PREVENTION AND TREATMENT OF IRON DEFICIENCY ANEMIA IN WOMEN AND CHILDREN: IRON HOMEOSTASIS AND OPTIMIZING ABSORPTION USING PREBIOTICS AND BREAST MILK COMPONENTS

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“It’s all a question of imagination. Our responsibility begins with the power to imagine.”

Haruki Murakami

dedicated to my grandma

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ABBREVIATIONS

2′-FL	2′-fucosyllactose
3′-FL	3′-fucosyllactose
3′-SL	3′-sialyllactose
^{54}Fe	Iron 54, with 26 protons and 28 neutrons
^{56}Fe	Iron 56, with 26 protons and 30 neutrons
^{57}Fe	Iron 57, with 26 protons and 31 neutrons
^{58}Fe	Iron 58, with 26 protons and 32 neutrons
6′-SL	6′-sialyllactose
AAS	Atomic absorbance spectrometer
ABCB10	ATP-binding cassette subfamily B member 10
AGP	α-1-acid glycoprotein
AI	Adequate intake
ALA	5-aminolevulinate
APP	Acute phase protein
bLf	Bovine lactoferrin
BMI	Body mass index
BMP6	Bone morphogenic protein 6
BW	Body weight
CLA	Conjugated linoleic acid
CRP	C-reactive protein
CV	Coefficient of variation
DCytB	Duodenal cytochrome B
DFL	Difucosyllactose
DMT1	Divalent-metal transporter 1
DP	Degree of polymerization
DRI	Dietary reference intake
DSLNT	Disialyllacto-N-tetraose
EAR	Estimated average requirement
EIU	Erythrocytes iron utilization
Epo	Erythropoietin

ERFE	Erythroferrone
FDA	Food and drug administration
Fe	Iron
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
Fe_{abs}	Iron absorbed daily
Fe_{circ}	Circulating iron
FeFum	Ferrous fumarate
Fe_{gain}	Net iron gained daily
Fe_{loss}	Iron lost daily
Fe_{nca}	Non-circulating active iron
FePP	Ferric pyrophosphate
$FeSO_4$	Ferrous sulfate
Fe_{stor}	Iron in storage
FIA	Fractional iron absorption
FLVCR1	Feline leukemia virus subgroup C receptor 1 b
FOS	Fructo-oligosaccharides
FPN	Ferroportin
FTH1	Ferritin heavy chain
FTL	Ferritin light chain
FTN	Ferritin
Fuc	Fucose
FUT2	Fucosyltransferase 2
FUT3	Fucosyltransferase 3
Gal	Galactose
Glc	Glucose
GlcNAc	N-acetylglucosamine
GOS	Galacto-oligosaccharides
GRAS	Generally recognized as safe
Hb	Hemoglobin
HEP	Hepcidin
HFE	Human hemochromatosis protein

HIF	Hypoxia-inducible factor
HJV	Hemojuvelin
HM	Human milk
HMOs	Human milk oligosaccharides
HMOX1	Heme oxygenases 1
HMOX2	Heme oxygenases 2
Hp	Haptoglobin
Hpx	Hemopexin
IAEA	International Atomic Energy Agency
ICP-MS	Inductively-coupled mass spectrometry
ID	Iron deficiency
IDA	Iron deficiency anemia
IOM	Institute of Medicine
IRE	Iron-response element
IRP	Iron regulatory protein
IS	Internal standard
ISC	Iron–sulphur clusters
JAK1	Janus kinase 1
k_{abs}	rate of change of body iron
k_{loss}	rate of decrease in body iron
LAZ	Length-for-age Z-score
Lf	Lactoferrin
LfR	Lactoferrin receptor
LIP	Labile iron pool
LNDFH	Lacto-N-difucohexaose
LNFP	Lacto-N-fucopentaose
LNnT	Lacto-N-Neotetraose
LNT	Lacto-N-tetraose
LOQ	Limit of quantification
MCV	Mean corpuscular volume
MFRN1	Mitoferrin 1
MFRN2	Mitoferrin 2

MI	Mutual information
MNP	Micronutrient powder
MOS	Mannanoligosaccharide
NaFeEDTA	Sodium iron ethylenediaminetetraacetic acid
NCOA4	Nuclear receptor coactivator 4
NRAMP1	Natural resistance-associated macrophages protein 1
NTBI	Non-trasferrin-bound-iron
PBG	Porphobilinogen
PCBP1	Poly(rC)–binding protein 1
PF	Plasma ferritin
PPIX	Protoporphyrin IX
Pro-E	Proerythroblasts
PSC	Pre-school children
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
RBV	Relative bioavailability
RDA	Recommended dietary allowance
RES	Reticuloendothelial system
RNA	Ribonucleic acid
RNI	Recommended nutrient intake
SCFAs	Short-chain fatty acids
SD	Standard deviation
SF	Serum ferritin
Sia	Sialic acid
SMAD	Sons of mothers against decapentaplegic
SNR	Signal-to-noise ratio
STAT	Signal transducer and activator of transcription
STEAP3	Six-transmembrane epithelial antigen of prostate 3
sTfR	Soluble transferrin receptor
Tf	Tranferrin
TfR1	Transferrin-receptor 1
TfR2	Transferrin-receptor 2

Th	T-helper cells
TIBC	Total iron-binding capacity
TIMS	Thermal ionization mass spectrometry
TSAT	Transferrin saturation
UL	Tolerable upper intake level
UTR	Untranslated regions
WAZ	Weight-for-age Z-score
WHO	World Health Organization
WLZ	Weight-for-length Z-score
WRA	Women of reproductive age
xCGE-LIF	Multiplexed capillary-gel-electrophoresis with laser-induced-florescence detection
XOS	Xylooligosaccharide
YLDs	Years lived with disability
ZnPP	Zinc protoporphyrin

SUMMARY

Background

One of the World Health Organization (WHO) global targets by 2025 aims at achieving a relative reduction of 50% of the number of non-pregnant women of reproductive age affected by anemia (1). Although several programs address its prevention and control, anemia still represents a major public health problem, especially in infants, children, and women of reproductive age (2). Iron deficiency accounts for more than half of the anemia cases, and optimizing its intake represents the main target of the nutrition-specific solutions proposed by the WHO (3). The success of nutritional plans currently in place is restricted by various technical, physiological, and socio-economical constraints. These include the difficulties in finding appropriate food vehicles to use as fortificants (4), poor iron absorption from oral iron supplements and fortificants (5), limited access to iron-rich food (6), and the occurrence of side effects when iron intake is increased (7). Additionally, a few studies have reported an increase in physiological iron losses with iron supplementation (8,9), which could further limit the efficacy of nutritional interventions.

Objectives

The overall goal of the studies included in this thesis was to implement and examine existing strategies, potentially laying the foundation for new approaches to the prevention and treatment of iron-deficiency anemia in vulnerable population groups. Specifically, we aimed to: I) confirm whether iron losses occur during iron supplementation in infants, quantify their magnitude, and provide insights on the underlying mechanism (**Manuscript 1**); II) determine if the enhancing effect of galacto-oligosaccharides (GOS) on iron absorption is consistent at different prebiotics or iron dosages than what was reported previously, and to assess whether other prebiotics have similar enhancing effects on iron absorption (**Manuscripts 2&3**); and III) verify if the addition of GOS and/or bovine lactoferrin (bLf) to an iron fortificant would lead to a greater improvement in iron and anemia status when compared to the effect of the fortificant alone (**Manuscript 4**).

Methods

In equilibrated subjects, where the isotopic signature is considered equal in all body compartments, the decrease in the concentration of the tracer in circulating blood is proportional to the quantity of iron absorbed and the decrease in the amount of the tracer is proportional to the quantity of iron lost (10). In Manuscript 1, we assessed daily iron balance over 3 months of iron supplementation in Kenyan toddlers using the stable iron isotopic dilution methodology. Additionally, we estimated heme/porphyrin fecal losses with an HemoQuant assay based on fluorescence quantitation, and we assessed lower and upper gastrointestinal tract inflammation by measuring fecal calprotectin and intestinal fatty acid-binding protein, respectively, with two different ELISA kits. We used Pearson's correlation to identify potential relationships between daily iron doses administered, median daily iron absorbed and median daily iron lost per kg body weight in all published studies using the stable iron isotope dilution methodology (8–11).

The effect of a single administration of the prebiotics GOS, fructo-oligosaccharides (FOS), and acacia gum on acute iron absorption in adults (Manuscript 2), and GOS and human milk oligosaccharides (HMOs) in infants (Manuscript 3) was determined in two cross-over randomized controlled trials using the erythrocyte iron incorporation method with stable iron isotopes. The amounts of isotopic tracers in whole blood after the administration of the supplement formulations were calculated based on the shift in iron isotope ratios and on the estimated amount of iron circulating in the body. For the calculation of fractional iron absorption, an 80% erythrocyte isotope incorporation of the absorbed iron was assumed (12). Furthermore, in Manuscript 3 we assessed the influence of maternal HMO profile on fractional iron absorption in infants. The HMO composition of the human milk samples was determined by capillary-gel-electrophoresis with laser-induced-florescence detection.

To establish the effect of the addition of GOS and bLf, alone or in combination, to an iron fortificant on iron, anemia, and inflammation status, we performed a randomized controlled clinical trial with a 2-by-2 factorial design (Manuscript 4). Biomarkers of iron (hemoglobin, plasma ferritin, soluble transferrin receptor) and inflammation status (C-reactive protein (CRP), alpha-1-acid glycoprotein (AGP)) were measured in plasma

samples using a multiplex immunoassay (13). Anemia was defined as hemoglobin < 11 g/L, and iron deficiency as plasma ferritin < 12 µg/L or/and soluble transferrin receptor > 8.3 mg/l. Systemic inflammation was defined as CRP > 5 mg/L or/and AGP > 1g/L, respectively.

Results

In Manuscript 1, more than half of the quantity of absorbed iron was lost (57%) over 3 months of iron supplementation in Kenyan infants. Contrary to our initial hypothesis, there were no measurable increases in the heme/porphyrin losses during the supplementation period and no changes in the biomarkers of intestinal health. We observed a strong significant correlation between daily median iron absorbed and lost per kg body weight from all studies using the stable iron isotope dilution methodology to assess iron balance.

In Manuscripts 2 and 3, the single administration of the prebiotic GOS acutely and consistently enhanced iron absorption from iron fumarate, when administered with a high supplemental iron dose in iron-depleted women (Manuscript 2), and when added in low dosage to an iron-fortified maize porridge in Kenyan infants (Manuscript 3). Single FOS administration in iron-depleted women led to an increase in iron absorption similar to that of GOS (Manuscript 2). No effect on iron absorption from acacia gum (Manuscript 2) and HMOs (Manuscript 3) administration was detected, and the HMO maternal profile did not predict the iron absorption from iron-fortified maize porridge in infants (Manuscript 3).

After 6 months of daily fortification with a low dose iron-micronutrient powder (MNP) alone, or with individual or combined provision of GOS and Lf, all treatments were associated with an increase in Hb and a decrease in the prevalence of anemia and iron deficiency anemia, but there was no effect on iron status and iron deficiency prevalence, in Kenyan infants (Manuscript 4). When compared to the same MNP with iron alone, or with GOS and bLf added individually, the co-provision of GOS and bLf induced a further improvement in Hb concentration, and anemia and iron-deficiency anemia prevalence (Manuscript 4).

Conclusions

Since estimated losses in Kenyan infants agree with results from earlier supplementation studies in labelled Gambian infants and Swiss women, the results from Manuscript 1 provide additional evidence that iron supplementation increases iron losses. We did not detect a clear pattern of increased heme/porphyrin loss during supplementation. Notably, we found a strong positive correlation between the median daily iron lost and the amount of iron absorbed in our and previously published trials. These findings suggest that the amount of iron lost increases based on the amount of iron absorbed, rather than on the dose of iron given. The pathophysiological mechanism responsible for our observations is yet to be defined but, if confirmed, an increase in iron losses during iron supplementation will need to be considered when implementing iron interventions.

In iron-depleted women (Manuscript 2) and infants (Manuscript 3), GOS was confirmed as an acute enhancer of iron absorption, both from high and low doses of iron fumarate, and when given as a fortificant at a low dosage. In women, FOS, but not acacia gum, may also represent a promising new enhancer of supplemental iron absorption (Manuscript 2). In infants, a single administration of HMOs did not affect iron absorption (Manuscript 3), and the maternal HMO profile did not predict iron absorption. These findings argue against a major role of a prebiotic-dependent decrease in colonic pH as the basis for the prebiotic enhancement of iron absorption.

The findings from Manuscript 4 suggest that the addition of GOS and bLf in combination could be a successful approach to enhance the efficacy of in-home iron fortification programs, in areas where anemia represents a public health problem. We reported that in Kenyan infants where the prevalence of anemia was above 50%, the daily administration of low-dose iron-containing MNPs successfully decreased anemia and iron-deficiency anemia prevalence, but the reduction was steeper with co-fortification with GOS and bLf. These results, when combined with data on the effect of the same MNPs formulations on morbidity, intestinal inflammation, and microbiota composition, could provide comprehensive evidence on the efficacy and safety of the addition of these two components to iron-containing MNPs.

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RIASSUNTO

Contesto

Uno dei “Global targets” dell'Organizzazione Mondiale della Sanità (OMS) entro il 2025 si prefigge di raggiungere una riduzione relativa del 50% nel numero di donne in età riproduttiva, non gravide, affette da anemia (1). Sebbene esistano innumerevoli programmi volti al suo controllo e alla sua prevenzione, l'anemia rappresenta ancora un significativo problema per la salute pubblica, soprattutto nei neonati, nei bambini e nelle donne in età riproduttiva (2). La carenza di ferro è responsabile di oltre la metà dei casi di anemia, e l'ottimizzazione del suo apporto è l'obiettivo principale delle varie strategie nutrizionali proposte dall'OMS (3). Tuttavia, il successo delle strategie attualmente in atto è limitato da vari fattori tecnici, fisiologici e socioeconomici. Tra questi, la difficoltà di reperire cibi e matrici alimentari appropriate da utilizzare come fortificanti (4), lo scarso assorbimento fisiologico del ferro fornito da integratori e fortificanti orali (5), l'accesso limitato agli alimenti naturalmente ricchi di ferro (6), e la comparsa di effetti collaterali quando l'assunzione di ferro aumenta (7). Inoltre, alcuni studi hanno riportato un aumento delle perdite fisiologiche di ferro con l'integrazione di ferro (8,9), che potrebbero limitare ulteriormente l'efficacia degli interventi nutrizionali.

Obiettivi

L'obiettivo generale degli studi inclusi in questa tesi è stato quello di implementare ed esaminare le strategie esistenti, e potenzialmente di gettare le basi per nuovi approcci per la prevenzione e il trattamento dell'anemia da carenza di ferro in soggetti vulnerabili. In particolare, ci siamo proposti di: I) confermare se si verificano perdite di ferro durante un periodo di supplementazione negli infanti, quantificarne eventualmente l'entità e fornire indicazioni sul meccanismo responsabile (Manoscritto 1); II) determinare se l'incremento dell’assorbimento del ferro ad opera dei galacto-oligosaccaridi (GOS) persiste a diversi dosaggi, sia del prebiotico che del ferro, rispetto a quanto riportato in precedenza, e valutare se altri prebiotici hanno effetti simili sull'assorbimento del ferro (Manoscritto 2&3); e III) verificare se l'aggiunta di galacto-oligosaccaridi e/o lattoferrina bovina a un

fortificante di ferro porti ad un ulteriore miglioramento dei livelli di ferro e una riduzione nella prevalenza dell'anemia, rispetto all'effetto del fortificante da solo (Manoscritto 4).

Metodi

Nei soggetti in equilibrio, ovvero in cui la distribuzione isotopica è considerata uguale in tutti i compartimenti corporei, la diminuzione della concentrazione del tracciante nella circolazione sanguigna è proporzionale alla quantità di ferro assorbito, e la diminuzione della quantità del tracciante è proporzionale alla quantità di ferro perso (10). Nel Manoscritto 1, abbiamo valutato il bilancio giornaliero del ferro in 3 mesi di supplementazione in bambini kenioti utilizzando la metodologia della diluizione degli isotopi stabili del ferro. Inoltre, abbiamo stimato le perdite fecali di eme/porfirina con un HemoQuant test, basato sulla quantificazione della fluorescenza, e abbiamo valutato l'infiammazione del tratto gastrointestinale inferiore e superiore misurando la calprotectina fecale e la proteina intestinale legante gli acidi grassi, rispettivamente, con due diversi kit ELISA. Abbiamo utilizzato la correlazione di Pearson per identificare le potenziali relazioni tra le dosi giornaliere di ferro somministrate, la mediana del ferro giornaliero assorbito e la mediana del ferro giornaliero perso per kg di peso corporeo in tutti gli studi pubblicati utilizzando la metodologia della diluizione degli isotopi stabili del ferro (8–11).

L'effetto di una singola somministrazione di prebiotici GOS, di fruttoligosaccaridi (FOS) e gomma arabica sull'assorbimento acuto del ferro negli adulti (Manoscritto 2) e di GOS e oligosaccaridi del latte umano (HMO) negli infanti (Manoscritto 3) è stato determinato in due studi cross-over, randomizzati e controllati, utilizzando il metodo di incorporazione del ferro negli eritrociti con isotopi stabili del ferro. Le quantità di traccianti isotopici nel sangue dopo la somministrazione delle varie formulazioni di integratori sono state calcolate in base alla variazione dei rapporti fra i vari isotopi del ferro e alla quantità stimata di ferro circolante nell'organismo. Per il calcolo dell'assorbimento relativo del ferro, è stata ipotizzata un'incorporazione isotopica del ferro assorbito negli eritrociti pari all'80% (12). Inoltre, nel Manoscritto 3 abbiamo valutato l'influenza del profilo oligosaccaridico nel latte materno sull'assorbimento relativo del ferro negli infanti. La composizione oligosaccharidica nei campioni di latte umano è stata determinata mediante elettroforesi su gel capillare con rilevamento a fluorescenza indotta da laser.

Per stabilire l'effetto sui livelli di ferro, anemia e infiammazione dell'aggiunta di GOS e lattoferrina bovina (bLf), da soli o in combinazione, a un fortificante a basse dosi di ferro, abbiamo svolto uno studio clinico controllato e randomizzato con un disegno fattoriale 2 per 2 (Manoscritto 4). I marcatori del ferro (emoglobina, ferritina plasmatica, recettore solubile della transferrina) e dello stato infiammatorio (proteina C-reattiva (PCR), alfa-1-glicoproteina acida (AGP)) sono stati misurati in campioni di plasma utilizzando un saggio con anticorpi (13). L'anemia è stata classificata come emoglobina < 11 g/L, e la carenza di ferro come ferritina plasmatica < 12 µg/L o/e recettore solubile della transferrina > 8,3 mg/l. L'infiammazione sistemica è stata definita come CRP > 5 mg/L o/e AGP > 1g/L.

Risultati

Nel Manoscritto 1, più della metà del ferro assorbito è stato perso (57%) nel corso dei 3 mesi di supplementazione di ferro degli infanti kenioti. Contrariamente alla nostra ipotesi iniziale, non ci sono stati aumenti misurabili nelle perdite di eme/porfirina durante il periodo di supplementazione, né cambiamenti significativi nei marcatori della salute intestinale. Abbiamo rilevato una forte correlazione significativa fra le mediane del ferro giornaliero assorbito e quella del ferro perso per kg di peso corporeo da tutti gli studi che hanno utilizzato la metodologia della diluizione degli isotopi stabili del ferro per valutare il bilancio del ferro.

Nei Manoscritti 2 e 3, una singola somministrazione dei prebiotici GOS ha aumentato l'assorbimento di ferro dal fumarato di ferro, sia quando è stato somministrato con un'alta dose di ferro supplementare in donne che ne erano carenti (Manoscritto 2), sia quando è stato aggiunto con un basso dosaggio a un porridge di mais fortificato con ferro in infanti kenioti (Manoscritto 3). La singola somministrazione di FOS in donne carenti di ferro ha portato a un aumento dell'assorbimento di ferro simile a quello indotto dai GOS (Manoscritto 2). Non è stato rilevato alcun effetto sull'assorbimento del ferro a seguito della singola somministrazione della gomma arabica (Manoscritto 2) e degli HMOs (Manoscritto 3), e il profilo oligosaccharidico del latte materno non ha predetto l'assorbimento del ferro dal porridge di mais fortificato negli infanti (Manoscritto 3).

Dopo 6 mesi di assunzione giornaliera di un multivitaminico in polvere a basso dosaggio di ferro (MNP) da solo, o con l'aggiunta, singola o combinata, di GOS e Lf, tutte le

formulazioni sono stati associate a un aumento dell'Hb e a una diminuzione della prevalenza di anemia e anemia da carenza di ferro. Tuttavia, non c'è stato alcun effetto del trattamento sui livelli di ferro e sulla prevalenza di carenza di ferro negli infanti kenioti (Manoscritto 4). Rispetto allo stesso MNP con il solo ferro, o con GOS e bLf aggiunti singolarmente, la somministrazione congiunta di GOS e bLf ha determinato un ulteriore miglioramento della concentrazione di Hb e della prevalenza di anemia e anemia da carenza di ferro (Manoscritto 4).

Conclusioni

Considerato che le perdite di ferro stimate negli infanti kenioti sono consistenti con i risultati di precedenti studi sulla supplementazione del ferro svolti in infanti del Gambia e in donne svizzere, i risultati del Manoscritto 1 forniscono un'ulteriore indicazione che la supplementazione di ferro può aumentare le perdite di ferro. Non abbiamo rilevato un pattern chiaro nelle perdite di eme/porfirina durante la supplementazione. Inoltre, abbiamo riscontrato una forte correlazione positiva tra le mediane del ferro perso giornalmente e quello assorbito dai risultati del nostro studio e di quelli pubblicati in precedenza. Questi risultati suggeriscono che la quantità di ferro perso aumenta in base alla quantità di ferro assorbito, invece che alla dose di ferro somministrata. Il meccanismo fisiopatologico responsabile delle nostre osservazioni deve ancora essere definito ma, se confermato, un aumento delle perdite di ferro durante l'integrazione dovrà essere preso in considerazione quando si pianificheranno strategie nutrizionali future.

Nelle donne con carenza di ferro (Manoscritto 2) e negli infanti (Manoscritto 3), i GOS sono stati confermati come sostanze che favoriscono l'assorbimento del ferro, sia da dosi elevate che da dosi ridotte di fumarato di ferro, e se somministrati con un basso dosaggio. Nelle donne, i FOS, ma non la gomma arabica, possono rappresentare promettenti potenziatori dell'assorbimento del ferro da formulazioni orali (Manoscritto 2). Negli infanti, una singola somministrazione di HMO non ha avuto alcun effetto sull'assorbimento del ferro (Manoscritto 3) e il profilo oligosaccaridico del latte materno non ha predetto l'assorbimento del ferro. Questi risultati sembrano declassare il ruolo di un'ipotetica diminuzione del pH del colon dovuta alla fermentazione dei prebiotici come meccanismo esplicativo dell'effetto dei prebiotici.

I risultati del manoscritto 4 suggeriscono che l'aggiunta combinata di GOS e bLf potrebbe rappresentare un approccio utile per il miglioramento dell'efficacia dei programmi di fortificazione del ferro nelle aree in cui l'anemia rappresenta un serio problema di salute pubblica. Infatti, negli infanti kenioti, che riportavano una prevalenza di anemia superiore al 50%, la somministrazione giornaliera di MNP a basso dosaggio di ferro ha ridotto con successo la prevalenza dell'anemia e dell'anemia da carenza di ferro. Tuttavia, la riduzione è stata più marcata con la fortificazione congiunta con GOS e bLf. Questi risultati, se combinati con i dati sull'effetto delle stesse formulazioni di MNP sulla morbosità, sull'infiammazione intestinale e sulla composizione del microbiota, potrebbero fornire prove complete sull'efficacia e sulla sicurezza dell'aggiunta di questi due componenti ai MNP contenenti ferro.

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INTRODUCTION

The ease with which iron can donate or acquire electrons is a major reason for its involvement in a variety of chemical reactions, making iron essential for virtually all forms of life (1). Although a trace element in humans, iron is required at a substantially higher level than that of other transition minerals (2). Nonetheless, due to its high reactivity, iron excess can produce severe damage, and its acquisition and retention are carefully controlled to avoid toxicity (1).

In 1937-8, a series of milestone studies by Widdowson and McCance on iron homeostasis found no evidence for an active iron excretion mechanism and concluded that maintenance of a normal quantity of iron in humans relies on the regulation of iron absorption (3,4). These studies were followed by 60 years of investigations and insights into iron metabolism and its regulation, and eventually led to the discovery of the iron-regulatory hormone, hepcidin, in 1998 (5). Hepcidin expression is reduced by iron deficiency, hypoxia, or increased erythropoietic demand, and is enhanced by iron overload, inflammation, or infection. Through its interaction with ferroportin, the sole known cellular iron export channel, hepcidin regulates the absorption and distribution of body iron (6).

Despite remarkable progress in understanding the regulation of iron metabolism and its role in physiology and disease, iron disorders remain a major public health problem (6). Iron deficiency is the most common nutritional deficiency worldwide and is generally considered to account for approximately 60% of anemia (7). Iron-deficiency anemia can result in a wide variety of adverse consequences, including reduced work capacity, immune dysfunction, negative pregnancy outcomes, and neurocognitive impairment (8). Multiple strategies for improving iron and anemia status have been proposed by the World Health Organization (WHO) and have been implemented in many countries, but their efficacy is greatly limited by a series of technical, socioeconomical and physiological constraints that require further action to be addressed.

The overall aim of this thesis was to evaluate and implement some of the current approaches to the prevention and treatment of iron-deficiency anemia in vulnerable

population groups. The first section of this thesis is a review of the literature that is divided into four chapters. Chapter 1 provides an overview of iron homeostasis. Chapter 2 describes different methods to measure iron status and to assess iron absorption, loss, and balance. Chapter 3 discusses the prevalence, etiology, and consequences of anemia, iron deficiency and iron-deficiency anemia, together with current strategies to manage these conditions. Chapter 4 reviews potential methods to optimize iron absorption from supplements and fortificants. The second section then presents the original research results of this thesis in four manuscripts. Manuscript 1 describes a stable iron isotope study estimating iron absorption and loss during a 3-month period of iron supplementation in Kenyan infants. Manuscripts 2 and 3 present two stable iron isotope trials, one in adults and one in infants, respectively, where the effects on iron absorption of a single administration of selected prebiotics and dietary fibers are quantified. Manuscript 4 describes a 2-by-2 factorial trial investigating the effects on iron, inflammation, and anemia status of the addition of galacto-oligosaccharides and bovine lactoferrin to an iron fortificant given to Kenyan infants daily for 6 months. In the third and final section of this thesis, the main findings of these studies are summarized and interpreted in the context of current knowledge. Overall limitations of the study designs, methods used, and study populations included are discussed. The thesis concludes with a consideration of the potential implications of our studies for future research.

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LITERATURE REVIEW

1. IRON METABOLISM AND HOMEOSTASIS

Iron (Fe) is the most abundant element on earth by mass (1) and it is present in the form of four stable isotopes (^{54}Fe, ^{56}Fe, ^{57}Fe, and ^{58}Fe), whose abundance is highly conserved (2). Due to its unique chemical properties, Fe is essential for almost all living organisms (3). Its ability to gain and lose electrons, switching between the ferrous, Fe^{2+}, and ferric, Fe^{3+} forms, are crucial for oxygen sensing, electron transfer, and catalysis (3). However, its advantageous redox potential promotes the formation of reactive oxygen species that might damage cellular macromolecules and lead to programmed cell death and tissue injury (4). The key to ensuring adequate iron availability for oxygen transport and activity of Fe-dependent enzymes, but to avoid overload (5), is the regulation of iron flux into plasma for transport by the glycoprotein transferrin (Tf) (4). In healthy individuals, Tf is about 30% saturated with iron (6). Transferrin binding maintains iron in a soluble form, limits its toxicity, and is central for cellular iron delivery (7,8). There are no regulated means to actively excrete iron; thus, the maintenance of adequate plasma levels relies on controlling dietary Fe absorption (9) and usage for erythropoiesis, and optimizing recycling (Figure 1) (4).

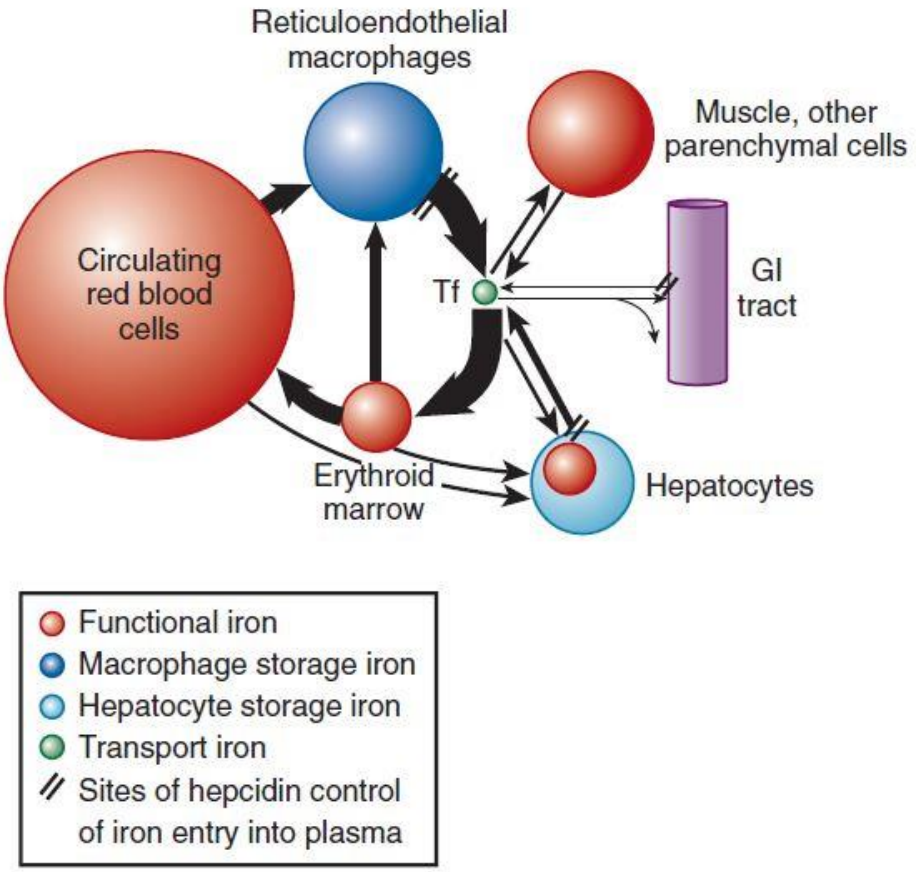

Figure 1: an overview of the routes of iron exchange in adults. The area of each circle is proportional to the amount of iron contained in the compartment, and the width of each arrow is proportional to the daily flow of iron from one compartment to another (4).

1.1 Iron absorption

Overall, dietary iron absorption normally accounts for only about 5% of the influx of iron into the plasma (10), compensating for 1-2 mg/day obligatory iron losses in an adult which mainly occur through sloughing of intestinal epithelial cells, exfoliation from epithelial surfaces, blood loss, or sweat (11). Dietary iron exists as heme or non-heme iron (12), with the non-heme form accounting for the great majority (90 to 95%) of iron supply (13). Heme iron is the most bioavailable source, with absorption ranging between 20–30%, in contrast with the 1–10% for the non-heme forms (13).

1.1.1 Non-heme iron

Inorganic dietary iron is predominantly absorbed in the proximal duodenum, where enterocytes maximize the absorptive surface area through finger-like microvilli at the luminal surface (14). Dietary non-heme iron predominately exists in the oxidized state (Fe^{3+}) and at pH higher than 3 precipitates as unabsorbable polymeric hydroxides (15).

Thus, ferric iron must be reduced either by the acidic gastric pH, chemically by specific enhancers (16), or by the membrane-associated ferrireductase duodenal cytochrome B (DCytB) before traversing the luminal membrane of the enterocytes through the divalent-metal transporter 1 (DMT1) (8,14). Effective iron transport by DMT1 depends on the amount of ferrous iron and on an acid microenvironment in the mucous layer (9), which is preserved by the activity of intestinal Na+/H+ exchangers (17). Once in the enterocytes, iron enters the poorly characterized cytosolic "labile iron pool" (LIP) and is either retained for immediate metabolic utilization, storage in ferritin, or export through the basolateral membrane via ferroportin (SLC40A1, FPN), the sole-known iron transporter (4). Iron release into circulation and loading onto Tf requires the hephastin-mediated oxidation of Fe^{2+} to Fe^{3+} (18). Cellular iron uptake can also occur through non-transferrin-bound-iron (NTBI) pathways mediated by calcium and zinc transporters (19), but evidence suggests that NTBI accumulation becomes prominent only under conditions of iron overload (20).

1.1.2 Heme iron

The acidic stomach pH, coupled with proteolytic digestion, is responsible for heme release from globins (21). The upper-duodenal heme-iron uptake, as well as its catabolism in the enterocyte, is not yet well understood (22). Nevertheless, there are two potential mechanisms hypothesized: 1) the existence of heme carrier proteins, transferring heme-iron intact across plasma membranes (22); 2) receptor-mediated endocytosis, with subsequent catabolism operated by heme oxygenase 1 (21) and export of the released iron to the labile iron pool. The iron is then thought to undergo identical transport into the circulation as for non-heme iron (22), as described above (paragraph 1.1.1).

1.2 Iron utilization

In conditions of iron sufficiency, at any given time, approximately 3 mg of Tf-bound-Fe is present in the adult circulation, but 10 times this amount moves daily through the plasma compartment (4). The uptake of iron from the circulation is mediated by the binding of the Tf-bound form with the ubiquitous Tf-receptor 1 (TfR1). The expression of TfR1 on the surface parallels the maturation of erythroid progenitors (8). The affinity between the TfR1 and its ligand depends on the Tf saturation, i.e. the proportion of iron-binding sites on the molecule occupied by Fe atoms, and pH (23). The internalization of the $Tf\text{-}Fe_2$/TFR1 complex happens through clathrin-coated vesicles (24). The proton pump-mediated

acidification of the endosomes induces structural changes in the Tf-Fe_2/TFR1 complex and consequent release of iron. The freed Fe^{3+} is then reduced to Fe^{2+} by the ferrireductase six-transmembrane epithelial antigen of prostate 3 (STEAP3) and released into the cytosolic LIP through DMT1 (18). The translocation of the Apo-Tf/TfR1 complex to the surface membrane and the exposure to the neutral plasma pH allows the reuse for further cycles of iron-binding and uptake (25) and is crucial for optimal hemoglobin production (6). Alternative pathways of iron uptake have been demonstrated *in-vitro*, with ferritin released from macrophages internalized through pinocytosis in erythroid precursors (26), but these findings are yet to be confirmed *in-vivo*.

1.2.1 Cellular iron storage

Unused iron from the LIP is delivered by the iron chaperone poly(rC)–binding protein 1 (PCBP1) to ferritin (27), a ubiquitous cytosolic heteropolymer made of self-assembled subunits of heavy (FTH1) and light (FTL) ferritin chains (28). The prevalence of each of the two different subunits varies depending on the tissue and the physiologic needs of the cells (29). FTH1 catalyzes the oxidation of Fe^{2+} to Fe^{3+}, whereas FTL lacks oxidase properties but improves structural stability and transfer of electrons across the protein shell of the polymer (30). Iron storage in ferritin (FTN) guarantees mobilization appropriate to cellular demands, probably through lysosomal degradation of the ferritin itself mediated via the cargo protein nuclear receptor coactivator 4 (NCOA4) (31). During erythropoiesis, NCOA4 levels increase to promote ferritinophagy and support the synthesis of increasing amounts of hemoglobin (32). Furthermore, by restricting the cytosolic accessibility to Fe, FTN limits Fe toxicity by acting as an antioxidant (28).

1.2.2 Erythropoiesis and Hemoglobin synthesis

Erythropoiesis and hemoglobin (Hb) synthesis represent the greatest drive of iron moving through the circulating plasma transferrin compartment (33) to the erythroid marrow. Each red blood cell (RBC) contains more than 1 billion Fe atoms, mostly incorporated in the tetrameric structure of hemoglobin (4). Daily production of 200 billion RBCs, compensating for the ones ending their lifespan (~120 days), requires about 20 to 25 mg of iron for Hb synthesis (18). Under normal conditions, approximately 1% of total RBCs are synthesized daily from multipotent hematopoietic stem cells (34). The maturation of

mature RBCs involves the progression of lineage-committed cells from progenitors, such as the burst-forming unit erythroid and colony-forming unit erythroid, to precursors, or proerythroblasts (Pro-E), which progressively accumulate Hb, decrease RNA content and condensate nuclear chromatin, to erythroblasts and normoblasts (35). Until this stage, the erythropoiesis is promoted by the "nurse" macrophage-erythroblast interaction within extravascular niches in the bone marrow, the erythroblastic islands (34). The final maturation of reticulocytes involves enucleation and mitochondrial, ribosomal, and Golgi apparatus clearance, and release into the bloodstream (36). After expelling its nucleus, the reticulocyte becomes a mature RBC through a reduction in the cell area and the elimination of any remaining organelles via an autophagy/exosome-combined pathway (6).

The heme biosynthetic pathway begins in the Pro-E and is illustrated in **Figure *2*** (37). It is composed of 8 sequential steps starting in the mitochondrial matrix with the condensation of glycine with succinyl-CoA to form 5-aminolevulinate (ALA), catalyzed by the enzyme ALA synthase (38). ALA molecules are then exported into the cytoplasm, where the porphobilinogen (PBG) synthase-mediated condensation of two ALA leads to the formation of PBG (**Figure 2**) (37). The catalysis by PBG-synthase requires four zinc ions, which may be replaced by lead in lead poisoning, resulting in inhibition of the enzyme (39) and potential impairment of erythropoiesis. Three additional cytoplasmic steps are required to convert 4 molecules of PBG into the tetrapyrrole coproporphyrinogen III, through the formation of hydroxymethylbilane and uroporphyrinogen III and catalysis of the enzymes hydroxymethylbilane synthase, uroporphyrinogen synthase, and uroporphyrinogen decarboxylase, respectively (37). Coproporphyrinogen III is then transported back into the mitochondrial intermembrane space, where a double oxidative decarboxylation leads to the formation of protoporphyrinogen IX, then oxidized to protoporphyrin IX (PPIX). The terminal step of heme biosynthesis requires the ferrochelatase-mediated insertion of Fe^{2+} into the protoporphyrin macrocycle (37). To satisfy the high demand for iron during this step, iron is translocated from the LIP into mitochondria via mitoferrin 1 (MFRN1, MFRN2 in non-erythroid tissues), and this process is facilitated by the structural stabilization of MFRN1 by ABCB10 (ATP-binding cassette, subfamily B, member 10) (40). Deficiency of either MFRN1 or ABCB10 eventually causes anemia (41). Iron may also be directly transported from Tf-Fe^{2+} containing endosomes into mitochondria by direct contact of the two organelles (42), although the relevance of this

pathway remains to be clarified. After iron conjugation with PPIX, the newly formed heme molecule is exported from the mitochondria for incorporation into hemoproteins or globin subunits that jointly form hemoglobin (8). Intracellular heme trafficking remains poorly understood, with the ABC transporter ABCB6 and the feline leukemia virus subgroup C receptor 1 b (FLVCR1b) transporter as putative key players for the heme efflux between mitochondria and the cytoplasm (43).

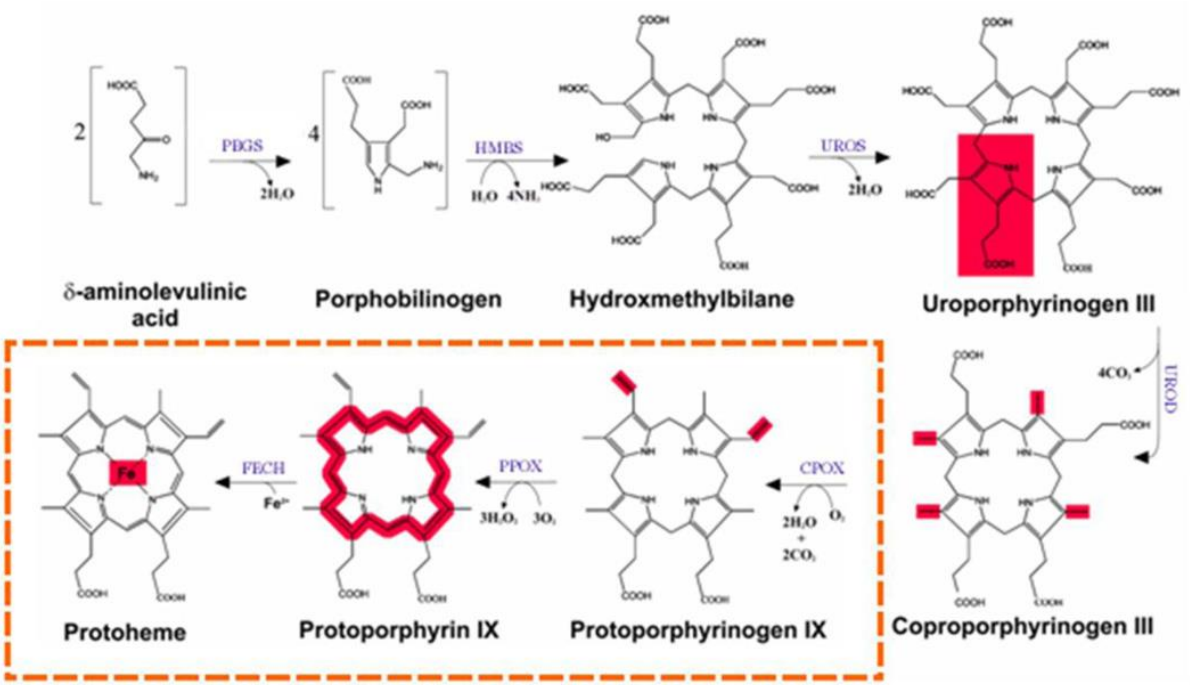

Figure 2: The mammalian heme biosynthetic pathway. Steps occurring in the mitochondrion are enclosed in the dashed box (37).

Iron availability modulates erythropoiesis both directly, being essential for enzymes involved in Hb synthesis (paragraph 1.2.3), and indirectly, through TfR2-mediates sensing of iron-loaded Tf (44). In addition to the liver, where it helps modulate hepcidin synthesis in relation to circulating Tf-Fe2, TfR2 is expressed on the plasma membrane of erythroid progenitors and is required for the efficient stabilization of the hormone erythropoietin (Epo)-receptor, which is crucial for proliferation and differentiation to RBC (44).

1.2.3 Iron-Sulphur clusters

Iron–sulphur clusters (ISC)-containing proteins are indispensable cofactors for a variety of biosynthetic processes, including electron transport (45), promotion of enzymatic catalysis, and metabolic sensing (46). The synthesis and maturation of Fe-S clusters proteins take place both in mitochondria and in the cytoplasm and occur in two phases:

1) synthesis of the ISC on a scaffold protein and (2) the transfer of the ISC to their client apoproteins (46). Protoporphyrin synthesis and Fe-S cluster assembly are not independent (41). The enzyme responsible for the metalation of PPIX is itself an ISC protein (6). Furthermore, Frataxin, a mitochondrial matrix protein, seems to act as an iron chaperone in ISC formation and as an iron sensor and metabolic switch during heme biosynthesis (47).

1.3 Iron recycling

In a steady-state, dietary heme and nonheme iron absorption contributes only up to 10% of the daily iron requirements (6). In humans, over 360 billion RBCs each day reach their lifespan (48) and, in normal conditions, erythropoiesis is balanced by erythrocyte disposal (49). The major pathway of iron recycling from senescent RBCs, i.e. erythrophagocytosis, is catabolized by reticuloendothelial system (RES) macrophages (33), which are predominantly located in the bone marrow, liver (Kupffer cells) and spleen (50). Approximately 120 days after erythropoiesis, aging RBCs start displaying markers of senescence, such as modifications of the membrane protein Band 3 (51), and phosphatidylserine on the outer side of the cell surface, increased membrane rigidity, and loss of sialic acid (52). The clustered Band 3 protein and the presence of phosphatidylserine on the plasma membrane can trigger conventional antibody- and complement-mediated macrophagic erythrophagocytosis (52). The internalization of phagocytic vesicles is followed by enzymatic catabolism of the RBC components and oxygenation of heme via heme oxygenases 1 and 2 (HMOX1/2) (48). Heme is exported into the cytosol by the transporter HRG1 (48). NRAMP1 (natural resistance-associated macrophages protein 1), homologous to DMT1, facilitates the iron efflux from phagolysosomes (53). FPN mediates the release of iron from macrophages into circulation (54). Despite erythrophagocytosis being the predominant mechanism of RBCs disposal (80-90%), a portion of erythrocytes undergo hemolysis, which induces heme and Hb release in the circulation (33). Specific plasma proteins, i.e. haptoglobin (Hp) and hemopexin (Hpx), bind and clear free Hb and heme, respectively, neutralizing their toxicity (50).

As iron deficiency develops, the expression of a FPN transcript (FPN1b) on RBCs that is not subject to transcriptional regulation (see paragraph 1.5), unlike the ubiquitous FPN1a, allows iron to be released into the circulation, even in conditions of systemic iron

deficiency (38). This mechanism preserves the body tissues to become iron deficient due to the prevailing iron consumption by erythropoiesis.

