezkorovainy and Solberg 1989, Bezkorovainy et al. 1996), but the majority of bifidobacteria do not produce siderophores or other active iron carriers. Abundant bifidobacteria, lactobacilli and other beneficial bacteria in the colon provide an
important ‘barrier effect’ against colonization and invasion by pathogens (Coconnier et al. 1997, Lievin et al. 2000, Weinberg 1997). Our findings suggest that +FeMNPs weaken this protective effect. If +FeMNPs promote expansion of enterobacteria, this may be important because abundances of closely related species can predict susceptibility to intestinal colonization by pathogenic bacteria (Stecher et al. 2010). In our study, this effect may have encouraged colonization by potentially pathogenic members of the genus Escherichia/Shigella species, evidenced by the higher abundances of this genus in the +FeMNPs at endpoint and in particular of the five pathogenic E. coli subgroups (EPEC, ETEC LT, ETEC ST, EHEC stx1, and stx2).

Calprotectin is a calcium-binding and zinc-binding protein found in the cytosol of neutrophils, monocytes and activated macrophages (Gisbert and McNicholl 2009). Fecal calprotectin levels mainly reflect migration of neutrophils into the gut mucosa, and are a non-specific marker of intestinal inflammation elevated in children with gastroenteritis (Berni Canani et al. 2004). Fecal calprotectin is markedly higher during infancy than in later childhood (Konikoff and Denson 2006). In healthy Ugandan children, median fecal calprotectin were 278 µg/g at 3-6 months, 183 µg/g at 6-12 months of age, and fell to 28 µg/g at school-age (Hestvik et al. 2011). We used a different assay than the Ugandan study, but found comparable levels of fecal calprotectin at baseline. In -FeMNP groups, fecal calprotectin decreased as expected over the 4 month intervention while in +FeMNP groups, and particularly in +12.5mgFeMNP, levels remained significantly elevated. In the Ivorian fortification study, where school-age children received ≈9 mg of iron per day, fecal calprotectin increased sharply and was correlated with the increase in gut enterobacteria (Zimmermann et al. 2010). Although we found no significant difference in systemic concentrations of most cytokines, circulating IL-12 concentrations were significantly higher in +2.5mgFeMNP versus -2.5mgFeMNP at endpoint. Gut microbial antigens can stimulate secretion of IL-12 by gut macrophages and induce development of Th1 cells (Maynard et al. 2012). Thus, in our study, IL-12 secretion may have been triggered by invasive pathogens or dysbiosis resulting from increased luminal iron; notably, in +2.5mgFeMNP versus -2.5mgFeMNP, the Escherichia/Shigella taxon was significantly higher at endpoint, an effect not seen in +12.5mgFeMNP. Furthermore, dietary iron has shown to increase intestinal inflammation in this study and others (Carrier et al. 2006, Zimmermann et al. 2010) through different potential pathways such as neutrophil infiltration, lipid peroxidation,
NF-κB activation and proinflammatory cytokines (Carrier et al. 2006). Electron acceptors generated as by-products of host inflammatory response have been proposed to favor facultative anaerobes, in particular, enterobacteria (Winter et al. 2013).

The +FeMNPs we used in this study contained iron as two different forms, ferrous and ferric, and the ferric iron was bound to a chelator (EDTA). Our data do not clarify whether the form of dietary iron is a potential determinant of the changes induced in the gut microbiota, as the relative concentration of ferrous versus ferric iron in the human colon resulting from dietary iron ingestion is unknown. However, even if different forms of iron are ingested, it is likely that most of the unabsorbed dietary iron that enters the colon is in the oxidized, ferric (Fe$^{3+}$) form, mainly as ferric oxides or hydroxides (Simpson et al. 1992). In the colon, low-oxygen tension would favor the reduction of ferric to ferrous (Fe$^{2+}$) iron. Systems for acquisition of ferrous and ferric iron have been identified in many gastrointestinal pathogens. Although ferric ion has a solubility of only $10^{-17}$ M at pH 7, and bacteria generally require iron at around $10^{-7}$-$10^{-5}$ M to achieve optimal growth (Andrews et al. 2003), bacteria can reduce ferric iron to the more soluble ferrous form and/or use extracellular ferric iron chelators, such as siderophores, as solubilizing agents prior to uptake (Koster 2001). Many enteric pathogens, including Salmonella species and Escherichia/Shigella species (Naikare et al. 2006), take up iron-siderophore complexes via specific outer membrane receptors. Most enteric gram-negative bacteria also have active transport systems for ferrous iron (Andrews et al. 2003), and iron transporter FeoB-mediated ferrous iron acquisition may increase virulence (Boyer et al. 2002).