1.4 Systemic iron homeostasis

The liver, the major iron storage organ, is the key player in controlling systemic iron balance (55). Due to its dual blood supply from the portal and systemic circulation, the liver can integrate multiple signals reflecting systemic plasma iron concentrations, newly absorbed iron in the portal circulation, erythropoietic activity, and inflammatory/infectious states (4,56). All these signals converge into the production of the iron-regulating hormone hepcidin (HEP), a 25-amino acid peptide generated from the cleavage of 84-amino acid prepropeptide, predominantly in the hepatocytes (**Figure 3**) (55).

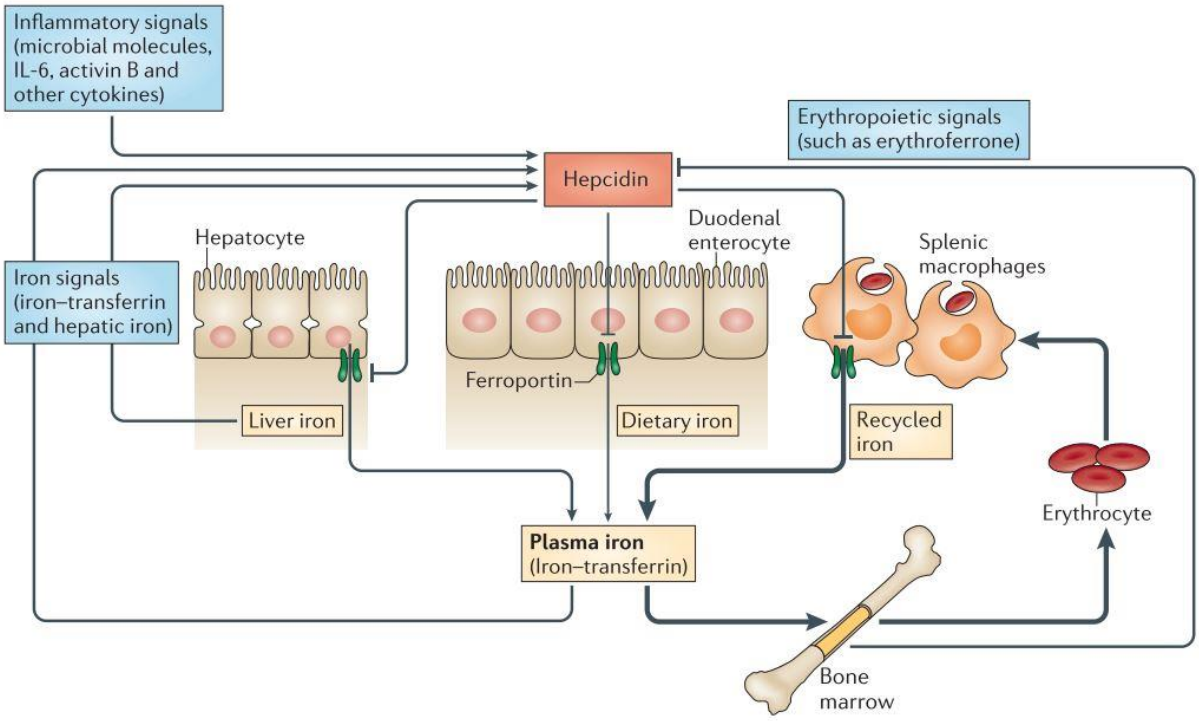

Figure 3: Integration of iron, inflammatory, and erythropoietic signals for hepcidin synthesis (10).

Hepcidin controls the inflows of iron into circulation by direct binding with the sole iron transporter Ferroportin (57). FPN expression is restricted to specialized groups of cells that can export iron, such as placenta syncytiotrophoblast, duodenal enterocytes, hepatocytes, and splenic and hepatic macrophages (58). FPN is also found in different isoforms in erythrocytes precursors in the bone marrow, where the expression of the isoform FPN1b seems to evade the iron-response elements (IREs)/ iron regulatory

proteins (IRPs) transcriptional control (see paragraph 1.5) and enables Fe export in the circulation even with low iron concentration (38). The binding between FPN and HEP induces inactivation of FPN, either through ubiquitination, endocytosis, and degradation of the FPN-HEP complex or by physical occlusion of FPN, in both cases leading to a decreased iron supply to plasma (59). Thereby, low HEP concentrations allow high rates of iron to enter plasma, whereas high HEP concentrations result in a fall in plasma iron concentrations (57).

1.4.1 Regulation of hepcidin levels

Hepcidin expression exclusively relies on transcriptional regulation (60). The main factors influencing hepcidin synthesis are systemic iron availability, erythropoietic activity, and inflammation (Figure 4) (56).

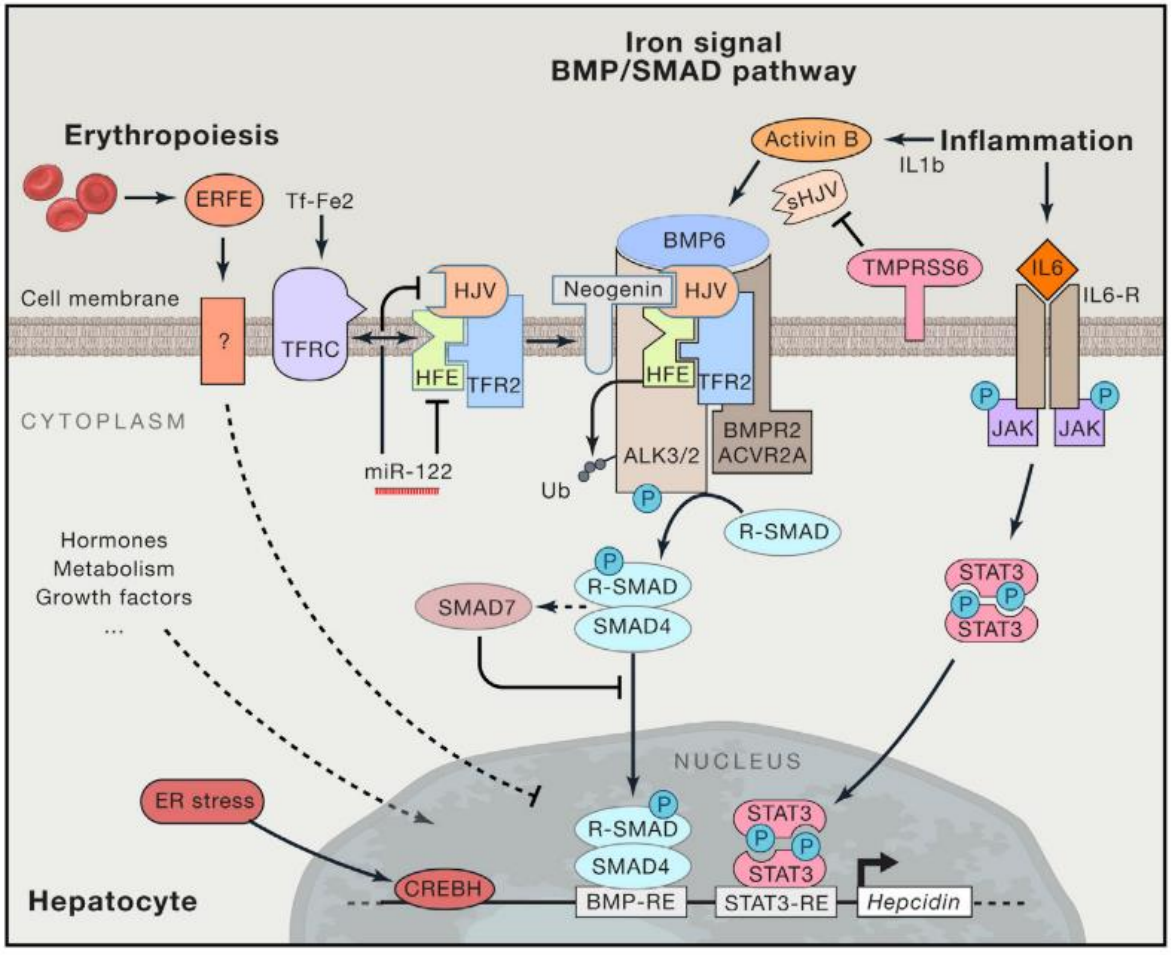

Figure 4: Pathways involved in transcriptional hepcidin regulation (6).

Iron accumulation in the liver and absorption of dietary iron seem to promote the synthesis of Bone Morphogenic Protein 6 (BMP6), the essential and specific BMP receptor-ligand

involved in iron signaling, in non-parenchymal cells of the liver (61) and duodenal enterocytes (4). BMP6, together with its co-receptor hemojuvelin (HJV), activates a signaling cascade leading to the phosphorylation of receptor-activated Smad (Sons of mothers against decapentaplegic) proteins and the formation of transcriptional complexes with SMAD4, promoting hepcidin synthesis. Moreover, high concentrations of holo-transferrin induce displacement of human hemochromatosis protein (HFE) from TfR1, which then complexes with TfR2 and HJV to again promote BMP/SMAD signaling to hepcidin (6). The BMP-SMAD pathway also seems to be involved in the reduction of hepcidin synthesis under increased erythropoietic activity (62), although some evidence supports the independence of erythropoietic hepcidin suppression from the BMP pathway (63). The proliferation of erythroblasts is greatly enhanced by the hypoxia-inducible factor (HIF)-2-mediated increases in the synthesis of Epo (64). Epo supports the maturation of erythroid precursors and stimulates those proerythroblasts to secrete erythroferrone (ERFE), which in turn sequesters BMP ligands away from their cell surface receptors and limits the activation of the SMAD pathway, thereby reducing hepcidin transcription in the nucleus (64). Lastly, hepcidin concentration is increased under inflammatory conditions through IL-6-mediated activation of the signal transducer and activator of the transcription (STAT) signaling pathway (65). The binding of IL-6 to its receptor induces the phosphorylation of STAT3 through Janus kinase 1 (JAK1) (65) and the coupling of a dimer of phosphorylated STAT3 to motifs in the hepcidin gene promoter, increasing its transcription (10).

1.5 Cellular iron homeostasis

Within each cell, iron regulatory proteins 1 (IRP1) and 2 (IRP2) optimize Fe cytosolic concentration through modulation of post-transcriptional expression of iron metabolism genes (66). IRPs interact with IREs, mRNA stem-loop structures which are found in the 5' or 3' untranslated regions (UTR) of ferritin, TfR, and several other iron metabolism genes (67). In iron depletion, IRPs have a high conformational affinity for IREs and the binding stabilizes the otherwise unstable mRNA (68). In iron-repleted cells, direct binding of iron promotes IRPs ubiquitination and subsequent degradation by the proteasome (69). Depending on the location of the IRE within the 5'- or 3'-UTR in the target mRNA, the binding of IRPs might enhance or inhibit the synthesis of proteins (68). When iron is limited, IRPs bind with high affinity to 5′-UTR IRE mRNAs of ferritin and ferroportin and other target

proteins that coordinate iron efflux and storage, repressing their translation, and to 3′ IREs in TfR1 and DMT1 mRNA to block their degradation, thus facilitating Fe uptake (70). Moreover, the IRP1/IRE system also coordinates the translation of the transcription factor HIF2 under hypoxia, when the enhanced Epo transcription can be attenuated and adjusted to iron bioavailability (71).

1.6 Interplay between systemic and cellular iron homeostasis

From paragraphs 1.4 and 1.5, it can be concluded that the main regulators of iron homeostasis are the HEP-FPN axis and the IRE/IRP system (8). The interconnection between these two pathways is guaranteed by: a) the post-transcriptional (hepcidin) and post-translational (mRNA, IRE/IRP) regulation of FPN expression (72); b) the indirect action of IRPs on DMT1 expression through their binding to the IRE on HIF-2α mRNA (73); c) the modulation of TfR-1 expression by the IRP binding to its IRE and its role as an iron sensor regulating hepcidin synthesis (8).

1.7 Iron homeostasis during inflammation and infection

Iron is an essential element for nearly all human pathogens (74). Iron is mostly stored or complexed intracellularly, or bound with Tf, and is thus not freely accessible (75). However, microbes have evolved scavenging strategies to obtain iron and meet their metabolic requirements, such as the secretion of the iron-chelating molecules siderophores and the ability to directly acquire heme from host proteins (76). Therefore, mammalians have developed a series of additional defense mechanisms falling under the name of “iron-targeted nutritional immunity” to deprive pathogens of iron (74). As mentioned above (paragraph 1.4.1), within hours of infection or inflammation, a cytokine-mediated increase in hepcidin synthesis induces a reduction in plasma iron levels (56), which is referred to as “hypoferremia of inflammation” (74). The primary and most immediate response to infection and inflammation relies on the release of proinflammatory cytokines by helper T cells and macrophages (77), which initiates a signaling cascade eventually leading to the transcriptional modulation of the so-called “acute-phase” proteins (APPs), mostly by the liver (78). Some of the APPs are also involved in iron trafficking, such as transferrin, the transport/redox protein ceruloplasmin, haptoglobin, hemopexin, and ferritin (79). An additional layer of physical protection is provided by the presence of fluids containing the

iron and siderophores-chelators lactoferrin and lipocalin 2 on the mucosal epithelia (74). Pro-inflammatory cytokines can also directly act on the bone marrow by inhibiting steady-state erythropoiesis and promoting erythrocyte turnover in the periphery (80).

Additional alterations in iron homeostasis might occur in case of infection with specific pathogens depending on their etiology and clinical manifestation. For example, *Plasmodium falciparum*, the parasite causing malaria, digests hemoglobin in the infected RBCs and synthesizes hemozoin, which is responsible for the production of inflammatory mediators and the inhibition of erythropoiesis (81). Hookworm and Schistosoma infestation affect more the 200 million people worldwide (82,83) and their infiltrations in the intestinal wall and urinary tract might cause consistent iron losses through bleeding (84,85).

2. DETERMINATION OF IRON STATUS, REQUIREMENTS, AND METABOLISM

In healthy adult subjects, the total body iron is 3-4 g (86), with premenopausal women having lower amounts than men due to menstrual losses (87). More than two-thirds of the body's iron resides in the erythrocytic compartment as heme iron; the rest is stored within reticuloendothelial macrophages (~600 mg), in muscle fibers as myoglobin (~130 mg) (88), at the cellular level as iron-sulfur or heme-containing enzymes (~150 mg) (88), in the liver as ferritin/hemosiderin (~1000 g) (86) or in the bone marrow (~300 mg) (89,90). Only a negligible amount of iron (3-4 mg) is present in circulation, bound to transferrin (91). As mentioned previously (paragraph 1.3), in a steady state, only 1-2 mg Fe per day are absorbed from the diet (92); thus, the vast majority of daily iron demand (20–25 mg/day) is covered by iron recycling from senescent RBCs. In periods of rapid growth and development, such as during fetal life, late infancy, and puberty, iron demand increases (88) and the contribution of dietary iron becomes more relevant (9).

2.1 Quantification of total body iron

Total body iron is conceptualized as composed of three different compartments, namely circulating iron (Fe_{circ}), non-circulating active iron (Fe_{nca}), and iron in storage (Fe_{stor}) (93,94). In the assessment of Fe_{circ}, the contribution of circulating iron-containing enzymes and transport protein is ignored, and the estimate exclusively relies on the heme-iron in RBCs (93). Fe_{circ} is calculated as Hb concentration x iron content in Hb (3.47 mg/g) x blood volume (93). Different methods exist for the estimation of blood volume. Traditional methods for the exact quantification of blood volume are either impractical or invasive and can use radioisotopes, exposing the study subjects to radiations (95,96). The optimized CO-rebreathing method, despite being less invasive than the common CO-rebreathing method ($Proc_{com}$) (96), still requires blood sampling and specific equipment which might be impractical in some research settings (96). Most frequently, especially in infants and children, the estimates rely on age-specific equations based on body weight and height (97,98). Fe_{nca} accounts for the tissue iron content and is estimated at 5-7 mg/kg body weight (94,99). Fe_{stor} reflects the iron stored in ferritin and hemosiderin. Different approaches have been proposed for the determination of Fe_{stor} (93,99–101). Among others, the two that are mostly used depend on equations derived from the results of a

phlebotomy study by Skikne et al., aiming at defining the relationship between iron stores and serum iron biomarkers (102). In the first approach, Hallberg et al. calculated a linear regression between the logarithm of serum ferritin values and iron stores reported by Skikne et al., corrected for increased iron absorption during phlebotomy (100), deriving the following regression equation:

$$\boldsymbol{Iron\ stores_{[\mu g/kg]} = 9380 \times log(SF_{[\mu g/L]}) - 11260}$$

SF concentrations < 15.9 µg/kg would result in negative values, indicating the absence of iron stores.

The second approach is based on the linear regression between the logarithm of the ratio of serum transferrin receptor/serum ferritin and body iron (101), according to the following equation by Cook et al.:

$$\boldsymbol{Body\ iron_{[mg/kg]} = -[\log(sTfR_{[\mu g/L]}/SF_{[\mu g/L]}) - 2.8229]/0.1207}$$

Despite being identified as "body iron", Cook's estimate represents a quantitative estimate of body iron stores when positive, and, when negative, an estimate of a functional iron deficit, i.e., the amount of iron that would be needed before iron stores are replenished (101). The Cook's functional iron deficit, in contrast with negative values derived from the Hallberg equation (100), could be attributed to reduced circulating iron, tissue iron, or both.

2.2 Assessment of iron status

The tripartite division of total body iron is also used to classify iron status inadequacy (103), with increasing severity of depletion progressively affecting the different body iron compartments (104). For this thesis, more focus will be placed on the assessment of iron deficiency (ID), but the biomarkers described below are used for the definition of the entire range of body iron status. Transient variations in iron needs or inadequate intakes are compensated by the release of iron from the ferritin stored in hepatocytes and macrophages (103). Absolute ID is defined as the complete utilization of body iron stores (105). The gold standard test for diagnosis of absolute ID is bone marrow aspiration, which is routinely replaced by serum ferritin (paragraph 2.2.1) due to its practical feasibility (106). Other blood biomarkers commonly used in the diagnosis of ID are transferrin saturation,

which indicates low plasma iron bioavailability to tissues when under 16%, sTfR (paragraph 2.2.2), an indicator of the iron supply to bone marrow (107), and mean corpuscular volume (MCV) (108). When a prolonged uncompensated negative iron balance occurs, the depletion of body iron stores might impair erythropoietic activity (103). In this case, a low blood hemoglobin concentration, in combination with evidence of ID, is a key indicator of iron deficiency anemia (IDA) (paragraph 2.2.3) (109). When the stores are adequate, but increased erythropoietic demands are not compensated by proper mobilization of iron, anemia is promoted (110). This condition is defined as functional iron deficiency and is frequently observed in inflamed subjects, where hepcidin upregulation prevents cellular iron export and promotes iron withholding from the plasma, eventually leading to iron-deficient erythropoiesis (111). Functional and absolute iron deficiency are not mutually exclusive, and functional iron deficiency might eventually cause absolute iron deficiency by restricting iron uptake (105). To conclude, each biomarker reflects the status of different body iron compartments and is specific for different stages of ID, and can be influenced by various factors, e.g., inflammation, to different extents. Therefore, to monitor iron status in populations, and to evaluate the impact of public health programs, the World Health Organization (WHO) recommends the use of multiple indicators (107).

2.2.1 Serum ferritin

Ferritin is the main iron-storage protein (107). Ferritin synthesis is regulated post-transcriptionally through the IRE/IRP system, with high cytosolic iron concentrations promoting its synthesis (112). In healthy subjects, serum ferritin positively correlates with iron stores in the liver (113). In adults, 1 µg/l is considered equivalent to 8-10 mg of storage iron/kg body weight (114), whereas in infants the same amount corresponds to 120 µg/kg body weight (115). Serum ferritin concentration is the most common indicator of iron deficiency but the thresholds indicating ID in different population groups have been uncertain (116). The WHO's recommended cut-off values for the definition of ID and iron overload (Table 1) (107,117) rely on qualitative expert opinion based on clinical studies (118) and were intended to be indicative of the absence of stainable reticular iron in the bone marrow (116).

Table 1: WHO proposed cut-off values of SF for iron deficiency and overload (107).

	Serum Ferritin (µg/L)[a,b]			
	Iron deficiency		Risk of iron overload	
	Apparently healthy individuals[c]	Individuals with infection or inflammation	Apparently healthy individuals	Non-heathy individuals
Infants and young children (0-23 months)	< 12	< 30	-	-
Children under 5 years of age (24-59 months)	< 12	< 30	-	-
Children (5 to less than 10 years)	< 15	<70	>150 females >200 males	>500[d]
Adolescents (10 to less than 20 years)	< 15	<70	>150 females >200 males	>500
Adults (20-59 years)	< 15	<70	>150 females >200 males	>500
Older persons (60+ years)	< 15	<70	>150 females >200 males	>500
Pregnant women	< 15 (first trimester)[e]	-	-	-

[a]From previous WHO recommendations and new evidence.
[b]Markers of inflammation should be assessed along with the ferritin concentration, and ferritin adjusted as necessary.
[c]For the purposes of this guideline, an apparently healthy individual is defined as an individual with physical well-being for their age and physiological status, without detectable diseases or infirmities.
[d]In adult, non-healthy populations, a ferritin concentration exceeding 500 µg/L may indicate risk of iron overload or other disease. This cut-off value indicates the need for further clinical and laboratory evaluation to establish the diagnosis and underlying cause of the ferritin levels
[e]There are several physiological changes occurring in pregnancy that may contribute to the variation in thresholds of iron deficiency in pregnancy as defined by serum ferritin, including a physiological rise in acute phase proteins secondary to pregnancy; second-trimester plasma volume expansion; and changes in inflammatory measures in the final trimester of pregnancy.

A recently developed approach proposes revised SF cut-offs for the definition of ID, based on the strength of the associations between ferritin and hemoglobin, and ferritin and sTfR (25 µg/L in women and 20 µg/L in children) (118). The definition of ID would then reflect the point where the first response to ID, i.e., the onset of iron deficient-erythropoiesis, could be detected (118). Recently, it has been suggested that at a ferritin level of 50 µg/L in young women, iron absorption starts being upregulated (119). The use of this higher threshold in young women would maximize the sensitivity and specificity to identify iron deficiency (119). SF is also an acute-phase protein (120) and assessment of ID in the presence of infection or inflammation might bias estimates of iron stores and mask ID. In a meta-analysis of healthy subjects, inflammation has been reported to increase ferritin by approximately 30%, leading to a 14% underestimation of iron deficiency (120). Several strategies have been proposed to assess ID by adjusting SF concentrations for inflammation. These approaches require the concurrent measure of SF with indicators of

inflammation or infection (α-1 acid glycoprotein (AGP) and C-reactive protein (CRP)): 1) the use of a higher cut-off value (<30 µg/L) for ID in individuals (or populations) with inflammation; 2) the removal of individuals with elevated CRP (>5 mg/L) or AGP (>1.0 g/L) (107); 3) the use of correction factors derived from the classification of SF based on different levels of inflammation (reference, incubation, early convalescence, and late convalescence) (120); 4) the regression correction approach, with coefficients derived from a linear multiple regression between SF and CRP and AGP concentrations and malaria incidence (dichotomous) as independent variables (121). When assessing ID, the adjustment of SF values for infection and inflammation led to an increase in the prevalence of depleted iron stores by 7 in pre-school children (PSC) and 25 absolute median percentage points in women of reproductive age (WRA), and an increment of 2 and 8 absolute median percentage points in PSC and WRA, respectively, depending on which approach was used (121).

2.2.2 sTfR

Transferrin receptor exists in two isoforms (TfR1 and 2), with TfR1 mediating the cellular uptake and internalization of Tf-bound iron from plasma (122). The TfR1 concentration on the cell surface is inversely proportional to the available intracellular iron (123). TfR1 is almost ubiquitously expressed in mammalian tissues, and the highest densities are detected on cells with the greatest requirement for iron, i.e., immature erythroid cells (124). The soluble transferrin receptor (sTfR) is a cleaved and truncated form of the TfR1 molecule (125), which originates from intracellular proteolysis of TfR occurring during its internalization and recycling in the acidic endosomes (126). Thus, sTfR concentration in circulation reflects total body TfR1 (123). Considering that the erythroid marrow accounts for 70 to 80% of the total body content of transferrin receptors, elevated levels of sTfR are indicative of enhanced erythropoiesis and iron deficiency (127). Therefore, sTfR can be used as a direct measure of ID, provided no other causes for abnormal erythropoiesis, such as ineffective erythropoiesis or hemolysis, are present (128). The relative stability of sTfR levels in the presence of infection or inflammation is crucial for the differentiation between IDA and the anemia of inflammation and the detection of iron deficiency in inflamed patients (129). There is no consensus on an international standard reference for sTfR, but in healthy subjects, the mean sTfR concentration ranges from 5 mg/L to 8 mg/L (130).

2.2.3 Hemoglobin

Hb has been extensively used for ID screening, but both its sensitivity and specificity are low (131). Low Hb values might be determined by other causes than ID, such as inflammation, infection, malignancy, and toxins such as lead (131). Hb is the main biomarker for the diagnosis of anemia (132). Anemia is a condition characterized by reduced quantity and/or oxygen-carrying capacity of RBCs (133). Individuals are defined as anemic when their Hb values are below two standard deviations from the mean Hb in a healthy population of the same age and gender, measured at the same altitude (132). The WHO age- and sex-specific cut-off values for anemia are listed in Table 2.

Table 2: WHO proposed cut-off values for anemia at sea level (134).

	Anemia			
Population	Non-Anemia	Mild	Moderate	Severe
Children 6-59 months of age	110 or higher	100-109	70-99	Lower than 70
Children 5-11 years of age	115 or higher	110-114	80-109	Lower than 80
Children 12-14 years of age	120 or higher	110-119	80-109	Lower than 80
Non-pregnant women (15 years of age and above)	120 or higher	110-119	80-109	Lower than 80
Pregnant women	110 or higher	100-109	70-99	Lower than 70
Men (15 years of age and above)	130 or higher	110-129	80-109	Lower than 80

As mentioned previously, anemia etiology is multifactorial, with about 25% (135) to 50% of the cases due to iron deficiency (136). When low Hb values are determined by impaired erythropoiesis due to iron restriction, anemia is classified as IDA, the most severe manifestation of ID (137). IDA requires the measurement of additional markers of ID in combination with Hb for its diagnosis, such as SF and sTfR, or, in some cases, a therapeutic trial of iron (132).

2.2.4 Further iron indices

As explained previously, the diagnosis of ID is complex and requires the use of several iron status indicators in combination (109). In addition to SF, sTfR, and Hb, which are the most commonly used, several additional laboratory markers of iron status are available, including the measurement of serum or plasma iron, transferrin saturation (TSAT), mean corpuscular volume (MCV), and zinc protoporphyrin concentration (ZnPP) (107).

2.2.4.1 Serum Iron and TSAT

Iron turnover in circulation takes around 40-50 minutes (138); thus, a measurement of serum iron reflects the dynamic movement of iron to and from tissue, which is very responsive to the release of inflammatory biomarkers from immune cells and to the post-prandial release of iron absorbed by enterocytes into circulation (138). Due to the substantial daily and within-the-day variation (139), when used alone, serum iron levels might lead to a misclassification of iron status and wrong estimation of the prevalence of ID. TSAT is calculated as the ratio between serum iron and total iron-binding capacity (TIBC), i.e. the total amount of iron that maximally saturates all plasma transferrin (140). Values of TSAT below 20% suggest ID (141). However, since TSAT is subjected to the same variation as serum iron, it is not specific nor sufficient for ID diagnosis, but it can be used in functional deficiency when serum ferritin is unreliable (137). A TSAT below 20% in combination with an elevated TIBC increases the likelihood of ID.

2.2.4.2 Mean corpuscular volume

MCV is an important marker in circulating RBCs for detecting established iron-deficient erythropoiesis (142). The MCV can be used to classify anemia based on the average size of circulating RBCs (143). When the MCV falls under 80 fL, anemia is classified as microcytic anemia, a condition predominantly seen in IDA, the anemia of chronic disease, sideroblastic anemia, and thalassemia trait (143). Limitations in using MCV to identify ID include RBC having a normal MCV during early stages of ID or when coexisting conditions occur simultaneously with ID (144), such as concurrent vitamin B12 or folic acid deficiency (145).

2.2.4.3 Zinc protoporphyrin

The conceptual basis for the measurement of ZnPP is that, in case of inadequate delivery of iron to the erythroid marrow, zinc is incorporated into protoporphyrin-IX and results in a measurable increase in the concentration of zinc protoporphyrin (138). ZnPP is a reliable and easy-to-measure marker for ID (146). Being the first clinically measurable biochemical change in erythrocytes with the onset of iron-deficient erythropoiesis, ZnPP offers higher sensitivity and specificity for ID than hemoglobin measured alone or with MCV, especially in children (147). When the ZnPP concentration is within the physiological range,

regardless of the ferritin concentration, which can indicate low iron stores even with adequate tissue supplies, the erythropoiesis is assumed to be adequately supplied with iron (148). However, if ZnPP is increased, the combination with ferritin and hemoglobin values is recommended for the diagnosis of ID due to other factors determining impaired iron utilization, e.g. infection, inflammation, anemia of chronic disease, and chronic lead exposure (148).

2.3 Definition of iron requirements

In healthy subjects, iron requirements depend on the daily iron losses and the changing needs for growth throughout the lifespan (149). The balance between requirements and absorption defines the iron status of an individual, which is reflected in the amount of iron stored in the body (150). In newborns, body iron at birth is approximately 75 mg/kg (151). In a healthy, term infant, this accounts for a total of about 250-300 mg iron, mostly as Hb, but with a consistent percentage in stores (25%) (152). Erythropoiesis almost ceases shortly after birth, leading to a steep decline in Hb during the first 6 weeks of life and an increase in stores size due to iron recycling from RBCs (153). Until 4 to 6 months, the tissue growth and RBC expansion can be sustained by the mobilization of iron from the stores and exclusive breastfeeding is sufficient to meet iron requirements, despite the low iron concentration in breast milk (0.3 mg/L) (154). Subsequently, complementary foods are needed to meet iron needs, which rise markedly up to 1 year of age. If iron stores are depleted, the infant becomes fully dependent on dietary iron to cover its daily requirements (155). Iron requirements per kg body weight in late infancy (6-23 months) are higher than during any other life period (153), especially in relation to energy intake (149). Healthy infants double their birth weight by 4-6 months of age and triple it by 1 year of age. Between 6 and 24 months, total body iron increases from 300 mg to 600 mg, reaching a concentration of 50 mg/kg at 2 years of age (88). Later childhood is characterized by a decreased growth velocity compared to infancy, which is reflected in reduced iron requirements per kg body weight (149). In adolescence, the pubertal growth spurt, and the consequent expansion of blood volume, requires an increase of approximately 1000 mg of iron for erythropoiesis (88). After menarche, despite their lower growth rate, adolescent females present higher iron requirements than their male counterparts due to blood losses during menstruation (156). Upon completion of growth, total body iron reaches 3-4 g in adults (chapter 2). In non-pregnant adult women, basal

iron losses and losses due to menstruation are the only factors defining requirements (149). During pregnancy, to expand the maternal Hb mass, meet fetal requirements, and compensate for losses at delivery, iron needs increase gradually from 0.8 mg/day in the first trimester to 7.5 mg/day in the third trimester (157). Overall, this leads to a total of approximately 1040 mg of iron required to cover gestational iron needs for an average woman, which raises to 1190 when the blood losses at delivery are included (158). In lactating women, daily iron losses in milk (0.3 mg) are added to the basal iron losses (0.8 mg) for the estimation of iron requirements (149). Lastly, although physiological iron requirements do not differ between adult and physically-active elderly men, and post-menopausal and elderly women (159), the reduction of physical activity and blood volume induces a shift in the iron used from erythropoiesis to storage (160), implying a reduction in estimated requirements (149).

2.3.1 Derivation of iron requirements

The WHO and the US Institute of Medicine (IOM) derived theoretical values of physiological iron requirements using a factorial estimate of nutritional needs (149,161). The "factorial model" is based on modeling each contributor to iron requirements according to their distribution of the target population, and then summing their median percentile to estimate the average requirement for absorbed iron (149,161). The 95th (for the WHO) and 97.5th (for the IOM) percentiles are used to calculate the total absolute iron requirements of 95 and 97.5%, respectively, of the population (149,161). As mentioned previously, the nutritional iron needs are determined by the basal iron losses (including menstrual losses in WRA) and by the requirements for tissue growth and RBCs expansion. In infants between 0 to 6 months, the response to dietary iron intake has not been related to any functional criteria (161). Thus, instead of a recommended dietary allowance (RDA), an adequate intake (AI) reflecting the observed mean iron intake from breastmilk is given (161). For the definition of basal iron losses, starting from 7-months old infants, both the WHO and the IOM rely on the measurements provided by the radio-iron isotopes study by Green and colleagues (162), adapted to different age groups. The estimates of iron requirements for growth differ slightly between the WHO and the IOM, with different sets of Hb values per age group, and different calculations for age-specific blood volumes and tissue iron content used as references (

Table 3) (149,161). Although the physiologic iron requirements are based on the minimum amount needed to maintain a sufficient total body iron, but only minimal stores (SF=15 µg/L), IOM also adds a term for increases in storage iron in children into their calculation of iron needs for growing tissues (161).

Table 3: iron required to cover needs for growth up to 18 years of age, basal iron losses and menstrual losses, and absolute total requirements, estimated by WHO (149).

	Age	BW	Required iron intake for growth	Basal iron losses	Menstrual losses		Absolute requirements[a]	
	(years)	mean (kg)	(mg/d)	median (mg/d)	median (mg/d)	95th percentile (mg/d)	median (mg/d)	95th percentile (mg/d)
Infants and children	0.5 – 1	9	0.55	0.17	-	-	0.72	0.93
	1 – 3	13	0.27	0.19	-	-	0.46	0.58
	4 – 6	19	0.23	0.27	-	-	0.50	0.63
	7 – 10	28	0.32	0.39	-	-	0.71	0.89
Males	11 – 14	45	0.55	0.62	-	-	1.17	1.46
	15 – 17	64	0.60	0.90	-	-	1.50	1.88
	18+	75	-	1.05	-	-	1.05	1.37
Females	11 – 14[b]	46	0.55	0.65	-	-	1.20	1.40
	11 – 14	46	0.55	0.65	0.48[c]	1.90[c]	1.68	3.27
	15 – 17	56	0.35	0.79	0.48[c]	1.90[c]	1.62	3.10
	18+	62	-	0.87	0.48[c]	1.90[c]	1.46	2.94
Post-menopausal		62	-	0.87	-	-	0.87	1.13
Lactating		62	-	1.15	-	-	1.15	1.50

[a]Total absolute requirements = requirements for growth + basal losses + menstrual losses
[b] Pre-menarche
[c]Effect of normal variation in hemoglobin concentration not included in these data

2.3.2 Dietary Reference Intakes

Along the whole range of intake in a target population, a set of four nutrient-based dietary reference intakes (DRIs) are estimated (163) (Figure 5). According to the WHO, the DRIs are defined as intake levels that meet specified criteria for adequacy, thereby minimizing the risk of nutrient deficit or excess (149). These criteria cover a wide range of biological effects and clinical manifestations, occurring over the whole nutrient intake distribution (149). The DRIs are used to assess individual and group iron status, evaluate the efficacy of prevention programs, and plan future nutritional interventions (161). The estimated average requirement (EAR) is the average daily nutrient intake level estimated to meet the requirement of 50% of healthy individuals in a particular age and gender group (161). When the standard deviation (SD) of the distribution of nutrient requirements is known, by adding two SD to the EAR it is possible to calculate the RDA, or recommended nutrient

intake (RNI) (149,161). The RDA is defined as the average daily dietary nutrient intake level sufficient to cover the requirements of 97.5% of healthy individuals in a specific age and gender group (149). When data about the variation of the intake distribution are scarce and the SD cannot be estimated, a coefficient of variation (CV) of 10-12.5% is assumed and the RDA is calculated as 2 times the CV added to the EAR (161). If there is no scientific evidence to estimate an EAR, an adequate intake (AI) is set (161). The AI is "the recommended average daily intake level based on observed or experimentally determined approximations or estimates of nutrient intake by a group (or groups) of apparently healthy people that are assumed to be adequate" (149). At the end of the acceptable range of intake, the tolerable upper intake level (UL) represents the intake of a nutrient at which no risk of adverse health effects is posed in 97.5% of the healthy individuals (149).

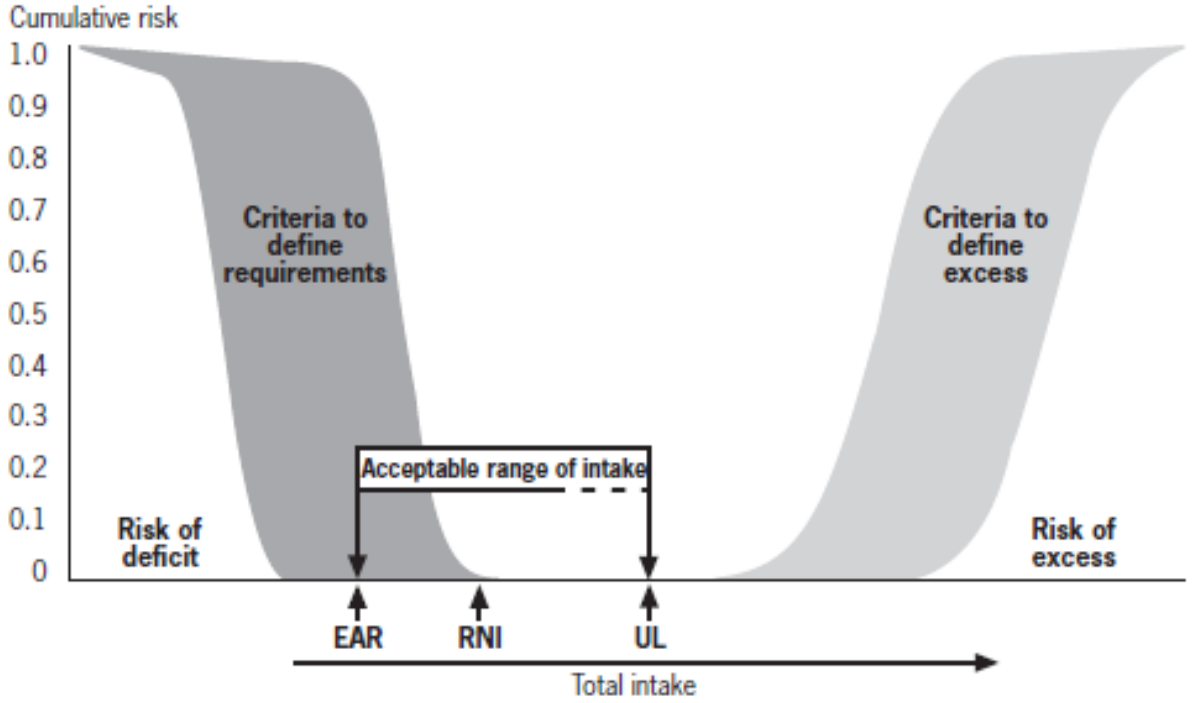

Figure 5: Risk function of deficiency and excess for individuals in a population related to nutrient intake (149).

2.3.3 Conversion of physiological iron requirements into DRI

Iron absorption is dependent on a series of homeostatic mechanisms reflecting the iron status of the subjects, the erythropoietic activity, and inflammatory/infectious states (paragraph 1.4). Moreover, iron bioavailability, e.g., the proportion of ingested iron that is absorbed by the intestine and metabolized, or stored (164), varies widely according to the

type of diet consumed, even from relatively similar diets (165), due to the enhancing or inhibiting effect that specific dietary factors have on iron absorption (166). Therefore, the DRIs are calculated according to distinct dietary iron bioavailabilities, ranging from 5 to 18% (149,161). Several algorithms to derive absorption factors for the conversion of physiologic iron requirements into DRIs have been described, but no consensus exists between different institutions on which one to adopt (167). The DRIs set by IOM and WHO are presented in

Table 4.

Table 4: Population RDA values set by the IOM and the WHO (149,161).

	Age	IOM[a]	WHO			
		18%	15%	12%	10%	5%
	(years)	(mg/d)	(mg/d)	(mg/d)	(mg/d)	(mg/d)
Infants and children	0.5 – 1	11 (6.9)	6.2[b]	7.7[b]	9.3[b]	18.6[b]
	1 – 3	7 (3.0)	3.9	4.8	5.8	11.6
	4 – 6	-	4.2	5.3	6.3	12.6
	4 – 8	10 (4.1)	-	-	-	-
	7 – 10	-	5.9	7.4	8.9	17.8
	7 – 11	-	-	-	-	-
Males	9 – 13	8 (5.9)	-	-	-	-
	11 – 14	-	9.7	12.2	14.6	29.2
	12 – 17	-	-	-	-	-
	14 – 18	11 (7.7)	-	-	-	-
	15 – 17	-	12.5	15.7	18.8	37.6
	18+	8 (6)	9.1	11.4	13.7	27.4
Females	9 – 13	8 (5.7)	-	-	-	-
	11 – 14[c]	-	9.3	11.7	14.0	28.0
	11 – 14	-	21.8	27.7	32.7	65.4
	12 – 17	-	-	-	-	-
	14 – 18	15 (7.9)	-	-	-	-
	15 – 17	-	20.7	25.8	31.0	62.0
	18+	-	19.6	24.5	29.4	58.8
	19 – 50	18 (8.1)	-	-	-	-
	50+	8 (5)	-	-	-	-
Postmenopausal		-	7.5	9.4	11.3	22.6
Lactating		9 (6.5)[d]	10.0	12.5	15.0	30.0

[a]Estimated average requirements in brackets.
[b]Bioavailability of dietary iron during this period varies greatly.
[c]Pre-menarche.
[d]10 (7) mg for women between 14 – 18 years.

2.4 Assessment of iron metabolism

As presented in paragraph 2.2, various indicators can be used for the assessment of iron status. Given that an appropriate sample size and study duration are selected, individual indirect biomarkers, e.g. SF and sTfR, and their combination, can provide an estimate of the iron balance during a specified period of time (101,168). However, the accuracy and

reliability of these indices might be biased in some population subgroups and in specific environmental conditions, such as in high infection pressure areas (169). Before the 1960s, the assessment of basal iron losses and iron absorption, defined as the iron secreted in feces subtracted from the original iron dose administered (170), relied on chemical balance studies. Losses were estimated from the fecal iron content in subjects on an iron-restricted diet and in iron balance (171,172). The agreement among different studies was challenged by the difficulties in distinguishing between the small quantities of iron lost endogenously and the ones originating from sample contamination (161). Moreover, even if valuable for other nutrients, due to the very small fraction of iron absorbed and lost, estimates from these studies could be biased by small errors in the recovery of unabsorbed iron (161).

As mentioned in chapter 1, iron presents four stable isotopes (^{54}Fe, ^{56}Fe, ^{57}Fe, and ^{58}Fe), but can also exist in the form of various radioactive isotopes (among others, ^{52}Fe, ^{55}Fe, and ^{59}Fe) (173). The difference in isotopic forms relates to the number of neutrons in the nucleus, whereas the number of protons is constant. The distribution of an element among each form is called the natural abundance and is highly conserved (174). The purification of one specific isotope, and its subsequent addition to food or its administration in a vector in known amounts, allows precise determination of iron bioavailability and absorption from the test condition (2), and of plasma iron turnover and RBCs utilization (175). The first isotopes used as labels, or tracers, in iron metabolism and bioavailability studies, were radioactive isotopes (176). To be used as such, an iron label has to be metabolized as the natural iron and the amount of label given should not affect the process under study, e.g., iron bioavailability from the test meal (2). The radioactive iron labels, specifically ^{55}Fe and ^{59}Fe, were used for almost all initial studies defining body iron pools and iron bioavailability (175). The main advantage of radioisotopes as labels is that even minimal amounts are easily detected with common research instrumentation (2). However, their finite half-lives might introduce time limitations for data collection and analysis, and the radioactive isotopes expose the subjects to ionizing radiations, restricting their use to adult, non-pregnant individuals (2). Since 1963, stable iron isotopes have been increasingly used instead of radioisotopes in iron nutrition studies (177). One disadvantage of stable isotopes is that being present in nature, they need to be administered in significant amounts to be detected (178). However, the major advantage over radioisotopes is that they do not emit radiation (176). Thus, they can be used in any population with no known

adverse risks (174), and consequently also in those most vulnerable to ID, such as pregnant women and children. Moreover, the differences in mass among the stable isotopes allow the quantification of bioavailability from multiple test conditions, when simultaneous administration of multiple stable isotopes occurs (179).

2.4.1 Stable iron isotopes

The use of stable iron isotopes as labels is considered the gold standard to assess iron absorption and bioavailability (180). Acute iron absorption can be determined by assessing the appearance of the administered tracer in: I) excreted feces in metabolic balance studies (fecal recovery method); II) plasma samples collected in the hours immediately after dosing to assess appearance kinetics (plasma appearance method); and III) whole blood samples collected from 2 weeks post-dosing to assess the amount of tracer incorporated in the RBCs (erythrocyte iron incorporation method) (2). Long-term iron absorption and losses can be estimated using the iron isotopes dilution method (paragraph 2.4.1.2), both with radio and stable isotopes. The use of isotopic labels with the same premises of chemical balance studies adds precision, since the tracer presence in the fecal samples can be distinguished from iron endogenous losses (2). On the other hand, compliance is crucial, because even small losses of excreted tracer can lead to a significant overestimation of the absorbed iron (2). The plasma appearance method is used to investigate the rate, quantity, and pattern of iron absorption from iron compounds (181). It requires multiple blood withdrawals within 6 hours post tracer administration; thus, it is time-consuming and a high burden for the study participants (182,183). Moreover, as for the fecal recovery method, plasma appearance only allows the determination of absorption, but not bioavailability. Therefore, both methods have largely been replaced by the erythrocyte incorporation method (2).

2.4.1.1 Erythrocytes incorporation approach

The concept behind the erythrocytes' incorporation method is shown in Figure 6.

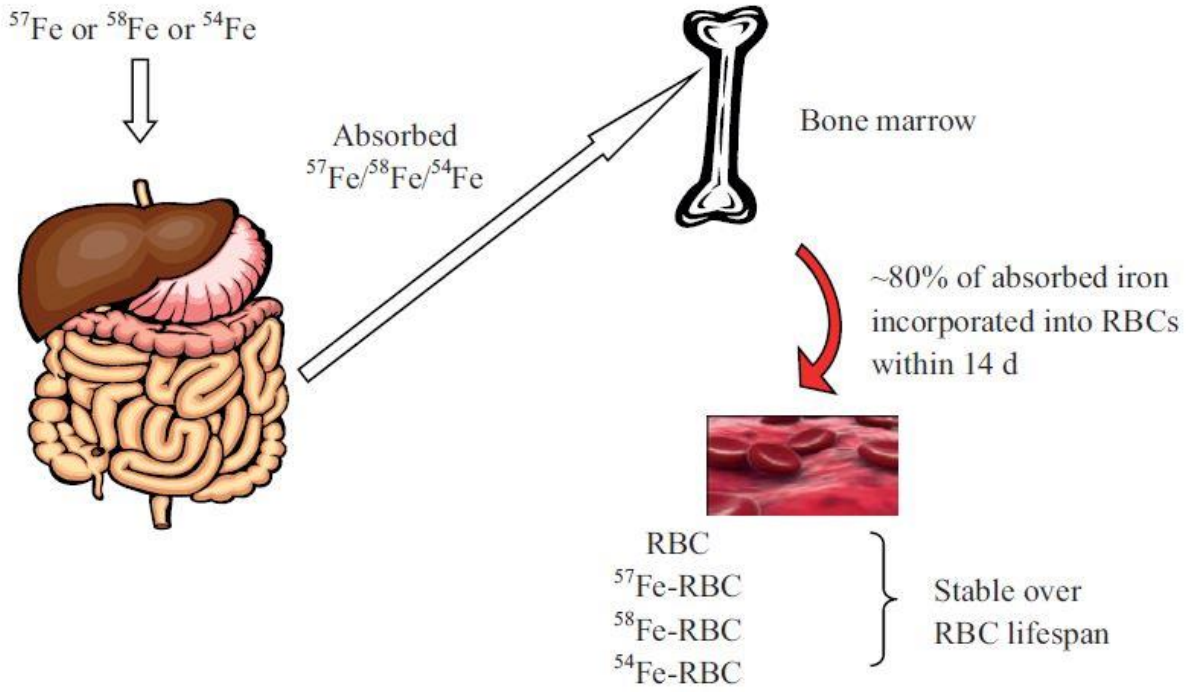

Figure 6: schematic representation of the physiology of the erythrocytes incorporation approach (2).

After the administration of the stable iron isotopes tracer, a consistent portion of the absorbed iron is transferred to the bone marrow, where it is used for Hb synthesis during erythropoiesis (paragraph 1.2.2, Figure 4). The quantification of the absorbed iron relies on the measurement of the shift in the iron isotopic abundances (from the natural abundance) in RBCs after incorporation of the label (184). The iron isotopic abundances are expressed as ratios between the molar amount of a certain isotope (usually ^{57}Fe and ^{58}Fe, being the least abundant) over the total amount of ^{56}Fe (the most abundant isotope) (184). For each isotope, its molar amount is given by its natural isotopic abundance plus its isotopic abundance in the labels used (184). This depends on the fact that stable iron isotopes are present in nature and that the labels are not 100% enriched (monoisotopic), meaning that the ^{57}Fe contains small portions of the ^{54}Fe, ^{56}Fe, and ^{58}Fe, and vice-versa (178). From the measured isotopic ratios, the amount of the circulating tracer then can be calculated when the natural iron isotopic abundance (constant) and the tracer isotopic abundance are known, and the molar amount of iron of natural isotopic composition in the blood of the subject (Fe_{circ}, see paragraph 2.1) is estimated (184). Considering that erythropoiesis takes between 9 and 12 days (34), 10 to 14 days after tracer ingestion the isotopic ratios can be measured using inductively-coupled mass spectrometry (ICP-MS)

or thermal ionization mass spectrometry (TIMS). The erythrocytes incorporation method is based on several assumptions (2):

I. the fraction of iron incorporated in RBCs from the Fe absorbed can be measured precisely after intravenous administration of a tracer but may be considered to be about 80% for most studies (constant). For precise assessment of iron bioavailability, the estimation of the fraction of iron that is absorbed but not incorporated in RBCs is required (2). Several studies have measured erythrocytes iron utilization (EIU) in different populations and age groups, with results ranging from 60 to 93 % (166,185–188). According to the International Atomic Energy Agency (IAEA), an EIU of 80% should be considered for the calculation of bioavailability (2). In certain conditions, the EIU rate might differ from 80% (2). In these cases, in addition to the oral dose, it is recommended to administer a tracer intravenously to measure directly EIU and to proceed with a precise estimation of iron bioavailability (2).

II. The fraction of iron in Hb in the erythrocytes is constant. In stable iron isotopes studies, the Hb iron content is assumed to be 3.47 mg Fe/g Hb (94,184), resulting from the direct division of the molar weight of 4 iron atoms to that of Hb A.

III. There is a negligible exchange between the iron incorporated in the erythrocytes and the plasma iron over the whole lifespan of the RBCs. This assumption cannot be fulfilled in presence of hemolytic conditions with substantial intravascular hemolysis, such as sickle-cell disease, thalassemia, infection-induced anemia, and malaria (189). Furthermore, the finding that erythroblast-specific FPN might play an essential role in iron homeostasis by releasing a significant amount of iron into the bloodstream under specific conditions (190) could potentially challenge the postulate. The extent to which iron exchange between RBCs and plasma affects erythrocyte iron incorporation measurement has not been investigated further.

When using the erythrocyte incorporation approach in studies assessing iron bioavailability from a test condition, some key factors must be considered to ensure the reliability and reproducibility of the results (2). Among others, the characteristics of the study population (health and iron status, age, sex, etc.), the study design (fasting state of the subjects, composition and native iron content of the test condition, etc.) and the type of label used (dose, intrinsic vs extrinsic) (2). The amount of stable isotope added to the

test condition should not interfere with the absorption of the native or non-labeled iron (2). Balancing between using a low dose and achieving a measurable enrichment with the mass spectrometer is more critical when the native amount of iron of the condition being tested is very low, such as in breastmilk (191,192) or iron-fortified food (193). The addition of the isotopic label extrinsically, by the simple addition of the tracer to the test condition, or intrinsically, by incorporation into the food during the growth process (194–196), has been reported to be comparable (197–199).

2.4.1.2 Dilution of stable iron isotopes

The dilution of stable iron isotopes methodology is based on principles used in radioisotope studies in adults (162,200,201). The intravenous injection of the radioisotope iron tracer in adults results in a distinct peak in levels of RBC radioactivity reflecting erythrocyte iron incorporation, followed by a steep fall of radioactivity in the first 12 months due to the recycling of iron from senescent RBC and its redistribution in other body compartments (162,200). In adult subjects, after approximately 1 year, the radioactivity decays exponentially in a linear fashion when plotted semi-logarithmically against time (201). The linear exponential decay with time indicates that full isotopic equilibration between all body compartments has occurred (162,200). Studies of long-term iron absorption and losses in adults in iron balance, using iron isotopes in the absence of iron supplementation, assume that daily iron absorption is equivalent to losses (162,200). In 2005, Fomon and colleagues for the first time extended the method by accounting for iron needs for growth in children while estimating isotopically iron absorbed, lost, and gained (94). With the use of stable iron isotopes, iron balance in toddlers could be estimated after oral administration of the isotopic tracer (94). The same method was then applied to other populations (200,202). After the iron tracer has reached equilibration with the total body iron, a modified isotopic signature is obtained (94). The modified signature, i.e., the shift in isotopic abundance, remains detectable over several years after the administration of the tracer. As the erythrocyte-iron compartment has a slow turnover the decrease (dilution) in the isotopic tracer concentration can occur only with the addition of iron with its natural composition through absorption (94). Conversely, the decrease in the absolute amount of the isotopic tracer is proportional to the amount of isotopic tracer removed, which can only occur through iron losses (94) and correlates with the absolute amount of iron lost from the body (**Figure 7**: schematic representation of the dilution of stable iron

isotopes methodology. 7-to-12 months after administration of the 58Fe ^{isotopic} tracer (1), full equilibration of the tracer with the total body iron has occurred (2). After equilibration, the iron losses have the same isotopic composition of the subject body iron (3). Iron losses are the only determinant of a decrease in the absolute amount of the tracer. Iron losses are replaced by the iron absorbed from the diet, which has the natural isotopic composition (4). Over time, the absorption of iron with a natural isotopic abundance determines the dilution of the tracer (5).) (93).

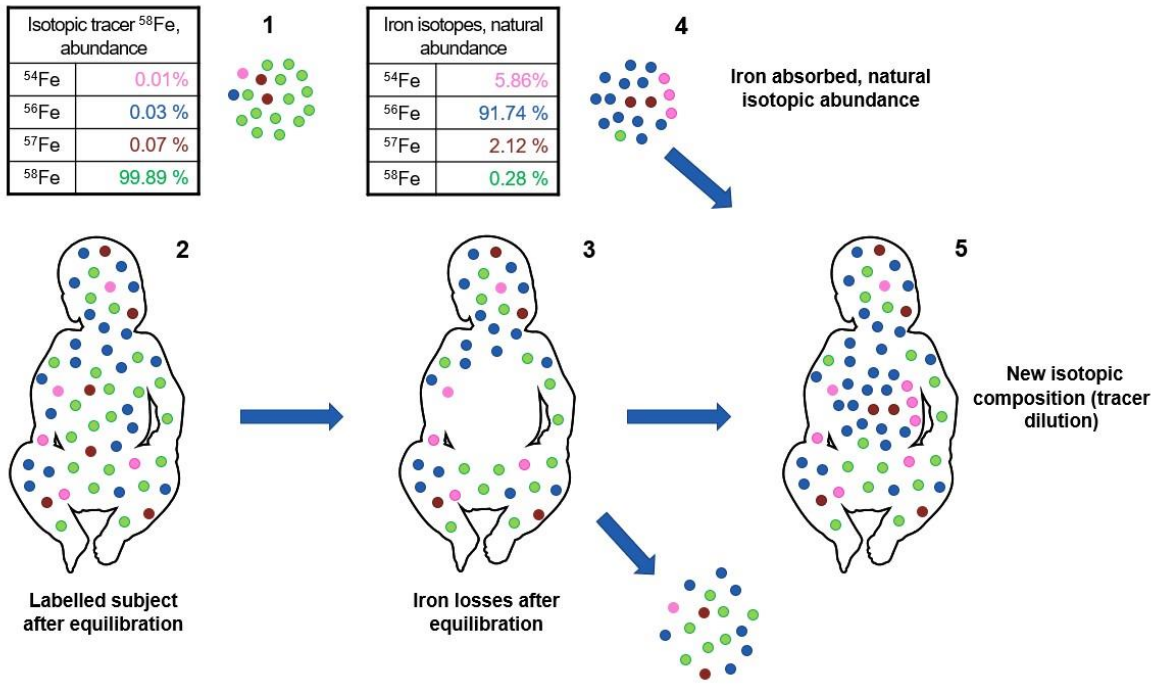

Figure 7: schematic representation of the dilution of stable iron isotopes methodology. 7-to-12 months after administration of the ^{58}Fe isotopic tracer (1), full equilibration of the tracer with the total body iron has occurred (2). After equilibration, the iron losses have the same isotopic composition of the subject body iron (3). Iron losses are the only determinant of a decrease in the absolute amount of the tracer. Iron losses are replaced by the iron absorbed from the diet, which has the natural isotopic composition (4). Over time, the absorption of iron with a natural isotopic abundance determines the dilution of the tracer (5).

Therefore, the fraction of total body iron absorbed per unit of time (k_{abs}) can be calculated as the slope of the natural logarithm of the concentration of the iron isotope tracer plotted against time (203). Similarly, the fraction of total body iron lost per unit of time (k_{loss}) can be calculated as the slope of the natural logarithm of the amount of the iron isotope tracer plotted against time (203). The mean iron absorbed over the period of analysis is derived as follows:

$$\boldsymbol{Fe_{abs} = -k_{abs} \times Fe_{tot}}$$

where Fe_{tot} represents the mean total body iron over the period of interest. Mean iron loss (Fe_{loss}) is calculated correspondingly and net iron balance (Fe_{gain}) is determined by subtracting Fe_{loss} from Fe_{abs} (203).

The stable iron isotope dilution method has also been recently used to quantify absorption and losses during iron supplementation in women and toddlers (204,205). In this context, the daily administration of iron supplements may induce an increment in iron stored but not yet equilibrated with the other body iron compartments (204,205). Thus, the difference between the newly absorbed, non-equilibrated pool of iron and the equilibrated total body iron might lead to an underestimation of absorption and losses (204). In a recent study where this methodology was applied, sensitivity analyses have reported that when the difference between the two pools stays below 5%, the error in the estimation of iron absorption and losses is negligible (204).

3. ANEMIA, IRON DEFICIENCY, AND IRON DEFICIENCY ANEMIA

Anemia, or a decreased oxygen-carrying capacity caused by a low hemoglobin concentration (206), is a public health problem that affects 1.8 billion people from low-, middle- and high-income countries (207,208). In 2019, despite a reduction in the age-standardized anemia prevalence in the previous decades, anemia accounted for more than 50 million of the total years lived with disability (YLDs) worldwide (208). In addition to adverse morbidity, anemia's consequences also exert a substantial economic burden, especially in developing countries (209). Mean hemoglobin concentrations vary widely among countries. The highest rates of anemia are reported in central and western sub-Saharan Africa, although Asia bears the greater absolute number (210). Within the same region, the determinants of the prevalence and distribution of anemia involve a variety of political, economic, social, and biological factors (211). In most settings, socioeconomic disadvantage correlates with anemia, with the poorest and least educated highly exposed to risk factors for anemia and its consequences (211). The most vulnerable groups to anemia are infants, children under 5 years of age, non-pregnant WRA, and pregnant women, with prevalence ranging from 29 to 43% in 2011 (207). Consequently, interventions and policies aiming at controlling and reducing anemia mostly target women and children (211). The global estimated prevalence of anemia in 2019 among infants and children younger than 5 years of age is shown in **Figure 8** (1). Any condition leading to a reduction in hemoglobin, or an alteration in the number of RBCs may cause anemia. Therefore, the pathophysiology of anemia is diverse and often multifactorial (210).

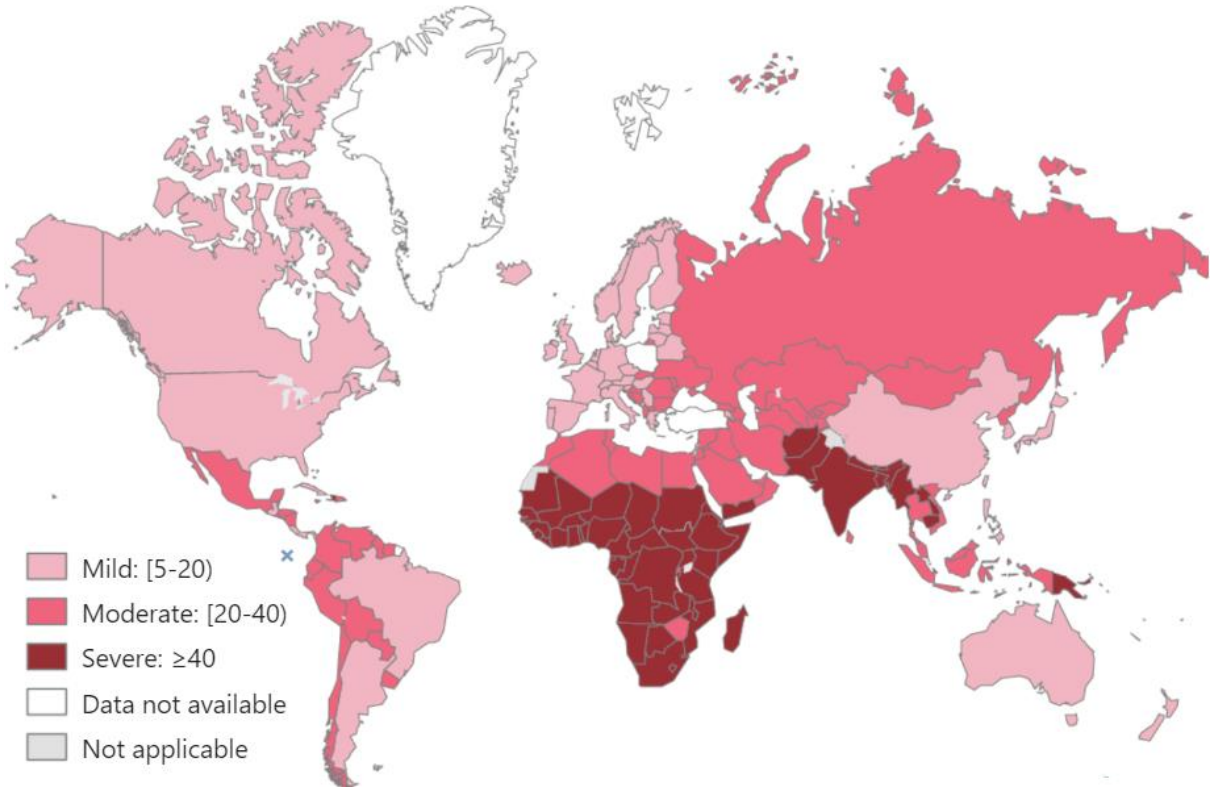

Figure 8: Most recent WHO global anemia estimates in children below 5 years old. Downloaded from: [https://www.who.int/data/gho/data/themes/topics/anaemia_in_women_and_children].

3.1 Etiology of anemia

Anemia is the result of an imbalance between erythrocyte production and loss, either due to inadequate or ineffective erythropoiesis (impaired proliferation of red-cell precursors or ineffective maturation of erythrocytes), and/or excessive loss of erythrocytes (hemolysis, blood loss) (133). Anemia can be classified by the morphology of RBC, or by the biological cause (132). The top three causes of anemia worldwide are malaria, hemoglobinopathies, and nutritional deficiencies (132). During malaria infection, the hemolysis of unparasitized erythrocytes accounts for the great majority of the acute anemia from a single infection (212). This manifestation is followed by erythropoietic dysfunction during and immediately after the acute illness, which might persist for weeks after the beginning of the treatment (213). Furthermore, the upregulation of hepcidin limits the supply of iron to the bone marrow through redistribution of iron from the systemic circulation to macrophages and reduction of the dietary iron uptake (214). Genetic hemoglobin disorders include abnormalities of hemoglobin synthesis (b- and a-thalassemia) and structure (Hb S, C, and E) (215). Structural hemoglobinopathies, such as sickle cell diseases, result in a chronic

hemolytic anemia due to the shorter lifespan of the erythrocytes (215). Reduced production of globin chains, typical of the thalassemias, can induce hypochromic and microcytic anemias with variable severity depending on the number of deletions or mutations in the globin genes (215). Nutritional anemias result from insufficient absorption of nutrients involved either in hemoglobin or erythrocyte synthesis or in both (216). Shortage of specific micronutrients, such as iron, Vitamin A, B_6, B_{12}, C, folate, and copper can lead to the development of anemia, either singularly (217) or due to a synergistic effect of multiple deficiencies within the same individual (211).

3.2 Iron deficiency and iron deficiency anemia

Iron deficiency is the most prevalent micronutrient deficiency and is the leading cause of anemia (218), accounting for about 66% and 57% of the burden in males and females, respectively (208). Although the global prevalence of anemia attributable to dietary iron deficiency is the highest among all other causes, the specific proportion of cases is heterogeneous across different economic statuses, age groups, and epidemiologic characteristics of a specific population (219). The global prevalence of iron deficiency without anemia remains elusive, although it is estimated that ID is at least twice as common as IDA (137). Iron deficiency results when iron losses or requirements exceed absorption and is often multifactorial (220). Iron deficiency can ensue if one or more of the following conditions occur: increased body needs, limited dietary intake, chronic inflammation, and chronic blood loss (221). Population groups with high iron demand include infants, preschool children, young menstruating women, and women in the second/third trimester of pregnancy and postpartum (137). In infants and children, the consumption of iron stores accumulated during gestation, and the rapid growth leads to a marked increment in iron needs (222). After childhood, adolescent girls have greater iron requirements than their male counterparts because of menstrual blood losses (222). During pregnancy, iron needs are tripled because of erythroid expansion and maintenance of the maternal-placental-fetal unit (222). Increased blood loss from gastrointestinal parasites, as well as consumption of monotonous plant-based diets with little meat, and high concentration of phytates, polyphenols, and calcium, can exacerbate nutritional iron deficiency, especially in developing countries (223). Moreover, in the same setting, a high prevalence of infection and inflammation can further reduce gastrointestinal iron uptake (223). In Western countries, additional risk factors for ID include vegetarian

and vegan dietary regimens, and causes of iron malabsorption, such as coeliac disease (137,222). ID development can be divided into 3 consecutive stages: iron depletion, iron-deficient erythropoiesis, and IDA (152), which was thought to occur upon complete emptying of iron stores (paragraph 2.2). However, recent evidence supports the existence of a mechanism downregulating erythropoiesis to help preserve iron distribution to all tissues under iron scarcity, rather than direct suppression of RBC synthesis due to iron stores exhaustion (38).

3.3 Diagnosis of Anemia, IDA, and ID

Common clinical symptoms of anemia, such as weakness and fatigue, are limited, unspecific, and often neglected (137). Therefore, several hematological and biochemical indicators are used alone or in combination to assess anemia and establish the underlying cause (109). As mentioned previously (paragraph 2.2.3), the clinical diagnosis of anemia is made based on hemoglobin or hematocrit levels. Age- and sex-specific Hb cut-offs are listed in Table 2. The major diagnostic challenge is to differentiate between the different types of anemia. For cases of anemia with a straightforward etiology, the diagnosis can be suspected from the size of RBCs (224). Although many anemias have a specific RBC appearance, multiple factors can result in similar RBC sizes and shapes, and specific hematological manifestations of a particular cause can coexist (225). The reticulocyte count can be used to estimate the degree of effective erythropoiesis and can help differentiate between decreased RBC production and increased RBC destruction or blood loss (225). Since iron deficiency is the leading cause of anemia, additional tests of iron status are also useful (paragraph 2.2) (211). As described in paragraph 2.2, the diagnosis of iron deficiency may be complex, and each biomarker of iron status has strengths and weaknesses. The use of several iron status indicators in combination may provide the best assessment of ID and related conditions, such as IDA (109).

3.4 Consequences of anemia, IDA, and ID

The association between low hemoglobin and various health outcomes, such as maternal mortality, perinatal mortality, and cognitive outcomes has been investigated in different cross-sectional, case-control, and prospective studies. Thus, despite highly suggestive evidence, the causal relationship, and the attributable fraction of anemia to the development of these conditions, is not fully defined (211). Nonetheless, it is well

documented that anemia decreases the physical capacity and increases fatigue, with detrimental consequences on work productivity (226). The symptoms of anemia depend on the severity of the disease, the rapidity of its onset, and the age and health status of the subject (227). Common symptoms of mild to moderate anemia include shortness of breath, dizziness, arrhythmias, headache, cold hands and feet, and pallor (227). Severe anemia, when developed in early pregnancy, can result in low birth weight after a premature delivery and may increase the risk of perinatal mortality (228). A U-shaped association was observed between maternal hemoglobin concentrations and birth weight (229). Many correlational studies report a negative correlation between IDA and cognitive and motor development in children that may be irreversible despite later iron repletion (230). The clinical consequences of nonhematologic effects of ID without anemia remain poorly understood. Due to its crucial role in various enzymes and structural proteins, iron deficiency alone might disturb the mitochondrial electron transport chain, impairing the functionality of tissues with high energy demands, such as the myocardium (231). Furthermore, since the adaptive immune system is highly sensitive to iron deprivation, ID can impair responses to vaccination and infection (232).

3.5 Approaches to prevent and treat anemia, IDA, and ID

Effective strategies for the treatment and prevention of anemia require a clear overview of the most relevant contributing factors to the disease in a specific environment (132). According to WHO, the potential approaches can be divided into two categories: nutrition-sensitive solutions, which aim at targeting underlying causes of anemia from a vast range of sectors, such as disease control (e.g. malaria, intestinal parasites, etc.), water, sanitation, and hygiene; nutrition-specific solutions, focusing specifically on improving the dietary intake of hematopoietic nutrients, like iron (132). For this thesis, we will focus on the nutrition-specific strategies used to prevent and correct ID and IDA, which are: I) dietary modification and/or diversification; II) iron fortification; and III) iron supplementation (223).

3.5.1 Dietary diversification

Dietary diversification aims at improving access and consumption of foods rich in highly bioavailable micronutrients, through changes in food production, selection, and preparation (233). Animal products, such as red meat, fish, and poultry, contain high

amounts of the most bioavailable form of iron, heme-iron (234). Many non-heme iron-rich foods, such as lentils, beans, soy, whole grain products, seeds, and nuts present high concentrations of iron absorption inhibitors, like phytic acid and polyphenols (235,236). Dietary modifications for preventing ID and IDA can involve an increased intake of highly-bioavailable iron-rich food, and the combination of non-heme iron-rich foods with meat, or with vegetables and fruits containing ascorbic acid (237), a well-known iron absorption enhancer (238). Despite being the most sustainable long-term approach, dietary diversification can be challenging especially in low-resource settings due to the expensive, limited access, difficulties in storing food rich in highly bioavailable iron, strict dietary preferences and habits, and religious beliefs (233).

3.5.2 Food fortification

Food fortification is defined as the addition of one or more essential nutrients to processed food to prevent a documented nutritional inadequacy (237). A requirement is that the fortified food(s) is consumed in an appropriate amount by the group targeted (239). Food fortification with iron is considered the most sustainable and cost-effective strategy to control ID at a population level (240). Food fortification can be differentiated between mass and targeted fortification (241). Mass fortification consists of the addition of one or more micronutrients to foods consumed by the whole population, and it is mainly adopted in settings where the risk of developing a specific deficiency is high and well documented, or the majority of the population, even if not clinically deficient, is not prone to develop overload (242). Targeted fortification is restricted to foods consumed by population groups that are more prone to develop a nutritional deficiency (239). In the case of ID, these groups would include infants, children, female adolescents, pregnant women, and WRA. Iron addition to food is challenging, because the high reactivity of the most bioavailable forms promotes chemical interactions with other food ingredients that produce undesirable organoleptic changes, such as rancidity and color shift (240). Thus, in addition to the iron status of the target group, the success of an iron fortification policy strongly depends on the choice of the appropriate vehicle and iron compound, as well as the dose adequate to meet a significant portion of the physiological iron requirements (237). A proper vehicle should be affordable, widely used, and present in a predictable and appropriate amount within the diet of the target group (237). The most commonly used vehicles for targeted fortification are staple cereal flours (243).

3.5.2.1 Choice of the iron compound

Numerous iron compounds can be used for fortification, and each fortificant has its advantages and disadvantages in terms of solubility, bioavailability, stability, oxidation, and costs (244). Four categories of iron fortificants can be identified: I) water-soluble iron compounds; II) poorly water-soluble iron compounds that are soluble in dilute acid; III) poorly water- and acid-soluble iron compounds; and IV) chelated iron compounds (245). Ferrous sulfate ($FeSO_4$) is a water-soluble iron compound that presents the highest relative bioavailability (RBV) (100%) among conventional fortificants (245). Ferrous sulfate has the additional advantage of being the least expensive but, depending on the storage time and the food matrix to which it is added, it might induce rancidity, color changes, and oxidized off-flavors (240). The most common poorly water- but diluted acid-soluble iron compound used in food fortification is ferrous fumarate (FeFum). It causes far fewer organoleptic consequences than water-soluble compounds, and its RBV is comparable to the one of $FeSO_4$ in adults (246,247). Inconsistent results have been reported in infants and children, with studies suggesting a lower acute iron absorption by infants and young children from ferrous fumarate-fortified cereal foods, compared to ferrous sulfate (248). Differences in sensitivity of FeFum and $FeSO_4$ to iron enhancers and inhibitors in the food matrix (249), in the iron status of the study participants (249), and a higher postprandial gastric pH in infants, perhaps not allowing the complete dissolution of FeFum in the stomach (250), might account for the conflicting findings. Nonetheless, long-term feeding studies report that ferrous fumarate-fortified cereals improve the iron status of infants (248), and two Cochrane reviews concluded that home fortification with iron-containing micronutrient powders (MNPs), with various compounds including ferrous fumarate, can be as efficacious as daily iron supplementation in reducing anemia and ID in infants (251) and children (252). The efficacy of in-home fortification in infancy with FeFum-containing MNPs is reviewed in section 3.4.2.2. One disadvantage of using FeFum might be the cost, which is higher than $FeSO_4$ (248). The use of poorly water- and acid-soluble compounds is common in the food industry due to their inexpensiveness and poor chemical reactivity but is limited by their low RBV. Their absorption varies widely depending on the factors influencing their dissolution in gastric juice, i.e., the size, shape and surface of the iron particles (243), as well as on the presence of enhancers and inhibitors in the food matrix (239). Therefore, higher doses of poorly soluble compounds need to be added to

compensate for the reduced absorption rate (239), potentially inducing unwanted health side effects, such as dysbiosis in the colon due to a substantial portion of unabsorbed iron (253). The two main groups within this category are iron phosphate compounds such as ferric pyrophosphate, and elemental iron powders such as electrolytic iron (240). The use of chelated iron compounds, such as sodium iron ethylenediaminetetraacetic acid (NaFeEDTA), may increase iron absorption in phytate-rich foods compared to $FeSO_4$ or FeFum by preventing the binding between iron and its inhibitors (244). However, the acceptable daily intake of EDTA raises concerns about its extended use in foods in the general population (242), and especially in infants and young children from developing countries, where the high burden of malnutrition might lead to intakes above the recommended levels in a non-negligible proportion of subjects (254).

3.5.2.2 Point-of-use fortification

Point-of-use fortification with MNPs has been proposed by WHO as a public health intervention to reduce micronutrient deficiencies in infants and children (255). In this method of fortification, a powdered premix containing iron and other vitamins and minerals is added to foods while or immediately after cooking (252). In infants, the most commonly used MNPs are Sprinkles®, sachets containing a daily dose of several micronutrients in powder form, which can be easily added to any food prepared at home (256). In these preparations, iron is encapsulated in a lipid layer which prevents unwanted organoleptic changes (218). In the first trials investigating the effect of the administration of FeFum-containing Sprinkles®, in comparison with the same amount of iron given as ferrous sulfate drops, the efficacy of two treatments in reducing the prevalence of anemia and IDA was comparable (257,258). A series of consecutive studies testing Sprinkles® with different dosages of FeFum concluded a lower dose of 12.5 mg might be as efficacious as the higher doses in reducing IDA in infants and children (259,260), with the advantage of fewer side effects and better compliance compared to the same iron administered as drops (260). These results were confirmed by further efficacy trials performed in infants and children, where Sprinkles® containing 12.5 mg FeFum were administered daily for periods ranging between 2 (261,262) and 12 months (263,264). The evidence gathered in these studies contributed to the development of WHO guidelines for point-of-use food fortification in infants and young children up to 12 years of age, which recommend consumption of 90 sachets containing 10-12.5 mg of iron over 6 months in areas where

the prevalence of anemia is 20% or higher (255). This approach has proven to be consistently effective across different regions and populations, meaning that the dosage of 12.5 mg iron is sufficient to overcome the negative effect of inhibitory food matrices, or elevated plasma hepcidin levels in areas with a high burden of infections (265) on iron absorption. In the past, the safety of this iron dosage, either as a supplement or fortificant, was questioned because several trials reported an increased risk of serious adverse events and hospitalization (266), respiratory episodes, and diarrhea (267). Because of concerns about the increased severity of malaria infections in malaria-endemic areas, in 2006 the WHO recommended the use of high-dose iron MNPs in these regions only in diagnosed anemic children and children at high risk of ID (268). The subsequent interruption of iron supplementation programs, causing the exposure of millions of children to the risk for the adverse impacts of IDA (269), lead to an amendment to these guidelines in 2011, where it was recommended to implement the provision of iron in conjunction with prevention and management services for malaria, in malaria-endemic areas (270). This approach has been successful, with no harm observed for iron-treated children when initial screening for malaria was performed and antimalarial treatment was provided when infected (271,272). Furthermore, according to the most recent Cochrane reviews investigating the effect of home fortification with iron-containing MNPs in children under 2 years of age (251) and children (252), the subjects receiving the intervention were exposed to the same risk of diarrhea as those receiving no intervention or placebo. Nonetheless, in both cases, the quality of the evidence was rated as low, due to the paucity and the high heterogeneity of studies reporting on diarrhea (251,252). Unabsorbed iron from MNPs entering the colon promotes the growth of many potential enteric pathogens, such as Salmonella, Shigella, and pathogenic Escherichia coli (273), being crucial for their expression of virulence and colonization factors (274,275). Pathogenic *E. coli* is reported to have a crucial role in the development of diarrhea and bacteremia among infants (276,277), and a rise in Enterobacteriaceae and/or a decrease in Bifidobacteriaceae could also play a role (278). The daily ingestion of large amounts of dietary iron, compared to what is usually provided by the diet alone, might impair the composition and metabolism of the intestinal microbiota, at the expense of beneficial barrier bacteria such as lactobacilli and bifidobacteria (253,273), whose growth is partially or fully independent from iron (279). In addition to the unfavorable effect of dysbiosis on local inflammation and diarrhea, alterations in the microbiota might also be implicated in

the development of a variety of psychiatric, neurological, and neurodegenerative diseases, as supported by the most recent highlights on microbiota-mediated communication between the gut and the brain (gut-brain axis) (280,281).

3.5.3 Iron supplementation

Oral iron supplementation is considered the first line of prevention and treatment of ID and IDA among the most vulnerable groups due to its safety and cost-effectiveness, but guidelines vary (282). The WHO guidelines for the prevention of ID and nutritional anemia change according to the target (children up to 2 years of age, preschool and school-age children, WRA, pregnant women, postpartum women, and low-birth-weight and very-low-birth-weight infants) and the prevalence of anemia in the region (132). Moreover, little consensus has been reached upon the ideal dosing regimen, or iron preparation to use (282). In general, ferrous iron salts are preferred because of their favorable ratio between costs and bioavailability (108). In adults, a daily total amount of 100 to 200 mg of elemental iron, divided into 3-4 administrations per day, is recommended as oral iron therapy; in children, daily 3 mg of iron per kilogram of body weight represents the standard of care (283). The efficacy of iron supplementation protocols is jeopardized by the occurrence of iron-related common adverse effects, such as nausea, vomiting, and constipation, which reduce compliance to the treatment and extend its duration (137). These symptoms might occur due to: 1) free radical generation in the gut lumen and at the mucosal surface due to the chemical reactivity of iron, which increases local inflammation (284,285), and 2) as mentioned in the previous paragraph, harmful alterations in the gut microbiota composition or metabolism. Alternate-day schedules could increase the efficacy of the treatment, by resulting in a higher fractional iron absorption with a lower incidence of gastrointestinal side effects, when compared to consecutive day dosing (286,287). In subjects who are not responding to oral iron, or whose needs cannot be met with oral therapy due to chronic bleeding or hemodialysis, or who are affected by iron-refractory iron-deficiency anemia, malabsorption syndromes, or chronic inflammation, oral iron supplements may be replaced by parenteral iron therapy (283). Intravenous iron administration is infrequent in the general population due to its potential to cause strong allergic reactions or serum sickness-like reactions (283), regardless of the product used (288).

4. Optimization of iron bioavailability from Fe supplements and iron fortified food: potential use of prebiotics and lactoferrin

As described in the previous chapter, the safety and efficacy of iron interventions to treat and prevent anemia, ID and IDA, have been questioned, especially in populations with a high burden of infections (289). Point-of-use fortification with iron-containing MNPs has been proposed by WHO as a public health intervention to reduce anemia and IDA in children (252). However, iron absorption from MNPs or other fortificants is blunted by the abundance of absorption inhibitors (e.g., phytic acid, polyphenols) in the complementary food to which the fortificants are added, or which is used as a vehicle (290). Furthermore, the sharp increase in colonic iron that follows the provision of iron fortificants or supplements favors the growth of pathogenic strains over healthy barrier strains, potentially leading to an increased risk of diarrhea, especially in infants and children (253). Supplementation with daily doses of 100–200 mg of elemental iron has been widely recommended in adults as an effective treatment for IDA (110). Nonetheless, fractional iron absorption from oral supplements is generally low, varying from 2% to 13% when consumed with food versus 5%–28% when consumed fasting (282). Furthermore, the consumption of oral iron supplements has been associated with the occurrence of several gastrointestinal adverse effects, such as erosive mucosal injury in the upper gastrointestinal tract, nausea, constipation and diarrhea (291). A relationship has been observed between the number and severity of side effects and the dose of iron administered (292). The maximization of fractional iron absorption, and the reduction of the iron dose to the lowest effective to prevent ID and IDA, would increase the safety and tolerability of oral iron supplements and fortificants (290,291).

4.1 Strategies to increase iron bioavailability

The methods currently in use to improve iron bioavailability from fortificants and supplements, and to reduce side effects, involve various approaches, i.e., the optimization of the iron compound, the addition of enhancers to the supplement/fortificant, or the degradation of potential inhibitors from the vehicle used for fortification (293). In general, these strategies aim at limiting the interaction between iron and the inhibitors of its

absorption, or at promoting the reduction of ferric to ferrous iron, the most suitable form for duodenal uptake (293). Some examples of the most common procedures to increase iron bioavailability are listed below.

4.1.1 Removal of inhibitors from the food matrix

The main inhibitors of iron absorption are phytates, polyphenols, and calcium. Phytates are present in relevant amounts in whole cereals and legumes, are strong chelators of iron and other metal ions, and form insoluble complexes that cannot be digested, thus reducing the bioavailability of minerals and trace elements (294). Their effect is dose-dependent and occurs also at very low concentrations; thus, mechanical (milling) or enzymatic (phytase) dephytinization has been suggested as a strategy to enhance iron absorption from cereal- and/or legume-based complementary foods (295). Complete enzymatic dephytinization has been reported to increase iron absorption from single meal studies up to 12-fold, but the enhancing effect was overcome by the use of milk instead of water to reconstitute the food, and was poorly efficient when even very low concentration of phytates resisted degradation (296).

4.1.2 Use of novel iron compounds: NaFeEDTA

Among other iron compounds, the use of NaFeEDTA has been widely recommended to fortify high phytate staple foods, such as cereal flours, as a substitute for $FeSO_4$ or FeFum (244). NaEDTA is a strong chelator of iron, especially at acidic pH, thus preventing the formation of non-absorbable complexes with dietary inhibitors in the stomach. Iron is then exchanged with other metals at the higher pH of the duodenum and is available for absorption (240). Nonetheless, the acceptable daily intake of EDTA limits the daily iron intake to ≈0.36 mg iron/kg body weight/day, corresponding to only 2-3 mg iron/day that can be provided to infants, which is well below the recommendations of the WHO (297).

4.1.3 Limitation of the interaction between iron and the food matrix

The separation of the iron compound from the food matrix can occur also through microencapsulation, i.e., coating the iron compound with hydrogenated oils or other substances to protect against oxidation (298). This approach should have the advantage of allowing the addition of iron forms with high bioavailability to difficult-to-fortify food vehicles without inducing organoleptic changes (299). Several iron compounds, including

$FeSO_4$ and FeFum, are available commercially in encapsulated form (300). In anemic children, the administration of encapsulated ferrous fumarate increased iron status and Hb concentration (257), even when given at a lower dose of 12 mg (260). However, the effect of encapsulation on Fe bioavailability is a concern, with factors such as capsule material, the ratio of material to Fe, and the technology and process used for encapsulation potentially affecting absorption (299). Moreover, in some food products, fortification with encapsulated iron has caused off-color development (301).

4.1.4 Addition of enhancers: ascorbic acid

The most studied iron absorption enhancers are vitamin C, folic acid, citric acid, peptides rich in amino acid cysteine, and vitamin A (302). Ascorbic acid is the strongest enhancer of non-heme absorption and it operates by converting ferric to ferrous iron (303), thus facilitating iron uptake from the duodenal DMT-1 (paragraph 1.1.1). In short-term studies, the addition of ascorbic acid and iron in a 2:1 molar ratio increased iron absorption from foods 2- to 3-fold in adults and children (293). In adults, ascorbic acid has a negligible effect on iron balance after long-term supplementation with vitamin C (238). A major limitation in the use of ascorbic acid is that is susceptible to losses when exposed to heat and oxygen during food storage and preparation (304). Recently, the focus has been placed on the use of novel compounds acting as enhancers of iron absorption while reducing the adverse impact of iron fortification or supplementation on the gastrointestinal tract. Two potential candidates, prebiotics and lactoferrin, are described in detail in the next paragraphs.

4.2 Prebiotics

A prebiotic was originally defined as “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health” (305). To be classified as a dietary prebiotic, a food ingredient needed to meet the following criteria: I) resistance to gastric acidity; II) resistance to hydrolysis by mammalian enzymes and to gastrointestinal absorption; III) fermented by the intestinal microflora; and IV) selective stimulation of growth and/or activity of intestinal bacteria associated with health and wellbeing (306). In recent years, after several revisions, the focus has expanded from the selective targeting of specific bacterial strains, i.e. bifidobacterial and lactobacilli, to the support of a broad

consortium of microorganisms and functional characteristics that are beneficial for the physiology of the host (307). This has led to the possibility of including under the definition of prebiotics non-carbohydrate substances, such as polyphenols and polyunsaturated fatty acids, and extending the prebiotic action to microbially colonized body sites other than the gastrointestinal tract, such as the vaginal tract and skin (308). Some substrates alter microbiota and metabolic composition without involving microbial utilization and are not classified as prebiotics. Among others, these substances include antibiotics, minerals, vitamins, and bacteriophages (Figure 9) (308).

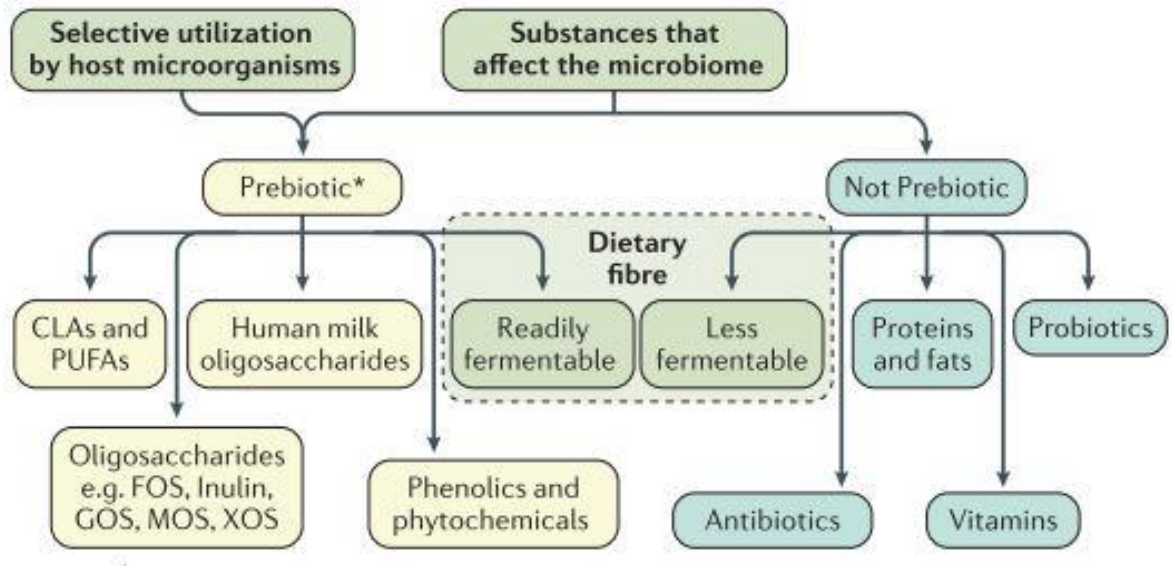

Figure 9: Distinction between substances that can be or cannot be considered as prebiotics despite their influence on the microbiota. The figure shows candidates as well as accepted prebiotics. CLA, conjugated linoleic acid; PUFA, polyunsaturated fatty acid; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides; MOS, mannanoligosaccharide; XOS, xylo-oligosaccharide (308).

A prebiotic-like effect has been reported for a vast number of fermentable carbohydrates, but the strongest evidence for positive effects on host physiology exists for the non-digestible oligosaccharides fructans and galactans (309). Fructo-oligsaccharides (FOS) and galacto-oligosaccharides (GOS) dominate the prebiotic field due to their massive application in infant formulas. In addition to GOS and FOS, a narrow range of confirmed prebiotic substances exists, but several intestinal microbiome-modulating carbohydrates and other substrates, such as vitamins, minerals, and fatty acids have been proposed as potential candidates for prebiotic status (310). Among others, human milk oligosaccharides (HMOs) are among the strongest candidates due to their known effect on the gastrointestinal health and immune development of breastfed babies (308). The

main limitation in their use, and consequently in providing supportive evidence of their prebiotic nature, was the difficulty in their synthesis and manufacturing (311). However, recent advances in the development of enzymatic, whole-cell, and living-cell systems have allowed the production of a growing number of HMOs (312). The structural units of GOS, FOS, and HMOs are displayed in **Figure 10** (313).