In our study, the ferrous fumarate-containing +12.5mgFeMNP tended to cause greater modifications of the gut microbiome and more intestinal inflammation; this was likely due to the fact that its iron dose was fivefold that of the chelated ferric iron in the +2.5mgFeMNP. However, it is not clear from our findings if the higher iron dose was more detrimental: although +12.5mgFeMNP resulted in lower abundances of Bifidobacterium, and higher levels of fecal calprotectin, as well as a trend towards higher rates of treated diarrhea, the effect on Escherichia/Shigella, the ratio of enterobacteria to bifidobacteria, and on the increase of pathogenic E. coli was stronger with +2.5mgFeMNP. Therefore, our data do not clarify whether the lower iron dose MNP would have a better safety profile.
In accordance with previous studies on human gut microbiota (Backhed et al. 2005, De Filippo et al. 2010, Fallani et al. 2011, Qin et al. 2010), the four dominant phyla in our infants at baseline were Actinobacteria (63%, mainly Bifidobacteriaceae), Firmicutes (22%), Bacteroidetes (9%), and Proteobacteria (4%). We found more distinct treatment effects after 4 months than after 3 weeks, likely due to the rapid fluctuations in the gut microbiome at 3 weeks caused by the introduction of the complementary feeding (Vael and Desager 2009). This finding underscores the importance of allowing the gut microbiome time to adapt to changes in diet when evaluating an intervention and comparing different trials. Our data from the gut microbiome of African infants are comparable with those reported in Dutch infants using qPCR methods that found similar abundances of bifidobacteria, E. coli, and lactobacilli (Penders et al. 2006). However, by contrast, many of our infants carried enteropathogens, with over half of fecal samples containing C. difficile, S. aureus, members of the C. perfringens group, enteropathogenic and/or enterotoxigenic E. coli, and nearly one in four harboring B. cereus and/or Salmonella. Our data also suggest the baseline gut microbiome of anemic versus non-anemic infants may differ, as may their response to +FeMNPs. Anemic infants showed higher Prevotella (Bacteroidetes) and lower Actinomycetales (Actinobacteria) and Streptococcus (Firmicutes) abundances than non-anemic infants. A previous study comparing the gut microbiome of older children from Burkina Faso and Italy found higher concentrations of Bacteroidetes (especially Prevotella and Xylanibacter) and lower concentrations of Firmicutes and Escherichia/Shigella in the Burkinabé than Italian children (De Filippo et al. 2010). Thus, our findings suggest higher rates of anemia in African children may contribute to differences observed in gut microbiota in African versus European children.

In rats, the availability of colonic iron may modulate gut microbiota metabolites and, in particular, production of caecal SCFAs (Dostal et al. 2012). This could have important gut modulatory effects, as SCFAs have been reported to inhibit growth or reduce concentrations of Salmonella enteritidis, Salmonella enterica serovar Typhimurium, E. coli, Shigella flexneri and Campylobacter jejuni (Ricke 2003, Topping and Clifton 2001), and butyrate can down-regulate expression of genes involved in Salmonella invasion at low doses (Van Immerseel et al. 2006). In in vitro fermentations, low iron concentrations decreased numbers of SCFA-producers, including Roseburia species/E. rectale, Clostridium Cluster IV members and Bacteroides species, and decreased
butyrate and propionate concentrations in the effluent (Dostal et al. 2013). In the present study, we found no effect of iron-containing MNPs on fecal SCFA concentrations. This difference may be due to the fact that 95-99% of SCFAs produced by bacterial fermentation are absorbed in the colon, so concentrations measured in feces likely do not reflect levels in the colonic lumen (Scheppach 1994). In Swedish infants, fecal butyrate and propionate concentrations steadily increased over the first 2 years of life, while fecal acetate increased rapidly in the first few weeks after birth and then plateaued (Midtvedt and Midtvedt 1992). A similar pattern from 6 months to 10 months of age was visible in the Kenyan infants in this study.

Data on the effect of iron interventions on diarrheal incidence in children disagree. In a systematic review of controlled trials of oral iron supplementation or fortification, provision of iron was associated with an 11% higher risk of developing diarrhea (p=0.04) (Gera and Sachdev 2002). Four food fortification studies reported diarrheal outcomes, three provided iron-fortified infant formula (Brunser et al. 1993, Power et al. 1991, Singhal et al. 2000), and one provided an iron-fortified infant food (Javaid et al. 1991). Since that review, two small iron fortification trials done in school-age children have not reported an increased risk for diarrhea (Manger et al. 2008, Moretti et al. 2006). However, a controlled trial in Swedish and Honduran infants providing iron supplements from 6 to 9 months, among infants with Hb ≥110 g/L, iron treatment increased risk for diarrhea (Dewey et al. 2002). Recent controlled iron supplementation trials (12.5-15 mg Fe/day) in Peru (Richard et al. 2006) and Bangladesh (Chang et al. 2010) reported a significant increase in diarrhea. Two large trials of iron and folic acid supplementation in infants and children (subjects aged 12-35 months received 12.5 mg Fe/day, younger children received half the dose) in Nepal (Tielsch et al. 2006) and Tanzania (Sazawal et al. 2006) reported no difference in diarrhea incidence. However, in a controlled study in Ghanaian children that used the same +12.5mgFeMNP as in our study, there were significantly more hospital admissions in the iron group (RR (95% CI) 1.23 (1.02 to 1.49)), and based on data from the outpatient register, 83% of the additional cases in the iron group were due to diarrhea, but this was not significant (RR (95% CI) 1.12 (0.86 to 1.46)) (Zlotkin et al. 2013). A recent study in Pakistan found an increase in diarrhea in infants receiving +12.5mgFeMNP, with or without zinc, compared to untreated controls (without zinc: OR (95%CI) 1.15 (1.00 to 1.3); with zinc: 1.31 (1.13 to 1.51)) (Soofi et al. 2013). In the present study, there was a trend towards a higher rate of
diarrhea requiring treatment in the infants receiving the +12.5mgFeMNP. Therefore, the available data suggest oral iron supplements and +FeMNPs may modestly increase in risk for diarrhea in infants. Our findings of a shift in the gut microbiome towards a potentially more pathogenic profile along with the increase in intestinal inflammation could provide a potential mechanism for this adverse effect.