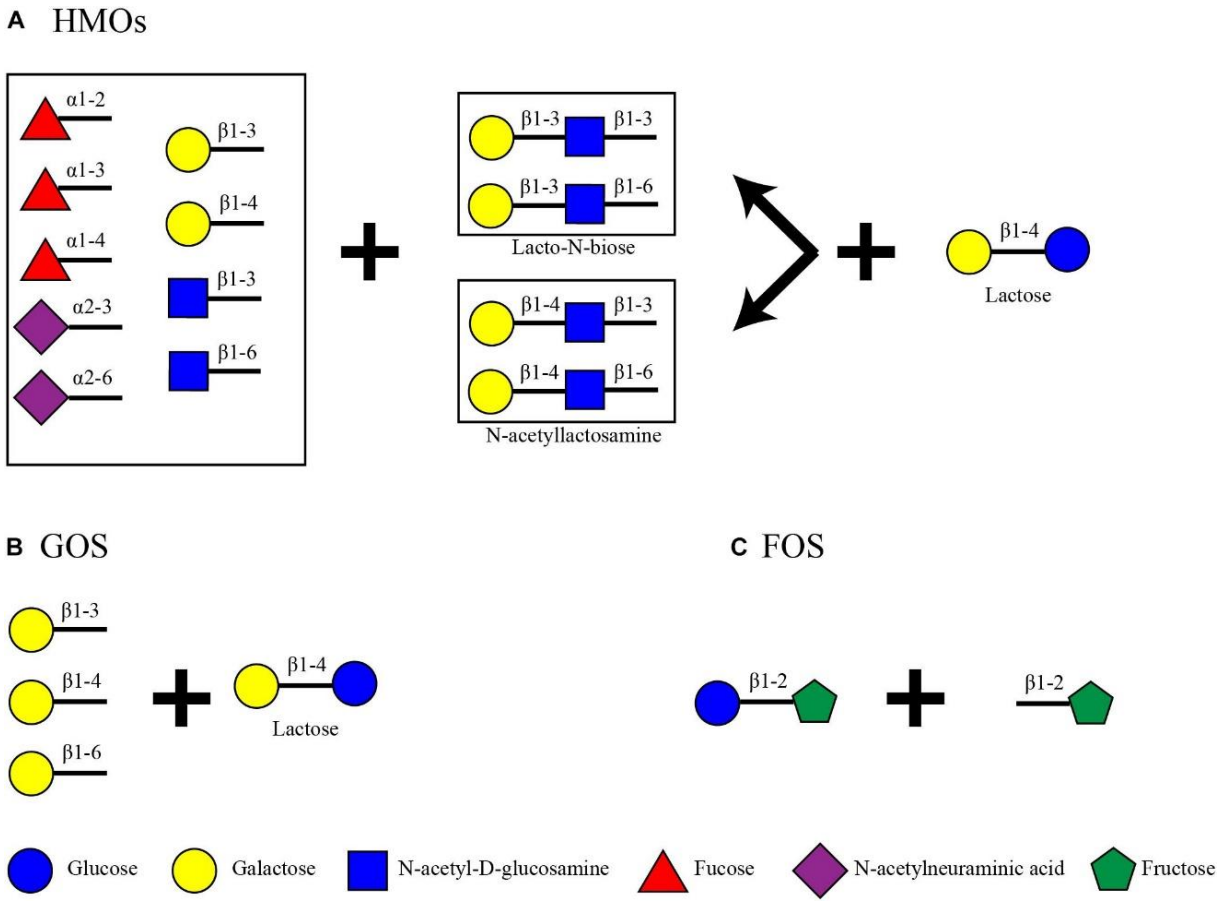

Figure 10: Structural backbone of (A) HMOs, (B) GOS, and (C) FOS.

4.2.1 Galacto-oligosaccharides and Fructo-oligosaccharides

GOS are a mixture of β(1→3)-, β(1→4)- and β(1→6)-linked di- and oligosaccharides of galactose units (between 2 and 20) and a galactose or glucose unit at the reducing end (galactose$_n$- galactose and/or galactose$_n$-glucose) (**Figure 11**) (314). GOS can be produced by the enzymatic transgalactosylation of lactose by the hydrolase β-galactosidase, through a 2-step reaction that starts with the formation of a galactosyl-enzyme complex (315). In the second step of the reaction, water competes as an acceptor of the galactosyl-enzyme complex with lactose or GOS (315). Therefore, GOS synthesis is kinetically controlled by the competition between hydrolysis and

transgalactosylation, with the first being favored after reaching a maximum GOS concentration, and the second when lactose is available at high concentrations (314).
FOS are β(2→1) and β(2→6)-linked fructose monomer units ranging between 2 and 60 with a terminally attached glucose unit (glucose-$(fructose)_n$ structure) (316). FOS can be found in plants such as onion, chicory, garlic, asparagus, banana, and artichoke (317), from which they have been extensively extracted in the past (316). Currently, FOS are produced either through the catalytic biosynthesis by the enzymes frutosyltransferases or β-fructofuranosidases, which use sucrose as a substrate, or by enzymatic degradation of inulin (318).

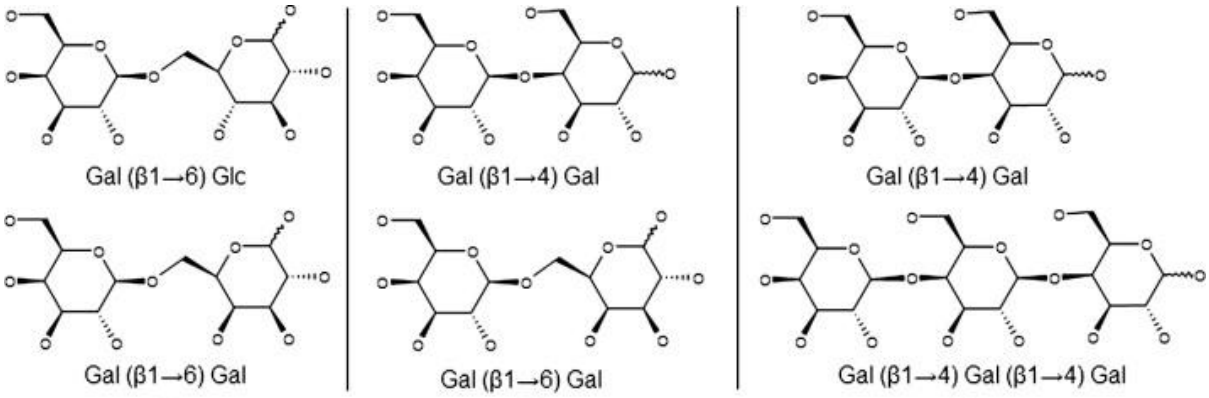

Figure 11: Some of the possible GOS structures. Differences can exist in saccharide composition, regiochemistry of the glycosidic linkages, and degree of polymerization (319).

In the European Union, GOS and FOS (320) can be used as a non-Novel Food ingredients in food products; in the U.S., GOS and FOS (321) have received GRAS (Generally Recognized As Safe) status by the U.S. Food and Drug Administration (FDA); in China, Brazil, Canada, and Japan, GOS and FOS have received official approval for use in food products (322). The commercial interest in GOS is due to the presence of galactose-based oligosaccharides in human milk and the structural similarity between commercial GOS and some of the HMOs (323). In fact, despite the high variation in oligosaccharides composition and concentration throughout lactation, in the first few months GOS account for 60 to 90%, and FOS for 10 to 40%, of the oligosaccharides in human milk (324). Thus, infant formulas, follow-on formulas, and infant foods represent the majority of GOS-containing products (322). FOS, alone or in a prebiotic mixture with GOS, has also been extensively used as an analog of HMO in infant formulas due to its well-proven nutritional and health benefits (325). Apart from its bifidogenic effect and its action as a dietary fiber,

FOS is widely used as a sucrose substitute (318) due to its lower sweetness intensity, its non-digestibility by human enzymes, which makes it “calorie-free”, and its non-cariogenic property (317).

4.2.1.1 Potential action of GOS and FOS on the gut microbiota and clinical outcomes

As briefly introduced, the rationale for supplementing infant foods with GOS and FOS is to mimic the bifidogenic effect and the implied advantages of a breastfed-like flora (326). Among other factors, the development of the microbiome from infancy to childhood strongly depends on breastfeeding, which is associated with higher levels of Bifidobacterium species (327). Together with human milk bacterial composition and abundance (328), HMOs have proven to be a crucial factor in the establishment and shaping of the neonatal gut microbiota (329). In fact, most HMOs reach undigested the intestinal tract, where they are utilized as a primary carbon and energy source by specific microbiota strains. In particular, HMOs provide a competitive growth advantage to Bifidobacterium and Bacteroides spp. over other commensal bacteria (329). GOS and FOS are well-established prebiotics (305), with high selectivity toward Bifidobacterium spp. (330). The enhancing effect of GOS and FOS on bifidobacterial growth has been extensively reported *in vitro* (331–335) and *in vivo* studies. Trials in infants showed that daily consumption of approximately 1–6 g/day or approximately 0.2–3 g/kg body weight (BW) of GOS, or GOS and FOS, increased the abundances of bifidobacteria (336–340). The same results were reported when infants consumed daily for 4 weeks infant formula fortified with FOS alone (341). The abundance of bifidobacteria in the microbiota provides several benefits, including: I) fermentation of non-digestible carbohydrates leading to the production of beneficial metabolites (342,343), such as short-chain fatty acids (SCFAs) (344); II) increased antimicrobial activity; III) anti-inflammatory effects on the intestinal epithelial cells (345); and IV) immune development (346). The most important end products of colonic bacterial fermentation are SCFAs, mainly acetate, propionate, and butyrate (347). The great majority of SCFAs is quickly absorbed from the intestinal lumen and they are either metabolized by colonocytes, or delivered to the liver via the hepatic portal vein (348). In addition to their role as an energy source for intestinal epithelial cells, SCFAs are sensed by specific G protein-coupled receptors, which are involved in the regulation of lipid and glucose metabolism (347). Furthermore, SCFAs exert various effects on different cellular components in the inflammatory and immune responses, via

inhibition of expression of adhesion molecules and reductions in chemokine production, suggesting SCFAs anti-inflammatory activities (349). Lastly, the increase in SCFA production in the colon decreases gut pH (338) and provides a less favorable growth environment for enteric pathogens (350). The SCFAs-mediated reduction of colonic pH constitutes only one of the possible mechanisms of resistance to pathogenic colonization that have been indirectly attributed to GOS and FOS. An increased bifidobacterial abundance might strengthen competitive metabolic interactions at the expense of potential pathogens, and can enhance the stimulation of the host immune system through the recognition of microorganism-associated molecular patterns of the commensal microbiota (351,352). These hypotheses are supported by in vitro studies reporting a reduced growth of C. difficile and a lower adherence of EPEC to Hep-2 and Caco-2 cells after the addition of GOS, FOS, and inulin to the fermentation medium (353,354). Whether the effect of GOS and FOS administration on morbidity, and specifically on gastrointestinal infections/diarrhea, can be clinically relevant is still unclear, because the available evidence is inconclusive (355). Nonetheless, some studies reported a reduction in diarrhea episodes, or lower incidence of gastrointestinal infections in infants when GOS, or a combination of GOS and FOS, was administered (356–358).

4.2.2 Human milk oligosaccharides

HMOs are a group of structurally diverse unconjugated glycans which constitute the third most abundant solid component of human milk (359). HMOs are composed of the five monosaccharides galactose (Gal), glucose (Glc), N-acetylglucosamine (GlcNAc), fucose (Fuc), and the sialic acid (Sia) derivative N-acetyl-neuraminic acid (359). All HMOs have lactose (Galβ1→4Glc) at the reducing end, which can be extended by β1→3- or β1→6-linkage by two different disaccharides, either Galβ1→3GlcNAc (type 1 chain) or Galβ1→4GlcNAc (type 2 chain) (360). Depending on the expression of the gene *Se*, which is translated into a functional fucosyltransferase 2 (FUT2) and classifies women as "secretors", Fuc and other fucosilated HMOs can be added to the HMO backbone (360). The frequency of the secretor phenotype is estimated at ~80% worldwide, but variation is high between different geographical regions (361). A separate enzyme called fucosyltransferase 3 (FUT3), codified by the *Le* gene, catalyzes the addition of Fuc in α1→3/4-linkage (362). FUT3, as FUT2, can also be inactive in parts of the population depending on the presence of mutations in one or both alleles of the *Le* gene (Le^a and Le^b)

(362). Women are classified as “Lewis negative” if they do not express an active form of FUT3, or if they only express it in traces (363). This results in four common milk profiles reflecting phenotypic differences: Se^{+}/Le^{a-b+}, Se^{-}/Le^{a+b-}, Se^{+}/Le^{a-b-}, Se^{-}/Le^{a-b-} (361). To date, more than 200 different HMOs structures have been identified (363). In addition to the variation introduced by maternal genetics and area of origin, the other known factors influencing the composition and abundance of HMOs are the gestational time and the stage of lactation (363), with colostrum having the highest absolute HMO concentration (9–22 g/L), followed by lower concentrations in transitional milk (8–19 g/L) and a subsequent decline as lactation progresses (6 months after birth, 4–6 g/L) (364).

HMOs were originally identified as the “Bifidus factor” in human milk (359), and their prebiotic nature has been extensively confirmed by *in-vitro* studies reporting resistance to acidic gastric conditions and upper GIT degradation, and selective promotion of growth of certain strains of Bifidobacteria and Lactobacilli in the colon (365,366). Moreover, in addition to their bifidogenic activity, studies in murine and human intestinal epithelial cells models exposed to HMOs reported decreased bacterial attachment and dextran permeability when challenged by enteric pathogens (367–369), giving insights into how specific HMOs may modulate intestinal barrier function. Lastly, the fact that approximately 1–2% of ingested HMOs are found intact in the urine and plasma of breastfed infants, suggests that they can also exert systemic effects, such as modulation of host immune response (370). This hypothesis is supported by *in-vitro* evidence reporting reduced expression of pro-inflammatory cytokine production in lymphocytes, potentially leading to a more balanced T-helper cells (Th) 1/Th2 response, and in LPS-stimulated macrophages, and decreased neutrophil activation upon HMOs addition in the cell medium (359). Because of the high complexity and low efficiency of biological production, HMOs have been generally absent from infant formulas (371). Most of the studies investigating their properties and mechanisms of action are observational studies on breastfeeding infants, correlating HMOs in breastmilk with various health outcomes (372), such as diarrhea (373) and respiratory tract infections (374). Nonetheless, seven HMOs have obtained the GRAS status from the U.S. FDA, including 2′-fucosyllactose (2′-FL), 3-fucosyllactose (3-FL), difucosyllactose (DiFL), lacto-N-Neotetraose (LNnT), lacto-N-tetraose (LNT), 3′-sialyllactose (3′-SL), and 6′-sialyllactose (6′-SL) (375). A few placebo-controlled studies with HMOs have been performed in infants, mostly focusing on the safety of administration and clinical outcomes (376). According to the available data, infant formulas

supplemented with 2′-FL and LNnT are as safe and well-tolerated as non-supplemented formulas (377,378). Moreover, when compared to the control, infants fed the HMO-fortified formula had a significantly lower incidence of respiratory tract infections, reduced use of antibiotics (378), and developed a gut microbiota that was closer to that observed in breastfed infants (379).

4.2.3 GOS, FOS and HMOs and iron absorption

Prebiotics can enhance the absorption of calcium and other minerals in animals and humans (380), an effect thought to be mediated by a combination of factors, including I) an enlargement of the absorption surface, II) an increase in cell density, III) a decrease in colonic pH, preventing complexation with negatively charged metabolites such as phytates (381), and IV) an increase in the soluble and ionized fraction of minerals in the colon, potentially promoting mineral diffusion via the paracellular route (382). The creation of a reducing environment due to a SCFAs-mediated reduction in luminal pH, the stimulation of the expression of genes codifying iron-regulatory proteins, and the enhancement of epithelial proliferation are also among the postulated mechanisms by which prebiotics could increase iron absorption (383). The findings of several animal studies investigating the effect of prebiotic administration support some of these proposed hypotheses. Different trials have reported a reduction of cecal pH and increased Hb concentration in iron-deficient anemic rats after FOS and iron administration (384), an increased gene expression of DMT1, ferritin, ferroportin, and TfR in the duodenum and the colon of anemic piglets after inulin supplementation (385), and cecal enlargement and increased cell proliferation in iron-deficient anemic FOS-fed rats, associated with an overall increase in absorbed iron (386). Several human studies have investigated whether prebiotics, specifically GOS and FOS, enhance iron absorption. GOS, and FOS did not increase significantly iron absorption in adults when administered with iron as $FeSO_4$ for three weeks, both in women and men regardless of their iron status at baseline (350,387). Nonetheless, acute GOS administration has been concisely reported to enhance iron absorption from FeFum in several studies in iron-depleted women (388–390), and FeFum and NaFeEDTA in one study in Kenyan infants, after a pre-feeding period of 3 weeks (250). These results were not confirmed in the same population when the pre-feeding with GOS was not performed. The addition of GOS to a single iron-fortified maize porridge test meal in Kenyan infants did not significantly increase iron absorption, both from FeFum and

NaFeEDTA or from the same dose of iron as $FeSO_4$ (391). In a study investigating the kinetics of iron absorption from FeFum given with and without GOS in iron-depleted women, no delayed peak in isotope appearance in blood, which would correspond to colonic uptake, was observed after GOS administration (392).

This, combined with the results from the long-term supplementation studies in adults (350,387) point towards a negligible role in colonic iron absorption compared to the duodenal uptake in adults. By contrast, the absence of an acute enhancing effect of GOS on iron absorption in infants, without prior GOS consumption, seems to indicate that long-term feeding of GOS is necessary to enhance iron absorption at this age (391).

To date, no trials have been published on the acute or long-term administration of HMOs on iron absorption. Nonetheless, in Kenyan infants, the maternal HMO profile modulates the microbiota response to fortificant iron, with the maternal secretor status influencing *Bifidobacterium* abundance after the iron intervention, and with a higher longitudinal prevalence of diarrhea among infants of non-secretor compared to infants of secretor mothers in the group not receiving iron (393).

4.3 Lactoferrin

Lactoferrin (Lf) is a multifunctional glycoprotein of approximately 691-696 amino acids and 78 kDa that is present in milk, saliva, and seminal fluids (394). Lf reaches its highest concentration in milk, ranging from 1–2 g/L in mature milk to 7 g/L in colostrum (395). Its single polypeptide chain is folded into two symmetrical and highly homologous (33-41%) globular lobes, the N-terminal and the C-terminal (396). Each lobe presents a binding site for a cation, predominantly iron, which is coordinated by four amino acid side chains and stabilized by two oxygen atoms from a carbonate ion (CO_3^{2-}) (Figure 12) (397).

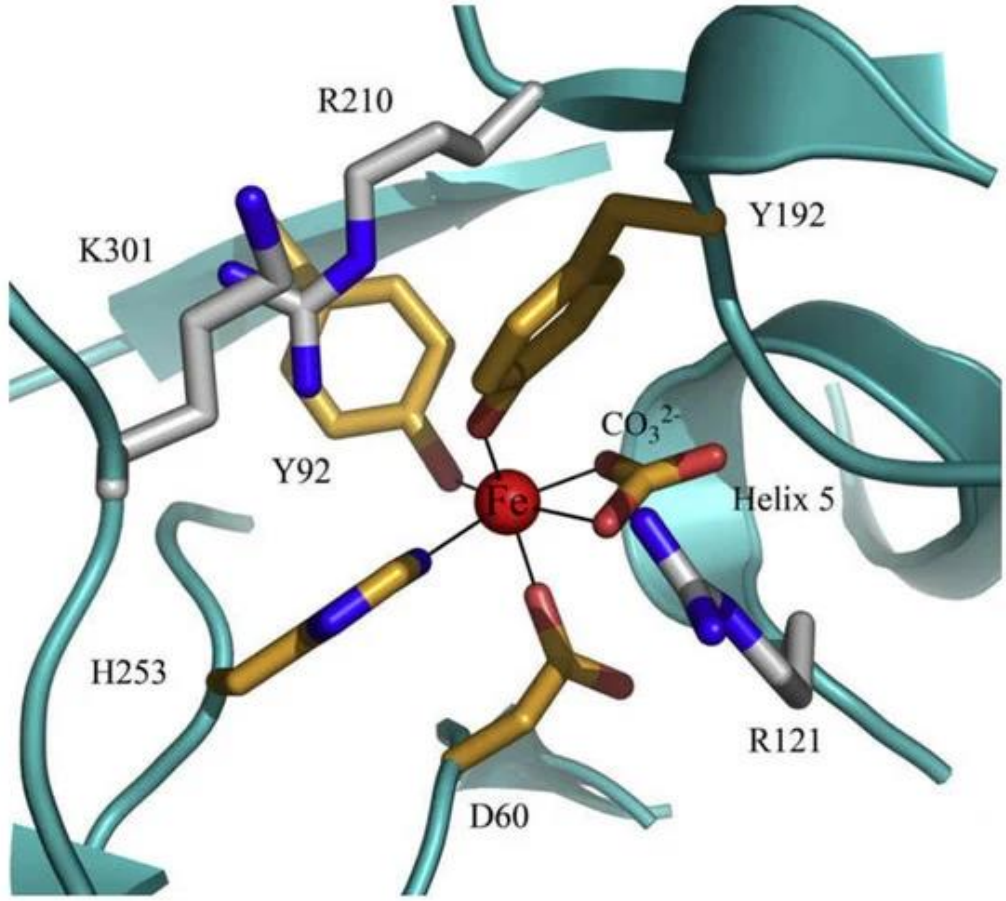

Figure 12: Lactoferrin iron-binding site. Iron-binding site in the N-lobe: two tyrosine (Y92 and Y192), one aspartic acid (D60), one histidine (H253) and one carbonate anion together with the arginine residue (R121) (398).

Lf has a very high affinity for iron, with an equilibrium dissociation constant value (Ka) of about 10^{-22} at a physiological pH (394). Despite their almost identical iron binding site (399), compared to other transferrins, Lf can retain its iron atoms down to a more acidic pH of approximately 3 due to its higher Ka (400). Based on iron-saturation levels, Lf can exist in three different forms: iron depleted (apo) form, iron saturated (holo) form, with one iron atom in each lobe, and partially iron saturated (mono) form, with one iron atom attached either to the C or N lobe (394). Multiple biological activities of Lf depend on the structural integrity of the protein or its active fragments (401), and the presence of a specific receptor (Lf receptor, LfR) at the surface of target cells (402). LfR has been detected in multiple tissues and cell types with differential levels of expression depending on age. LfR mRNA has the highest concentration in the small intestine during fetal development, and in cardiac tissues in adults (402). LfR binds both apo- and holo-Lf, and facilitates their cellular uptake via clathrin-mediated endocytosis (403), with affinity being the highest at pH 6.5-7.0 (404). Depending on the degree of iron saturation (405) and the

age of the subject, Lf presents a different degree of resistance to proteolytic degradation by trypsin and trypsin-like enzymes in the gastrointestinal tracts (406). Gastric survival of Lf and functional fragments of Lf occur both in adults and infants and depends on the digestion conditions, such as pH, gastric emptying times, and enzymatic activity (394). In infants, intact Lf or larger fragments can be detected in significant quantities in the feces (407). In adults, the evidence is contrasting, with *in-vitro* and *in-vivo* studies reporting various percentages of Lf degraded during the actual or simulated gastric stage of digestion (394).

Human Lf shares approximately 69% amino acid sequence identity with bovine Lf (bLf), which has been shown to have analogous cellular uptake and metabolism (408) and to exert similar biological functions compared to human Lf *in-vitro* and in clinical studies (409,410). The intestinal uptake of bLf is thought to occur through the binding with the LfR, although the efficiency is reduced due to differences in the tertiary structures potentially affecting the internalization (410). Due to its easy availability and production on large scale, bLf has been extensively used as a food additive, especially in infant formulas to mimic the effect of human Lf, and has obtained GRAS status in the U.S. (411).

4.3.1 Biological functions of lactoferrin

Most of the biological activities of Lf depend on its high affinity with iron (412), and the role of Lf in iron absorption from breastmilk have been extensively debated (413). The majority of the iron in breast milk is bound to Lf, but the overall Lf saturation reaches only 3% due to its high abundance and low iron concentration (414). The existence of the Lf receptor and its density in the infant's intestine has been used as a potential explanation for the high absorption of iron from breast milk (415), but the available evidence is conflicting. Older iron isotope studies in infants (192,416) and women (417) reported that Lf either had no enhancing effect, or inhibited iron absorption, when compared to control. Nonetheless, a recent stable isotope study in infants reported that the amount of iron absorbed from holo-Lf was comparable to that of $FeSO_4$, and the addition of apo-Lf to the test meal containing $FeSO_4$ increased iron absorption (418). Long-term intervention trials evaluating the effect of Lf administration on iron status in infants are also equivocal (419,420), whereas in pregnant women Lf was more efficacious than iron preparation in treating moderate, but not mild, iron deficiency anemia (421). The putative mechanism(s) of how Lf, and especially apo-Lf, can facilitate iron absorption remain(s) uncertain. The

LfR-mediated uptake of iron in holo-Lf could also apply to intact apo-Lf chelating iron in the common non-heme iron pool in the gut lumen, thereby forming holo-Lf (422). Alternatively, the iron binding to apo-Lf could limit the interaction between iron and dietary inhibitors and favor its duodenal uptake. Lastly, Lf fragments originating from partially digested apo-Lf could increase iron solubility, as already reported for other amino acids and peptides (423). Whether Lf binds iron to facilitate its absorption and/or to sequester iron from potential enteropathogens is not known. A review of clinical trials with Lf (mostly bLf) found a positive protective effect of its administration on the prevention of infant infections (424). Moreover, fortification with human Lf of an oral rehydration solution had a beneficial effect on infants with diarrhea (425).These results were confirmed in a recent randomized, placebo-controlled intervention trial in infants receiving high iron doses, where co-provision of bLf reduced both the incidence and duration of diarrhea (426). It was thought that the positive bacteriostatic effect depended exclusively on the avidity of Lf for iron, which could reduce its availability for iron-dependent enteropathogens (394). Recently, it has been shown that Lf exerts both iron-dependent (427,428) and iron-independent antimicrobial activity (429,430). Among others, one of the iron-independent mechanisms relies on the competitive binding to host cells and/or to microbial surface components, such as the lipopolysaccharides of Gram-negative and the lipoteichoic acid of Gram-positive bacteria (398). Lf binding results in an increase in membrane permeability and disruption of virulence proteins anchored to the outer membrane that limits adherence and biofilm formation and can eventually result in bacterial death (398). An even stronger antibacterial effect has been reported for Lf-derived peptides, when compared to the full-length protein (431). In addition to its antimicrobial activity, Lf can also act as a mediator for both innate and adaptive immune responses through a series of processes, among which are the ability to recruit neutrophils and monocytes/macrophages, the interaction with pathogen-associated microbial patterns and their receptors, which eventually inhibits LPS-induced activation of cells at inflammatory sites, and direct interaction with immune cells surface components (432).

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MANUSCRIPT 1

Stable iron (^{58}Fe) isotopic measurements in Kenyan toddlers during 3-months of iron supplementation demonstrate that more than half of the iron absorbed is lost

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Abstract

Increased iron losses may reduce the effectiveness of iron supplementation. Iron absorption and losses can be quantified by long-term changes in the body iron isotopic signature. The objective of this study was to determine if daily oral iron supplementation increases iron losses, measured using a stable isotope of iron (^{58}Fe). We enrolled and dewormed 24 iron-depleted Kenyan children, 24–27 months of age, whose body iron was enriched and equilibrated with ^{58}Fe given at least one year earlier. Over 3 months of supplementation (6 mg iron/kg body weight (BW)/day), median (interquartile range) iron absorption was 1.07 (0.98; 1.27) mg/day. During supplementation, 0.61 (0.26, 0.79) mg/kg BW was lost, equal to 57% of the amount of absorbed iron. Supplementation did not increase biomarkers of enterocyte damage or gut inflammation and did not increase heme/porphyrin fecal losses. To examine the relationship between iron dose, absorption, and loss with results from other long-term iron isotopic studies, we compared the 6 mg/kg BW dose in Kenyan toddlers with doses of 0.8–1.2 mg/kg BW in Gambian toddlers, women in Benin and Switzerland, and with dietary iron alone in Malawian children and U.S. toddlers. Overall, the daily iron loss was closely correlated with iron absorption (Pearson r=0.94, p=0.005) but not with iron dose (p=0.36). The results of this study provide evidence that increased iron losses with oral iron supplementation are a function of the amount of iron absorbed rather than the dose of iron administered. This study was registered at ClinicalTrials.gov as NCT04721964.

Introduction

The goal of our study was to measure and identify the source of increased iron losses associated with oral iron supplementation. Conventional means of evaluating the impact of iron supplementation using iron biomarkers can estimate net changes in body iron but cannot separately determine iron absorption and loss. Single meal or short-term studies using radioactive or stable iron isotopes do not assess loss and evaluate only acute iron absorption (1). Older, long-term iron isotopic studies in adults under steady-state conditions with negligible changes in total body iron reported that daily obligatory losses were equivalent to the amount of iron absorbed (2,3). The first study using long-term stable isotopic labeling to measure iron absorption and loss under non-steady-state conditions was in U.S. toddlers consuming their customary diets (4). This long-term method depends upon uniform labelling of total body iron after oral administration of a stable isotope, and requires an equilibration period of up to one year or more, depending on the age of the subject (4,5). Thereafter, iron absorption is proportional to the rate of decrease in the concentration of isotopic tracer in blood, while iron loss is proportional to the rate of decrease in the amount of the tracer. In a recent long-term isotopic study, iron absorption and loss in iron-sufficient 5- to 7-year-old children in Malawi (6) consuming their customary diets were similar to those in U.S. toddlers (4), when expressed as mg iron per kg BW.

The first long-term isotopic study with oral iron supplementation was done in iron-deficient Gambian toddlers (7). During the control period on their customary diet, absorption and loss in the toddlers were similar to those in U.S. toddlers and in Malawian older children, when expressed as mg iron per kg BW (7). Unexpectedly, iron losses increased 3.4-fold during iron supplementation compared to the control period, and 75% of the iron absorbed during the supplementation period was lost (7). Subsequently, similar results were observed in iron-depleted Swiss women given iron supplements, in whom iron losses increased almost 3-fold during supplementation compared to the pre-intervention period (8). In these studies, the source of the increased iron losses during supplementation was

not identified. Body iron loss is not regulated and mainly results from cell desquamation; iron loss cannot be increased physiologically even in the presence of iron overload (2).

Episodic occult gastrointestinal blood loss caused by iron supplements might explain the increased iron losses in the above studies (7,8). Excess unabsorbed iron in the gut can cause mucosal irritation, inflammation and bleeding (9–11). Therefore, the present study in Kenyan toddlers was designed to assess potential iron-induced gastrointestinal blood loss as the source of increased iron losses during supplementation. We gave an oral iron dose recommended by the Kenyan pediatric protocol that contained a 5-fold higher iron dose per kg BW than the micronutrient powder used in the Gambian toddlers, potentially increasing iron losses. We hypothesized that this higher dose of iron, given for 3 months, would increase iron losses compared to the Kenyan baseline and produce higher losses than those in the Gambian toddlers (7). Along with the measurement of endogenous iron losses using the long-term isotopic method, we assessed potential gastrointestinal blood losses by measuring intact heme and porphyrins in repeat fecal collections.

Methods

Study site and participants

We conducted the study between February and May 2021 in Kwale County, Kenya. In order to study children who had enriched and equilibrated body iron isotopic composition (2,4,8), we recruited children who previously participated in stable iron isotopes studies between July 2019 and January 2020. Designs of the two studies are provided in Supplemental Material (Data S1). Inclusion criteria at the baseline of our study were: 1) participation in a stable iron isotopes absorption study in which isotope administration occurred ≥ 1y before the current study; 2) 9.0 g/dL< hemoglobin (Hb) < 11.5 g/dL; 3) iron depletion [mean corpuscular volume (MCV)< 80 fL] (12,13); 4) written informed consent signed by the caregiver; 5) planned residence in the area for the study duration; 6) no chronic gastrointestinal, renal, and/or metabolic disease; 7) not severely underweight [Z-score weight-for-age ≤ -3]; 8) not severely wasted [Z-score weight-for-height ≤ -3]; 9) no use of iron supplements in the 2 weeks before screening, and 10) no antibiotic treatment in the 7 days before screening. The ethics committees of ETH Zurich (EK 2020-N-152)

and Jomo Kenyatta University of Agriculture and Technology in Nairobi (JKU/2/4/896B) approved the study. The trial was registered at clinicaltrials.gov as NCT04721964.

Study design

The study had a prospective experimental design, in which all subjects consumed daily oral iron supplements for 91 days (Figure 1). One week before baseline, 47 children were screened for eligibility (Day 0). We collected a venipuncture or finger-prick blood sample (3 mL) for iron isotopic analyses, determination of iron (Hb, MCV, PF, sTfR), and inflammatory biomarkers (CRP, AGP) (Data S2), and malaria parasitemia (Giemsa staining method). If the malaria smear was positive, we treated the child with artemether/lumefantrine. Twenty-four children met the inclusion criteria and were enrolled in the study (Figure 1). Upon enrolment, to prevent potential blood loss from intestinal parasitosis, we gave the toddlers 400 mg oral albendazole (Cadila Pharmaceuticals Ltd, Ahmedabad, India) and gave a repeat dose of albendazole at the third study visit (Day 63). Starting from the morning of Day 4 (Baseline; pre-supplementation), the caregivers were provided plastic diapers and carefully instructed to collect into a clean plastic container all of the child's stool for 72 hours. We collected each stool sample from the caregivers, recorded stool sample ID, number, and weight, and stored the stools at -20 °C. The first stool collection period ended on the morning of Day 7, and on that day during a clinic visit, trained field workers instructed the caregivers on how to give the oral iron syrup and supervised the first syrup administration (Data S2). The iron dose was calculated based on body weight and the specific dose was marked on a disposable syringe given to each caregiver (Data S2). The iron syrup was given daily for 91 days from Day 7 until Day 98 (Endpoint). Compliance for the syrup consumption was estimated by recording the number of bottles distributed and fully consumed by each participant, and by weighing the syrup left in the latest bottle open at every hospital visit. Throughout the intervention, there were three additional 72 h stool collection periods (Study Days 32-35, 60-63, and 95-98), each followed by a clinic visit (Days 35, 63, and 98 respectively). At each hospital visit, we collected a venipuncture or finger-prick blood sample from the toddlers for iron isotopic analyses and determination of Hb, serum ferritin (SF), soluble transferrin receptor (sTfR), C-reactive protein (CRP,) and alpha-1-acid glycoprotein (AGP). We defined

anemia as Hb ≤11.0 g/dL, iron deficiency (ID) as sTfR >8.5 mg/L, and iron-deficiency anemia (IDA) as Hb <11.0 g/dL and sTfR >8.5 mg/L (14). Inflammation was defined by CRP and AGP concentrations of >5 mg/L and >1 g/L, respectively.

Fecal samples analysis

Stool samples collected from the plastic diapers were weighed and stored frozen at -20 °C until shipment. All the samples collected within each 72h period from the same participant were manually homogenized by stirring and weighted at ETH Zurich. We collected four aliquots of 2 ml from each homogenized sample. We sent one aliquot to Mayo Clinic Laboratory (Rochester, USA) for the determination of blood losses in stools using a HemoQuant assay with fluorescence quantitation (15). Hemoglobin and heme released by hemoglobin degradation in the gut are converted into porphyrins, which are then quantified by fluorescence measurement after extraction of any interfering fluorescing substances (15). The lower detection limit of the HemoQuant assay was 0.3 mg Hb/g feces, corresponding to 1.04 mg Fe/g feces. From a second aliquot, we assessed lower gastrointestinal tract inflammation by measuring fecal calprotectin with an ELISA kit (Eurospital, Trieste, Italy). From the same aliquot, we analyzed fecal pH using a digital pH meter (Metrohm, Herisau, Switzerland).

Sample size calculation

Sample size calculation for within-subject comparisons indicated that, with an SD of 0.4 derived from iron losses data from a previous stable iron dilution study in our laboratory (7), 15 participants were needed to detect a 50% (0.25 mg/d) increment in iron losses, with a type I error rate of 5% and 80% power. Considering the lack of data on occult blood loss and its potential variability during oral iron supplementation, the expected high attrition due to study duration, and that around 100 participants of former isotopes study (JKUAT/IERC No. 301019 and JKU/2/4/896B) were eligible for screening, we increased the study sample size to 24 participants.

Statistical analysis

We used REDCap electronic data capture tools (Vanderbilt University, Nashville, Tennessee) to collect study data and conducted statistical analyses using R (R 4.0.2.

software; R Core Team) statistical programming environment. We used the BRINDA package to adjust SF values for inflammation (16), using within-study internal regression coefficients for the adjustment. We estimated iron absorbed and lost based on isotopic measurements as described by Speich et al. (7) (Data S3). We used unpaired Student t-tests for comparisons between daily iron losses in our study and in Gambian toddlers (7). We used Pearson's correlation to assess the strength of the relationships between daily iron doses administered and median daily iron lost per kg body weight, and median daily iron absorbed and median daily iron lost per kg body weight, in different populations from all published studies using the same stable iron isotope dilution methodology to asses iron balance (4,6–8). When iron doses, and/or daily iron absorbed and lost per kg body weight were not published, they were calculated from available data from the publication.

For our study, we calculated descriptive statistics for all variables at baseline, 1 month, 2 months, and endpoint and assessed normality by visually inspecting the histogram and by testing the distribution of continuous variables against a normal distribution using the Shapiro-Wilk W test. Normally distributed data were expressed as mean ± SD; non-normally distributed data as medians and IQR. We investigated the effect of study visit (ordinal) on each indicator of interest by fitting a linear mixed-effect model using the CRAN package "lmerTest". The factor study visit (4 levels: baseline, 1 month, 2 months, and endpoint) was added to the models as a fixed factor and subject as a random factor. We visually inspected the normal Q-Q-Plot and the Tukey-Anscombe plot to verify the assumptions of homoscedasticity and normality of residuals. In case of a significant effect of the fixed factor, we used a post-hoc paired samples t-test with Bonferroni adjustments, with significance set at $p < 0.05$.

Results

After screening, 24 children met our criteria (**Figure 1**) and were included in the study. One subject withdrew consent before the baseline stool collection, and 23 began iron supplementation on day 7. Before the second 72h stool collection period, one subject was dropped from the study due to poor compliance with the supplement. A total of 22 subjects completed the study. One subject was excluded from the analysis of iron and

inflammation parameters at the study endpoint, and from estimates of iron balance during the supplementation period, due to an episode of malaria just before the last study visit. Despite having participated in the previous EK 2018-N-84 study, one subject had not received the isotopic label and was excluded from the calculation of iron absorption, losses, and net iron balance during supplementation.

Baseline characteristics of the study participants are shown in **Table 1**. According to the inclusion criteria of a low MCV and a Hb <11.5 g/l, all 23 participants at baseline were considered iron depleted. The mean (±SD) amounts of iron absorbed, lost, and gained during the supplementation period calculated with the stable iron isotopes dilution method are displayed in **Figure 2**. The median (IQR) amounts of iron absorbed were 1.07 (0.98; 1.27) mg/day; 0.61 (0.26; 0.79) mg Fe/d were lost, leading to a net iron balance of 0.64 (0.22; 0.77) mg/d.

Hb, iron, and inflammation status biomarkers at the different study visits are shown in **Table 2**. There was a significant increase in Hb, unadjusted and adjusted SF, BIS, total body iron, circulating iron, and storage iron after 1 month of iron supplementation ($p<0.05$ for all). There was a significant decrease in sTfR after 2 months of supplementation ($p=0.04$). There were no significant changes in CRP and AGP during supplementation. There were no significant changes through the intervention period in the different biomarkers of intestinal health (pH, I-FABP) and inflammation (fecal calprotectin) (**Table 3**). Also, there was evidence of iron losses from occult blood only in n=7, 3, 6, and 6 stool samples at baseline, 1 month, 2 months, and endpoint, respectively (Table 3), as estimated from fecal heme and porphyrin concentrations above the limit of detection. In the stool samples with fecal iron concentrations above the detection limit, no statistical analysis was performed on fecal iron concentration and time elapsed since the start of iron supplementation, due to the limited number of samples above the limit of detection and their randomness.

The characteristics of the study populations, the daily iron doses, the daily median iron absorbed and lost per kg body weight, and the proportion of absorbed iron that was lost during the study period, from our study and all the other long-term isotopic studies are

listed in **Table 4** (4,6–8). There was no significant difference in the distributions of individual daily iron losses between Kenyan and Gambian toddlers (p=0.09). Overall, there was no significant correlation (p=0.36) between daily iron dose and daily median iron lost per kg BW in our study, in Gambian toddlers, in women in Benin and Switzerland, and no significant correlation between dietary iron and daily median iron lost per kg BW in Malawian children and U.S. toddlers. Similarly, there was no significant correlation (p=0.18) between daily median iron absorbed per kg BW and daily iron dose administered, or dietary iron in these studies. In contrast, the correlation between daily median iron absorbed and lost per kg BW from our study, and from these previous studies was highly significant (r = 0.94, P = 0.005, **Figure 3**).

Discussion

Our results provide evidence that increased iron losses during oral iron administration are a function of the amount of iron absorbed rather than the dose of iron administered. During 3 months of daily oral iron supplementation in the Kenyan toddlers, we gave a five-fold higher dose of iron than in the earlier, similar trial in Gambian toddlers and found that over half the iron absorbed was lost (7). Contrary to our hypotheses, we did not detect gastrointestinal blood loss as the source of iron loss, and the higher dose of iron in Kenya did not increase iron losses compared to those observed in the Gambian toddlers. The proportion of iron absorbed from the higher dose of iron given in Kenya is lower than that in The Gambia, and the amount of iron absorbed did not differ in the two studies. This is likely due to decreased fractional iron absorption with the higher dose of iron in Kenya and downregulation of iron absorption in the Kenyan toddlers because of their higher body iron than in The Gambia (17).

Because the iron losses did not differ between these two studies with similar iron absorption, we then examined the relationships between iron dose, absorption, and loss in all six available isotopic studies of iron absorption and loss. These isotopic studies included (i) toddlers in Kenya and The Gambia (7) and women of reproductive age in Benin and Switzerland (8) who were given a variety of ferrous iron preparations for periods ranging from months to years, and (ii) young children in Malawi (6) and toddlers in the

United States (4) consuming their customary diet with no added iron, a total of 133 subjects (Table 4). Expressed in terms of body weight, we found no significant correlation between daily iron loss and the dose of iron among these groups. In contrast, daily iron loss was closely correlated with iron absorption (Pearson r=0.94, p=0.005) (Figure 3), despite the heterogeneity of the groups, the duration of the trials, and the different non-heme iron preparations studied. Both non-heme and heme iron absorption are maximal in the proximal duodenum and then decrease distally along the duodenal portion of the small intestine (18). The close relationship between iron absorption and iron losses in these stable iron isotopic studies suggests that the primary site of iron losses may be at the site of maximal iron absorption in the proximal duodenum.

Our data do not support the hypothesis that increased iron losses during supplementation are due to occult gastrointestinal bleeding from mucosal damage. Supplementation did not increase I-FABP, a reliable measure of small intestinal enterocyte integrity/damage (19). Also, no clear patterns were found of increased heme/porphyrin loss during the supplementation period, and no clear association was present between the length of supplementation or total iron dose and heme/porphyrin loss. The HemoQuant fluorometric assay quantifies occult blood losses by measuring the concentration of heme and heme-derived porphyrins in stool samples (20). The heme-porphyrin test is little affected by other dietary components with the exception of myoglobin deriving from meat (21,22). We did not assess food intake in the days prior and during stool collection, but meat is rarely consumed in our study population (23,24). We cannot exclude the possibility that stool collection over the repeated 72h periods throughout the study was incomplete. If the potential blood losses induced by iron supplementation did not occur continuously, but as multiple intermittent episodes (25), missing samples could lead to underestimation of blood losses. Iron losses of a similar magnitude as in the Gambian toddlers were confirmed in our study population by the isotopic measurements (7). Nonetheless, if we assume a similar increase in the quantity of losses during iron supplementation, in comparison to pre-intervention, and we attribute most of it to occult bleeding, the porphyrin concentration in our stool samples would be ~20 times higher than the detection

limit of the assay. Therefore, it seems unlikely that the absence of detectable amounts of blood in our samples is due to incomplete compliance.

There are other potential pathophysiological mechanism(s) responsible for increased iron losses with iron supplementation. One possibility is that iron uptake into enterocytes increases enterocyte senescence, by ferroptosis or by other forms of programmed cell death (26,27) and the enterocytes are subsequently shed at an increased rate. Gastrointestinal cellular desquamation has been recognized as a source of pathological iron loss for a half century (28). In the gastrointestinal tract, the small intestine, with the highest epithelial cell turnover combined with the largest surface area, is responsible for an estimated 90% of the total intestinal cell loss (28). Small intestine cell turnover can increase further in response to inflammatory, infectious, chemical, and other agents that injure enterocytes (29). Increases in gastrointestinal cell turnover of up to 8-fold have been reported in celiac disease, infections, and other disorders (28,30,31). Other recent studies in Kenyan infants have shown that even the doses of iron used in dietary fortification increase intestinal inflammation and enterocyte damage (32,33). As shown in Table 4, isotopically measured median daily iron losses in U.S. toddlers and older Malawian children consuming diets with no added iron were similar when expressed as milligrams of iron per kilogram of body weight. An increase in gastrointestinal iron loss of up to 4-fold could account for the increased losses observed with iron supplementation in Kenya and The Gambia.