This is the first controlled intervention trial to examine the effect of +FeMNPs on the African infant gut microbiome and our findings need confirmation in other settings and populations. IDA and diarrhea are major causes of morbidity and mortality in infants in developing countries. In-home fortification with +FeMNPs has repeatedly been shown to reduce IDA rates in infants and children, but whether they increase risk for diarrheal disease remains uncertain. The limited available clinical evidence and our findings of their effects on the gut microbiome and inflammation, suggest +FeMNPs may not be entirely safe in settings with high burdens of infectious diseases. Currently, WHO recommends fortification using MNPs containing iron, vitamin A, and zinc for children aged 6-23 months irrespective of their iron status in settings with an anemia prevalence of >20%, and in conjunction with measures to treat malaria, promote improved sanitation, and improve overall management of diarrhea (WHO 2011). At the same time, WHO recommends iron supplementation only be targeted to infants with IDA, while providing adequate protection from malaria (Fontaine 2007). Our findings, together with others (Soofi et al. 2013, Zlotkin et al. 2013), suggest that, until safer formulations are available, +FeMNPs should be targeted to infants with IDA, while providing adequate protection from malaria and diarrhea.

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References


EFFECT OF IRON FORTIFICATION ON THE INFANT GUT MICROBIOME


SUPPLEMENTARY FILES

Supplementary Materials and Methods

Gut microbiome, short-chain fatty acids (SCFAs) and fecal calprotectin

Fecal DNA was extracted with the Fast DNA Spin Kit for Soil, including a bead-beating step (MP Biomedicals, Illkirch, France) and concentration was quantified with a Nanodrop 1000 spectrophotometer (Witec AG, Littau, Switzerland). For the preparation of the amplicon pool for pyrosequencing, the following universal primers were applied for amplification of the V3-V6 region of the 16S rRNA gene: a) forward primer, 5'-CCATCTCATCCCTGCGTGTCTCCGACTAGNNNNNNACTCCTACGGGAGGCAGCAG-3' (the italicized sequence is the 454 Life Sciences primer A, and the bold sequence is the broadly conserved bacterial primer 338F; NNNNNN designates the sample-specific six-base barcode used to tag each PCR product); b) reverse primer 5'-CCTATCCCTGTGCTTGGCAGTCTCAGCRRCACGAGCTGACGAC-3' (the italicized sequence is the 454 Life Sciences primer B, and the bold sequence is the broadly conserved bacterial primer 1061R). PCR amplification mixture contained: 1 μL fecal DNA, 1 μL bar-coded forward primer, 15 μL master mix (1 μL KOD Hot Start DNA Polymerase (1 U/μL; Novagen, Madison, WI, USA), 5 μL KOD-buffer (10x), 3 μL MgSO4 (25 mM), 5 μL dNTP mix (2 mM each), 1 μL (10 μM) of reverse primer) and 33 μL sterile water (total volume 50 μL). PCR conditions were: 95°C for 2 minutes followed by 35 cycles of 95°C for 20 s, 55°C for 10 s, and 70°C for 15 s. The approximately 750 bp PCR amplicon was subsequently purified using the MSB Spin PCRapace kit (Invitek) and the concentration was checked with a Nanodrop 1000 spectrophotometer (Thermo Scientific). A composite sample for pyrosequencing was prepared by pooling 200 ng of these purified PCR products of each sample. The pooled sample was purified using the Purelink PCR Purification kit (Invitrogen), with high-cutoff binding buffer B3, and submitted for pyrosequencing of the V3-V4 region of the 16S rRNA gene on the 454 Life Sciences GS-FLX platform using Titanium sequencing chemistry (GATC-Biotech, Germany). Targeted quantitative real-time polymerase chain reaction (qPCR) was performed using specific primers for bacterial subgroups most prevalent in the human gut and expected low-abundant pathogens (Supplementary Table S1). The enumeration of these bacterial groups was performed with a 7500 Fast Real-Time qPCR System (Applied Biosystems Europe BV, Zug, Switzerland) using SYBR Green PCR Master Mix (Applied Biosystems), and taxon-specific primers in a 25 μL volume. Duplicate sample
analysis and standard curves were routinely performed in each run. Data were analyzed using the 7500 Fast System Sequence Detection Software (Version 1.4, Applied Biosystems).

We measured fecal calprotectin using the Calprest ELISA assay for stools, following the manufacturer’s procedures (Eurospital, Trieste, Italy). For the measurement of the SCFAs (acetate, propionate, and butyrate), we homogenized 100-300 mg of stool in 1 mL 0.15 mM sulphuric acid and centrifuged at 9000 rpm and 2°C for 20 minutes (Underwood et al. 2009). The supernatant was transferred into a microconcentrator and filtered by centrifugation at 4700 rpm and 2°C for 90 minutes (Chen and Lifschitz 1989). The HPLC analysis was performed using a Phenomenex column (Rezex ROA-Organic Acid H+ (8%), 300*7.8 mm).

**Biochemical indicators**

Haemoglobin was measured immediately after collection with a HemoCue (HemoCue AB, Ängelholm, Sweden) or a HemoControl device (EKF diagnostics Sales GmbH, Barleben/Magdeburg, Germany). Serum was separated and frozen on collection day. The erythrocytes were washed thrice with normal saline, and zinc protoporphyrin to haem ratio (ZPP) was measured using a calibrated AVIV hematofluorometer (AVIV Biomedical, Lakewood, USA). Serum ferritin (SF), soluble transferrin receptor (sTfR) and C-reactive protein (CRP) were analyzed at Lancet Laboratories in Nairobi using the Cobas Integra (Roche, Basel, Switzerland). We converted the Roche sTfR concentration to the Flowers assay (Flowers et al. 1989) using the regression equation by Pfeiffer (Flowers=1.5*Roche +0.35) (Pfeiffer et al. 2007). Serum hepcidin-25 was measured in Nijmegen (hepcidinanalysis.com) by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (Kroot et al. 2010; Jaeggi et al. 2013). Body iron stores were calculated according to the equation by Cook et al. (body iron (mg/kg)=-[log10 (sTfR*1000/SF) -2.8229]/0.1207)(Cook et al. 2003), and total body iron stores by multiplying with body weight.

Serum levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFNγ, TNFα, and GM-CSF were determined by using a human cytokine multiplex kit (Cytokine 10-plex panel, Invitrogen, Breda, Netherlands), and IL-12 (p40/p70) and IL-17 were determined by using Singleplex bead kits (Invitrogen) at Radboud University Medical Center, Nijmegen, Netherlands.
Statistical analysis
Data were analyzed using IBM SPSS Statistics 20.0.0 (SPSS Inc., Chicago, IL) and Microsoft Office EXCEL 2010 (Microsoft, Redmond, WA). Data were double entered and distribution checked for normality; not normally distributed data were log transformed. Normally distributed data were expressed as means and standard deviations (SD) or standard errors of the mean (SEM). For log transformed data, we obtained geometric means (GM) and corresponding standard deviations (SD) for absolute concentrations by taking the antilog of these values. Correlations of gut microbial subgroups and intestinal inflammation were done using crude values and Kendall’s tau. Pyrosequencing data were analyzed with a workflow based on QIIME v1.2 (Caporaso et al. 2010), and reads were filtered for chimeric sequences using Chimera Slayer (Haas et al. 2011). OTU clustering was performed with settings as recommended in the QIIME newsletter of December 17th 2010 (http://qiime.wordpress.com/2010/12/17/new-default-parameters-for-uclust-otu-pickers/) using an identity threshold of 97%. Diversity metrics were calculated as implemented in QIIME 1.2. Hierarchical clustering of samples was performed using UPGMA with weighted UniFrac as a distance measure as implemented in QIIME 1.2. The RDP classifier version 2.2 was performed for taxonomic classification (Cole et al. 2009). Visualization of differences in relative abundance of taxa between different study groups was done in Cytoscape (Shannon et al. 2003). The baseline gut microbiome composition was illustrated using the approach presented by Sundquist et al. (Sundquist et al. 2007). Statistical analysis of the pyrosequencing data was done with SciPy (www.scipy.org). Differences in relative abundance between groups at a single time point (cross-sectional) were compared by Mann-Whitney U (MWU) testing. Comparisons of targets of our primary interest (the phyla Firmicutes and Bacteroidetes, and the taxa lactobacilli, *Roseburia* spp., *Clostridium* spp., bifidobacteria, and enterobacteria) were not corrected for multiple testing. Longitudinal effects of intervention were statistically assessed by comparing change over time, which were calculated by dividing the relative abundance of a taxon at 4 months or 3 weeks by the relative abundance of a taxon at baseline. These changes over time for two groups were compared by MWU. Changes over time of ratios of enterobacteria to bifidobacteria or lactobacilli (ratio of relative abundances) were compared the same way. The development of specific taxa and phylogenetic diversity over time was assessed by paired testing using the Wilcoxon matched-pairs signed-
rank test. Multivariate redundancy analysis (RDA) was performed in R (http://www.R-project.org) using the vegan package (Oksanen et al. 2012).

For the qPCR analysis, a total of 22 bacterial targets were tested for a treatment effect in univariate general linear models (GLM) for the two MNPs, using baseline variables as covariates (Supplementary Table S1). Moreover, we assessed treatment effects of any iron fortification by pooling data from the two iron groups (+FeMNP) and control groups (-FeMNP) and using univariate GLM, including baseline values as covariates. A summary variable was created for the pathogenic *E. coli* community by summing copy numbers of the *eaeA* (*E. coli* attaching and effacing) gene detecting EPEC and EHEC strains, and of the heat-labile and heat-stable enterotoxin gene detecting ETEC LT and ETEC ST strains (Fukushima et al. 2003).

Treatment effect on weight and height were assessed using GLM with baseline variables as covariates. Treatment effects on the incidence of diarrhea, malaria, and RTI were assessed using logistic regression. Baseline differences in iron status markers, inflammation markers, and hepcidin-25 were assessed using independent samples *t*-tests. Estimated intervention effects on iron status and hepcidin-25 were assessed with univariate GLM using baseline values as covariates. *P* values <0.1 were considered as a trend towards significance, and *p* values <0.05 as statistically significant.
References Supplementary Materials and Methods