Our study has limitations. We used a convenience sample of toddlers who previously participated in stable iron isotopes studies, restricting the generalizability of the results. Moreover, although determination of the k_{abs} only requires isotopic measurements, k_{loss}, and the estimates of the amount of total body iron absorbed or lost per unit of time, Fe_{abs} and Fe_{loss} respectively, need the determination of Fe_{total}, that relies on measurements of conventional iron indices. Our study has also strengths. We used a prospective within-subject design, which reduces the potential effect of high inter-individual variability. The isotopic dilution method provides accurate and direct quantification of long-term changes in body iron (4) and is less subject to the influence of factors affecting conventional iron

indices, such as inflammation (34). The HemoQuant assay is a robust and sensitive method to quantify blood fecal occult losses (35).

In conclusion, this study provides evidence that, during iron supplementation, the amounts of iron lost increase with the amount of iron absorbed rather than the dose of iron given. In general, limited net iron gains during iron supplementation have been attributed to internal influences, such as inflammatory increases in hepcidin, or to external effects, such as poor adherence or inhibition from dietary phytates, polyphenols or other components. The findings of these long-term stable iron isotopic measurements suggest a potential alternative explanation, that increased iron losses during iron supplementation reduce the effectiveness of iron interventions. Finally, together with all similar long-term isotopic studies, our results provide evidence that the site of iron losses coincides with the site of absorption.

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Authors contributions

The authors' contributions were as follows—MBZ, GMB, SK, CS, NS and AG: designed the research study; AG, CN, and CZ: performed the research; GMB, AG and CZ: analyzed the data; AG wrote the first draft of the manuscript, and all authors edited, read, and approved the final manuscript.

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Tables

Table 1: Baseline anthropometric characteristics, breastfeeding prevalence, and MCV of the participating children (n=23).

Characteristic	
Males, n (%)	15 (62)
Breastfeeding, n (%)	4 (17)
Age (mo)	24.9 (24.6; 27.2)
Height (cm)	85.1 ± 3.7
Weight (kg)	11.3 ± 1.3
BMI (kg/m^2)	15.6 ± 1.2
Weight-for-height z scores	-0.06 ± 0.85
Height-for-age z scores	-1.06 ± 1.08
Weight-for-age z scores	-0.54 ± 0.89
BMI-for-age z scores	0.10 ± 0.89
Hb (g/dL)	10.7 (10.2; 11.2)
MCV, (fL)	63.5 ± 7.0

For continuous variables, values are reported as means ± SD when normally distributed or medians (IQRs). For dichotomous variables, we reported absolute prevalence and percentages in brackets.
BMI, body mass index; Hb, hemoglobin; MCV, mean corpuscular volume.

Table 2: Participants' biomarkers of iron and inflammation status and estimates of body iron pools at the different study visits.

Characteristic	Baseline	1 month	2 months	Endpoint
n	23	23	22	21
Hb, g/l	10.7 (10.2; 11.2)	11.3 (10.9; 12.0)[a]	11.4 (10.8; 11.9)[a]	11.5 (11.1; 12.1)[a]
Anemia, % (n)	56.5 (13)	34.8 (8)	40.9 (9)	14.3 (3)
$SF_{unadjusted}$, ug/L	18.4 (12.4; 26.0)	18.4 (12.4; 26.0)[a]	30.9 (25.3; 44.9)[a]	36.2 (30.8; 48.5)[a]
SF_a*, ug/L	12.9 (8.3; 16.1)	20.1 (15.3; 26.5)[a]	22.4 (18.1; 26.3)[a]	26.2 (20.5; 29.9)[a]
ID, % (n)	39.1 (9)	4.3 (1)	0 (0)	4.8 (1)
IDA, % (n)	26.1 (6)	4.3 (1)	0 (0)	0 (0)
sTfR, mg/L	13.2 (9.6; 15.7)	10.5 (8.8; 13.9)	8.9 (7.5; 10.8)[a,b]	7.4 (6.4; 8.6)[a,b]
ID, % (n)	82.6 (19)	82.6 (19)	59.1 (13)	28.6 (6)
IDA, % (n)	47.8 (11)	30.4 (7)	31.8 (7)	14.3 (3)
Total Body Iron†, mg	340.8 (319.7; 372.1)	380.3 (335.9; 411.4)[a]	391.5 (349.3; 426.4)[a]	413.0 (379.2; 440.8)[a,b,c]
Circulating Iron, mg	270.0 (255.3; 299.1)	295.7 (246.9; 318.1)[a]	304.8 (275.8; 333.2)[a]	330.2 (300.2; 344.0)[a,b,c]

Tissue Iron, mg	67.2 (64.5; 73.5)	69.0 (65.1; 72.3)	69.0 (66.6; 74.9)[a]	72.6 (69.2; 76.8) [a,b,c]
Storage Iron, mg	0 (0; 0.76)	10.8 (0; 22.1)[a]	16.5 (6.2; 24.0)[a]	24.9 (11.9; 31.0)[a]
Total Body Iron/Body Weight, mg/Kg	30.1 (29.1; 31.8)	32.2 (31.2; 34.2)[a]	32.9 (31.0; 34.9)[a]	34.4 (32.0; 35.8)[a]
CRP, mg/L	0.80 (0.29; 4.82)	0.49 (0.16; 1.92)	0.93 (0.43; 4.01)	1.37 (0.34; 4.0)
>5 mg/L, % (n)	21.7 (5)	8.6 (2)	18.2 (4)	23.8 (5)
AGP, g/L	1.06 (0.76; 1.28)	0.96 (0.74; 1.22)	0.92 (0.82; 1.16)	1.08 (0.82; 1.29)
>1 mg/L, % (n)	60.9 (14)	47.8 (11)	36.4 (8)	57.1 (12)

All values for continuous variables are medians (IQR). Counts of ID, IDA, and systematic inflammation are reported as % of the study population and absolute number of cases in brackets (n). Differing superscript letters in a row indicate statistically significant differences between time points assessed with Bonferroni correction for post hoc pairwise comparisons. Specifically, [a] indicates difference with baseline, [b] difference with 1 month visit and [c] difference with 2 months visit. No statistical analysis applied on ID/IDA/systematic inflammation counts.
AGP, alpha-1-acid glycoprotein; BMI, body mass index; BIS, body iron stores; CRP, C-reactive protein; Hb hemoglobin; ID, iron deficiency; IDA, iron deficiency anemia; IQR, interquartile range; SF, serum ferritin; sTfR, soluble transferrin receptor.
*BRINDA adjusted SF concentrations calculated using the BRINDA package for the R software.
[†]Total body iron is calculated for each subject as the sum of the circulating iron, tissue iron and iron stores.

Table 3: Iron concentration in fecal samples from GI bleeding estimated from fecal porphyrin concentrations, and biomarkers of intestinal health (pH, I-FABP) and inflammation (CalPro) at different study visits.

Characteristic	n	Baseline	n	1 month	n	2 months	n	Endpoint	P
[Fe] in feces, µg/g*	7	1.39 (1.21; 2.08)	3	2.43 (2.08; 3.30)	6	1.91 (1.39; 3.73)	6	1.74 (1.04; 2.95)	-
I-FABP, pg/mL	20	483.9 (293.7; 745.2)	23	459.4 (310.0; 636.1)	22	510.0 (320.4; 715.3)	21	447.2 (330.5; 915.9)	0.59
CalPro, ug/g	21	125.2 (28.4; 159.7)	22	68.9 (29.2; 159.78)	22	85.9 (31.5; 144.8)	22	86.75 (39.4; 154.7)	0.72
Fecal pH	23	6.4 (5.8, 7.0)	22	5.9 (5.5; 6.3)	22	6.0 (5.5; 6.2)	22	6.0 (5.8; 6.2)	0.06

*only from values above LoD (Hb concentration > 0.3 mg/g feces)

All values are presented as medians (IQR). We tested the effect of study visit (ordinal) on I-FABP, CalPro and pH by fitting a linear mixed-effect model, with study visit (4 factor-level: baseline, 1 month, 2 months, and endpoint) as a fixed factor and subject as a random factor. There was no evidence of a significant effect of study visit on the variables reported above. No statistical analysis applied on [Fe] in stools.
[Fe], iron concentration; CalPro, fecal calprotectin; GI, gastrointestinal; I-FABP, intestinal fatty acids binding protein.

Table 4: Characteristics of the study populations, supplemental daily iron doses, daily median iron absorbed (Fe_{abs}) and lost (Fe_{loss}), and proportion of iron absorbed that was lost during the study period, from all stable iron isotopes dilution studies quantifying iron absorption and losses (4,6–8).

Author/publication year	Study Area	Study population (n)	Iron compound	Iron doses/day (mg/kg body wt)	Hb (g/L)	SF_a (µg/L)	Fe_{abs} (mg/kg body wt)	Fe_{loss} (mg/kg body wt)	Fe_{loss}/Fe_{abs} (%)
Giorgetti et al.	Kenya	Toddlers (20)	Ferric ammonium citrate	6	10.7	12.9	0.096	0.053	57
Speich et al./2021 (7)	The Gambia	Toddlers (15)	Ferrous sulphate	1.2	9.8	1.8	0.102	0.077	75
Speich et al./2021 (8)	Benin	WRA (11)	Ferrous sulphate	0.84	12.2	28.2	0.063	0.027	42
Speich et al./2021 (8)	Switzerland	WRA (10)	Ferrous sulphate	0.87	13.2	18.8	0.071	0.044	62
Fomon et al./2005 (4)	U.S.	Toddlers (29)	-	Dietary	12.1	25.6	0.042	0.021	-
Speich et al./2021 (6)	Malawi	5-7 yo children (48)	-	Dietary	11.4	23.4	0.030	0.017	-

Hb, hemoglobin; SF_a, serum ferritin corrected values calculated using the BRINDA package for the R software; WRA, women of reproductive age.

Figures

Figure 1: Study design and participant flowchart. Hb, hemoglobin; MCV, mean corpuscular volume.

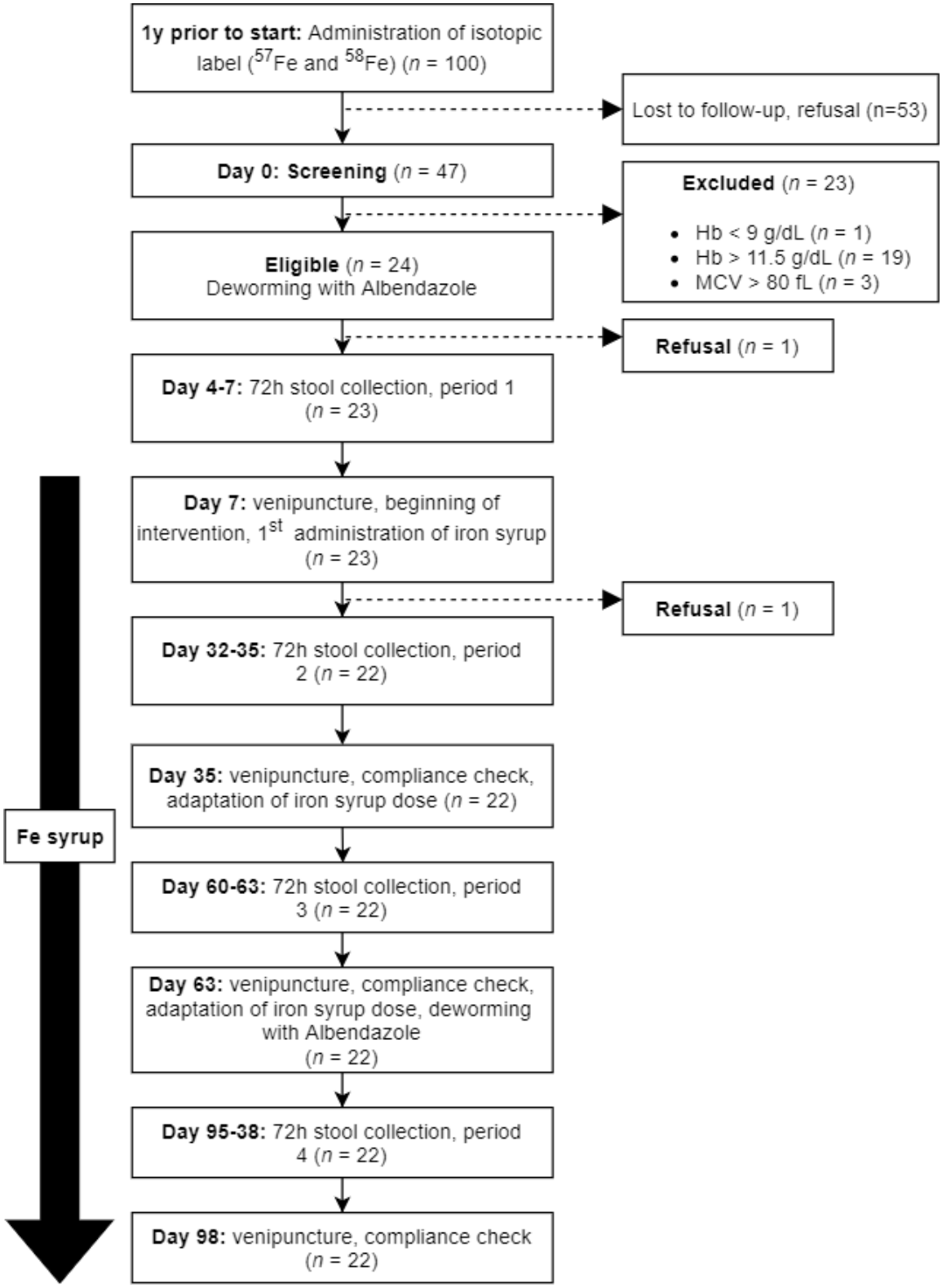


Figure 2: Mean (SD) daily amounts of iron absorbed (Fe_{abs}), lost (Fe_{loss}) and net iron balance calculated isotopically (Fe_{gain}) over the supplementation period of 91 days (n=20).

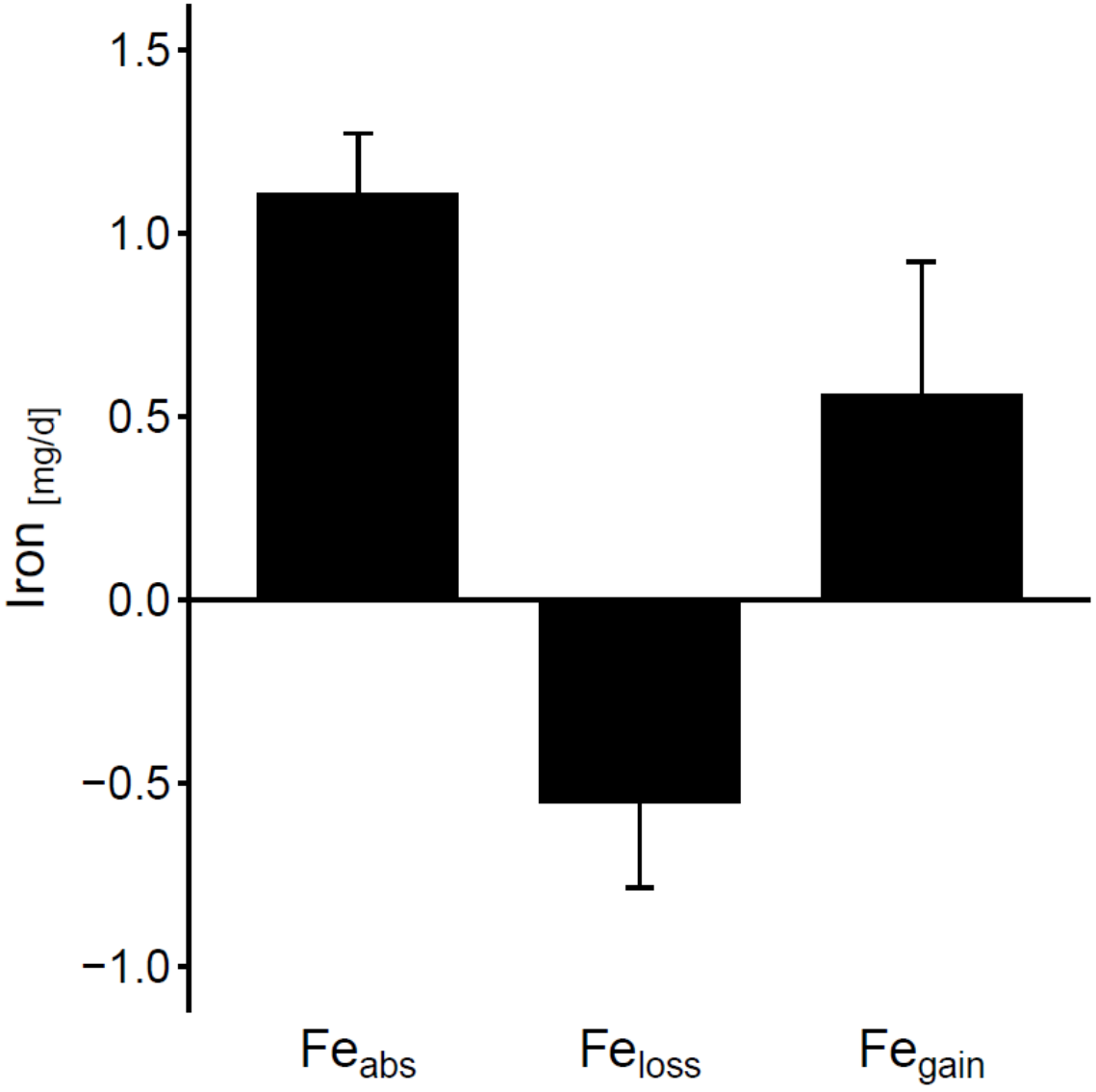
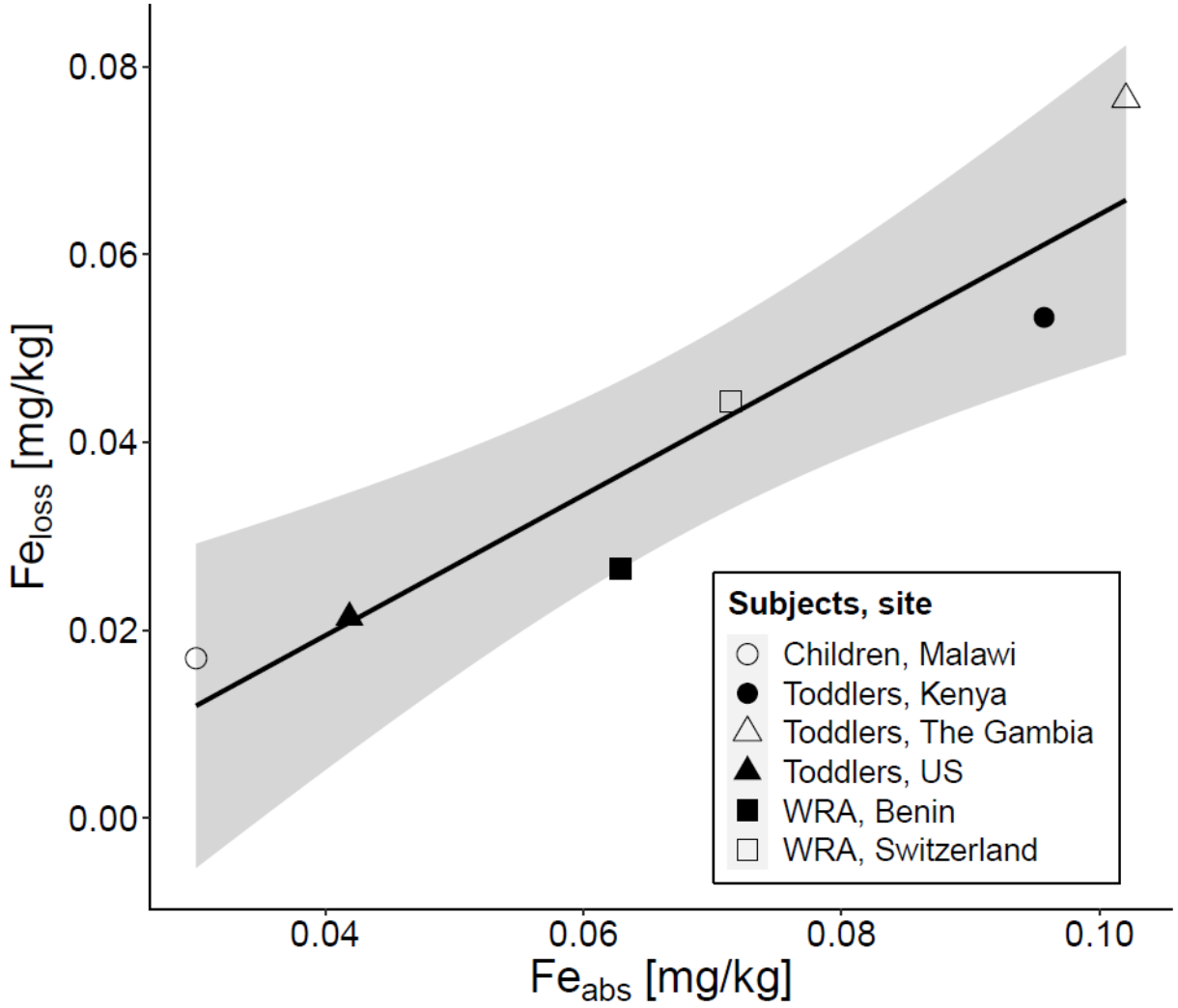


Figure 3: Correlation plot between median iron absorbed (Fe_{abs}) and lost (Fe_{loss}) per kg body weight [mg/kg] from all studies assessing iron balance using the stable iron isotopes dilution methodology (4,6–8). The confidence interval (light grey area) is set at 95%. r = 0.94, P = 0.005.



Supplemental material, section 1

Study design, acute iron absorption studies

The first study (EK 2018-N-84) was a single-blind, randomized, controlled, intervention trial in infants aged 6-11 months. The subjects were randomized to receive daily for 3 weeks a wheat-based instant cereal containing 3.6 mg ferrous fumarate, ascorbic acid, and either 7.5 g, 3 g, or no prebiotic mixture (Galacto-oligosaccharides (GOS)+ Fructo-oligosaccharides, 9:1). Moreover, they consumed four stable iron isotope-labeled cereal meals fortified with 3.6 mg FeFum (either as 3.6 mg ^{57}FeFum or 1.5 mg ^{58}FeFum and 2.1 mg ^{56}FeFum), without and with the prebiotic mixture, two before and two after the intervention. In the second study (EK 2019-N-59), the study subjects aged 8 to 12 months and they consumed 3 maize-porridge based test meals fortified with: 1) 5.0 mg iron as ^{54}Fe-labeled ferrous fumarate (FeFum); 2) 5.0 mg iron as ^{58}FeFum and 3.0 g GOS; and 3) 5.0 mg iron as ^{57}FeFum and 3.0 g human milk oligosaccharides.

Supplemental material, section 2

Composition and dosage of the iron supplement

The administered supplement was a ferric ammonium citrate (8.2 mg/ml) iron syrup (Ranbaxy Ranferon-12 Syrup, Ranbaxy Laboratories, Gurgaon, India), also containing 0.3 mg/ml Folic Acid, 10 µg/ml Vitamin B12 (Cyanocobalamin). Ranferon-12 syrup is a widely used supplement to treat anemia in infants and toddlers in the study area. Kenyan pediatric guidelines recommend daily administration of 3-6 mg iron per kg body weight to iron-deficient anemic children (36). We decided to administer 6 mg iron/kg body weight/day to ensure iron intake within the recommended range even with incomplete compliance. At each visit, the research coordinator updated the iron syrup doses to be administered daily based on the changes in body weight. By WHO guidelines for iron supplementation to treat and prevent IDA, the duration of the intervention was 13 weeks (14).

Analytical methods

Blood analysis

We collected venous or finger prick blood samples into heparin-coated tubes. We

measured Hb immediately after sample collection with the use of a HemoCue 300 analyzer (HemoCue); controls (Eurotrol) were measured on each visit day. We assessed malaria parasitemia using a rapid diagnostic test (Abbott Laboratories, Chicago, USA). Aliquots of whole blood samples were frozen at –20°C until further analyses. The remaining whole blood was centrifuged at 3000 rpm for 10 min and the plasma aliquots were frozen at –20°C until further analyses. We measured iron status (PF, sTfR) and inflammation (CRP, AGP) in plasma samples using a multiplex immunoassay (37). We assessed upper gastrointestinal tract integrity by measuring intestinal fatty acid-binding protein (I-FABP) using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Hycult Biotech, Uden, The Netherlands). Whole blood samples were mineralized in duplicates by microwave-assisted digestion in HNO_3 followed by iron separation from the blood matrix by anion-exchange chromatography and a subsequent precipitation step with ammonium hydroxide (38). We measured isotope ratios using inductively coupled plasma mass spectrometry (Neptune, Thermo Finnigan) equipped with a multi-collector system for simultaneous ion beam detection (38).

Supplemental material, section 3

Calculation of iron absorbed and lost

We estimated total body iron (Fe_{total}) as the sum of circulating iron (Fe_{circ}), tissue iron (Fe_{tiss}), and storage iron (Fe_{stor}) (4). We calculated Fe_{circ} based on the blood volume (39) and measured Hb concentrations at each time point for each participant, with 3.47 being the concentration of iron in Hb (mg/g). We estimated Fe_{tiss} as 6 mg/kg body weight (4,40). We calculated storage iron as Fe_{stor} (µg) = {9380 × log10[SF (µg/L)]—11,260} × body weight (41) if [SF] > 15.9 µg/L. Despite being derived from an adult population, the equation has previously been applied to children (4,6). For concentrations of SF < 15.9 µg/L, the Hallberg equation would result in negative estimates, that we replaced with 0 µg/L. For the calculations of Fe_{stor}, we used SF values adjusted for inflammation using the BRINDA approach (42).

The participants receive stable isotope tracers enriched in iron 57 (^{57}Fe) and iron 58 (^{58}Fe) >1 y before the baseline of our study. Iron isotope tracer administration causes an initial

isotopic enrichment in red blood cells (RBCs), followed by a decrease due to redistribution of the iron from the RBCs to the other body compartments. After approximately 10 months (2), the concentration of isotope tracer can be considered equal in all body compartments (43). When the steady-state of isotopic concentration is reached, the decrease in the concentration of the isotope tracer in circulating blood is proportional to the quantity of iron absorbed and the decrease in the amount of the isotope tracer is proportional to the quantity of iron lost (4). Consequently, we determined the fraction of total body iron absorbed per unit of time as the slope (k_{abs}) of the natural logarithm of the concentration of the ^{58}Fe and ^{57}Fe tracers (ppm) plotted against time (days). Similarly, we determined the fraction of total body iron lost per unit of time as the slope (k_{loss}) of the natural logarithm of the amount of the ^{58}Fe and ^{57}Fe tracers (mmol) plotted against time (days) (4). We calculated the amounts of ^{57}Fe, and ^{58}Fe isotopic tracers in whole blood collected at baseline (Day 0) and on Day 35, 63, and 98 based on the shift in iron isotope ratios and on the estimated amount of iron circulating in the body. The calculations were based on the principles of isotope dilution and took into account that iron isotopic labels are not monoisotopic (44).

We calculated the mean quantity of iron absorbed (Fe_{abs}) per day over the supplementation period as

$$Fe_{abs} = -k_{abs} \times Fe_{total}$$

and the mean quantity of iron lost (Fe_{loss}) per day over the supplementation period as

$$Fe_{loss} = -k_{loss} \times Fe_{total}$$

with Fe_{total} as the total body iron at intervention midpoint calculated from the linear regression of Fe_{total} against time.

We calculated daily iron balance over the supplementation period by using the estimates derived from the stable iron isotopes dilution method, as $Fe_{gain} = Fe_{abs} - Fe_{loss}$.

MANUSCRIPT 2

Prebiotic galacto-oligosaccharides and fructo-oligosaccharides, but not acacia gum, increase iron absorption from a single high-dose ferrous fumarate supplement in iron-depleted women

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Abstract

Background Prebiotic galacto-oligosaccharides (GOS) increase iron absorption from fortification-level iron doses given as ferrous fumarate (FeFum) in women and children. Whether GOS or other fibers, such as prebiotic fructo-oligosaccharides (FOS) and acacia gum, increase iron absorption from higher supplemental doses of FeFum is unclear.

Objectives In iron-depleted [serum ferritin (SF)< 25µg/L] women, we tested if oral co-administration of 15g of GOS, FOS or acacia gum increases iron absorption from a 100mg iron supplement given as FeFum.

Methods In a randomized, single-blind cross-over study, 30 women (median age 26.2 years, median SF 12.9µg/L) consumed a 100mg iron tablet labelled with 4mg of ^{57}Fe or ^{58}Fe, given with either: a) 15g GOS; b) 15g FOS; c) 15g acacia gum; or d) 6.1g lactose and 1.5g sucrose (control; matching the amounts of sucrose and lactose present in the GOS powder providing 15 g GOS), dissolved in water. The primary outcome, fractional iron absorption (FIA), was assessed by erythrocyte isotopic incorporation 14 days after administration. Data were analyzed using a linear mixed-effect model. We also tested, *in vitro,* iron solubility at different pH and dialyzability from the different supplement combinations administered *in vivo*.

Results FIA from FeFum given with GOS and FOS was significantly higher (+45% and 51%, respectively; p< 0.001 for both) compared with control; total iron absorption [median (IQR)] was 34.6 (28.4; 49.1); 36.1 (29.0; 46.2) and 23.9 (20.5; 34.0) mg, respectively. Acacia gum did not significantly affect FIA from FeFum (p=0.688). In vitro, iron dialyzability of FeFum + GOS was 46% higher than of FeFum alone (p=0.003).

Conclusions In iron-depleted women, both GOS and FOS co-administration with FeFum increase iron absorption by ~50% from a 100mg oral iron dose, resulting in an additional 10-12mg of absorbed iron. Thus, GOS and FOS may be promising new enhancers of supplemental iron absorption.

Introduction

It is estimated that anemia affects one third of the global population, with approximately half of the cases resulting from iron deficiency (ID) (1). Globally, ID anemia is 1 of the 5 leading causes of years lived with disability and the time lost due to premature mortality (DALYs) (2), and is the first cause in women (1). Although oral iron supplementation is considered the first line of treatment of ID in most women (3), fractional iron absorption (FIA) from commonly used iron salts is typically low and varies from 2% to 13% when consumed with food versus 5%–28% when consumed fasting (4). Unabsorbed iron may cause gut irritation, inflammation and dysbiosis (5): the most common adverse effects are epigastric pain, nausea, and constipation; these reduce compliance with therapy in 30%–70% of cases (6). Other than ascorbic acid, there are few enhancers of iron absorption available (7).

Prebiotics have been shown to increase iron bioavailability in *in vitro*, animal and human studies, but results are not consistent across iron compounds and fiber types (8–11). Prebiotic galacto-oligosaccharides (GOS) are enzymatically-synthesized galactose di- and oligo-saccharides with galactose or glucose units at the reducing end (12). Previous studies reported that GOS acutely enhances FIA from low doses of ferrous fumarate (FeFum), both in infants and adult women (11,13,14). Prebiotic fructo-oligosaccharides (FOS) are short-chain mixtures of sucrose oligomers and up to three terminal fructose (15). Acacia gum is an heterogeneous material whose structure can be divided in two main fractions, an arabinogalactan-peptide fraction and an arabinogalactan-protein complex (16), and may be a prebiotic fiber (17). The mechanism of the enhancing effect of GOS on FeFum and whether its effect occurs when higher supplemental doses of iron are administered is unclear. Moreover, it is unknown whether dietary fibers other than GOS, such as FOS and acacia gum, may affect iron absorption from FeFum.

Therefore, our study objectives were to determine: (a) *in vitro,* the effects of these three fibers on iron solubility and dialyzability; (b) in iron depleted [serum ferritin (SF)< 25µg/L] women, whether co-administration of GOS, FOS or acacia gum would affect FIA from high, supplemental oral doses of FeFum. Our hypotheses were that: (a) all three fibers would increase iron solubility *in vitro*, and (b) all three fibers would increase FIA from a 100 mg

oral iron dose as FeFum, with GOS having the strongest enhancing effect. The primary outcome, FIA, was assessed by erythrocyte incorporation of stable iron isotope labels, measured 14 days after administration.

Methods

Study site and participants

We conducted the study between August and October 2020 at the ETH Zurich, Switzerland. We recruited women from the staff and students of ETH Zurich and University of Zurich, Switzerland. Inclusion criteria were: 1) female aged 18 to 45 y; 2) SF < 25 µg/L; 3) Body Mass Index (BMI) 18.5 to 24.9 kg/m^2; and 4) body weight < 70 kg. Exclusion criteria were: 1) anemia [hemoglobin (Hb) < 11.7 g/dL]; 2) chronic gastrointestinal, renal, and/or metabolic disease; 3) known hemoglobinopathies; 4) use of chronic medication (except for oral contraceptives); 5) use of vitamin, mineral, and pre- and/or probiotic supplements within 2 wk of first supplement administration; 6) blood transfusion, blood donation, or significant blood loss over the past 4 mo; 7) difficulties with blood sampling; 8) antibiotic treatment in the previous month; 9) known hypersensitivity or intolerance to iron supplements, GOS, or lactose; 10) pregnancy (tested in plasma at screening) and/or lactation; 11) smoking; 12) alcohol consumption > 2 units/d and 13) inability to follow study procedures. The Ethics Committee of the Canton Zurich approved the study (2020-00643). The trial was registered at clinicaltrials.gov as NCT04194255.

Study design

The study had a crossover, single-blinded, controlled design, in which all subjects received all test conditions (**Figure**). The primary outcome was FIA from 4 standardized test supplements containing FeFum with either GOS, FOS, acacia gum or sucrose and lactose (control). Approximately 1 month before baseline, 142 women were screened for eligibility. The study procedure was explained in detail and all subjects gave written informed consent prior to the start of any measurements. We collected a venipuncture blood sample (4 ml) for determination of iron (Hb, SF) and inflammatory (C-reactive protein (CRP)) status and pregnancy testing. We measured weight to the nearest 0.1 kg and height to the nearest 0.5 cm. We used a standardized questionnaire to obtain demographics and assess eligibility. Forty-five women met the inclusion criteria and from

these, we enrolled the 30 women with the lowest CRP concentrations from the screening into the study (Figure 1).

The experimental phase lasted 43 days per participant. Each participant received 4 supplemental doses of 104 mg iron as FeFum containing 4 mg isotopically labelled iron as FeFum (^{57}Fe or ^{58}Fe). The use of vitamin, mineral, and pre- and/or probiotic supplements was not permitted two weeks prior to baseline and for the entire study duration. Information on menstruation cycle was not collected. Limited evidence exists about a potential effect of the different phases of the menstrual cycle on iron-status indicators, such as Hb and SF (18). Both parameters are included in the calculation for FIA and therefore, the potential effect of menstruation cycle is accounted for in our analyses. On day 1 and 22, the participants received the labelled iron as ^{57}FeFum. On day 4 and 25, the participants received the labelled iron as ^{58}FeFum. The iron supplements were given with 1) 15 g GOS; 2) 15 g FOS; 3) 15 g acacia gum or 4) 6.1 g sucrose and 1.5 g lactose (control). The 4 test conditions were administered with a randomized computer-generated sequence of letter codes (Excel, Microsoft Office 2016). The supplemental iron doses were administered between 07:00 and 09:00 after an overnight fast under supervision of the study team. After consumption, participants were instructed not to consume food or drinks for 3 h. We collected data about the effects of the previous supplement administration at each visit. On day 1, 22 and 43, we collected venous blood samples after an overnight fast for iron isotopic analysis and for determination of iron status (Hb, SF, soluble transferrin receptor (sTfR)) and inflammation (CRP, α-1-acid glycoprotein (AGP)). On the same days, we repeated the anthropometric measurements.

Stable isotope tracers

^{57}Fe- and ^{58}Fe-enriched FeFum were prepared, respectively, from ^{57}Fe- and ^{58}Fe-enriched elemental Fe (95.55% and 99.89% isotopic enrichment, respectively, Chemgas, Boulogne, France) by Dr. Paul Lohmann GmbH (Emmerthal, Germany). Each 104 mg iron dose contained 4 mg of iron tracer. We analyzed the labelled iron compounds for iron isotopic compositions and the tracer iron concentration via isotope-dilution mass spectrometry, as outlined in the analytical methods.

Composition of the test supplements

The supplemental iron doses were given as 100 mg iron as FeFum capsules (Ferrum Hausmann, Vifor Inc., St. Gallen, Switzerland) and 4 mg of isotopically labelled iron as FeFum, weighted and encapsulated at the Human Nutrition Laboratory, ETH Zurich. The recommended daily dose of elemental iron for treatment of ID and IDA in WRA is between 40 and 200 mg (3). We chose Ferrum Hausmann as a study supplement as it provides an iron dosage within this range and is commonly prescribed and available in Switzerland. The following products were dissolved in 200 mL ultrapure water and consumed together with the iron supplements: 1) Vivinal GOS Powder (Friesland Campina DOMO, Amersfoort, The Netherlands) containing GOS (69%), lactose (23%), monosaccharides (5%) and trace amount of minerals, nitrogen and sulphated ash; 21.7 g were administered containing 15 g of GOS; 2) Fibrulose F97™ FOS (Cosucra Groupe S.A., Warcoing, Belgium), containing oligofructose (min. 95%) and free fructose, glucose and sucrose (max. 5%); 15.5 g were administered containing 15 g of FOS; 3) Fibregum™, Acacia Gum (Nexira food, Rouen, France), containing arabinogalactan (min. 90%) and small amounts of proteins and trace minerals; 16.7 g were administered containing 15 g of acacia gum; 4) when no GOS, FOS or acacia gum were given, 6.1 g lactose and 1.5 g sucrose were dissolved in the ultrapure water for control. These amounts were based on the amounts of sucrose and lactose present in the administered portion of Vivinal GOS powder. The dose of 15 g prebiotics was based on previous studies reporting that 15 g of GOS improved FIA from ferrous fumarate (13). GOS is well-tolerated at intakes < 20 g per day. The same applies to FOS and acacia gum (9,19). GOS, FOS and acacia gum have GRAS (generally recognized as safe) status in the USA (12,17,20).

Analytical methods

In vitro experiments

We tested iron solubility and dialyzability of the following conditions: 1) FeFum; 2) FeFum + GOS; 3) FeFum + FOS and 4) FeFum + acacia gum. The required amounts of prebiotics were calculated to match the prebiotic/Fe ratios of the different supplement combinations administered *in vivo*.

To determine iron solubility, we tested all conditions in triplicates and at 3 different pH (2, 4 and 6). For each replicate, we weighted 200 ml of ultrapure water (18.2 MΩ • cm) only, or of ultrapure water in combination with either 2.1 g GOS, 1.5 g FOS or 1.6 g acacia gum. We adjusted the pH with HCl 3M and $NaHCO_3$ 1M and then split the solutions into 3 aliquots of 50 g in Erlenmeyer flasks. We transferred 7.5 mg FeFum into each flask after incubating them for 15 minutes in a water bath at 37°C. Starting from the addition of the first FeFum sample, we collected 1 ml of solution from each flask after 30 minutes. The aliquots were diluted appropriately with 0.1 M HCl. We measured iron concentration in the samples by flame-atomic absorption spectroscopy (AAS) (AA240FS, Agilent Technologies).

To determine iron dialyzability, we used the methods described by Hackl et al. (21) except for the incubation time after pancreatin addition, which was reduced to 1 h. Dialysis membranes SpectraPor 1 (20.4 mm O, 6-8 kDa, Spectrum Laboratories) were used. Samples for each condition were prepared by dissolving in 40 ml ultrapure water (18.2 MΩ • cm) 6.25 mg FeFum, corresponding to 2 mg elemental iron, with either 0.12 g of lactose and 0.03 g of sucrose (control condition), 0.43 g of GOS, 0.31 g of FOS or 0.33 g of acacia gum. We tested all four conditions in triplicate, starting from pH 2 for the gastric digestion step and adjusting it to pH to 7.5 to resemble the duodenal phase of the digestion. We measured the iron concentration in the dialysates by graphite-furnace absorption spectrophotometry (AA240Z, Agilent Technologies).

Determination of iron solubility and dialyzability

The percentage of soluble iron of each sample was calculated by dividing the total amount of iron measured in the respective aliquot, multiplied by 100, by the total amount of iron added to each flask. The total amount of iron in each dialysate was calculated by multiplying the measured iron concentration of the dialysate by AAS with the total weight of the dialysate. The percentage of dialyzable iron of each sample was calculated by dividing the total amount of iron in the respective dialysate by the total amount of iron added to each sample, multiplied by 100. We expressed iron dialyzability of each condition (FeFum + GOS, and FeFum + FOS, FeFum + acacia gum), as relative iron dialyzability (percentage), calculated by dividing the percentage of dialyzable iron of the respective

condition by the percentage of dialyzable iron of the control condition (FeFum with lactose and sucrose).

Blood analysis

We collected venous blood into EDTA-coated (4 mL) and serum-vacutainer (10 mL) tubes. We measured Hb in whole blood using a Sysmex XE_5000 (Sysmex Corporation, Kobe, Japan) analyzer. At screening, we measured SF and CRP using chemiluminmescent immunoassays (Immulite 1000, Siemens Healthcare, Erlangen, Germany). At study visits, we measured iron status (SF, sTfR) and inflammation (CRP, AGP) in serum using a multiplex immunoassay (22). Anemia was defined as an Hb < 12 g/dL (2). Iron deficiency was defined as SF < 15 mg/L (2) and/or sTfR > 8.3 µg/mL. CRP and AGP > 10 mg/L and > 1 g/L, respectively, were defined as inflammation (22). Whole blood samples were mineralized in duplicates by microwave-assisted digestion in HNO_3 followed by iron separation from the blood matrix by anion-exchange chromatography and a subsequent precipitation step with ammonium hydroxide (23). We measured isotope ratios using inductively coupled plasma mass spectrometry (Neptune, Thermo Finnigan) equipped with a multicollector system for simultaneous ion beam detection (23).

Calculation of iron absorption

We calculated the amounts of ^{57}Fe, and ^{58}Fe isotopic tracers in whole blood collected at baseline and 18 d after completion of the 2nd and 4th administrations (day 1, 22 and 43) on the basis of the shift in iron isotope ratios and on the estimated amount of iron circulating in the body. Circulating iron was calculated on the basis of the blood volume (24) and measured Hb concentrations (mean Hb value of baseline measurement and endpoint measurement) for each participant. The shift was calculated based on the isotopic ratio measured in the prior blood sample, e.g., the shift between the sample on day 22 and 43. For the first 2 supplement combinations, the natural isotopic distribution was used as baseline (based on data from 60 unlabeled blood samples from female subjects), unless the participant previously took part in iron isotope studies. In that case, the baseline sample was also measured. Incorporation of the absorbed isotopic labels into new erythrocytes reaches a plateau after 10–14 d and remains stable for ~120 d (25). The calculations were based on the principles of isotope dilution and took into account

that iron isotopic labels are not monoisotopic (26). For the calculation of FIA, we assumed an 80% erythrocyte isotope incorporation of the absorbed iron (25).

Sample size calculation

Sample size calculation for within-subject comparisons indicated that, considering a SD of 0.153 from logarithmically (log) transformed iron absorption from a previous GOS study in our lab (13), 24 participants were needed to detect a 25% difference in FIA with a type I error rate of 5% and 80% power. Considering the duration of the study and anticipating a drop-out rate of 20%, we enrolled 30 participants.

Statistical analysis

We analyzed biochemical and anthropometric data and iron absorption using R (R 4.0.2. software; R Core Team) statistical programming environment. We ran descriptive statistics for all variables at baseline and assessed normality by visually inspecting the histogram and by testing the distribution of continuous variables against a normal distribution using Shapiro-Wilk test. Normally distributed data were expressed as mean ± SD; non-normally distributed data as medians and IQR. Based on data distributions, appropriate parametric or non-parametric tests were selected for comparisons. The in vitro solubility results and in vitro dialyzability results were compared by using one-way ANOVA followed by Bonferroni post-hoc adjustments for pairwise comparisons. We reported adjusted p values. FeFum was used as the control group and the other supplement combinations were exclusively tested against the control. For solubility, we only perform comparisons within the same level of pH. We investigated the effect of treatment on FIA by fitting a linear mixed-effect model using CRAN package “lmerTest”. The treatment (4 levels: FeFum, FeFum + GOS, FeFum + FOS and FeFum + acacia gum) was added as a fixed factor to the model (model 1). We defined subject as the random factor in the model and we used compound symmetry structure matrix as covariance structure. We performed model diagnostics by visually inspecting the normal Q-Q-Plot and the Tukey-Anscombe plot to verify the assumptions of homoscedasticity and normality of residuals. We fitted two additional models to test for the effect of period and carryover on FIA. Since no significant effect was detected, model 1 was used for data analysis. Furthermore, we fit the model 1 to a database which did not include

measurements for SF and FIA from participants with elevated CRP and/or AGP. There was no significant difference in the fit of this model and the one which included all measurements. Therefore, we used the complete database for our analysis. Significance was set at $p < 0.05$.

Results

In vitro iron solubility

The iron solubility of all the test conditions is shown in **Figure 2**. There was a significant effect of the different compounds on iron solubility ($F=10.88$ $p<0.001$). Post-hoc analysis revealed that the solubility of FeFum + GOS was significantly lower than of FeFum alone both at pH 2 (p.adj = 0.027) and pH 6 (p.adj =0.018). At pH 2, the solubility of FeFum + FOS was lower than of FeFum alone (p.adj = 0.045).

In vitro iron dialyzability

The percentages reported are relative to dialyzed iron from FeFum in water, which was used as a reference (100% dialyzability). The addition of prebiotics significantly affected iron dyalizability ($F=92.52$, $p<0.001$). The relative iron dialyzability of FeFum + GOS was higher (146.2%) than of FeFum alone (p.adj =0.003). The addition of FOS to FeFum showed significantly lower dialyzability (61.6%) than of FeFum alone (p.adj=0.018). The dialyzability of FeFum + acacia gum did not differ significantly from FeFum alone (109.2%).

Iron Absorption study

Anthropometric and iron and inflammatory status of the participants at baseline are presented in **Table 1**. Based on an SF < 15 µg/L and an Hb < 12 g/dL, 18 women (60%) were classified as iron deficient and 1 (3%) as anemic. Fractional and total iron absorption from the different test conditions is shown in **Table 2** and **Figure 3**. Compared to the FeFum control, FIA was higher from the FeFum + GOS and FeFum + FOS (+45% and 51%, respectively; $p< 0.001$ for both). Total iron absorption from both FeFum + GOS and FeFum + FOS was higher than from FeFum control ($p< 0.001$ vs. control, for both). In contrast, there was no significant difference in FIA from FeFum + acacia gum compared to FeFum alone ($p=0.688$) (Table 2 and Figure 3).

Discussion

The main findings of this study are that when given with a single supplemental dose of 104 mg of iron as FeFum in iron-depleted young women: 1) 15 g of GOS significantly increased FIA by 45%; 2) 15 g of FOS significantly increased FIA by 51%; and 3) 15 g of acacia gum did not affect FIA. To our knowledge, this is the first study investigating the acute effect of prebiotic fibers on iron absorption from a high supplemental iron dose.

In two previous studies conducted in iron-depleted women, GOS administration increased iron absorption from a 14 mg iron dose as FeFum given in a capsule (11,13). In the first study, the administration of 15 g GOS in water significantly increased FIA by 61%. The same doses of GOS increased iron absorption from 14 mg of iron as FeFum given with a non-inhibitory test meal, before or after 4 weeks of daily 15 g GOS consumption (+28% and +29% in FIA, respectively) (13). In the second study, a lower dose of 7 g GOS, with or without ascorbic acid, significantly increased FIA from 14 mg iron as FeFum (+64% and +26%, respectively) (11). Our results confirm the enhancing effect of a single dose of 15 g GOS on FIA from FeFum in women, but at a much higher iron dose and with the iron in a capsule form.

The enhancing effect of prebiotic fibers may be compound specific for FeFum (8,9), as previous studies in adults that assessed the effect of long term administration of GOS, FOS and inulin on iron absorption from $FeSO_4$ did not detect a significant increase in absorption (8–10). Similarly, Paganini et al. (14) showed that in anemic Kenyan infants, 3 weeks of daily 7.5 g GOS consumption increased FIA (+62%) from a maize porridge supplemented with 2.5 mg of FeFum + 2.5 mg NaFeEDTA, but not when fortified with $FeSO_4$. In contrast, Mikulic et al. (27) assessed the acute effect of 7.5 g GOS on iron absorption in Kenyan infants from a maize porridge fortified with 5 mg iron as either FeFum + NaFeEDTA, or as $FeSO_4$, and did not find an enhancing effect on iron absorption.

Acacia gum is a widely used hydrocolloid additive for various applications within the food industry, such as stabilization, emulsification and flavoring (16). Acacia gum is an heterogeneous material, with about 90% consisting of an arabinogalactan-peptide fraction (16); it has been proposed as a prebiotic (17). In addition to evidence *in vitro*

(28,29), including its binding to divalent cations (30) , studies in adults have reported that daily consumption of a 5 to 40 g dose acacia gum for 10 days to 4 weeks increases abundances of bifidobacteria, lactobacilli and total lactic acid-producing bacteria (19,28). A rat study reported acacia gum supplementation increased calcium, magnesium, phosphorus, and zinc concentrations in the femur (31). Whether acacia gum influences iron absorption is uncertain, but our *in vitro* and *in vivo* data suggest it does not have this enhancing effect.

Previous studies hypothesized that the enhancing effect of GOS administration on FIA from FeFum was due to a reduction of colonic luminal pH caused by the production of SCFAs from prebiotic fermentation by the microbiota (13,14). A lower pH would increase iron dissolution from FeFum (which is only soluble at low pH), potentially favoring colonic iron absorption (13,14). However, a recent study by Husmann et al. (32) in young women reported that GOS predominantly affect iron absorption from the proximal gastrointestinal tract and that iron solubility from FeFum+GOS was higher than FeFum only at pH typical of the proximal duodenum. These results suggest that the potential microbiota-mediated enhancement of colonic iron absorption is negligible in adults. Further investigations would be needed to clarify if the interaction between prebiotic GOS, or other prebiotics, and the gut microbiota can influence iron absorption.

Several mechanisms may explain the acute enhancing effect of GOS and FOS on iron absorption from FeFum. GOS and FOS co-administration could increase gastric residence time of the iron and extend the time of exposure to low gastric pH (11). This would selectively benefit poorly water-soluble iron forms, such as FeFum, that require a pH ~2 for complete dissolution (33). However, a recently published study showed no delay in the peak of iron absorption when ferrous fumarate was given together with GOS (32). Moreover, our *in vitro* data suggest the absorption enhancing effects of GOS and FOS are not due to a simple increase in iron solubility, as iron solubility of FeFum + GOS and FeFum + FOS at pH 2 was lower than of FeFum alone (Figure 2). Nonetheless, this disagrees with the results from Husmann et al.(32), where no differences in solubility where detected at pH 2. In conditions mimicking physiologic digestion, iron dialyzability from FeFum + GOS was 46.2% higher than from FeFum alone ($p=0.003$), while iron

dializability from FeFum + FOS was 38.4% lower than from FeFum alone (p=0.02). Acacia gum did not affect iron dialyzability. The mechanism behind the opposite effect of GOS and FOS on iron dializability is uncertain and the discrepancy between *in-vivo* and *in-vitro* results for FeFum+FOS also requires further investigation. Dietary fibers interact with iron (34) and reducing sugars can form stable soluble complexes with iron (35,36), but the effects are variable across different fibers and remain poorly understood (37). Interaction of free iron dissociated from FeFum with the reducing units of GOS and FOS could prevent formation, and therefore precipitation, of insoluble iron hydroxides (38), which would favor iron absorption. The formation and stability of carbohydrate -Fe^{2+} or -Fe^{3+} complexes is affected by several variables, including pH, the concentration and chemical form of ligands that may form complexes or chelates with the metal iron, the saccharide composition of the fiber and its degree of polymerization (34,35,38). Despite the presence of reducing sugars in all the compounds used in this study (16,39), acacia gum has a more complex structure than GOS and FOS. Acacia gum is composed of polypeptide chains bearing hyperbranched polysaccharides, which might hinder accessibility of iron to the reducing sugars (40). In contrast, GOS and FOS are simpler oligosaccharides which have a comparable degree of polymerization and no branches. These marked structural differences may explain the varying effects of GOS and FOS, compared to acacia gum, on iron absorption.

Our study has several strengths. We used erythrocyte incorporation of stable iron isotopes to assess iron absorption, the reference method in humans, in a cross-over design, where all participants consumed all test conditions which allowed for within-subject comparisons. We studied mostly iron deficient women, who are the main target group for high dose iron supplementation (2) and compared three widely-used prebiotic fibers. We examined underlying mechanisms by assessing *in vitro* effects of these fibers on iron solubility and dialyzability. Our study has several limitations. Nearly all of our iron-depleted women were not anemic; our results might have been different had we included women with iron deficiency anemia. We did not test whether the fibers would affect absorption from supplemental iron compounds other than FeFum. Finally, we measured the enhancing effects of the fibers on absorption from a single supplemental iron dose; whether these

effects on iron absorption would be sustained during long-term iron supplementation is uncertain.

In conclusion, GOS and FOS represent promising enhancers of iron absorption from high-dose iron supplements. In addition to the beneficial effect on iron absorption, the co-consumption of prebiotic GOS and FOS with iron could mitigate iron-induced gut dysbiosis and gut inflammation (41) and have other beneficial effects on the gastrointestinal tract (5). We feel further studies are needed on: a) the mechanism of the acute effect of GOS and FOS on iron absorption; b) whether the enhancing effect of GOS and FOS on FIA would be maintained during long-term administration; c) if the prebiotic nature of GOS and FOS would lead to a reduction in gastrointestinal side effects from iron supplements, which might thereby improve compliance; and d) whether lower doses of GOS and FOS can enhance iron absorption.

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Authors contribution

The authors' contributions were as follows—MBZ, IHA, FMDH and AG: designed the study; AG, IH-A, and CZ: conducted the study; AG and CZ: analyzed the data and performed the statistical analyses; AG, IHA, and MBZ: participated in the data interpretation; AG wrote the first draft of the manuscript, and all authors edited the manuscript, and read and approved the final manuscript.

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Tables

Table 1: Baseline anthropometric characteristics, hemoglobin, iron and inflammation indices of the participating women.

Parameter	Value (n=30)[1]
Age (y)	26.3 ± 2.8
Height (cm)	168.9 ± 5.6
Weight (kg)	59.0 ± 5.5
Body Mass Index (kg/m^2)	20.9 ± 1.5
Hemoglobin (g/dL)	13.4 ± 1.0
Serum Ferritin (µg/L)	12.9 (10.0; 19.9)
Serum Soluble Transferrin Receptor (mg/L)	5.6 ± 1.2
Serum C-Reactive Protein (mg/L)	0.15 (0.03; 0.77)
Serum α-1-acid-glycoprotein (g/L)	0.42 ± 0.11

[1]Values are means ± SD or medians (IQRs)

Table 2: Fractional and total iron absorption from 100 mg oral iron as ferrous fumarate given alone and with 15 g of GOS, FOS, or AG in iron depleted women (n = 30).

	FeFum[2]	FeFum and GOS	FeFum and FOS	FeFum and AG
FIA (%)	23.0[1] (19.7; 32.7)	33.3 (27.3; 47.2)	34.8 (27.9; 37.1)	20.0 (14.8; 28.4)
Total iron absorption (mg)	23.9 (20.5; 34.0)	34.6 (28.4; 49.1)	36.1 (29.0; 46.2)	20.8 (15.4; 29.6)

[1]Median (IQRs), all such values.
[2]AG, acacia gum; FeFum, ferrous fumarate; FIA, fractional iron absorption; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides.

Figures

Figure 1: Study design and participant flowchart. AG, acacia gum; BMI, body-mass index; CRP, C-reactive protein; Hb, hemoglobin; FeFum, ferrous fumarate; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides; SF, serum ferritin.

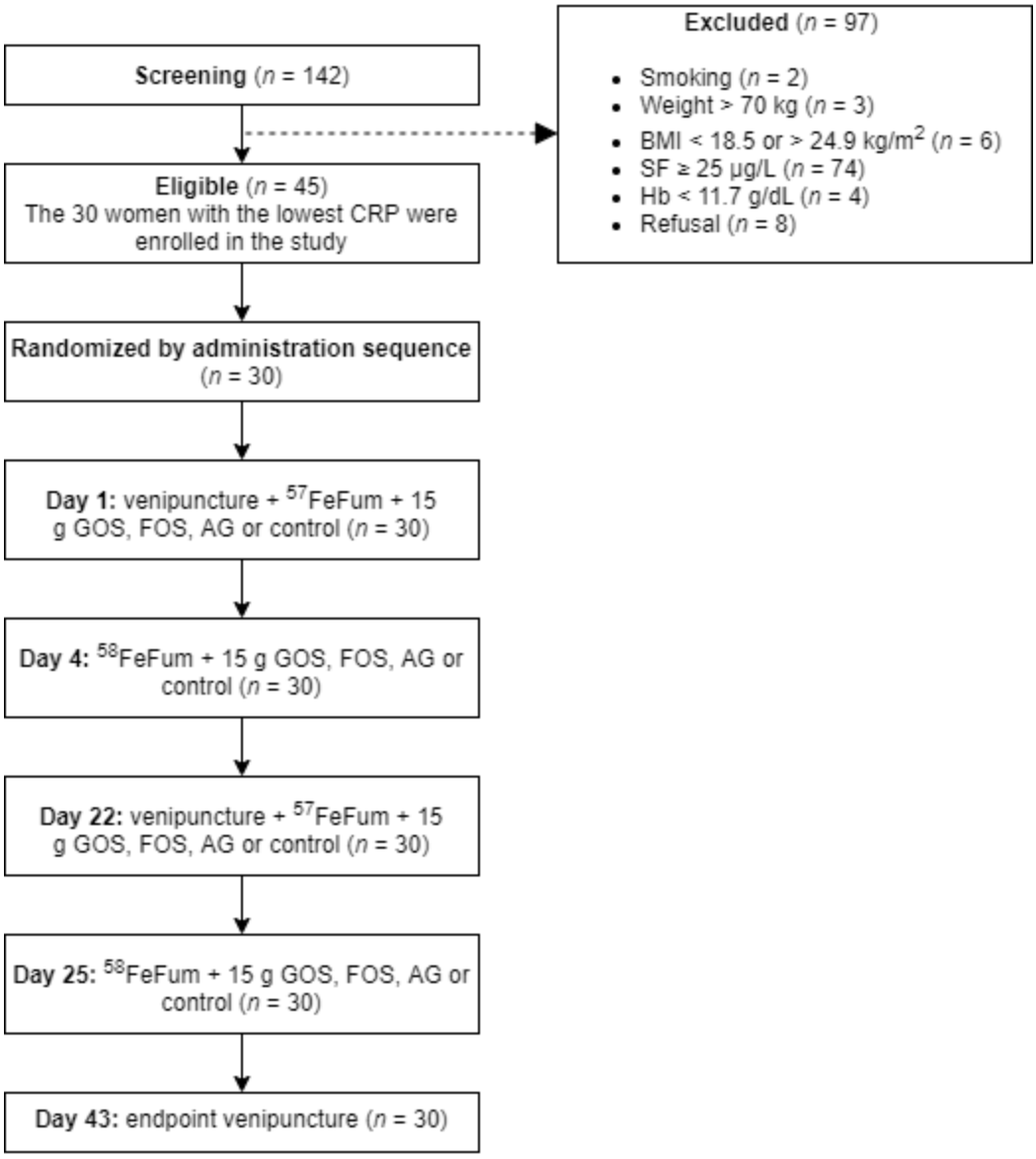


Figure 2: In vitro iron solubility of ferrous fumarate alone or with GOS, FOS, or AG at pH 2, 4 and 6. Values are mean ± SD, n=36. *Bracketed means differ significantly, p.adj <0.05. We used one-way ANOVA followed by Bonferroni post-hoc adjustments for pairwise comparisons. AG, acacia gum; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides.

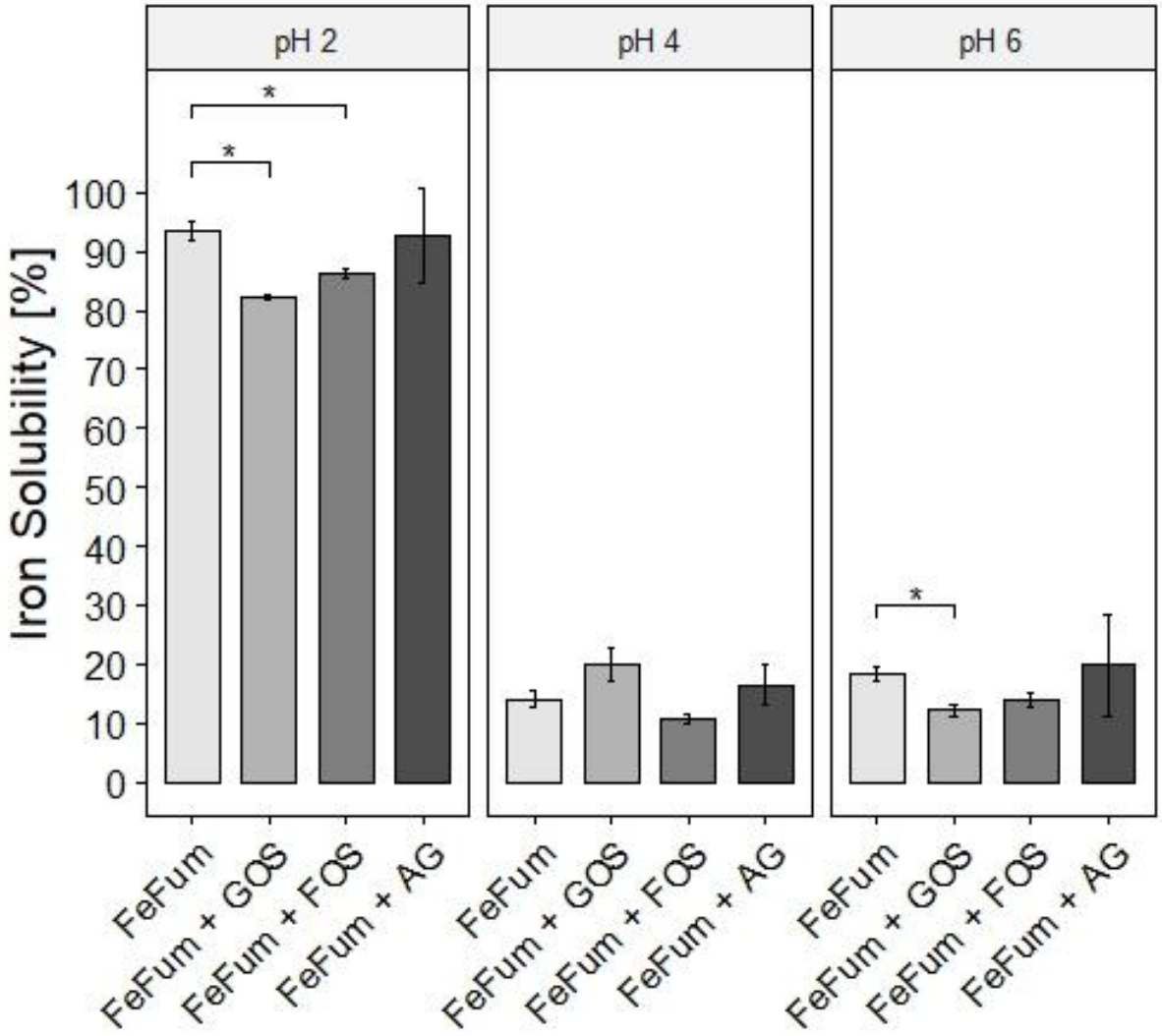
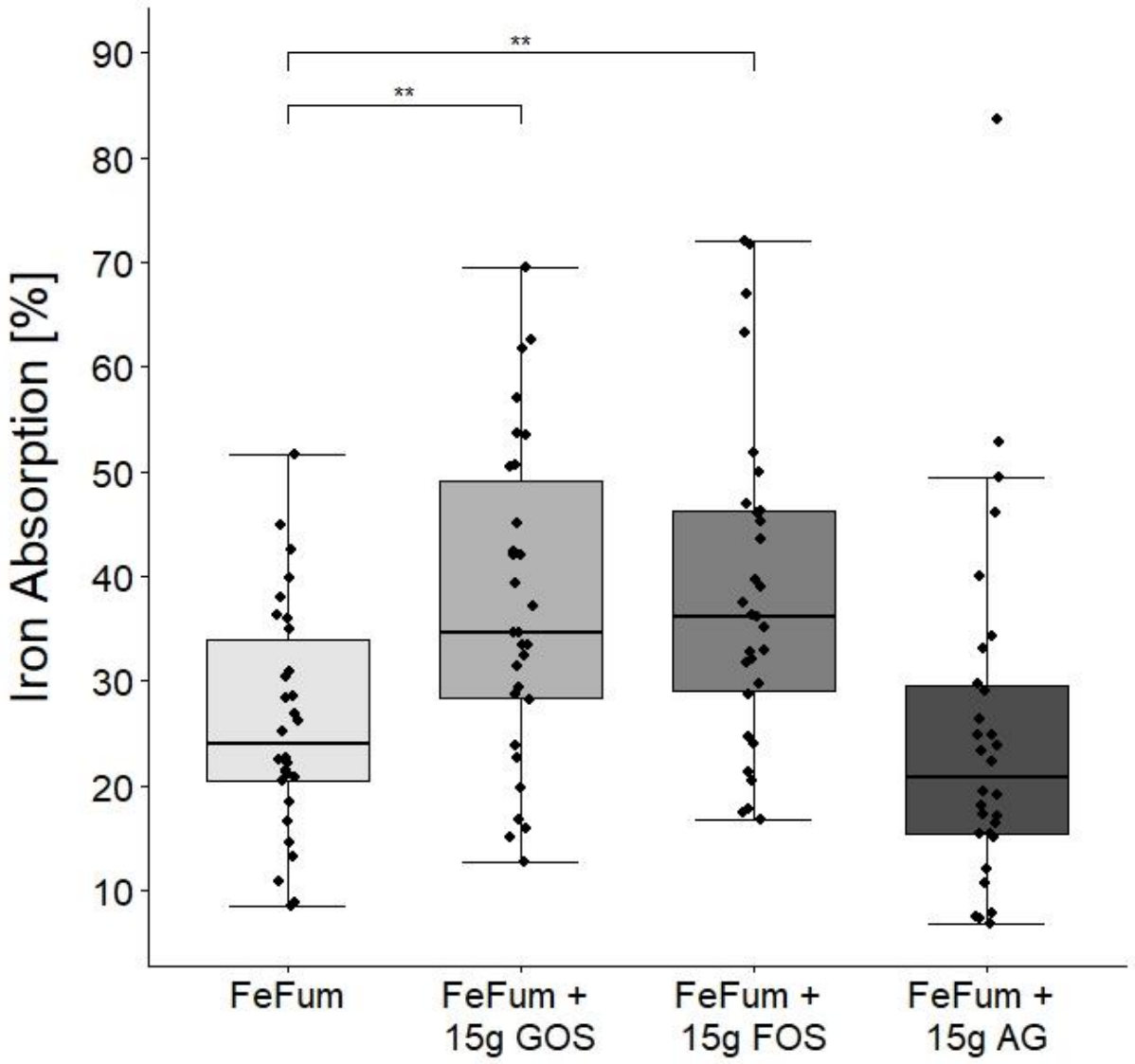


Figure 3: Fractional iron absorption from ferrous fumarate alone or in combination with 15 g of GOS, FOS, or AG in iron depleted women (n=30). The boxes show the medians with 25th and 75th percentiles. We investigated the effect of treatment on fractional iron absorption by fitting a linear mixed-effect model, with treatment (4 factor-level: FeFum, FeFum + GOS, FeFum + FOS and FeFum + AG) as the fixed effects and subject as the random effect. The whiskers extent to the furthest data point that is within 1.5 times the IQR; ** $p < 0.001$. AG, acacia gum; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides.



MANUSCRIPT 3

The effects of 2'-fucosyllactose and lacto-N-neotetraose, galacto-oligosaccharides and maternal human milk oligosaccharide profile on iron absorption in Kenyan infants

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Abstract

Background Whether prebiotic human milk oligosaccharides (HMO), such as 2'-fucosyllactose (2'-FL) and lacto-N-neotetraose (LNnT), enhance iron absorption in infants is unknown. Whether maternal HMO profile affects absorption of iron fortificants or the effects of prebiotic galacto-oligosaccharides (GOS) and/or HMO on iron absorption is uncertain.

Objectives We tested if consumption of 3.0 g of GOS or HMO enhances iron absorption from iron-fortified maize porridge in partially breastfed Kenyan infants, and whether maternal HMO profile modulates these effects.

Methods In a randomized, prospective cross-over study, 55 infants (aged 8–12 months) consumed test meals fortified with: 1) 5.0 mg iron as ^{54}Fe-labeled ferrous fumarate (FeFum); 2) 5.0 mg iron as ^{58}FeFum and 3.0 g GOS (FeFum+GOS); and 3) 5.0 mg iron as ^{57}FeFum and 2.0 g 2'-FL and 1.0 g LNnT (FeFum+HMO). Fractional iron absorption (FIA) was assessed by erythrocyte incorporation of iron isotopes. HMO profiles were determined by capillary-gel-electrophoresis with laser-induced-florescence detection (xCGE-LIF). Data were analyzed with mixed-effect models. Iron dialyzability was measured *in vitro*.

Results FIA from FeFum+GOS (median (IQR) 22.2% (16.5 – 25.9%)) was higher than FIA from FeFum (12.5% (9.5 – 20.9%)) (p=0.003). FIA from FeFum+HMO was 13.3% (7.1 – 24.4%) and did not differ from FeFum (p=0.727). Maternal HMO profile did not predict FIA or modulate the effects of GOS or HMO on FIA. Iron dialyzability ratios at pH 2 of FeFum+GOS to FeFum and FeFum+HMO to FeFum were 2.1 and 0.9 (p=0.001 and p=0.322), respectively.

Conclusions In Kenyan infants consuming FeFum-fortified maize porridge, co-provision of 3.0 g GOS increased FIA by 78%, while co-provision of 3.0 g HMO did not affect FIA. Variations in maternal HMO profile, including secretor (Se) and Lewis (Le) phenotype, did not predict FIA. These data argue against a physiological role for 2'-FL and LNnT in facilitating iron absorption in infancy.

Introduction

Iron absorption from iron fortificants is generally low (< 10%) (1) and new strategies to increase absorption by adding absorption enhancers would be valuable (2). Prebiotic galacto-oligosaccharides (GOS), a mixture of di- and oligosaccharides composed of galactose units and a galactose or glucose unit at the reducing end (3-5) are promising enhancers of iron absorption (6-8). In Kenyan infants and young Swiss women consumption of 7.0 – 15.0 g of GOS significantly increased fractional iron absorption (FIA) from ferrous fumarate (FeFum) given as fortificant in micronutrient powders (MNPs) or supplements (6-9). Whether GOS doses lower than 7.0 g would enhance FIA in infants has not been tested.

Infant formula is often supplemented with GOS with the aim of emulating the beneficial health effect of 'natural prebiotic' human milk oligosaccharides (HMO) (10, 11). HMO are linear and branched combinations of D-glucose, D-galactose, N-acetylglucosamine, L-fucose and sialic acid (12), and are major components of the total carbohydrate fraction of human milk (HM) (11). HMO composition is highly variable, and the presence or absence of a functional α-1-2-fucosyltransferase (FUT2) determines the secretor (Se) phenotype [secretor (Se+) or non-secretor (Se-)] and of a functional α-1-3/4-fucosyltransferase determines the Lewis (Le) phenotype [Lewis-positive (Le+) or Lewis-negative (Le-)] (13). A previous intervention study in Kenyan infants suggested that maternal Se status may modulate the efficacy of iron and GOS fortificants (14).

HMO, such as 2'-fucosyllactose (2'-FL), a fucosylated HMO present in HM of Se+ women, and lacto-N-neotetraose (LNnT), a non-fucosylated and non-sialylated neutral HMO found in HM of both Se+ and Se- women, can be synthesized and added to infant formula (12, 15). Whether prebiotic HMO, like prebiotic GOS, can enhance iron absorption has, to our knowledge, not been tested. Furthermore, if maternal HMO profile predicts absorption from iron fortificants, or whether the enhancing effect of GOS (or of HMO) on iron absorption in infants is modulated by maternal HMO profile is unknown.

Therefore, our primary objective was to determine in Kenyan infants the effects of 3.0 g of GOS and of 3.0 g of HMO, given as a mixture of 2.0 g 2'-FL and 1.0 g LNnT, on FIA

from a maize porridge fortified with FeFum. Our hypothesis was that addition of GOS or of HMO to the FeFum-fortified test meal would result in higher FIA compared to FIA of the FeFum-fortified test meal alone. Our secondary objectives were to: 1) investigate if maternal HMO profile affects iron absorption from FeFum-fortified porridge and/or the potential iron absorption enhancing effects of GOS and HMO; and 2) measure *in vitro* the effects of GOS and HMO on iron dialyzability.

Subjects and Methods

Study site and participants

We conducted the study between November 2019 and 2020 in Kwale County, southern coastal Kenya. Maize is the staple food crop in this area, and the principal complementary food for weaning infants is a thin maize porridge called "uji". We recruited mother-infant pairs in the catchment area of Msambweni County Referral Hospital. Inclusion criteria were: 1) age 8 – 12 months; 2) anticipated residence in the area for the study duration; 3) ability and willingness to follow study procedures. Exclusion criteria were: 1) severe acute or chronic illness; 2) use of vitamin and mineral supplements in 2 months prior to baseline; 3) severe anemia [hemoglobin (Hb) concentration < 70 g/L]; and 4) severe wasting [weight-for-length Z-score (WLZ) < −3.0]. Ethical review committees of ETH Zurich (EK 2019-N-59) and Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya (JKU/2/4/896B) approved the study. The trial was registered at clinicaltrials.gov. as NCT04163406.

Study design

The study had a single-blinded, randomized, prospective cross-over design. At baseline (Day 0 and Day 1), 60 infants were screened for eligibility (**Figure 1**). The study procedures were explained in detail and all caregivers gave written informed consent with either a written signature or a fingerprint prior to the start of any measurements. Fifty-five infants were eligible and enrolled into the study. We collected a venipuncture or finger-prick blood sample from all infants for determination of baseline iron isotopic composition, iron status [Hb, plasma ferritin (PF) and soluble transferrin receptor (sTfR)], and inflammation status [C-reactive protein (CRP) and alpha-1-acid glycoprotein (AGP)]. We measured infant

weight and length to the nearest 0.1 kg and 0.5 cm, respectively. We assessed demographic characteristics, brief medical histories and feeding habits of the infants using a standardized questionnaire and health records. Maternal HM samples for analyses of HMO were obtained by manual expression of HM by the mother into a clean plastic container.

After baseline, 30 infants consumed three different test meals (A, B, and C) on alternate test meal days (Day 2, Day 4, and Day 6) and 25 infants consumed two different test meals (A and C) on alternate test meal days (Day 2 and Day 4) (Figure 1). Test meal compositions are described in detail in the next paragraph. The sequence of the test meals was assigned with a randomized computer-generated sequence of letter codes. The test meals were fed to the infants between 0630 and 0830 on each test meal day. The caregivers were told to only feed breast milk after the previous evening meal and to not feed any breast milk to the infant ≤ 3 h before the test meals. Members of the study team or caregivers fed the test meals to the infants using a small spoon and a bowl. All utensils were rinsed carefully with water, which was then also consumed by the infant. After the feeding, the participants stayed at the study site for 2 h and were not allowed to eat, drink or be breastfed.

Seven days after the last meal (Day 11 and 13, respectively), we conducted a home visit to assess infant morbidity. Fourteen days after the last test meal (Day 18 and 20, respectively), we collected a venipuncture or finger-prick blood sample from all infants for determination of erythrocyte iron isotopic composition, Hb, PF, sTfR, CRP, and AGP. We repeated anthropometric measurements and assessed feeding habits of the infants as described at baseline.

Test meals

Each test meal portion consisted of 80.0 g unfortified maize porridge, composed of 5.7 g unfortified, refined maize flour, 2.5 g sugar and 71.8 g bottled water. We prepared the maize porridge in bulk on each test meal day according to a standardized protocol. Just before feeding, we added to test meal A (FeFum control test meal) 5.0 mg iron as FeFum (added as 2.5 mg ^{56}Fe + 2.5 mg ^{54}Fe), to test meal B (FeFum+GOS) 5.0 mg iron as FeFum

(added as 2.5 mg ^{56}Fe + 2.5 mg ^{58}Fe) and 3.0 g of GOS (added as 4.0 g of Vivinal GOS-75 Powder; Friesland-Campina, Wageningen, Netherlands), and to test meal C (FeFum+HMO) 5.0 mg iron as FeFum (added as 2.5 mg ^{56}Fe + 2.5 mg ^{57}Fe) and 3.0 g HMO (added as 2.0 g 2'-FL and 1.0 g LNnT; Glycom, Horsholm, Denmark).

Stable isotope tracers

^{54}Fe-, ^{57}Fe-, and ^{58}Fe-enriched FeFum were prepared from ^{54}Fe-, ^{57}Fe- and ^{58}Fe-enriched elemental Fe (99.4%, 95.6%, and 99.9% isotopic enrichment, respectively, Chemgas, Boulogne, France) by Dr. Paul Lohmann GmbH (Emmerthal, Germany).

Random assignment and masking

The sequence of the test meals was randomly assigned using a computer-generated list (Excel, Microsoft Office 2010; Microsoft Corp) using letter codes (ABC, ACB, BAC, BCA, CAB, CBA, AC, and CA) by two senior team members. The mothers-infants' pairs and the local study team were masked to the test meal sequence. Only the two senior members of the research team responsible for the addition of the isotopic iron and prebiotic compound to the meal were not masked.

Sample size calculation

This study was powered to detect a 40% difference in FIA between FeFum control and FeFum+GOS test meals and a 28% difference between FeFum control and FeFum+HMO test meals based on a SD of the difference of 0.26 from logarithmically (log) transformed iron absorption data from previous absorption studies in infants done by our laboratory, a type I error rate of 5%, and 80% power. This calculation yielded a total sample size of 50 infants (27 infants for the comparison FeFum vs. FeFum+GOS, and an additional 23 infants for the comparison FeFum vs. FeFum+HMO). Anticipating a dropout or exclusion rate of ~10%, 55 infants (30 infants for the comparison FeFum vs. FeFum+GOS, and additional 25 infants for the comparison FeFum vs. FeFum+HMO) were enrolled into the study.

Analytical methods

Blood samples

We collected venous or finger-prick blood samples into heparin-coated tubes. We measured Hb immediately after sample collection using an HemoCue 301 analyser (HemoCue, Ängelholm, Sweden); we measured quality controls (Eurotrol, BS Ede, Netherlands) before each assessment. We froze aliquots of whole blood at –20°C until shipment and further analyses at ETH Zurich, Switzerland. We centrifuged the remaining whole blood at 3000 rpm for 10 min and stored the plasma aliquots frozen at –20°C until shipment and further analyses at ETH Zurich, Switzerland. Whole blood samples were mineralized in duplicates by microwave-assisted digestion in HNO_3 followed by iron separation from the blood matrix by anion-exchange chromatography and a subsequent precipitation step with ammonium hydroxide (16). We measured isotope ratios using inductively coupled plasma mass spectrometry (ICP-MS) (Neptune, Thermo Finnigan, United States) equipped with a multicollector system for simultaneous ion beam detection (16). We measured PF, sTfR, CRP, and AGP in plasma samples using a multiplex immunoassay (17).

HM samples

We froze and stored aliquots of HM immediately after collection –20°C and shipped them on dry ice to the glycoanalytical laboratory (glyXera GmbH, Magdeburg, Germany) for analyses. The HMO composition of each individual HM sample was qualitatively and quantitatively determined using a glyXboxCE™ system (glyXera GmbH, Magdeburg, Germany) based on multiplexed capillary-gel-electrophoresis with laser-induced-florescence detection (xCGE-LIF) (18). In accordance with the glyXera GmbH kit protocol (KIT-glyX-OS.P-APTS, glyXera GmbH, Magdeburg, Germany), the pure HM samples were diluted 1:100, spiked with an internal standard (IS) and treated with a denaturation solution. Then, the free oligosaccharides were labeled with 8-aminopyrene-1,3,6-trisulfonic acid (APTS), then purified and finally measured on a glyXbox™ system. All measurements included the addition of a migration time alignment standard (glyXalign4; STD-glyXalign-4-S, glyXera GmbH) to the sample.

Stable-isotope labels

Labeled iron compounds (^{54}Fe-, ^{57}Fe-, and ^{58}Fe-labelled FeFum) were analyzed for iron isotopic composition and the tracer iron concentration by ICP-MS as described above.

Composition of the maize flour

Iron concentration of the maize flour used for the test meals was measured on five replicates using graphite-furnace atomic absorption spectrophotometry (AAS) (Varian AA240Z, Agilent, United States) after mineralization by microwave digestion.

In vitro experiments

We tested iron dialyzability *in vitro* of the following conditions: 1) FeFum alone; 2) FeFum+GOS; and 3) FeFum+HMO. Additionally, as standard reference, we tested iron dialyzability of ferrous sulphate ($FeSO_4$). The required amounts of GOS and HMO were calculated to match the GOS/Fe and HMO/Fe ratios of the different supplement combinations administered *in vivo*. To determine iron dialyzability, we used the methods described by Hackl et al. (19) except for the incubation time after pancreatin addition, which was reduced to 1 h. Dialysis membranes SpectraPor 1 (20.4 mm O, 6-8 kDa, Spectrum Laboratories, United States) were used. Samples for each condition were prepared by dissolving in 40 mL ultrapure water (18.2 MΩ • cm) 6.25 mg FeFum, corresponding to 2 mg elemental iron, alone (FeFum), with 1.2 g of GOS (FeFum+GOS), or with 0.8 g of 2'-FL and 0.4 g of LNnT (FeFum+HMO). We tested all three conditions in triplicate, starting from pH 2 or pH 4 for the gastric digestion step and adjusting it to pH to 7.5 to resemble the duodenal phase of the digestion. We measured the iron concentration in the dialysates by AAS (Agilent).

Data and statistical analyses

Iron absorption

We calculated the abundance of ^{54}Fe, ^{57}Fe, and ^{58}Fe isotopic tracers in whole blood 14 days after administration of the last test meal (endpoint at day 18 and 20, respectively) on the basis of the shift in iron isotope ratios (shift between natural isotopic ratio and isotopic ratio at endpoint) and the estimated amount of iron circulating in the body. We measured

the isotopic ratio of whole blood samples at baseline in a subgroup to confirm the natural isotopic ratio. Circulating iron was calculated by multiplying Hb concentration (mean Hb of baseline and endpoint) with blood volume, estimated from body weight (mean body weight of baseline and endpoint) (20), and with the total amount of iron present in Hb, assumed to be 3.47 mg iron per gram Hb (21). The calculations were based on the principles of isotope dilution and took into account that iron isotopic labels were not monoisotopic (22). For the calculation of FIA, we assumed a 75% incorporation of the absorbed iron, based on a previous study that measured erythrocyte incorporation from intravenously infused ^{58}Fe in African infants in the same age range as the infants in our study (1).

Anthropometrics, iron and inflammation indexes

We calculated the weight-for-age Z-score (WAZ), length-for-age Z-score (LAZ) and WLZ using WHO Anthro software (version 3.2.1; WHO). Anemia was defined as Hb concentration < 110 g/L (23). Iron deficiency (ID) in this setting with a high infection burden was defined as PF concentration < 30 µg/L (24) or sTfR concentration > 8.3 µg/mL. Iron deficiency anemia (IDA) was defined as a Hb concentration < 110 g/L and PF concentration < 30 µg/L or elevated sTfR concentration > 8.3 µg/mL. CRP and AGP values > 5 mg/L and > 1 g/L, respectively, were defined as indicating inflammation.

HMO profiling

Data processing, data analysis and evaluation of the *HMO Fingerprints* (normalized electropherograms) were performed via glyXtoolGUI™ software (Beta v0.8.11, glyXera GmbH, Magdeburg, Germany). The signal-to-noise ratio (SNR) of each *HMO Fingerprint* was used to determine the limit of quantification (LOQ) according to Ullsten et al. (25). The respective noise within each sample was determined after the migration time alignment of the unsmoothed data in the late migration time range (approximation range = degree of polymerization (DP) 18<DP<20). Consequently, the LOQ is specific with respect to sample and measurement. All peaks ≥ LOQ were considered, and their IS-normalized peak areas were calculated (as percentages relative to the peak area of the IS [% IS] (= nPA)). We assigned the four HM groups based on the presence and absence

of specific fucosylated HMO, according to Thurl et al. (26) and Kottler et al. (18). Mothers with HM groups I or III are defined as being Se+, mothers with HM groups II or IV as Se-, mothers with HM groups I or II as Le+, and mothers with HM groups III or IV as Le-.

Determination of iron dialyzability in vitro

The total amount of iron in each dialysate was calculated by multiplying the measured iron concentration of the dialysate by AAS with the total weight of the dialysate. The percentage of dialyzable iron of each sample was calculated by dividing the total amount of iron in the respective dialysate by the total amount of iron added to each sample, multiplied by 100. We expressed iron dialyzability of each condition (FeFum, FeFum+GOS, and FeFum+HMO), as relative iron dialyzability (%), calculated by dividing the percentage of dialyzable iron of the respective condition by the percentage of dialyzable iron of the reference standard ($FeSO_4$).

Statistical analyses

We analyzed the data using the R statistical programming environment (R 4.0.2. software; R Core Team). We ran descriptive statistics for all variables and assessed normality by visually inspecting the histogram and by testing the distribution of continuous variables against a normal distribution using Shapiro-Wilk W test. Normally distributed data were expressed as mean ± SD; non-normally distributed data as median and IQR. Based on data distributions, appropriate parametric or non-parametric tests were selected for comparisons. When departing significantly from normality, the variables were transformed to correct skewness before proceeding with further data analyses. We investigated the effect of treatment (fixed factor, 3 levels: FeFum test meal, FeFum+GOS test meal and FeFum+HMO test meal) and period (fixed factor, 3 levels: day 2, day 4, and day 6) on fractional and total iron absorption by fitting a linear mixed-effect model using CRAN package 'lmerTest'. We defined subject as the random factor in the model. To investigate the effect of maternal Se status, Le status and HM group on FIA from the different test meals, we added them to the model described above (2 factor-level: Se+ and Se-; 2 factor-level: Le+ and Le-; or 4 factor-level: HM group I to IV) as interaction with treatment. We performed model diagnostics by visually inspecting the normal Q-Q-plot and the

Tukey-Anscombe plot to verify the assumptions of homoscedasticity and normality of residuals. Significance was set at $p < 0.05$. We used Pearson correlation coefficient and Spearman's rank correlation coefficient to investigate possible linear or monotonic relationships between all HMO peaks (IS-normalized area under the curve) in the HM samples and FIA, overall and by treatment group. For all correlations significance was set at $p < 0.01$.To identify HMO showing dependence on FIA, feature selection was performed using mutual information (MI) (27). MI measures how much information is shared between two random variables: it quantifies the amount of uncertainty of one variable that can be reduced by knowing the other. MI = 0 if two random variables are independent. A high MI value indicates a large uncertainty reduction, whereas a low MI value indicates a small reduction of uncertainty. For the *in vitro* iron dialyzability tests results were compared by using one-way ANOVA followed by Tukey's post-hoc test for pairwise comparisons. Significance was set at $p < 0.05$.

Results

Characteristics of the participants

After the baseline screening, 55 mother-infant pairs were included in the study. One infant was excluded due to an acute illness between baseline and test meal day 2. Thus, 54 mother-infant pairs completed the study and were included in the data analyses (Figure 1). Demographics, anthropometrics, Hb and iron and inflammation indexes of the study participants at baseline are given in **Table 1**: 38 infants (70%) were anemic, 49 (91%) had ID, 37 (69%) had IDA; 13 (24%) and 23 (43%) had an elevated baseline CRP or AGP concentration, respectively. Median [min, max] duration of exclusive breastfeeding was 6 [3,11] months. During the period of the study, all infants were still being partially breastfed (89% of the mothers reported breastfeeding > 5 times per day, 11% between 1 – 5 times per day) and all were receiving complementary foods, mainly maize porridge.

Test meal composition

The native iron concentration in the maize flour used for porridge preparation was 7.15 ± 0.30 µg Fe/g dry matter (≈ 0.04 mg Fe/test meal).

Iron absorption

Median (IQR) FIA (%) from FeFum, FeFum+GOS, and FeFum+HMO were 12.5 (9.5 – 20.9), 22.2 (16.5 – 25.9), and 13.3 (7.1 – 24.4), respectively (**Figure 2**). Median (IQR) total iron absorption (mg) from FeFum, FeFum+GOS, and FeFum+HMO were 0.6 (0.5 – 1.0), 1.1 (0.8 – 1.3), and 0.7 (0.4 – 1.2), respectively. FIA from FeFum+GOS was significantly higher than from FeFum (estimate: 5.1, confidence interval [2.5% – 97.5%]: 1.9 – 8.4, p = 0.003). FIA from FeFum+HMO was not significantly different from FeFum (−0.48, CI: −3.1 – 2.1, p = 0.727). There was no significant effect of period on FIA (p = 0.480).

Maternal HMO profile

In total 49 HM samples were analyzed for their HMO composition; five samples could not be analyzed due to inadequate sample amount. Seventy-eight HMO peaks, corresponding to at least an equal number of different HMO structures, could be detected in the HM samples. The HM typing showed that 42.9% of the HM samples corresponded to HM group I (Se+, Le+), 24.5% to HM group II (Se-, Le+), 20.4% to HM group III (Se+, Le-), and 12.2% to HM group IV (Se-, Le-). After grouping by Se and Le status, we identified that 63.3 % of the mothers were Se+, 36.7% were Se-, 67.4% were Le+, and 32.6% were Le-.

Maternal HMO profile and iron absorption

There was no evidence for an effect of the maternal Se and Le status or HM group (p = 0.250, p = 0.512, and p = 0.491, respectively), or for an interaction of the maternal Se and Le status or HM group with treatment (p = 0.531, p = 0.175, and p = 0.278, respectively), on FIA. None of the 78 detected HMO peaks showed a significant linear or monotonic correlation with FIA, overall and by treatment. The HMO peaks with the highest MI value, independent of treatment, in descending order were: 3'-fucosyllactose (3'-FL), 2'-FL, lacto-N-tetraose (LNT), lacto-N-fucopentaose (LNFP) II, LNFP I, LNFP III, lacto-N-difucohexaose (LNDFH) I, difucosyllactose (DFL), disialyllacto-N-tetraose (DSLNT), LNnT and LNDFH II.