## Supplementary Tables

### Supplementary Table S1

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<td>Roseburia spp./ E. rectale</td>
<td>16S rRNA gene</td>
<td>RrecF</td>
<td>(Walker et al. 2005; Ramirez-Farias et al. 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rrec630mR</td>
<td></td>
</tr>
<tr>
<td>Clostridial Cluster IV</td>
<td>16S rRNA gene</td>
<td>Clep866mF</td>
<td>(Ramirez-Farias et al. 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clep1240mR</td>
<td></td>
</tr>
<tr>
<td>Eubacterium hallii</td>
<td>16S rRNA gene</td>
<td>EhalF</td>
<td>(Hold et al. 2003; Ramirez-Farias et al. 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EhalR</td>
<td></td>
</tr>
<tr>
<td>Faecalibacterium praunznitii</td>
<td>16S rRNA gene</td>
<td>Fprau223F</td>
<td>(Wang et al. 1996; Bartosch et al. 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fprau420R</td>
<td></td>
</tr>
<tr>
<td>Sulfate-reducing bacteria</td>
<td>Alpha subunit dissimilatory sulfite reductase</td>
<td>dsrA_290F</td>
<td>(Pereyra et al. 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dsrA_660R</td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Target gene (description)</td>
<td>Primer and sequence (5'-3')</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>---------------------------</td>
<td>------------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>Salmonella</td>
<td>invA (invasion)</td>
<td>InvA 139 GTGAATTTATCGCCACGTTCCGGGCAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>InvA 141 TCATCGCAGCCTCAAAAGGAACCC</td>
<td></td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td>Nuclease</td>
<td>SA-1 GCGATTGATGTTGATACCGTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SA-2 CAAGCCTGTGCACTAAAGAGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Brakstad et al. 1992; Fukushima et al. 2003)</td>
<td></td>
</tr>
<tr>
<td><strong>Bacillus cereus</strong></td>
<td>Hemolysin</td>
<td>BC-1 CTGTAGCGAATCGTGACTGTATC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BC-2 TACTGCTCCAAGCCACATTAC</td>
<td></td>
</tr>
<tr>
<td><strong>Clostridium difficile</strong></td>
<td>16S rRNA gene</td>
<td>cdF TTGAGCCGATTACTTCCGGTAAAGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>cdR CCATCCTGTACTGCTACACCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Rinttila et al. 2004)</td>
<td></td>
</tr>
<tr>
<td><strong>Clostridium perfringens group</strong></td>
<td>16S rRNA gene</td>
<td>cpF ATGCAAGTGGAGCGA(G/T)G</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>cpR TATGCCTTAATACCT(C/T)CTTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Rinttila et al. 2004)</td>
<td></td>
</tr>
<tr>
<td><strong>Vibrio cholera</strong></td>
<td>CT (cholera toxin)</td>
<td>CT-F ACAGGAATGATCTGTTTGAGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT-R ATACCATCATATATATGGGAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Fukushima et al. 2003; Walker et al. 2005)</td>
<td></td>
</tr>
<tr>
<td><strong>Enteropathogenic Escherichia coli</strong> (EPEC)**</td>
<td>eaeA (E. coli attaching and effacing)</td>
<td>Eae a ATGCTTGAATGCTGGTTTAGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eae b GCCCCCCATCATATGGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Fukushima et al. 2003)</td>
<td></td>
</tr>
<tr>
<td><strong>Enterohemorrhagic Escherichia coli (EHEC)</strong></td>
<td>stx1 (shiga toxin 1)</td>
<td>JMS1F GTCACAGTAACACCGTAACCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>JMS1R TCGTGGACTACTTTATCTGGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Fukushima et al. 2003)</td>
<td></td>
</tr>
<tr>
<td><strong>Enterohemorrhagic Escherichia coli (EHEC)</strong></td>
<td>stx2 (shiga toxin 2)</td>
<td>JMS2F CGACCCCTCTTGAAACATA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>JMS2R GATAACATCAAGCCTCGGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Fukushima et al. 2003)</td>
<td></td>
</tr>
<tr>
<td><strong>Enterotoxigenic Escherichia coli (ETEC)</strong></td>
<td>LT (heat-labile enterotoxin)</td>
<td>LT-1 AGCACATTTCCACCGGATCACC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LT-2 GTGCTGATATTTGGGTTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Fukushima et al. 2003)</td>
<td></td>
</tr>
<tr>
<td><strong>Enterotoxigenic Escherichia coli (ETEC)</strong></td>
<td>ST (heat-stable enterotoxin)</td>
<td>ST_f GCTAAACCCAGYAGRGTCTTCAAAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ST_rev CCCGGTACARGCAGGATTACAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Liu et al. 2013)</td>
<td></td>
</tr>
</tbody>
</table>
**Supplementary Table S2** Fecal short-chain fatty acid (SCFA) composition over the time of the iron-fortification trial

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Time point</th>
<th>SCFA (µmol/g)</th>
<th>Acetate (µmol/g)</th>
<th>Propionate (µmol/g)</th>
<th>Butyrate (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>+FeMNP</strong></td>
<td>baseline</td>
<td>96.1 ± 1.6</td>
<td>76.9 ± 1.4</td>
<td>12.4 ± 1.8</td>
<td>6.9 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>3 weeks</td>
<td>107.6 ± 1.6</td>
<td>83.3 ± 1.4</td>
<td>15.0 ± 1.7</td>
<td>8.8 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>106.2 ± 1.5</td>
<td>75.0 ± 1.3</td>
<td>19.4 ± 1.6</td>
<td>11.8 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>baseline</td>
<td>104.2 ± 1.5</td>
<td>83.0 ± 1.3</td>
<td>14.0 ± 1.5</td>
<td>7.2 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>3 weeks</td>
<td>110.3 ± 1.5</td>
<td>85.7 ± 1.3</td>
<td>14.4 ± 1.5</td>
<td>10.1 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>105.3 ± 1.6</td>
<td>74.5 ± 1.4</td>
<td>17.8 ± 1.8</td>
<td>13.0 ± 1.7</td>
</tr>
<tr>
<td><strong>-FeMNP</strong></td>
<td>baseline</td>
<td>104.2 ± 1.5</td>
<td>83.0 ± 1.3</td>
<td>14.0 ± 1.5</td>
<td>7.2 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>3 weeks</td>
<td>110.3 ± 1.5</td>
<td>85.7 ± 1.3</td>
<td>14.4 ± 1.5</td>
<td>10.1 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>105.3 ± 1.6</td>
<td>74.5 ± 1.4</td>
<td>17.8 ± 1.8</td>
<td>13.0 ± 1.7</td>
</tr>
<tr>
<td><strong>ALL</strong></td>
<td>baseline</td>
<td>99.7 ± 1.6</td>
<td>79.6 ± 1.4</td>
<td>13.1 ± 1.7</td>
<td>7.0 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>3 weeks</td>
<td>108.8 ± 1.5</td>
<td>84.6 ± 1.4</td>
<td>14.8 ± 1.6</td>
<td>9.4 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>4 months</td>
<td>105.7 ± 1.6</td>
<td>74.8 ± 1.4</td>
<td>18.6 ± 1.7</td>
<td>12.4 ± 1.7</td>
</tr>
</tbody>
</table>

Data are geometric means ±SD. There were no differences between +FeMNP and -FeMNP using GLM with baseline as covariate and p <0.05. In all infants, propionate (p=0.004) and butyrate (p=0.0001) increased significantly from baseline to endpoint using paired t-test; this was also seen in +FeMNP (propionate (p=0.029) and butyrate (p=0.022) and to some extent in -FeMNP (propionate (p=0.070) and butyrate (p=0.002)).
Supplementary Table S3: Hematological measurements and anthropometrics performed in study infants.

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Time point (mo)</th>
<th>Hb (g/L)</th>
<th>ZPP (µmol/mol heme)</th>
<th>SF (µg/L)</th>
<th>sTfR (mg/L)</th>
<th>CRP (mg/L)</th>
<th>Hepcidin (nM)</th>
<th>Body iron (mg/kg)</th>
<th>Weight (kg)</th>
<th>Length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2.5mg FeMNP</td>
<td>0</td>
<td>104.4 ± 10.6</td>
<td>90.5 ± 1.6</td>
<td>31.3 ± 1.9</td>
<td>8.6 ± 1.2</td>
<td>4.4 ± 1.5</td>
<td>3.3 ± 1.3</td>
<td>2.67 ± 1.0</td>
<td>7.2 ± 1.2</td>
<td>64.7 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>100.6 ± 10.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.2 ± 1.1</td>
<td>70.1 ± 1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>102.7 ± 10.7</td>
<td>94.0 ± 1.8</td>
<td>19.8 ± 1.6</td>
<td>9.6 ± 1.2</td>
<td>3.7 ± 2.0</td>
<td>2.5 ± 1.3</td>
<td>0.91 ± 1.0</td>
<td>8.7 ± 1.1</td>
<td>73.8 ± 1.0</td>
</tr>
<tr>
<td>-2.5mg FeMNP</td>
<td>0</td>
<td>105.4 ± 10.6</td>
<td>109.0 ± 1.8</td>
<td>25.8 ± 1.7</td>
<td>9.5 ± 1.2</td>
<td>3.4 ± 1.5</td>
<td>2.0 ± 1.2</td>
<td>1.98 ± 1.0</td>
<td>7.4 ± 1.1</td>
<td>65.2 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>103.1 (± 10.5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.4 ± 1.1</td>
<td>69.5 ± 1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>103.8 ± 10.7</td>
<td>111.9 ± 1.7</td>
<td>22.4 ± 1.6</td>
<td>11.2 ± 1.4</td>
<td>3.0 ± 1.4</td>
<td>2.1 ± 1.2</td>
<td>1.00 ± 1.0</td>
<td>8.7 ± 1.1</td>
<td>73.7 ± 1.1</td>
</tr>
<tr>
<td>+12.5mg FeMNP</td>
<td>0</td>
<td>96.0 ± 10.7</td>
<td>132.9 ± 1.9</td>
<td>29.0 ± 2.0</td>
<td>10.1 ± 1.2</td>
<td>3.2 ± 1.3</td>
<td>2.1 ± 1.2</td>
<td>1.98 ± 1.0</td>
<td>7.1 ± 1.1</td>
<td>63.1 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>109.9 ± 10.7</td>
<td>87.6 ± 1.8*</td>
<td>33.7 ± 1.7*</td>
<td>8.75 ± 1.2*</td>
<td>2.8 ± 1.3</td>
<td>3.5 ± 1.3</td>
<td>3.28 ± 1.0*</td>
<td>8.4 ± 1.2</td>
<td>70.2 ± 1.1*</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-12.5mg FeMNP</td>
<td>0</td>
<td>102.7 ± 10.8</td>
<td>113.6 ± 1.1</td>
<td>36.0 ± 2.1</td>
<td>10.7 ± 1.2</td>
<td>2.8 ± 1.3</td>
<td>2.5 ± 1.2</td>
<td>2.68 ± 1.0</td>
<td>7.0 ± 1.1</td>
<td>63.7 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>106.5 ± 10.7</td>
<td>101.7 ± 2.2*</td>
<td>21.9 ±1.6*</td>
<td>11.5 ± 1.2*</td>
<td>3.9 ± 1.5</td>
<td>2.0 ± 1.3</td>
<td>0.5 ± 1.0*</td>
<td>8.3 ± 1.2</td>
<td>68.1 ± 1.1</td>
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<td>6</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are geometric means ±SD. There were no differences at baseline using independent samples t-tests. *significant treatment effects (between iron and control of the same MNP, ±2.5mgFeMNP and ±12.5mgFeMNP) using GLM with baseline as covariate and p < 0.05.
References Supplementary Tables