In-vitro iron dialyzability

Median ± SD iron dialyzability (%, relative to $FeSO_4$) was significantly higher for FeFum+GOS than for FeFum at pH 2 (187.7 ± 1.1 and 91.5 ± 6.9, respectively) ($p = 0.001$) and at pH 4 (204.2 ± 5.1 and 140.1 ± 6.5, respectively) ($p < 0.000$). The ratio between iron dialyzability of FeFum+GOS and FeFum was 2.1 at pH 2 and was 1.5 at pH 4. Median ± SD iron dialyzability (%, relative to $FeSO_4$) from FeFum+HMO at pH 2 and pH 4 (86.3 ± 1.1 and 144.6 ± 16.2, respectively) was not higher than from FeFum at pH 2 and pH 4 ($p = 0.322$ and $p = 0.685$, respectively). The ratio between iron dialyzability of FeFum+HMO and FeFum was 0.9 at pH 2 and was 1.0 at pH 4.

Discussion

The main findings of this study are, in partially breastfed Kenyan infants: 1) consumption of a single low dose of 3.0 g GOS significantly enhanced FIA from maize porridge fortified with FeFum; 2) the iron absorption enhancing effect of GOS was independent of maternal HMO profile; 3) consumption of a single dose of 3.0 g HMO (2.0 g 2'-FL and 1.0 g LNnT) did not enhance FIA from the iron-fortified maize porridge, regardless of maternal HMO profile; 4) maternal HMO profile did not predict FIA from the FeFum-fortified maize porridge.

Several previous human studies reported GOS increases iron absorption from FeFum (6-9). In Kenyan infants, consumption of a dose of 7.5 g GOS daily for 3 weeks significantly increased FIA from a maize porridge fortified with iron as FeFum and sodium iron ethylenediaminetetraacetate (6), while a single dose did not (28). In young Swiss women, consumption of single doses of 15.0 g and 7.0 g GOS significantly increased FIA from FeFum (7-9), while a single dose of 3.5 g GOS did not (8). Our study shows that, in infants, co-administration of 3.0 g GOS sharply increases FIA from FeFum-fortified maize porridge. Use of this lower, but still efficacious GOS dose, would be beneficial for reducing bulk in the formulation of GOS-containing MNPs.

To our knowledge, our study is the first investigating the effect of HMO (2'-FL and LNnT) on iron absorption. We found no significant effect of HMO administration on FIA from FeFum in infants. Even though HMO are in general described as prebiotics, based on our

findings, they do not appear to enhance iron absorption like other prebiotics, such as GOS (6-9) and FOS (9). This discrepancy might be explained by differences in structural and/or physicochemical properties of GOS compared to HMO. GOS are linear linked galactose units with a galactose or glucose unit at the reducing end ($galactose_n$-galactose and/or $galactose_n$-glucose) with a DP between two and eight (3-5). In contrast, 2'-FL is only a tetrasaccharide but composed of three different monomers namely glucose, galactose, and fucose, and LNnT is composed of the four monomers glucose, two galactoses, and N-acetylglucosamine (12). Some monosaccharides can chelate iron forming stable soluble complexes and potentially facilitating iron absorption (29). Also, several sugars can act as reducing agents, and, if they could reduce Fe^{3+} to Fe^{2+} in the gut, this could facilitate iron absorption (30) because only Fe^{2+} is transported by the divalent metal transporter 1 in the enterocyte (31). The hypothesis that the structural characteristics of GOS are important for their iron absorption enhancing effect is supported by our *in vitro* results, showing iron dialyzability from FeFum was enhanced at lower physiological pH by GOS, but not by HMO.

A previous intervention study in Kenyan infants suggested that maternal Se status may modulate the efficacy of iron and GOS fortificants: after receiving iron and GOS daily for 4 months, infants breastfed by Se- mothers showed greater improvements in iron status compared to infants breastfed by Se+ mothers (14). In contrast, in our study, there was no evidence that the maternal HMO profile modified the effects of GOS on iron absorption. This difference may be due to differences in study design: the previous study was a 4-month intervention (14) while our study was a single-meal absorption study. Similarly, maternal HMO profile did not modify the effect of HMO on iron absorption in this study.

Strengths of our study are: 1) we used a prospective cross-over design, allowing for within-subject comparisons and avoiding high between subject variability, increasing statistical power; 2) we studied weaning infants in Africa with a high prevalence of ID and IDA, the target group of iron fortification programs; 3) we used maize porridge as the test meal, which is the typical complementary food in East Africa; 4) we used a high-performance glycoanalysis system based on xCGE-LIF to assess the HMO profile in HM; and 5) we tested a lower dose of GOS that would reduce bulk and increase usability of

GOS as a co-fortificant in MNPs. A limitation of our study is that we tested only two out of the many different HMO present in HM, and our results may not be generalizable to other HMO with different structural and chemical properties.

In conclusion, this study shows that consumption of a single low dose of GOS increases iron absorption from FeFum in infants, and this effect is independent of maternal HMO profile. Based on the absorption rate in this study, an MNP containing 5.0 mg of iron as FeFum, given with GOS, would provide enough absorbed iron to cover the daily iron requirement of 0.69 mg/day of 6 – 12 month-old infants (32). This would be a substantial reduction in iron dose from the currently used dose of 12.0 mg in MNPs (33). This lower, but well-absorbed iron dose might be preferred to the higher dose because it might induce less adverse gastrointestinal side effects, such as inflammation and gut dysbiosis (34). To our knowledge, this study shows for the first time that a single dose of prebiotic HMO (2'-FL and LNnT) does not enhance iron absorption from FeFum in infants, nor increase iron dialyzability *in vitro*. These data argue against a physiological role for 2'-FL and LNnT in facilitating iron absorption in infants. Future studies are needed to investigate the mechanism behind the effect of GOS on iron absorption, and to test the effect of other prebiotic compounds, including other HMO, on iron absorption in infants.

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Authors contributions

The authors' contributions were as follows—AG, DP and MBZ designed research; AG, DP, and SN conducted research; AG, DP, RK, MF, and MBZ analyzed data; AG and DP wrote the first draft of the manuscript; AG, DP, SN, RK, MF, SK, TH, and MBZ had primary responsibility for final content; All authors read and approved the final manuscript.

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Tables

Table 1: Age, gender, anthropometric measurements, hemoglobin, and iron and inflammation indexes in Kenyan infants (n = 54) at baseline.

Parameter	Value
Age (months)	9.9 ± 1.1 [1]
Male/Female (n)	29/25
Height (cm)	71.2 ± 2.8
Weight (kg)	8.5 ± 1.1
Length-for-age Z-score	−0.5 ± 1.0
Weight-for-age Z-score	−0.4 ± 1.1
Weight-for-length Z-score	−0.2 ± 1.0
Hemoglobin (g/dL)	10.5 (9.8 – 11.1) [2]
Plasma ferritin (µg/L)	10.9 (5.8 – 25.7)
Soluble transferrin receptor (mg/L)	13.7 (8.2 – 17.2)
C-reactive protein (mg/L)	0.7 (0.3 – 4.4)
Alpha-1-acid-glycoprotein (g/L)	0.9 (0.6 – 1.1)

[1] Mean ± SD, all such values. [2] Median (IQR), all such values.

Figures

Figure 1 Study design and participant flowchart.

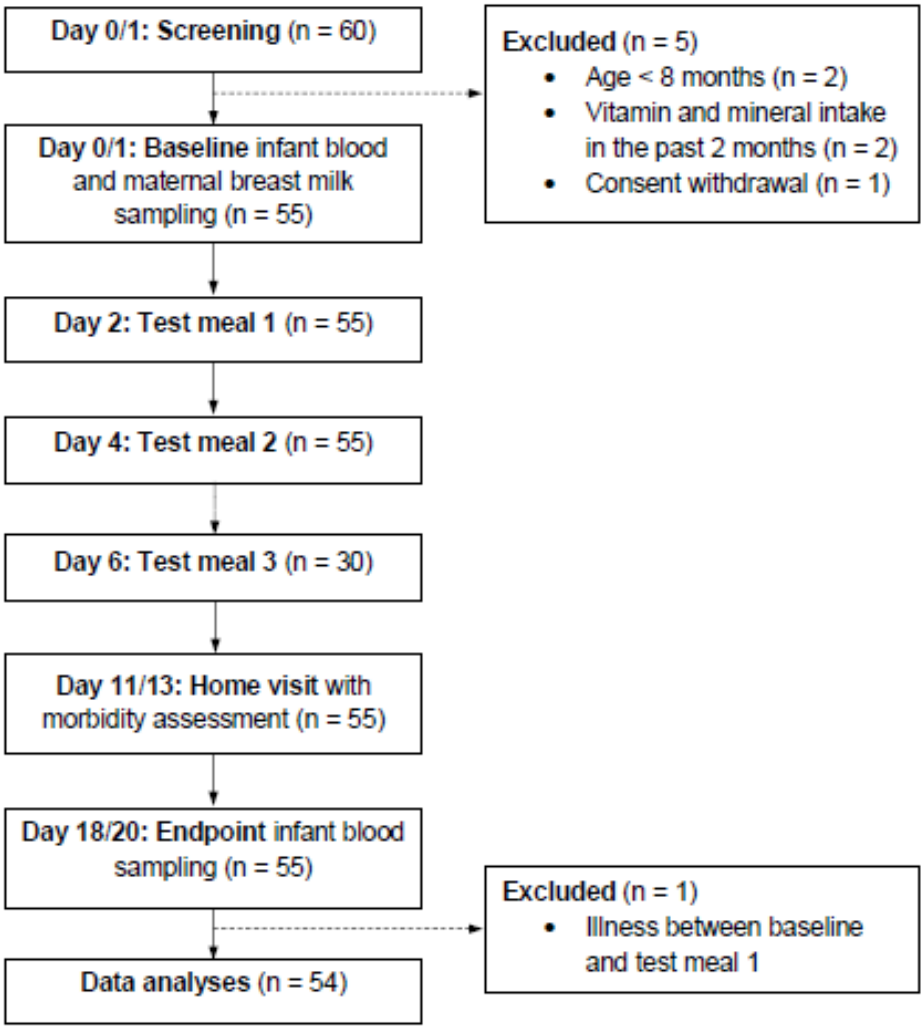
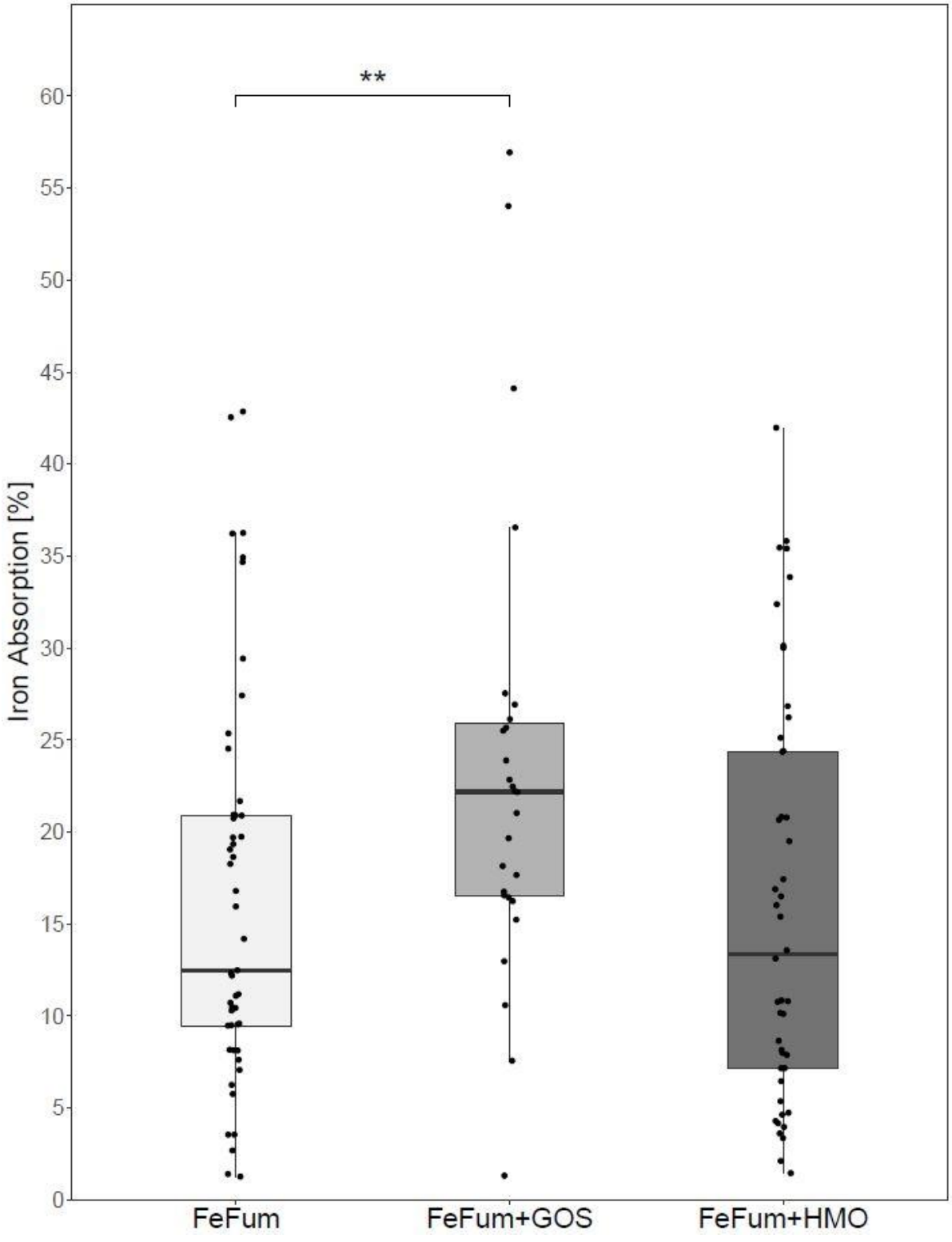


Figure 2: Fractional iron absorption (FIA) (%) from maize porridge fortified with 5.0 mg iron as ferrous fumarate (FeFum), with 5.0 mg iron as FeFum and 3.0 g galacto-oligosaccharides (GOS) (FeFum+GOS), and with 5.0 mg iron as FeFum and 3.0 g human milk oligosaccharides (HMO) given as 2.0 g 2'-fucosyllactose (2'-FL) and 1.0 g lacto-N-neotetraose (LNnT) (FeFum+HMO), in Kenyan infants (n = 54). Two test meals were excluded from FeFum+GOS and three test meals from FeFum+HMO before data analyses; the reason for exclusion were incomplete consumption and/or vomiting of the test meal by the infant. For five infants all consumed test meals (FeFum and FeFum+HMO) were excluded due to non-adherence to the protocol during test meal days. The boxes show the median (25^{th} and 75^{th} percentile). The whiskers extend to the furthest data point that is within 1.5 times the IQR. The dots show the individual values for each infant. We investigated the effect of treatment on FIA by fitting a linear mixed-effect model, with treatment (3 factor-level: FeFum, FeFum+GOS and FeFum+HMO test meals), and period (3 factor-level: test meal day 2, test meal day 4, and test meal day 6) as the fixed effects and subject as the random effect. There was evidence for higher FIA of FeFum+GOS than of FeFum ($p = 0.003$), but no evidence for higher FIA of FeFum+HMO than of FeFum ($p = 0.727$). There was no evidence for an effect of period on FIA ($p = 0.480$). ** $p < 0.01$.


**
Iron Absorption [%]
0
5
10
15
20
25
30
35
40
45
50
55
60
FeFum
FeFum+GOS
FeFum+HMO

MANUSCRIPT 4

Co-fortification of an iron-containing micronutrient powder with galacto-oligosaccharides and bovine lactoferrin improves hematologic outcomes in Kenyan infants compared to iron alone

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Abstract

Background High-dose iron-containing micronutrient powders (MNPs) (>10 mg iron/day) are recommended for infants in settings with an anemia prevalence >20%, but these may be associated with gut dysbiosis and inflammation.

Objectives To determine the efficacy of daily fortification with lower dose iron-containing MNPs (5 mg iron/day) with added galacto-oligosaccharides (GOS) and bovine lactoferrin (bLf), alone or in combination, in Kenyan infants.

Methods Using a randomized, controlled, double-blind 2-by-2 factorial design, 288 infants (aged 6 months ± 3 weeks) were randomly allocated to receive MNPs daily for 6 months, fortified with 5.0 mg iron as ferrous fumarate (Fe) alone (control), or with 7.5g GOS (Fe+GOS), 1g bLf (Fe+bLf), or 7.5g GOS and 1g bLf (Fe+GOS+bLf). At baseline, 1 and 6 months after beginning fortification, hemoglobin [Hb], iron status and systemic inflammation were assessed. We investigated the effects of time and treatment using linear mixed-effect models.

Results At baseline, 52% of the study population were anemic, 62% were iron deficient and 39% were iron-deficient anemic. Compliance was >90% in all groups with no between group differences. Attrition was 8% overall, with no between group differences. After 6 months, all treatments were associated with an increase in Hb (+0.52 g/L) and a decrease in the prevalence of iron deficiency anemia (IDA) (-12%) ($P<0.001$ for all). Compared to the Fe group, there were no significant time by treatment effects on Hb or iron status biomarkers in the Fe+GOS or Fe+Lf groups. In contrast, there were significant time by treatment effects on Hb ($t = 2.787$) and sTfR ($t = -2.118$) in the Fe+GOS+Lf group ($P < 0.05$ for all). There were also significant time by treatment effects on anemia prevalence ($z = - 1.998$) and IDA prevalence ($z = - 2.201$) in the Fe+GOS+Lf group compared to the Fe group ($P < 0.05$ for both), but not for Fe+GOS, or Fe+bLf.

Conclusions In Kenyan infants, the co-addition of GOS and bLf to an MNP containing 5 mg of iron given daily for 6 months was well-tolerated and increased Hb and reduced anemia and IDA, compared to the same MNP with iron alone.

Introduction

The World Health Organization (WHO) recommends point-of-use fortification of complementary foods with iron-containing micronutrient powders (MNPs) in regions where the prevalence of anemia in infants is 20% or higher (1). The MNP should contain 10 to 12.5 mg elemental iron, with a target of 90 doses given over a six-month period (1). A recent Cochrane systematic review found that MNPs with an iron content of ≥12.5 mg improved hematologic outcomes to a greater extent compared to those with lower daily doses (2) but recommended further research on other strategies, such as prebiotics or other means to improve iron bioavailability. Moreover, data on morbidity from iron in-home fortification are scarce (2), with some trials reporting an increased risk of diarrhea and bloody diarrhea in infants receiving MNPs with iron (3,4). Daily administration of iron containing MNPs to Kenyan infants increased potentially pathogenic intestinal microbiota and intestinal inflammation (5,6).

Prebiotics galacto-oligosaccharides (GOS) are promising enhancers of iron absorption (7). When given together with ferrous fumarate (FeFum), an increase in iron absorption has been consistently reported at varying dosages of GOS in women and children (8–11). However, a four-month trial of adding GOS to a MNP with sodium iron ethylenediaminetetraacetate (EDTA) and FeFum found no effect on iron biomarkers or anemia, compared to iron alone (12). In addition to their iron absorption enhancing properties, GOS can partially mitigate the adverse effects of iron-induced dysbiosis by enhancing the growth of barrier populations of bifidobacteria and lactobacilli in infants (12).

Lactoferrin (Lf) is a multifunctional glycosylated protein highly abundant in the whey protein fraction of milk (13). Lf exists in two forms: an apo (metal-free) state or an holo-form, with one atom of iron bound to each of the two globular lobes (14). Unlike transferrin, which binds iron tightly only at near neutral pH, Lf can retain iron at pH as low as 3.5 (15). Lf exerts multiple functions in breastfeeding infants, including bacteriostatic, bifidogenic (16) and anti-inflammatory effects, and can modulate gut immune function by enhancing production of several cytokines (17,18). Bovine lactoferrin (bLf) is highly homologous with human lactoferrin (19) and its effect on infant metabolism has been extensively

investigated (20). When administered orally, bLf has been shown to reduce the duration and severity of diarrhea episodes in children (21). Furthermore, acute administration of apo-bLf in Kenyan infants increased iron absorption from ferrous sulfate by >50% (22).

In this paper, we report a substudy of a parent trial designed to determine if combining GOS and bLf in an iron-containing MNP for African infants could protect against the adverse gastrointestinal effects of iron, including dysbiosis and inflammation, while at the same time increasing iron efficacy against anemia. Here, we present only the effects of the intervention on iron status and Hb, as well as systemic inflammation.

Methods

Study site and participants

We conducted the study between January 2020 and December 2021 in Kwale County, Kenya. We identified potential participants from pediatric vaccination clinics at Msambweni and Kwale Hospitals and through community health workers from villages in the surrounding area. Inclusion criteria were: 1) age 6 months ± 3 weeks; 2) caregivers ≥ 15 years of age; 3) breastfed from birth; 4) already consuming complementary foods; 5) anticipated residence in the study area for the study duration; 6) ability of the caregiver to provide informed consent; 7) not severely anemic [hemoglobin (Hb) < 70 g/L]; 8) not severely underweight [weight-for-age Z-score (WAZ) < -3]; 9) not severely wasted [weight-for-length Z-score (WLZ) < -3]; 10) no maternal or infant chronic illnesses; 11) no use of iron supplement 2 weeks prior study start; and 12) no antibiotic treatment 7 days prior to study start. The Ethics Commission of the Swiss Federal Institute of Technology (ETH) (EK 2019-N-04) and the Kenya Medical Research Institute (KEMRI) Scientific and Ethics Review Unit (SERU) (KEMRI/RES/7/3/1 no.656) reviewed and approved the study. An independent Data Safety Monitoring Board (DSMB) monitored the study, which was registered at clinicaltrials.gov as NCT03866837.

Study design

The study had a randomized, controlled, double-blind 2-by-2 factorial design. Infants were randomly allocated to receive MNPs daily for 6 months, fortified with: 1) 5 mg iron as

ferrous fumarate iron (Fe group); 2) 5 mg iron as Fe and 7.5 g GOS (Fe+GOS group); 3) 5 mg iron as Fe and 1 g bLf (Fe+Lf group); and 4) 5 mg iron as Fe, 7.5 g GOS and 1 g bLf (Fe+GOS+Lf) (Figure 1). We identified and screened 317 infants between January 2020 and April 2021. The study procedure was explained in detail and all caregivers gave written informed consent prior to the start of any measurements (Day 0). We took anthropometric measurements (weight to the nearest 0.1 kg and height to the nearest 0.5 cm using a digital scale and a rigid measurement board, respectively) and calculated WAZ and WLZ. We used a standardized questionnaire to obtain demographics and assess eligibility. We collected a venipuncture or finger-prick blood sample (3 mL) for the determination of iron [Hb, plasma ferritin (PF), serum transferrin receptor (sTfR)], and inflammatory biomarkers [c-reactive protein (CRP), alpha-acid glycoprotein 1 (AGP)]. 288 infants met the inclusion criteria and were randomly assigned to one of the 4 treatment arms (Fe, Fe+GOS, Fe+Lf, Fe+GOS+Lf) (Figure 1). At baseline (Day 1), the caregivers and infants returned to the hospital and received instructions and the first portion of maize porridge fortified with the assigned MNP, which was fed to the infant under close supervision of the study team. From baseline, we dispensed seven MNP sachets and 2 kg of refined maize flour (Mombasa Maize Millers Ltd, Kenya) every week for 6 months for the daily in-home preparation of fortified porridge. During the first 6 months, we assessed compliance weekly by collecting and weighing the unused MNP sachets from the previous week at the weekly home visits. We also assessed infant morbidity weekly with a standardized questionnaire performed during the home visits in the first 6 months, and by alternating home visits and phone calls bi-weekly during the 3 months of follow-up. At each hospital visit after baseline, we repeated the same procedures performed on Day 0.

Composition of the test meals

Maize is the staple crop in this region, and thin maize porridge, “uji”, is the typical local complementary food. We instructed the caregivers at baseline on how to prepare and administer MNP-fortified uji. The ingredients of the MNPs are a mixture of vitamins and minerals delivered in a maltodextrin carrier. In our intervention, maltodextrins were partially replaced by GOS (given as Vivinal GOS-75 Powder; Friesland-Campina, The

Netherlands), Lf (given as apo-bovine Lf, Armor Proteins, France), or both, except for the control group. The composition of the MNPs is shown in Table 1.

Analytical methods

Blood analyses

We collected venous or finger prick blood samples into heparin-coated tubes. We measured Hb immediately after sample collection with the use of a HemoCue 300 analyzer (HemoCue, Sweden); controls (Eurotrol, the Netherlands) were measured on each visit day. Aliquots of whole blood samples were frozen at –20°C until further analyses. The remaining whole blood was centrifuged at 3000 rpm for 10 min and the plasma aliquots were frozen at –20°C until further analyses. We measured PF, sTfR, AGP, and CRP in plasma samples using a multiplex immunoassay (23). Anemia was defined as Hb < 11 g/L, iron deficiency (ID) was defined as adjusted (see below) plasma ferritin $(PF)_a$ < 12 µg/L or/and sTfR > 8.3 mg/l, respectively. Iron deficiency anemia (IDA) was defined as Hb ≤ 11 g/L and PF_a < 12 µg/L or/and sTfR > 8.3 mg/L, respectively. Systemic inflammation was defined as CRP > 5 mg/L or/and AGP > 1g/L, respectively.

Calculation of body iron pools

We estimated total body iron (Fe_{total}) as the sum of circulating iron (Fe_{circ}), tissue iron (Fe_{tissue}), and storage iron (Fe_{stores}) (24). We calculated Fe_{circ} on the basis of the blood volume (25) and measured Hb concentrations at each time point for each participant, with 3.47 being the concentration of iron in Hb (mg/g). We estimated Fe_{tissue} as 6 mg/kg body weight (24,26). We calculated storage iron as Fe_{stores} (µg) = {9380 × log10[PF (µg/L)]—11,260} × body weight (27) if [PF] > 15.9 µg/L. Despite being derived from an adult population, the equation has previously been applied to children (24,28). For concentrations of PF < 15.9 µg/L, the Hallberg equation would result in negative estimates, that we replaced with 0 µg/L. For the calculations of Fe_{stores}, we used PF values adjusted for inflammation using the BRINDA approach (29).

Randomization and masking

We randomly assigned infants to the 4 intervention groups (n = 72 per group) using a computer-generated randomly permuted list with blocks of size 2, 4 or 6 with stratification by gender (Excel, Microsoft Office 2016; Microsoft Corporation). The MNPs were packed in identical appearing color-coded sachets (each containing one daily dose) at the factory, and the codes were kept solely by the Data and Safety Monitoring Board (DSMB) and the producer; study participants and investigators were blinded to group assignment.

Sample size calculation

This study focuses on the analysis of several of the secondary outcomes of the main trial, specifically on the assessment of the effect of treatment and time on iron and inflammation status, and anemia, ID, IDA, and inflammation prevalence. The primary outcome was the ratio of the abundances of potentially harmful to beneficial bacterial groups at 1 month. The sample size calculation was based on the preliminary data from the study by Paganini et al. (12). This study was powered to detect a standard deviation of the ratios of harmful pathogens and beneficial bacteria in the study groups receiving GOS, Lf, or both, compared to the group receiving iron alone, between 0.0004 and 0.02, with 80% power, and a type I error rate of 0.05. Anticipating a dropout rate or exclusion rate of 5%, a total of 288 subjects were enrolled in the study, divided equally across the 4 intervention groups.

Data and statistical analysis

We used REDCap electronic data capture tools (Vanderbilt University, Tennessee) to collect study data and conducted statistical analyses using R (R 4.0.2. software; R Core Team) statistical programming environment. We used the CRAN package “BRINDA” to adjust SF values for inflammation (30), using within-study internal regression coefficients for the adjustment. We calculated WAZ, WLZ, and length-for-age z-score (LAZ) for each infant using the WHO Anthro software (version 3.2.2; WHO). We calculated descriptive statistics for all variables at baseline, 1, 6, and 9 months and assessed normality by visually inspecting the histogram and by testing the distribution of continuous variables against a normal distribution using Shapiro-Wilk W test. Normally distributed data were

expressed as mean ± SD; non-normally distributed data as medians and IQR. Prevalence was expressed as % (95% CI). When departing significantly from normality, the variables were LN-transformed to correct skewness. We investigated the main effect of time (continuous independent variable) and its interaction with administration of GOS and of Lf (2-level factors: yes, no), either individually or combined, on each indicator of interest during the intervention period by fitting a linear mixed-effect model using the CRAN package "lme4". We investigated the main effect of time (continuous independent variable) and its interaction with administration of GOS and of Lf (2-levels factors: yes, no), either individually or combined, on proportions during the intervention period (0 to 6 months) by fitting a mixed effects logistic regression using the CRAN package "glmer". All models included "sex" as fixed factor and "study site" and "subject in study site" as random factors. We visually inspected the normal Q-Q-Plot and the Tukey-Anscombe plot to verify the assumptions of homoscedasticity and normality of residuals. When the interaction between time and GOS or Lf administration, or both, was significant, we used post-hoc paired samples t-tests with Bonferroni adjustments, with significance set at $p < 0.05$.

Results

After screening 317 infants, 288 met our criteria (**Figure 1**) and were randomly allocated to the 4 treatment groups. In the Fe, Fe+GOS, Fe+Lf, and Fe+GOS+Lf groups, the dropout rates were: (a) between baseline and 1 month: 3% (n = 2), 3% (n = 2), 0% (n = 0), and 3% (n = 2), respectively, and 2% overall (n = 6); (b) between baseline and 6 months: 6% (n = 4), 10% (n = 7), 8% (n = 6), and 10% (n = 7), respectively, and 8% overall (n = 24). In total, 264 participants completed the intervention period. There was no significant difference in dropout rates between groups. The mean adherence to treatment during the intervention was 94%; compliance was 97%, 94%, 95%, and 91% in the Fe, Fe+GOS, Fe+Lf, and Fe+GOS+Lf groups, respectively. There was a significantly lower compliance in the Fe+GOS+Lf group compared to the Fe group ($t = 2.70$, $p = 0.048$), but in both groups adherence to the treatment was >90%.

Baseline characteristics of the study participants are shown in **Table 2**. The four groups in the trial did not differ significantly at baseline with respect to any measured parameter. According to the WHO criteria (1), 52% of the study population at baseline were anemic. Based on sTfR > 8.3 mg/L, or PF_a < 12 µg/L, 62% of the participants were iron deficient and 39% were iron-deficient anemic at baseline. In the linear mixed effect models testing the effect of time and its interaction with the administration of GOS, Lf or both, the main effect of time was significant for Hb concentration (t = 4.732, P < 0.001), with an increment in the total study population of 0.52 g/L over 6 months intervention. Moreover, the total prevalence of anemia (from 52 to 33%, z = - 3.433, P < 0.001) and IDA (from 39 to 27%, z = - 2.319, P = 0.020) decreased significantly between baseline and 6 months. There was no significant change in the prevalence of ID between baseline and 6 months (62 vs 63%, z = 0.089, P = 0.929).

Biomarkers of iron and inflammation, body iron pools and prevalence of anemia, ID, IDA and systemic inflammation by group are reported in **Table 3**. Compared to the Fe group, there were no significant time by treatment effects on Hb and TfR concentrations in the Fe+GOS or Fe+Lf groups during the intervention. In contrast, there were significant time by treatment effects on Hb (t = 2.787) and sTfR (t = -2.118) in the Fe+GOS+Lf group compared to the Fe group (P < 0.05 for all) (Table 3). There were also significant time by treatment effects on anemia prevalence (z = - 1.998) and IDA prevalence (z = - 2.201) in the Fe+GOS+Lf group compared to the Fe group (P < 0.05 for both) (Table 3). The mean (± SD) daily iron gains, calculated as the Fe_{tot} at baseline subtracted from Fe_{tot} at endpoint, were 0.209 (± 0.160) mg Fe/day in the total study population, and did not differ across study groups (F = 1.596, P = 0.191).

Sub-group analyses are shown in **Figure 2**, together with the mean distributions of Hb, PF_a, sTfR, Fe_{tot}, AGP and CRP by group, during the whole study period. There were differences between timepoints in Hb in the Fe (time 0 vs time 6, t = 5.969, p = 0.004), and in Fe+GOS+Lf group (time 0 vs time 6, t = 3.693, p < 0.001). AGP differed significantly between 0 and 6 months in the Fe+GOS group (t = 4.372, p < 0.001) and in the Fe+Lf group (t = 3.033, p = 0.043). The output of post-hoc analyses performed on anemia and

IDA prevalence, as well as the distributions of the mean prevalence (%) of anemia, ID and IDA, are displayed in **Figure 3**. A significant decrease in anemia prevalence was reported for the Fe+GOS+Lf group (time 0 vs time 6, t = -1.684, p = 0.002); IDA decreased significantly in the Fe+GOS+Lf group between baseline and 6 months (t = -1.537, p = 0.023).

Discussion

This randomized, double-blind trial found that the co-addition of GOS and bLf to an MNP containing 5 mg of elemental iron (as ferrous fumarate), given daily for 6 months, compared to the same MNP with iron alone, resulted in: (i) a greater increment in Hb concentration (0.85 vs 0.48 g/dL, respectively) and (ii) a 10% greater decline in the prevalence of iron deficiency anemia (23% vs 13%, respectively). In contrast, the addition of GOS or bLf alone to the MNP did not have a significant effect on Hb or the prevalence of iron deficiency anemia compared to iron alone. All of the iron containing MNPs increased Hb and reduced IDA prevalence compared to baseline, suggesting a 5 mg dose of FeFum may be efficacious in an MNP, and the efficacy of this low dose was enhanced by the co-addition of the GOS and bLf.

Previous studies in Kenyan infants have suggested that both GOS alone (10) and bLf alone (22) enhance iron absorption. Paganini et al. reported an enhancing effect of GOS on FIA from single meals after a pre-feeding phase with GOS of 3 weeks. The authors suggested that GOS fermentation by the microbiota may have increased colonic short-chain fatty acid production, thereby reducing colonic pH and favoring solubility and colonic absorption of FeFum (10). However, other authors argue that, at least in adults, colonic iron absorption is negligible (9,31). Another postulated mechanism for the increase in absorption with GOS involves the interaction of the iron released from FeFum with the reducing sugars of GOS, enhancing iron solubility and preventing the formation of insoluble iron hydroxides (7). Long-term consumption of GOS has been suggested to be required to increase iron absorption (32). In a recent study by Mikulic et al., the addition of GOS to a single test meal did not enhance FIA in Kenyan infants. It has also been proposed that GOS has anti-inflammatory effects in the colon (33,34), which could

facilitate iron absorption by decreasing hepcidin circulation (35); however, there is little evidence of this in humans.

The enhancing effect of Lf on iron absorption may be due to several mechanisms. Lf can bind iron at the pH values found throughout the small and large intestine (15). An intact Lf-iron complex potentially could be absorbed through the putative intestinal Lf receptor (36). Studies investigating the absorption of iron from holo-Lf reported its absorption is similar to iron from ferrous sulfate (22,37,38). These results suggest that iron may dissociate from Lf in the duodenum, join the common non-heme iron pool, and be absorbed through the non-heme absorption pathway (22). Still another possibility is that peptide fragments resulting from Lf partial digestion could temporarily bind iron, reducing iron interaction with iron absorption inhibitors and facilitating uptake by DMT-1 (22).

Despite evidence from single meal iron stable isotope absorption studies in Kenyan infants that GOS and bLf can individually increase iron absorption, there was no significant effect of the GOS- or bLf-containing MNPs on Hb or iron status in this efficacy study, compared to iron alone, despite high compliance. It is well known that single meal absorption studies tend to exaggerate the effects of enhancers and inhibitors on iron absorption, compared to long-term studies (39–41). This effect may at least partially explain our findings: it is possible that the absorption enhancing effects of GOS or bLf alone given over 6 months, were too small to differentiate from iron alone. However, when given together, the enhancing effects were sufficient to improve iron status and reduce IDA compared to iron alone. Authors have suggested that the anti-inflammatory properties of Lf and/or GOS may be responsible for reported increases in iron absorption (42–44). However, there was no significant decrease in inflammation as assessed by the prevalence of elevated CRP or AGP in the GOS, Lf, or GOS+Lf groups compared to iron alone, suggesting this proposed mechanism did not contribute to our results. Clearly, the mechanisms responsible for our findings need further investigation.

The WHO recommends point-of-use fortification of complementary foods with iron-containing micronutrient powders that contain 10 to 12.5 mg elemental iron, given in 90 doses over a six-month period (1). Our trial provided a similar amount of iron but given

daily as a lower dose of iron, 5 mg, over six months. Our study design did not include a group given MNPs without iron because our previous study found that no decrease in anemia or improvement in Hb concentration is found in groups of infants given MNPs without iron (45). Direct comparisons are not possible between the increases in Hb and iron status from baseline to six months in our study to those from trials that compare outcomes with placebo or no interventions. Still, the magnitude of the increases in Hb concentration with each of our treatments compares favorably with those reported for point-of-care fortification in systematic reviews (2).

Six months of administration of iron fortified MNPs was sufficient to improve the Hb status and IDA prevalence in each treatment group. After the intervention, median Hb increased by 0.52 g/L overall and the prevalence of anemia and IDA decreased significantly. In contrast, the prevalence of ID did not change over time in any of the treatment groups. Despite the significant improvements in Hb status, at the end of the intervention, anemia persisted in 33% of the participants and was still higher than the percentage that WHO uses (20%) as a threshold to recommend iron fortification programs in infants and children (1). Similarly, the proportion of subjects with IDA after 6 months-fortification remained at approximately one third, Surprisingly, there was no overall effect of the intervention on ID prevalence. The WHO estimates the daily median iron requirements as 0.72 mg/day in infants between 6 months and 1 year of age, and 0.46 mg/day in infants and toddlers between 1 and 3 years (46). In our population, we estimated mean daily iron gains of around 0.20 mg during the 6 months intervention. These daily iron gains might not have been sufficient to sustain both increased iron needs for erythropoiesis and, at the same time, the needs for tissue growth and building iron stores. This might explain why Hb concentrations, anemia, and IDA were improved at the end of intervention, while iron status and ID prevalence were not.

Our study has limitations. First, we did not include a control group receiving MNPs without iron due to the absence of a significant effect of the MNPs without iron on anemia prevalence and Hb concentrations in a previous study in Kenyan infants. It is likely unethical to withhold supplemental iron from weaning infants known to be at high risk for

IDA and its detrimental effects. Second, we did not assess hepcidin concentration throughout the study, which may have provided insights into our findings. Last, we were unable to verify compliance for complete stool collection. Our study has also strengths, including: 1) the 2 x 2 factorial design, which permits the assessment of the effect of the administration of GOS and Lf individually and in combination, 2) high compliance and low attrition, and 3) we studied infants from a region with high prevalence of IDA, a target group for iron containing MNPs.

In conclusion, this study demonstrates that the co-fortification of an iron-containing MNP with GOS and bLf induces greater gains in Hb levels and a steeper reduction in IDA prevalence, when compared to MNP with iron alone. These findings suggest that the addition of GOS and bLf in combination might be a successful approach to enhance the efficacy of in-home iron fortification programs, in areas where anemia represents a public health problem. Finally, combining these findings with the data from our parent trial on the effect of the different MNPs formulations on intestinal inflammation and microbiota composition will provide comprehensive evidence on not only the efficacy, but also the safety of the addition of GOS and bLF, alone or in combination, to iron containing MNPs.

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Authors contributions

The authors' contributions were as follows—GMB, MBZ, SK, NS, AG: designed the study; AG, SN, MM: conducted the study; GMB, VG, AG: analyzed the data and performed the statistical analyses; AG, VG, MBZ and GMB: participated in the data interpretation; AG and MBZ wrote the first draft of the manuscript, and all authors edited the manuscript, and read and approved the final manuscript.

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Tables

Table 1: Composition of the MNPs by groups.

Ingredient	Control	GOS	Lf	GOS-Lf
Vitamin A	400 µg	400 µg	400 µg	400 µg
Vitamin D	5 µg	5 µg	5 µg	5 µg
Vitamin E	5 mg	5 mg	5 mg	5 mg
Thiamine	0.5 mg	0.5 mg	0.5 mg	0.5 mg
Riboflavin	0.5 mg	0.5 mg	0.5 mg	0.5 mg
Vitamin B6	0.5 mg	0.5 mg	0.5 mg	0.5 mg
Folic Acid	90 µg	90 µg	90 µg	90 µg
Niacin	6 mg	6 mg	6 mg	6 mg
Vitamin B12	0.9 µg	0.9 µg	0.9 µg	0.9 µg
Vitamin C	30 mg	30 mg	30 mg	30 mg
Copper	0.56 mg	0.56 mg	0.56 mg	0.56 mg
Iodine	90 µg	90 µg	90 µg	90 µg
Selenium	17 µg	17 µg	17 µg	17 µg
Zinc	4.1 mg	4.1 mg	4.1 mg	4.1 mg
Phytase	190 FTU	190 FTU	190 FTU	190 FTU
Iron (as Fe)	5 mg	5 mg	5 mg	5 mg
GOS[1]	0 g	7.5 g	0 g	7.5 g
Bovine lactoferrin	0 g	0 g	1 g	1 g
Maltodextrin carrier	11 g	1 g	10 g	0 g

[1]as 10 g Vivinal GOS 75 Powder

Abbreviations: Fe, ferrous fumarate; FTU, phytase units; GOS, galacto-oligosaccharides; Lf, lactoferrin; MNPs, micro-nutrients powders.

Table 2: Baseline characteristics of study participants.

Characteristic at baseline	n	Overall population
Age, mo	288	6.11 ± 0.40
Weight, kg	287	7.55 ± 1.01
Height, cm	287	66.31 ± 2.39
BMI, kg/m^2	287	17.13 ± 1.79
Weight-for-height z scores	287	-0.18 ± 1.1
Height-for-age z scores	287	0.05 ± 1.15
Weight-for-age z scores	287	-0.25 ± 1.08
Hb, g/l	288	10.81 ± 1.28
$Ferritn_{unadjusted}$, µg/L	286	21.00 (10.32; 43.73)
$Ferritin_a$, µg/L	286	14.85 (9.02; 30.87)
sTfR, mg/L	286	8.25 (6.74; 12.82)
CRP, mg/L	286	0.53 (0.18; 2.01)
AGP, g/L	286	0.75 (0.59; 1.00)
Total Body Iron, mg	287	128.25 (113.65; 141.73)
Anemia, %	288	52 (46; 57)
ID, %	286	62 (56; 67)
IDA, %	286	39 (34; 45)

Data are presented as mean (±SD) for normally distributed data, or median (IQR) for non-normally distributed data, and as % (95% CI) for proportions. Anemia is defined as Hb < 11 g/L; ID as PF_a < 12 µg/L and/or sTfR > 8.3 mg/L; IDA as Hb < 11 g/L and PF_a < 12 µg/L and/or sTfR > 8.3 mg/L.

AGP, alpha-1-acid glycoprotein; BMI, body mass index; CRP, c-reactive protein; Hb, hemoglobin; ID, iron deficiency; IDA, iron-deficiency anemia; sTfR, soluble transferrin receptor.

Table 3 Participants' iron and inflammation status during intervention.