Supplementary Figures

Supplementary Figure S1 Alpha diversity of the infant gut microbiome over the time course of the trial in the pooled groups (+FeMNP and -FeMNP). Phylogenetic diversity was not influenced by +FeMNP intervention, but increased significantly over time (p = 0.004 for baseline to 3 weeks and p=0.005 for 3 weeks to 4 months). Boxplots are displayed with the 10-90th percentiles.
Supplementary Figure S2 Changes over time from baseline to endpoint in gut microbiome composition of infants in the -FeMNP group. Nodes represent taxa; edges link the different taxonomic levels. The fold increase is calculated as the 2log of the ratio of the relative abundance at the age of ten months old and six months (0=no difference between baseline and endpoint, 1=twice as abundant at endpoint). The significance is expressed as the p value of a Mann-Whitney U test. The node-size corresponds to the relative abundance. In this explorative analysis, the significance is expressed as the p value of a Mann-Whitney U test. The node-size corresponds to the relative abundance. Taxa (that is, nodes) were included in this visualization if they met the following criteria: all samples together have an average relative abundance of > 0.1% for the taxon and the sample groups have a fold-difference of at least 0.5 with a significance of p < 0.05 or the taxon has a child (that is, more specific taxonomic classification) meeting the criteria.
CONCLUSIONS AND PERSPECTIVES

The overall aim of this thesis was to explore efficacious and safe ways to improve the iron status of Sub-Saharan African infants. More specifically we aimed to: (1) investigate the determinants of hepcidin in Kenyan infants with a high burden of both iron deficiency and infection; (2) in a one-year RCT, assess the efficacy of in-home fortification using a low dose iron-containing MNP on iron status and growth in infants; (3) explore the effect of a low and a high dose iron containing MNPs on infants gut microbiota and metabolites, gut inflammation and morbidity.

In a cross-sectional study in 6 month-old rural Kenyan infants, we investigated parameters determining serum hepcidin-25 concentrations. There were high rates of inflammation (30.7%, likely due to infection), anemia (70.5%) and iron deficiency (25.7%) among the infants. The regression analysis identified both iron status (SF, sTfR, ZPP) and inflammation (CRP and IL-6) as significant predictors for serum hepcidin-25, while biomarkers of gut inflammation (fecal calprotectin, IL-12, and IL-17) were not significant predictors. We thus conclude that hepcidin regulatory pathways seen in adults, driven by iron status and inflammation/infection, are already functional in 6 month-old infants.

Serum hepcidin-25 concentrations in iron deficient anemic infants with and without inflammation/infection differed significantly but only marginally, suggesting stimuli that reduce hepcidin (iron status, hypoxia and erythropoietic stimuli) predominate over stimuli that increase it (inflammation/infection) in this population. Therefore, we suggest more research is needed before serum hepcidin can be used as a single point-of-care biomarker (Prentice et al. 2012) providing sufficient discriminatory power to target iron interventions to non-infected African infants. We showed that serum hepcidin-25 values in girls were significantly higher than in boys (7.2 ng/mL vs. 4.9 ng/mL); and propose gender-specific reference values for serum hepcidin-25 in iron-replete infants without inflammation (6.4 ng/mL, girls: 9.0 ng/mL, boys: 4.1 ng/mL), using a WCX-TOF MS method (Kroot et al. 2010).

This study contributes to the understanding of serum hepcidin regulation in infants. However, it is difficult to compare serum hepcidin-25 values from studies using different hepcidin assays; which generate widely differing values. Although two inter-laboratory comparisons have been conducted, without use of the standardized
HEPCON1 values for all laboratories and methods, it is impossible to compare serum hepcidin data among studies (Kroot et al. 2009, 2012). Improved standardization should be a focus of future research.

In the one-year RCT, we investigated the effects of a maize porridge fortified with either an MNP including 2.5 mg iron as NaFeEDTA (+2.5mgFeMNP) or the same MNP without iron (-2.5mgFeMNP) on iron status, inflammation, morbidity and growth in Kenyan infants. The low dose of iron, which was expected to be highly bioavailable (Troesch et al. 2011), did not reduce anemia prevalence nor improve iron status compared to the MNP without iron. We suggest this lack of efficacy may be due to a combination of low iron absorption, due to the high infection prevalence, and the low iron dose provided. This conclusion is supported by findings from a recent study in Kenyan preschool children with a 9-fold lower prevalence of infections, where the same dose of 2.5 mg iron as NaFeEDTA significantly reduced IDA (Macharia-Mutie et al. 2012). Also, a higher dose (12.5 mg iron as ferrous fumarate) given for only 4 months significantly improved iron status in our study population (manuscript 3).

After one year, iron fortification of complementary food using +2.5mgFeMNP increased infant weight compared -2.5mgFeMNP. Previous studies using iron fortification and supplementation have shown inconsistent results on child growth. A meta-analysis, suggested a trend for an increase in weight through iron supplementation in children from malarial endemic areas (Sachdev et al. 2006). There are several possible explanations for this higher weight in the +2.5mgFeMNP after the one year study, such as: (A) differences in feeding practices, however this study was randomized and we do not expect this; (B) EDTA increasing zinc bioavailability, improving zinc status and increasing weight (Brown et al. 2009); or (C) by increasing the population of short-chain fatty acid producing gut bacteria and the subsequent energy extraction from their metabolites (McNeil 1984).

In-home iron fortification with MNPs containing 12.5 mg iron/day effectively reduce anemia rates in infants, but they increase severe diarrhea in Pakistani infants (Soofi et al. 2013), and hospital admissions in Ghanaian infants (Zlotkin et al. 2013). These findings are concerning and suggest the need to reassess the risk-benefit ratio of untargeted high dose iron MNPs in infants in developing countries. In our one-year RCT using a lower dose of iron (2.5 mg NaFeEDTA), we found no difference in the prevalence
of diarrhea, malaria and respiratory tract infections between the two treatment groups, although the study was not powered for clinical outcomes.

A recent controlled study of food fortification with a high dose iron (20 mg/d, 4 d/wk) in Ivorian schoolchildren reported changes the gut microbiota towards a more pathogenic profile and an increase in gut inflammation (Zimmermann et al. 2010). To further investigate the effects of iron fortificants on the gut microbiota, we studied the effects of two widely used low and high dose iron-containing MNPs in Kenyan infants. We hypothesized that iron fortification would: a) increase fecal enterobacteria and their ratio to bifidobacteria and/or lactobacilli; b) favor colonization by other potentially pathogenic strains; and c) increase gut inflammation.

To test these hypotheses we performed two RCTs in 6 month-old Kenyan infants. Participants received a maize porridge fortified with either: a) an MNP containing 2.5 mg iron as NaFeEDTA (+2.5mgFeMNP) b) the identical MNP without iron (-2.5mgFeMNP) c) an MNP containing 12.5 mg iron as ferrous fumarate (+12.5mgFeMNP), d) the identical MNP without iron (-12.5mgFeMNP), daily for 4 months. Iron status (SF, sTfR, ZPP, and body iron stores) was significantly increased by +12.5mgFeMNP, but not by +2.5mgFeMNP. Using pyrosequencing, +FeMNPs (pooled +2.5mgFeMNP and +12.5mgFeMNP) increased the enterobacteria, particularly Escherichia/Shigella, the enterobacteria to bifidobacteria ratio, and Clostridium compared to -FeMNPs. Most of these effects were confirmed using qPCR; e.g., +FeMNPs increased pathogenic E. coli strains. +FeMNPs also increased fecal calprotectin. During the trial, 27.3% of infants in +12.5mgFeMNP required treatment for diarrhea versus 8.3% in -12.5mgFeMNP. There were no study-related serious adverse events in either group.

Comparing the effects in the two +FeMNPs separately, the low dose iron MNP (+2.5mgFeMNP) had a stronger effect on the increase of Escherichia/Shigella, the pathogenic E. coli abundance and the ratio of enterobacteria to bifidobacteria, whereas the high dose iron MNP (+12.5mgFeMNP) resulted in lower levels of Bifidobacterium, higher levels of fecal calprotectin and perhaps higher rates of diarrhea episodes that needed treatment. Therefore, our data do not clarify whether the lower dose iron MNP would have a better safety profile, although this remains a possibility that should be tested in future research.
Our study was the first controlled study to examine the effect of +FeMNPs on the African infant gut microbiome and our findings need confirmation in other settings and populations. Both IDA and diarrhea are major causes of morbidity and mortality in infants in developing countries. In-home fortification with +FeMNPs has shown to reduce IDA rates in infants and children, but whether they increase diarrheal disease remains uncertain. WHO recommends the use of MNPs containing iron, vitamin A, and zinc for children aged 6-23 months irrespective of their iron status in settings with an anemia prevalence of >20% and in conjunction with measures to treat malaria, as well as to promote overall sanitation and management of diarrhea (WHO 2011). At the same time, WHO recommends iron supplementation only to be targeted to infants with IDA, while providing adequate protection from malaria (Fontaine 2007). Our findings, together with others (Soofi et al. 2013; Zlotkin et al. 2013), suggest that, until safer formulations are available, this latter recommendation may also be a prudent strategy for +FeMNPs: in areas with high infectious disease burden, they could be targeted to infants with IDA, while providing concurrent monitoring and treatment of malaria and diarrhea. A priority for future research is to develop +FeMNP formulations, and other in-home strategies such as crushable tablets or fat-based spreads, which are both effective and safe.

We are now planning a follow-up study in Kenyan infants from the same study area, in which we will evaluate the safety and efficacy of in-home fortification with an iron-containing MNP that includes a prebiotic. The findings from this study will elucidate if the addition of a prebiotic to an iron-containing MNP can favor selection of beneficial commensal barrier bacteria, such as bifidobacteria, and thereby balance the potential iron-mediated detrimental effects on the gut microbiota and gut inflammation.
References

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