Characteristic	n	Baseline	n	1 month	n	6 months	Intervention			
							p time	p time*GOS	p time*Lf	p time*GOS*Lf
Hb, g/l										
Fe	72	10.96 ± 1.12	70	10.96 ± 1.15	68	11.44 ± 1.02	0.000	0.321	0.216	0.006
Fe+GOS	72	10.82 ± 1.35	70	10.92 ± 1.03	65	11.23 ± 1.26				
Fe+Lf	72	10.96 ± 1.23	72	10.94 ± 1.03	66	11.30 ± 1.25				
Fe+GOS-Lf	72	10.48 ± 1.37	70	10.66 ± 1.04	65	11.33 ± 0.97				
$Ferritin_{unadjusted}$, ug/L										
Fe	72	20.44 (9.71; 30.09)	70	22.90 (15.08; 34.75)	68	21.30 (12.18; 29.86)	0.973	0.408	0.312	0.711
Fe+GOS	71	20.29 (11.55; 43.03)	69	23.93 (13.89; 49.12)	65	19.02 (12.10; 29.75)				
Fe+Lf	72	22.61 (9.10; 53.35)	72	24.37 (12.45; 38.93)	66	18.73 (12.42; 33.81)				
Fe+GOS-Lf	71	19.61 (11.34; 44.89)	70	27.94 (13.36; 55.68)	65	20.71 (13.41; 40.41)				
$Ferritin_a$*, ug/L										
Fe	72	14.27 (9.04; 22.76)	70	18.10 (11.52; 24.65)	68	14.65 (10.65; 23.34)	0.782	0.092	0.087	0.285
Fe+GOS	71	15.07 (10.24; 30.20)	69	17.87 (11.10; 34.73)	65	15.75 (8.55; 22.98)				
Fe+Lf	72	17.20 (7.88; 36.44)	72	20.02 (10.65; 30.75)	66	12.22 (8.32; 23.40)				
Fe+GOS-Lf	71	14.72 (8.99; 34.61)	70	21.48 (11.16; 39.22)	65	13.90 (9.54; 25.77)				
sTfR, mg/L										
Fe	72	7.94 (6.56; 12.03)	70	8.81 (6.32; 13.41)	68	8.40 (6.93; 10.68)	0.496	0.538	0.430	0.035
Fe+GOS	71	8.35 (6.84; 10.89)	69	8.32 (6.53; 12.65)	65	8.23 (6.92; 10.46)				
Fe+Lf	72	8.06 (6.38; 13.95)	72	8.06 (6.45; 11.41)	66	8.61 (7.39; 11.93)				
Fe+GOS-Lf	71	9.02 (6.94; 13.59)	70	8.74 (7.23; 13.93)	65	8.11 (6.90; 9.72)				

Total Body Iron†, mg/kg BW										
Fe	72	19.01 (22.29; 26.31)	70	19.00 (22.64; 26.19)	68	19.72 (22.80; 25.73)	0.586	0.034	0.035	0.007
Fe+GOS	71	18.21 (22.45; 27.00)	69	19.26 (23.05; 26.77)	65	18.76 (22.54; 25.41)				
Fe+Lf	71	18.47 (22.72; 27.13)	72	19.29 (22.74; 26.10)	66	19.20 (22.51; 25.78)				
Fe+GOS-Lf	71	17.85 (21.84; 27.06)	69	18.63 (22.71; 27.07)	65	17.58 (23.00; 26.43)				
CRP, mg/L										
Fe	72	0.71 (0.29; 1.93)	70	0.53 (0.25; 1.47)	68	0.61 (0.20; 2.42)	0.776	0.156	0.657	0.483
Fe+GOS	71	0.35 (0.16; 1.42)	69	0.52 (0.22; 1.89)	65	0.60 (0.21; 3.11)				
Fe+Lf	72	0.51 (0.19; 2.29)	72	0.53 (0.19; 1.57)	66	0.57 (0.22; 1.53)				
Fe+GOS-Lf	71	0.58 (0.15; 2.44)	70	0.58 (0.19; 3.58)	65	1.03 (0.25; 3.67)				
AGP, g/L										
Fe	72	0.80 (0.57;1.02)	70	0.85 (0.65; 0.99)	68	0.85 (0.70; 1.02)	0.303	0.020	0.043	0.036
Fe+GOS	71	0.71 (0.58; 0.91)	69	0.80 (0.64; 1.06)	65	0.92 (0.70; 1.25)				
Fe+Lf	72	0.76 (0.60; 1.07)	72	0.73 (0.64; 0.99)	66	0.92 (0.73; 1.16)				
Fe+GOS-Lf	71	0.77 (0.60; 1.00)	70	0.88 (0.63; 1.10)	65	0.91 (0.75; 1.15)				
Anemia (%)										
Fe	72	0.49 (0.37; 0.60)	70	0.56 (0.44; 0.67)	68	0.31 (0.21; 0.43)	0.001	0.116	0.726	0.046
Fe+GOS	72	0.43 (0.32; 0.55)	70	0.43 (0.32; 0.55)	65	0.35 (0.25; 0.48)				
Fe+Lf	72	0.54 (0.43; 0.65)	72	0.49 (0.37; 0.60)	66	0.33 (0.23; 0.45)				
Fe+GOS-Lf	72	0.61 (0.50; 0.72)	70	0.57 (0.45; 0.68)	65	0.31 (0.21; 0.43)				
ID (%)										
Fe	72	0.58 (0.47; 0.69)	70	0.59 (0.47; 0.69)	68	0.60 (0.48; 0.71)	0.929	0.959	0.419	0.582
Fe+GOS	71	0.66 (0.55; 0.76)	69	0.59 (0.48; 0.70)	65	0.65 (0.52; 0.75)				
Fe+Lf	72	0.61 (0.50; 0.72)	72	0.58 (0.47; 0.69)	66	0.78 (0.56; 0.78)				
Fe+GOS-Lf	71	0.61 (0.49; 0.71)	70	0.60 (0.48; 0.71)	65	0.60 (0.48; 0.71)				

IDA (%)										
Fe	72	0.39 (0.28; 0.50)	70	0.37 (0.27; 0.49)	68	0.26 (0.17; 0.38)	0.020	0.210	0.337	0.028
Fe+GOS	71	0.34 (0.24; 0.45)	69	0.30 (0.21; 0.42)	65	0.29 (0.20; 0.41)				
Fe+Lf	72	0.39 (0.28; 0.50)	72	0.31 (0.21; 0.42)	66	0.30 (0.21; 0.42)				
Fe+GOS-Lf	71	0.45 (0.34; 0.57)	70	0.39 (0.28; 0.50)	65	0.22 (0.13; 0.33)				
Inflammation (CRP > 5 mg/L (%))										
Fe	72	0.14 (0.08; 0.24)	70	0.14 (0.08; 0.24)	68	0.18 (0.10; 0.28)	0.472	0.323	0.924	0.627
Fe+GOS	71	0.08 (0.04; 0.17)	69	0.12 (0.06; 0.21)	65	0.18 (0.11; 0.30)				
Fe+Lf	72	0.15 (0.09; 0.25)	72	0.11 (0.06; 0.20)	66	0.17 (0.10; 0.27)				
Fe+GOS-Lf	71	0.15 (0.09; 0.26)	70	0.13 (0.07; 0.23)	65	0.20 (0.12; 0.31)				
Inflammation (AGP > 1 g/L (%))										
Fe	72	0.26 (0.18; 0.38)	70	0.20 (0.12; 0.31)	68	0.32 (0.22; 0.44)	0.176	0.067	0.239	0.065
Fe+GOS	71	0.17 (0.10; 0.27)	69	0.28 (0.18; 0.39)	65	0.46 (0.35; 0.58)				
Fe+Lf	72	0.31 (0.21; 0.42)	72	0.22 (0.14; 0.33)	66	0.47 (0.37; 0.60)				
Fe+GOS-Lf	71	0.24 (0.16; 0.35)	70	0.31 (0.22; 0.43)	65	0.40 (0.29; 0.52)				

Data are presented as mean ± SD for normally distributed data, as median (IQR) for non-normally distributed data, as % (95% CI) for proportions. Continuous data were analyzed using linear mixed effect models with non-transformed (for normal data) or LN-transformed (non-normal data) dependent variable, and proportions were analyzed using mixed effects logistic regression. During intervention: testing the main effect of time (from baseline to 6 months), the interaction effect of time by GOS (GOS vs no GOS), the interaction of time by Lf (Lf vs no Lf), and the interaction effect of time by GOS and Lf, and controlling for sex. All models included the random terms “study site” and “subject in study site” to account for the effect caused by the nested structure of the data. Anemia is defined as Hb < 11 g/L, ID is defined as $Ferritin_a$ < 12 µg/L or/and sTfR > 8.3 mg/l, respectively. IDA is defined as Hb ≤ 11 g/L and $Ferritin_a$ < 12 µg/L or/and sTfR > 8.3 mg/L, respectively. Systemic inflammation is defined as CRP > 5mg/L or/and AGP > 1g/L, respectively.

*BRINDA corrections calculated using the BRINDA package for R R (R 4.0.2. software; R Core Team)

†Total body iron is calculated as the sum of the circulating iron, tissue iron and iron stores, with tissue iron calculated as 6 mg/kg BW *kg BW

AGP, alpha-1-acid glycoprotein; BW, body weight; CRP, C-reactive protein; Fe, iron fumarate; GOS, galacto-oligosaccharides; Hb hemoglobin; ID, iron deficiency; IDA, iron deficiency anemia; IQR, interquartile range; Lf, lactoferrin; sTfR, soluble transferrin receptor.

Figures

Figure 1: Study design and participant flowchart.

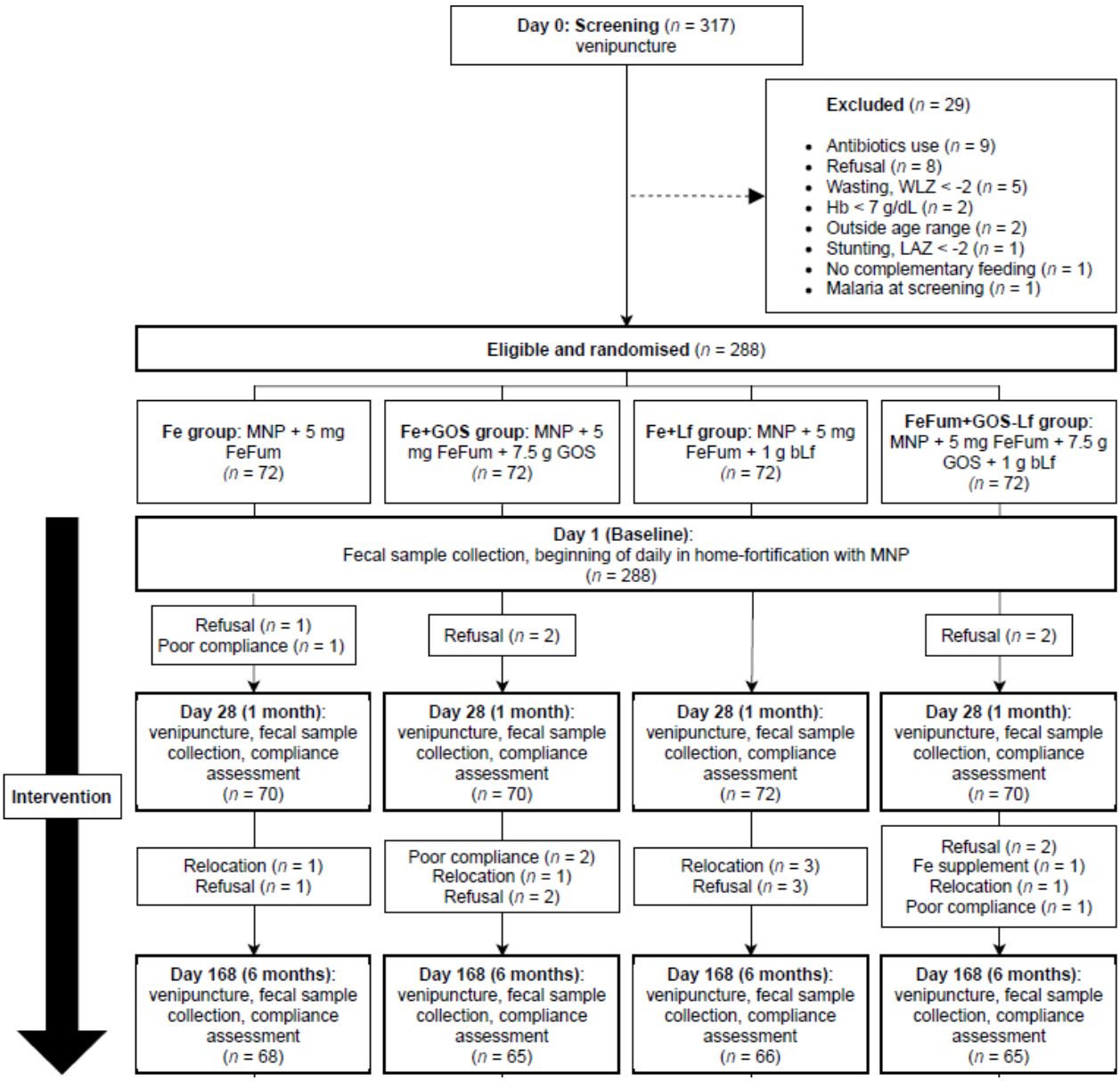


Figure 2: mean distributions of Hb, PF_a, sTfR, Fe_{tot}, AGP and CRP by group during the intervention (0-6 months).

A. Mean distribution of Hb during intervention. Post-hoc test (Bonferroni adjusted): differences between time points are in Fe group (time 0 vs time 6, $p<0.001$), and in Fe+GOS+Lf group (time 0 vs time 6, $p<0.001$; time 1 vs time 6, $p<0.001$).

B. Mean distribution of PF_a during intervention. Since no interactions between time, GOS administration or Lf administration were detected in the linear mixed effect model, no post-hoc analysis were performed.

C. Mean distribution of sTfR during intervention. No differences detected with post-hoc test (Bonferroni adjusted) between time points, or groups at the same time point.

D. Mean distribution of Fe_{tot}/kg BW over time. No differences detected with post-hoc test (Bonferroni adjusted) between time points, or groups at the same time point.

E. Mean distribution of AGP during intervention. Post-hoc test (Bonferroni adjusted): differences between time points are in Fe+GOS group (time 0 vs time 6, $p<0.001$), and in Fe+Lf group (time 0 vs time 6, $p=0.043$).

F. Mean distribution of CRP during intervention. Since no interactions between time, GOS administration or Lf administration were detected in the linear mixed effect model, no post-hoc analysis were performed.

AGP, alpha-acid glycoprotein 1; FeFum, ferrous fumarate; Fe_{circ}, circulating iron; Fe_{stor}, iron in stores; Fe_{tot}, total body iron; GOS, galacto-oligosaccharides; Hb, hemoglobin; Lf, lactoferrin; sTfR, serum-transferrin receptor.

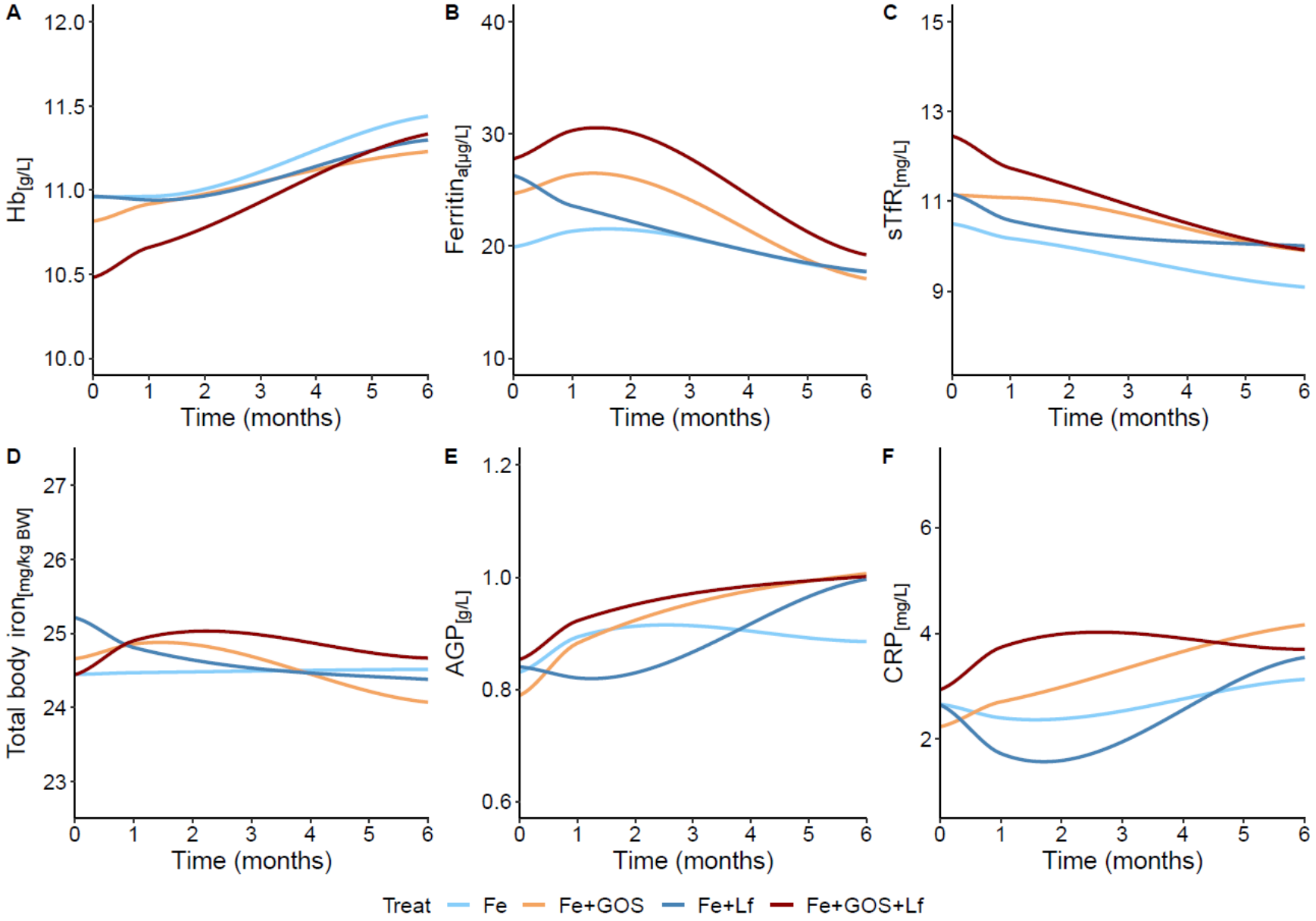

A
Hb[g/L]
12.0
11.5
11.0
10.5
10.0
0 1 2 3 4 5 6
Time (months)
B
Ferritin[µg/L]
40
30
20
10
0 1 2 3 4 5 6
Time (months)
C
sTfR[mg/L]
15
13
11
9
0 1 2 3 4 5 6
Time (months)
D
Total body iron[mg/kg BW]
27
26
25
24
23
0 1 2 3 4 5 6
Time (months)
E
AGP[g/L]
1.2
1.0
0.8
0.6
0 1 2 3 4 5 6
Time (months)
F
CRP[mg/L]
6
4
2
0 1 2 3 4 5 6
Time (months)
Treat
Fe
Fe+GOS
Fe+Lf
Fe+GOS+Lf

Figure 3 Distributions of the mean prevalence (%) of ID and IDA by group, during the intervention (0-6 months).

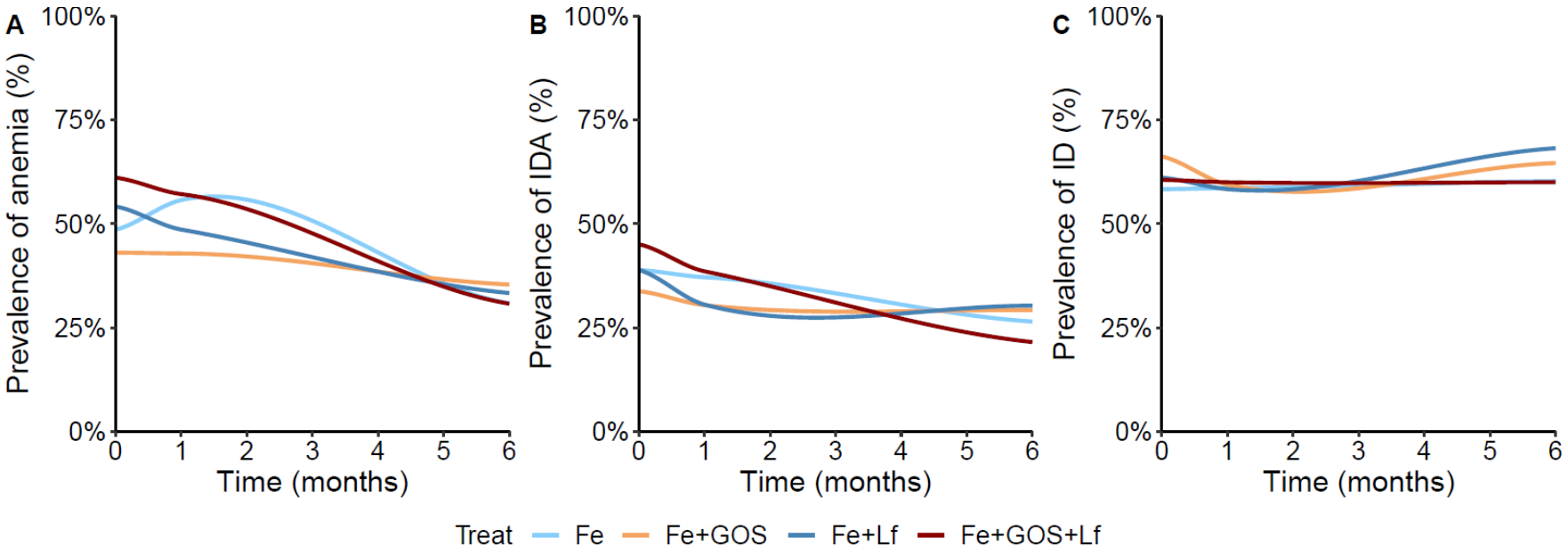
Anemia is defined as Hb < 11 g/L, ID as $Ferritin_a$ < 12 µg/L and/or sTfR > 8.3 mg/L, and IDA as Hb < 11 g/L and $Ferritin_a$ < 12 µg/L and/or sTfR > 8.3 mg/L.

A. Mean prevalence of anemia over time. Post-hoc test (Bonferroni adjusted): differences between time points are in Fe+GOS+Lf group (time 0 vs time 6, p=0.002).

B. Mean prevalence of IDA over time. Post-hoc test (Bonferroni adjusted): differences between time points are in Fe+GOS+Lf group (time 0 vs time 6, p=0.023).

C. Mean prevalence of ID. Since no interactions between time, GOS administration or Lf administration were detected in the logistic regression model, no post-hoc analysis were performed.

Hb, hemoglobin; ID, iron deficiency; IDA, iron-deficiency anemia; FeFum, ferrous fumarate; $Ferritin_a$, BRINDA-adjusted plasma ferritin; GOS, galacto-oligosaccharides; Lf, lactoferrin; sTfR, soluble transferrin receptor.


A
B
C
Prevalence of anemia (%)
Prevalence of IDA (%)
Prevalence of ID (%)
100%
75%
50%
25%
0%
0
1
2
3
4
5
6
Time (months)
Treat
Fe
Fe+GOS
Fe+Lf
Fe+GOS+Lf

DISCUSSION

General discussion and conclusions

The WHO has proposed various strategies for the prevention and treatment of anemia, with the ones targeting IDA mostly focusing on improving the dietary intake of iron. In particular, nutrition-specific solutions include dietary diversification, aiming at increasing the intake of highly-bioavailable iron-rich food, food fortification with iron preparations, and iron supplementation (1). As highlighted in chapters 3 and 4, the success of each approach is threatened by various constraints, including the difficulties in finding appropriate food vehicles to use as fortificants, the lack of an ideal iron preparation to use, the generally low fractional iron absorption from oral iron supplements, and the development of multiple common iron-related adverse effects when iron intake is increased, especially in countries with a high burden of infections. Moreover, current recommendations for iron interventions are based on iron requirements that, for specific population groups, such as infants and children, are not measured directly; in fact, iron needs are estimated factorially from growth tables, median Hb concentrations, iron amounts in Hb, myoglobin, and enzymes, and extrapolation of losses from radioisotope studies performed in adults (2). The partial failure of iron programs worldwide is reflected in the fact that, despite the reduction in the age-standardized prevalence in the previous decades, anemia and IDA continue to represent major public health problems, with the first affecting 1.8 billion people worldwide (3), and ID considered to account for above 60% of the anemia cases (3). Infants, children under 5 years of age, non-pregnant WRA, and pregnant women are the most vulnerable groups (4).

The overall goal of the studies included in this thesis was to implement and examine existing strategies, potentially laying the foundation for new approaches to the prevention and treatment of iron-deficiency anemia in vulnerable population groups. Specifically, in **Manuscript 1**, we aimed to confirm whether iron losses occur during iron supplementation in infants, as previously reported (5), to quantify the magnitude of the reduction in the effectiveness of the treatment from any such losses, and to search for the underlying mechanism. In **Manuscripts 2 and 3,** our objectives were to determine if the known

enhancing effect of GOS on iron absorption was still present either at higher supplemental iron doses (Manuscript 2), or at lower doses of the prebiotic in infants (Manuscript 3). We also examined whether other prebiotics, such as FOS and acacia gum in adults (Manuscript 2), and HMOs in infants (Manuscript 3) would also have an enhancing effect on FIA and to assess if the maternal secretor status influenced the potential enhancing effect of prebiotics on FIA in breastfed infants (Manuscript 3). In Manuscript 4, our goals were to determine if the acute effects of GOS (6) and apo-bLf (7) on increasing FIA would persist in a 6-month iron fortification intervention in Kenyan infants, and result in a higher efficacy of the iron formulation with added GOS or bLf, or both, in ameliorating iron and anemia status.

To achieve these objectives, I) we quantified iron losses during a period of iron supplementation in a prospective study in Kenyan infants, using the stable iron isotopes dilution methodology while measuring fecal blood losses (Manuscript 1); II) we performed two cross-over stable iron isotope studies investigating the effect of a single administration of different prebiotics (GOS, FOS, and acacia gum in Swiss women, and GOS and HMOs in Kenyan infants) on fractional iron absorption (Manuscripts 2 and 3); III) we analyzed the HMOs composition of maternal breast milk and estimated its effect on FIA from a maize porridge fortified with FeFum, administered either alone, or with GOS or HMOs, in Kenyan infants (Manuscript 3); and III) using a 2-by-2 factorial design, we assessed the efficacy of daily fortification for 6 months with MNPs containing a low dose of iron with added GOS and bLf, alone or in combination, in Kenyan infants (Manuscript 4).

Main findings

In Manuscript 1, we found that over 3 months of iron supplementation (6 mg iron/kg BW/day) in Kenyan infants, 57% of the amount of absorbed iron was lost. Contrary to our hypothesis, evidence of iron losses from occult blood was detectable only in a minority of the samples, and no clear pattern of increased heme/porphyrin loss during the supplementation period was observed. Moreover, no significant changes were reported through the intervention period for the different biomarkers of intestinal health (pH, I-FABP) and inflammation (fecal calprotectin). Although the dose of iron administered to the Kenyan infants was higher, there were no significant differences in the distributions of

individual daily iron losses between our study population and the Gambian toddlers (5). Because of these findings, we assessed the relationships between iron dose, absorption, and loss in all six available isotopic studies of iron absorption and loss (2,5,8,9). We found a significant correlation between daily median iron absorbed and lost per kg BW, suggesting that the primary site of iron losses may be at the site of maximal iron absorption in the proximal duodenum. Therefore, we rejected our original hypothesis that increased iron losses with iron supplements were the result of episodic occult gastrointestinal bleeding. Overall, the results of Manuscript 1 provide evidence that increased iron losses during oral iron administration are a function of the amount of iron absorbed rather than the dose of iron administered.

In Manuscript 2 and 3, we confirmed that the single administration of the prebiotic GOS acutely enhances FIA from FeFum, given either with a high oral supplemental iron dose of 100 mg in iron-depleted women (Manuscript 2), or, in Kenyan infants, added to an iron-fortified maize porridge (5 mg iron as FeFum) (Manuscript 3), at a dose lower than that was used in previous trials (10,11). In Manuscript 2, a single administration of 15 g FOS also increased FIA from FeFum to a similar extent as GOS (~50%), resulting in an additional 10-12 mg of absorbed iron. In Manuscript 3, compared to control, the addition of 3.0 g GOS to the iron-fortified maize porridge determined a 78% increment in FIA, which corresponded to 0.5 mg of iron further absorbed from the test meal. Contrary to our hypotheses, the single administration of acacia gum in iron-depleted women (Manuscript 2), and the fortification of the iron-containing test meal with HMOs in Kenyan infants (Manuscript 3), did not affect FIA. Moreover, in infants, there was no evidence for an effect of the maternal HMO profile, or an interaction of the maternal HMO status with treatment, on FIA from any of the test meal conditions (Manuscript 3). To provide further insights on putative mechanisms explaining the *in-vivo* effect of the administration of the different prebiotics on iron absorption, we performed *in-vitro* dialyzability experiments, both in Manuscripts 2 and 3; the required amounts of prebiotics were calculated to match the prebiotic/Fe ratios of the different combinations administered *in-vivo*. The addition of GOS to the medium, in amounts similar to the prebiotic/Fe ratios of the test conditions from Manuscripts 2 and 3, significantly increased dialyzability from FeFum in water. This result suggested that the acute enhancing effect of GOS on iron absorption from FeFum

might depend on lower precipitation of iron as insoluble hydroxides, resulting in greater availability of iron in a form suitable for absorption (12). The results from the *in-vitro* dialyzability experiment for acacia gum (Manuscript 2) and HMOs (Manuscript 3) were also consistent with observations *in-vivo*. Nonetheless, a discrepancy between the in-vitro and in-vivo experimental evidence was observed for FOS, with iron dialyzability from FeFum + FOS being almost 40% lower than from FeFum alone (manuscript 2). The mechanism behind the opposite effect of GOS and FOS on iron dialyzability observed in Manuscript 2 is uncertain and would require further investigation. In conclusion, the results from Manuscripts 2 and 3 provide further evidence supporting the role of GOS as an acute enhancer of iron absorption from FeFum and provide an indication that FOS, but not acacia gum, might perform as well as GOS in iron-depleted women. In infants, the hypotheses that the single administration of the prebiotic HMOs would enhance iron absorption similarly to GOS, and that the effect of different prebiotics could be modulated by the maternal HMO profile in breast milk, must be rejected (Manuscript 3).

In Manuscript 4, the main finding was that co-addition of GOS and bLf to a low dose iron-MNP given daily for 6 months increased Hb and reduced anemia and IDA compared to the same MNP with iron alone, in Kenyan infants. Despite an overall significant increase in Hb concentration and a decrease in anemia prevalence over the 6 months intervention, no greater increment in Hb, or decline in anemia, was observed with the addition of GOS or bLf individually, when compared to a control of iron alone. The mean daily iron gains during the 6 months of intervention were approximately 0.20 mg, which are well below the WHO estimated iron needs in infants (13). This might explain why no overall effect of the intervention on iron status and ID prevalence was observed. Although unanticipated, the absence of individual enhancing effects of GOS and bLf, combined with the results from adding the combination of GOS and bLf on Hb concentration and anemia prevalence, point towards a role of the anti-inflammatory properties of lactoferrin (14) and GOS (15,16), which might facilitate iron absorption by decreasing systemic hepcidin concentrations (17); but the mechanisms responsible need further examination. In conclusion, these findings suggest that the addition of GOS and bLf in combination might be a useful approach to enhance the efficacy of in-home iron fortification programs, in areas where anemia represents a public health problem.

General limitations

As summarized above, the approaches currently used to prevent and treat iron-deficiency anemia are several. Each approach presents its own set of challenges and limitations and depends on the context in which these strategies are applied, and the technical implementation required. Existing gaps in knowledge impede further optimization of these approaches. In view of this, different research questions required various and specific study designs to be addressed. In this thesis, we performed: I) a long-term trial in infants using the dilution of stable iron isotope methodology; II) two stable iron isotope cross-over trials assessing acute iron bioavailability with the erythrocyte incorporation method, one in infants and one in young women; and III) a prospective efficacy trial with a 2-by-2 factorial design in infants. Each methodology and study design presents its unique series of limitations.

The dilution of stable iron isotope methodology used in **Manuscript 1** represents the most accurate and precise method to assess iron balance, given that uniform labelling of total body iron has been reached about one year after the administration of the stable iron isotope tracer (9). The conventional iron indices, such as Hb, sTfR, and SF, are subject to the confounding effect of infection, inflammation, and a variety of chronic disorders (18). By contrast, measuring the proportion of total body iron absorbed per unit of time, expressed as k_{abs}, derives from a direct measure of the shift of the isotopic ratios in blood and is independent of other external measures. (9). In contrast, k_{loss}, which measures the proportion of total body iron lost per unit of time, requires determination of the Hb concentration and measurement or estimation of blood volume to calculate the absolute amount of tracer that is lost. Translation of k_{abs} and k_{loss} into an amount of iron that has been absorbed over a unit of time, requires an estimate of total body iron, which in turn depends upon estimates of circulating blood iron, tissue iron, and body iron stores. The use of SF may have biased our estimates because, except for baseline, all the samples were collected on a day when high-dose oral iron supplementation was provided during weeks of iron supplementation. SF concentrations can stay elevated up to 6 days after iron supplementation, in a dose-dependent manner (19). The introduction of these factors in the calculations adds variability and some level of uncertainty. The use of k_{abs} alone to

assess the efficacy of a specific intervention, and its comparison with others, would still be possible if a control period prior to intervention would be included in the design, and a ratio of the two slopes (prior to, and during the intervention) could be calculated. Unfortunately, in our study, we did not assess k_{abs}, and daily iron absorbed and lost, before the start of the intervention. The absence of these measurements rules out the possibility of directly comparing slope ratios; thus, we could not avoid using iron status parameters and accepting several assumptions for the calculation of total body iron, for iron absorbed and lost, at different time points. Moreover, the lack of a control period restricts the comparisons with previous studies measuring absorption and losses before, during, and after a period of oral iron supplementation. Furthermore, the fact we do not have any baseline estimate of daily absorption and losses limits the chance of attributing a specific proportion of the iron losses during the iron intervention to the intervention itself.

We used the erythrocyte iron incorporation method with stable iron isotopes to estimate iron bioavailability from different supplemental formulations of FeFum and test meals fortified with FeFum, in young women (Manuscript 2) and infants (Manuscript 3), respectively. Despite being considered the gold standard to assess iron absorption and bioavailability (20), the use of stable iron isotopes has limitations. The fraction of iron incorporated in RBCs after oral administration can be measured precisely in conjunction with intravenous administration of a tracer. In general, the IAEA suggests that erythrocyte iron incorporation be estimated at 80% for studies examining absorption and bioavailability from an oral formulation. This might not be entirely accurate for all population groups and individuals (21–25). Nonetheless, this assumption is unlikely to represent a major source of bias in our study populations (25). Another assumption is that the exchange between the iron incorporated in the erythrocytes and the plasma iron is negligible over the whole lifespan of the RBCs. This assumption cannot be met in conditions where intravascular hemolysis occurs, such as sickle-cell disease, thalassemia, infection-induced anemia, and malaria (26). Some of these conditions are common in the Kenyan region where Manuscript 3 was performed. Furthermore, the recent finding that erythroblast-specific FPN might release a significant amount of iron into the circulation under specific conditions (27) could affect our calculations. Still, the magnitude at which

iron exchange between erythrocytes and plasma affects erythrocyte iron incorporation measurement has yet to be determined.

The study described in **Manuscript 4** was an efficacy study and had a randomized, prospective, 2 x 2 factorial design. The main advantages of this type of design are that it can provide evidence about the effect on the chosen outcome(s) of two different factors alone, or in combination, and quantify their interaction, with a smaller sample size than the one needed when testing all the 4 possible combinations individually with the same statistical power and level of significance. The study design *per se,* and the methodology used, were adequate for the research question we wanted to address. Nonetheless, in addition to the ones listed in the manuscript, a major study limitation might have been that the sample size was not optimized for the outcomes we reported, as these were secondary outcomes from a parent trial; and may have reduced the statistical power for the detection of a hypothetical individual effect of the addition of GOS and bLf to the iron-containing MNP on iron and inflammation status.

In general, an advantage of all trials reported in this thesis is that we always selected the population group representing the actual target of the iron treatment we wanted to implement or investigate. We enrolled infants from the African region (**Manuscripts 1, 3,** and **4**) and women of reproductive age (**Manuscript 2**), which are among the most vulnerable subjects to anemia and IDA, according to recent estimates (3,4). Furthermore, in our two intervention studies (**Manuscript 1** and **4**), the iron treatments were designed in agreement with the local pediatric guidelines and with the recommendations of the WHO. In addition, we used iron supplements and fortificant formulations commonly available and prescribed in the study area. Consistent with this, our acute single-meal study performed in Kenyan infants (**Manuscript 3**) assessed iron absorption from an iron-fortified maize porridge, which is the principal complementary food for weaning infants in the region. The iron supplement administered to iron-depleted women (**Manuscript 2**) provides an iron dosage within the recommended range and is commonly prescribed and available in Switzerland, where the trial was performed. Nonetheless, our exclusion criteria placed a limit on the severity of anemia allowed in all studies, with subjects severely anemic (**Manuscripts 3** and **4**), mildly anemic (**Manuscript 1**), and anemic (**Manuscript 2**)

being denied participation. It follows that being anemic, or IDA, was never a requirement for inclusion, and that only part of the study populations enrolled in our studies had anemia or iron-deficiency anemia. Therefore, despite their internal validity, our study results cannot be directly generalized to subjects affected by more severe conditions than those investigated in the trials, and confirmation of these findings in such groups would require further study.

Overall interpretation of the results and implications for future research

The results of this thesis may provide valuable indications for the improvement of the efficacy of existing approaches for the prevention and treatment of iron-deficiency anemia. Nonetheless, several points remained unsolved, and new questions arose from the interpretation of our findings. Among the various factors potentially limiting the success of iron supplementation and fortification programs, an increment in iron losses induced by the iron treatment itself has been barely considered (1). This probably derives from the lack of evidence, with only two studies assessing iron losses during an iron intervention, using the stable iron isotopes dilution methodology (5,8). Furthermore, the results from the study performed in Beninese and Swiss women are not all consistent in the two populations; in fact, iron supplementation was associated with a significant increase in protracted iron losses in the women in Switzerland, but not in Benin, and may have been due to differences in iron status at baseline in the two groups (8). Our Manuscript 1 strengthens the finding that increased iron losses occur during iron supplementation, as detected both in Swiss women (8) and in anemic Gambian toddlers (5). Moreover, we provided evidence supporting that losses would be a function of the amount of iron absorbed, and not the result of unspecific gastrointestinal bleeding from mucosal damage due to unabsorbed iron, as previously hypothesized (5). Body iron loss is not regulated, and cannot be increased physiologically even in the presence of iron overload (28). Gastrointestinal cellular desquamation, specifically from the small intestine, accounts for approximately 90% of the total intestinal cell loss (29) and can increase further in response to inflammatory, infectious, chemical and other agents that injure enterocytes (30). Increases in gastrointestinal cell turnover of up to 8-fold have been reported in celiac disease, infections and other disorders (29,31,32). Some mechanistic aspects of intestinal

shedding still need to be clarified; for example, it is not understood whether cell death triggers the initiation of detachment, or if detachment itself leads to cell death (33). To strengthen our hypothesis on increased iron losses, future *in-vitro* experiments and *in-vivo* trials would need first, to provide evidence on an increment in epithelial cell shedding from the human small intestine with oral iron supplementation, and second, to confirm our findings in different population groups. Ideally, the participants should be expected to absorb the highest amounts of iron, such as non-inflamed iron-deficient anemic subjects. The migration of intestinal epithelial cells from the base of the crypt to the villi, where they are lost, has been studied in animal models using *in-vivo* microscopy of living intestinal tissue, and in human tissue culture models *ex-vivo* (34). These models also allow the investigation of the epithelial layer in the small intestine under pathological conditions and upon exposure to endotoxins, such as Lipopolysaccharides (31). A few studies also assessed the loss of intestinal barrier function *in-vivo* in human subjects using fluorescein–aided confocal laser endomicroscopy, which allows the evaluation of changes in the structure of the gastrointestinal mucosal layer with high resolution, upon previous intravenous administration of a fluorescent dye (35,36). Before shedding, epithelial cells become permeable to fluorescein, and the fluorescence increases as shedding continues (35). Hypothetically, these methodologies could be used to examine the cellular response *ex-vivo* and *in-vivo* of human intestinal cells to the exposure to high doses of iron. An interesting design to be considered would be a crossover trial in labeled and equilibrated subjects evaluating the effect of an oral and an intravenous iron dose, matching the estimated quantity of iron absorbed orally, on iron losses. However, this design would require careful assessment. In fact, in oral supplementation studies it is assumed that if the difference between the non-equilibrated pool of absorbed iron (ingested orally) and the equilibrated body iron is below 5%, the underestimation of iron absorbed or lost introduced by k_{abs} and k_{loss} is negligible. It is not known whether this assumption still holds after intravenous administration of iron. To conclude, if our hypothesis would be confirmed, iron-induced iron losses would need to be considered among the main factors accounting for limited net iron gains during iron supplementation, both in developing and industrialized countries, together with inflammatory increases in hepcidin, low compliance with the intervention, or inhibition of iron absorption from phytates, polyphenols or other

components. Further *in-vitro* experiments and *in-vivo* trials are needed to provide evidence supporting this hypothesis.

In parallel with the official definition of the term "prebiotic", and with the classification of some non-digestible oligosaccharides (NDOs) as prebiotics, or prebiotic candidates (37), a series of studies were performed in animal models to evaluate the effect of NDOs on mineral bioavailability, specifically calcium, magnesium, zinc, and iron (38). Since then, technical advancements both in the synthesis and/or isolation of oligosaccharides with potential bifidogenic effects, such as HMOs, and their subsequent availability on a large scale (39–41), and the extension of the use of stable isotopes in bioavailability studies (42) have facilitated the investigation of the potential health benefits of NDOs compounds, both related with enhanced mineral uptake and utilization (43) and with their modulation of the gut microbiota and potential clinical benefits (44). As for other minerals, for what concerns iron, the two aspects appeared to be interconnected; in fact, it was proposed that the enhancing effect on iron absorption observed with co-administration of iron with prebiotics, both in animal models and in humans (6,45), would be linked to a decrease in colonic pH mediated by the prebiotic fermentation to SCFAs, mostly by Bifidobacteria and lactobacilli, that would favor colonic iron absorption. Nonetheless, the absence of an effect on iron absorption of the administration of acacia gum, a dietary fiber with bifidogenic activity (46), contrary to what was observed with GOS and FOS, raises concerns about the hypothesis of a microbiota-mediated effect on iron uptake, and about the relevance of colonic iron absorption in adults (Manuscript 2). Moreover, these findings are supported by a recent trial in iron-depleted women investigating the kinetic of iron absorption with or without GOS; in comparison with iron only, no delay in the peak of iron absorption when iron was given with GOS was observed, indicating that the role of colonic uptake may be negligible in adults (47). In Kenyan infants, in a first study investigating the effect of daily administration of iron-containing MNPs for 3 weeks, it was shown that GOS fortification enhanced iron absorption significantly (6). However, the study design did not allow differentiation between an acute or a long-term effect of GOS administration (6). Considering the beneficial changes that GOS supplementation induces in the gut microbiota of infants supplemented with iron (10), a potential prebiotic-induced change in colonic iron absorption appeared as a potential explanation (6). This hypothesis was

further supported by a recent trial performed in a similar population, that reported the single addition of GOS to an iron-containing MNP did not significantly increase iron absorption, when compared to control, suggesting that a pre-feeding period with GOS may be needed to enhance iron absorption at this age (11). Our findings from **Manuscript 3** again challenge this hypothesis, in agreement with **Manuscript 2**, given that the single co-provision of a low dose of GOS with iron was sufficient to improve its absorption, when compared to control, but this was not the case for HMOs, and that variations in maternal HMO profile did not predict FIA. In light of these results, it seems more appropriate to propose the chemical interaction of free iron dissociated from the supplement or the fortificant with the reducing units of GOS and FOS, thereby preventing the formation of insoluble iron hydroxides (12) and favoring duodenal uptake, as a possible explanation for the effect of GOS (**Manuscript 2** and **3**) and FOS (**Manuscript 2**) on FIA. Therefore, when considering potential iron supplements and fortificants formulations exclusively focusing on the optimization of iron absorption, it might be more appropriate to investigate in single-meals studies the effect of other reducing agents, such as fructose (48) (although evidence is contrasting (49,50)), rather than more complex oligosaccharides, or to restrict to ascorbic acid the use as iron absorption enhancer (51). Nonetheless, the efficacy of specific iron formulations in long-term interventions does not always reflect the observation in single meal studies, because short-term measurements of absorption tend to overestimate the effects of iron enhancers and inhibitors (22,52,53). This is also the case for ascorbic acid (54), and, in addition to its instability when exposed to heat and oxygen during food storage and preparation (55), this aspect decreases its appeal as a suitable ingredient of iron formulations for longer interventions. According to the results of **Manuscript 4**, an inflation of the effect of GOS and bLf on iron absorption when given in a single administration, rather than long-term, might have occurred, since both compounds were reported to acutely enhance FIA in Kenyan infants (6,7), but no improvements in iron and anemia status in a similar population were observed, when the two compounds were added individually to an iron-containing MNPs administered daily for 6 months. Regardless of the treatment group, in **Manuscript 4** we observed an overall increase in Hb concentration, and a decrease in the prevalence of anemia and IDA, which was greater with co-administration of GOS and bLf, but no changes in iron status. One of the proposed

explanations for our observations was that the anti-inflammatory properties of Lf and/or GOS may be responsible for a decrease in hepcidin leading to increases in iron absorption. However, no significant decrease in inflammation, assessed by the prevalence of elevated CRP or AGP, was reported and no measures of hepcidin, the main iron homeostatic hormone that is controlled, among other factors, by inflammation (56), were assessed. With the knowledge we gained from Manuscript 2, it would be valuable to perform a similar study in an isotopically-labeled equilibrated population, to assess if the co-administration of GOS and bLf produced any difference, when compared to the iron alone, or to the individual addition of GOS and bLf, on iron absorption and losses quantified with the stable iron-isotopes dilution method. However, due to the length of the equilibration period between label administration and equilibration, which requires one year or more depending on the age of the subjects (9,57), it would not be possible to include subjects aged 6 months, as we did in Manuscript 4, but toddlers of around 1.5-2 years would probably represent the earliest possible target.

We mentioned in chapter 3 that the efficacy of iron supplementation and fortification protocols is jeopardized by the occurrence of iron-related common adverse effects, and that their safety has been questioned, especially in areas with a high burden of infections. Although apparently not efficacious in enhancing iron absorption acutely (as for FOS, acacia gums, and HMOs), or in improving iron status after long term consumption (as for GOS and bLf when given individually), these compounds might still be valuable in mitigating the side effects of iron administration. If the results from our parent trial on the effect of the different MNPs formulations on intestinal inflammation and microbiota composition would provide evidence towards a protective role of GOS and bLf against iron-induced dysbiosis and mucosal impairment, it could be worthwhile to investigate if the same results could be achieved with the administration of the HMOs which can be currently synthesized and administered, such as 2-FL and LNnT. Although the effect on safety and tolerability of the addition of HMOs to infant formulas has been assessed, no studies have considered yet the consequences of long-term administration of HMOs with iron on the gut microbiota.

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