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REGULATION OF IRON ABSORPTION: EFFECTS OF *HELICOBACTER PYLORI*, THE *TMPRSS6* GENE, IRON ENCAPSULATION AND POLYPHENOLS

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"THE SIZE OF YOUR DREAMS MUST ALWAYS EXCEED YOUR CURRENT CAPACITY TO ACHIEVE THEM.
IF YOUR DREAMS DON'T SCARE YOU, THEY AREN'T BIG ENOUGH."

ELLEN JOHNSON SIRLEAF

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DEFINITIONS

Mutation vs. Polymorphism

Prior the era of Next Generation Sequencing, an arbitrary frequency threshold of 1% was established to distinguish from common polymorphisms to rare mutation variants.[1] Changes in the DNA sequence are called mutation, and if the mutation causes changes in the phenotype the corresponding individuals are called a mutants in contrast to wild-type individuals. Naturally occurring and rare sequence variants, which are clearly distinguishable from wild-type are also called mutations. Polymorphisms are variants of DNA sequences that co-exist in a population, at relatively high frequency, and no clear wild-type and mutant individuals can be defined.[2]

However, with growing advances in DNA sequencing, the definitions of mutation and polymorphism have blurred. The use of the two terms "mutation" and "polymorphism" for the same event (which is a difference to a reference) can lead to problems in the accuracy and interpretation of results. Recently, it has been proposed to define mutations as DNA variants obtained in a paired sequencing project including the germline and the somatic DNA. It is possible that germline sequences differ between individuals and this would constitute a polymorphism in a population, and importantly these genotypes should always be called variants, but never simply "mutation" without using an attribute. The term "mutation" should only be used if the germline sequence was used as a reference, and should always be accompanied by a prefix indicating whether the mutation occurs only in the somatic cells (somatic mutation, for example tumor cells), or also in the germline cells (germline mutation)[3]

Within this thesis – the "old" definition of mutation and polymorphism is used.

SNP rs855791

The nonsynonymous single nucleotide polymorphism (SNP) rs855791 on the *TMPRSS6* gene, encoding the serine transmembrane protease matriptase-2, has several notations. On protein level this is: p.Val736Ala (an A = Alanine to V = Valine substitution at position 736), and on DNA level: c.2207T>C (a C = Cytosine is substituted by a T = Thymine).[4] In this thesis, the notation on the DNA level is used. In variants carrying both C alleles (CC), the matriptase-2 shows no impaired protease function (in manuscript 2 defined as wild-type), whereas in

variants with two T alleles (TT) the protease function seems to be impaired (defined as mutation in manuscript 2).[5]

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ABBREVIATIONS

AI	Anemia of inflammation
BIS	Body iron stores
BMP	Bone morphogenetic protein
BMPR	Bone morphogenic protein receptor
CRP	C-reactive Protein
CUB	C1r/C1s, urchin embryonic growth factor, bone morphogenetic protein 1
CV	Cardiovascular
CVD	Cardiovascular disease
CYBRD1	Gene encoding DCYTB
DALY	Disability-adjusted life-year
DCYTB	Duodenal cytochrome b
DFS	Double fortified salt
DMT1	Divalent metal transporter 1
EDTA	Ethylenediaminetetraacetic acid
EFSA	European food safety authority
e.g.	Example given
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ERFE	Erythroferrone
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
Fe	Iron
FeFum	Ferrous fumarate
FePP	Ferric pyrophosphate
FIA	Fractional iron absorption
FPN	Ferroportin
$FeSO_4$	Ferrous sulfate
GAE	gallic acid equivalents
GOS	Galacto-oligosaccharides
GRAS	Generally recognized as safe
GWAS	Genome wide association studies
Hb	Hemoglobin
Hct	Hematocrit
HFE	Human hemochromatosis protein
HIF	Hypoxia induced factor
HJV	Hemojuvelin
HNO3	Nitric acid
HO-1	Heme oxygenase-1
IAEA	International Atomic Energy Agency
ID	Iron deficiency

IDA	Iron deficiency anemia
IL	Interleukin
IRIDA	Iron refractory iron deficiency anemia
IRPs	Iron regulatory proteins
I$	International dollar
JAK	Janus kinase
LDLRA	Low-density lipoprotein receptor class A
LIP	Labile iron pool
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MT	Matriptase
NRF-2	Nuclear erythroid factor 2
NTBI	Non-transferrin bound iron
PA	Phytic acid
PCBP1	Poly-(rC)-binding protein 1
PHep	Plasma hepcidin
PP	Polyphenols
RBC	Red blood cell
RBV	Relative bioavailability
RCT	Randomized controlled trial
RNI	Recommended nutrient intakes
ROS	Reactive oxygen species
RR	Risk ratio
SCFA	Short chain fatty acid
SEA	Sea urchin sperm protein, enteropeptidase, agrin
SF	Serum ferritin
SFe	Serum iron
SHep	Serum hepcidin
SMAD	Sons of mothers against decapentaplegic
STAT	Signal transducer and activator of transcription
sTfR	Soluble transferrin receptors
TF	Transferrin
TFR1, 2	Transferrin receptor1 and 2
TIBC	Total iron binding capacity
TM	Transmembrane
TMPRSS6	Transmembrane protease serine 6 (matriptase 2) encoding gene
TSat	Transferrin saturation
WHO	World Health Organization
ZIP14	Zinc transporter protein 14

SUMMARY

Background

Iron is an essential element for almost all biological systems. In humans, iron is involved in oxygen transport, DNA synthesis, electron transfer and has several functions in iron-containing enzymes. Due to iron's high reactivity leading to the formation of reactive oxygen species, iron metabolism in humans is tightly regulated. The hepatic hormone hepcidin is the main regulator of iron metabolism. Hepcidin regulates cellular iron export from enterocytes or macrophages into circulation.

The transcription of the *HAMP*, gene encoding hepcidin, is regulated by iron status, inflammation and erythropoiesis. Matriptase-2 (encoded by *TMPRSS6*) is a negative feedback regulator of the BMP-SMAD pathway regulating hepcidin expression. Mutations in *TMPRSS6* resulting in complete loss of function cause iron-refractory iron deficiency anemia which is characterized by increased hepcidin concentrations. Genome wide association studies and cross-sectional studies have associated the single nucleotide polymorphism (SNP) rs855791 (2321 C>T) with erythrocyte parameters, hepcidin, and iron status. The T-allele frequency in Britain is reported to be 0.41, 0.54 among Han Chinese, 0.10 in Gambians and 0.58 among Peruvians.

The etiology of iron deficiency and iron deficiency anemia is complex and has many determinants. Infection with *Helicobacter pylori* can be one factor. Current evidence indicates an increased risk for iron depletion in *H. plyori* infected individuals. Half of the world's population is infected with *H. plyori*, with large regional variation and a higher prevalence in low- and middle-income countries. The majority of infected individuals remain asymptomatic. However, whether the asymptomatic infection with *H. plyori* is associated with a decrease in iron absorption is unknown, and equivocal results have been reported in literature.

Food fortification can be a very cost-effective strategy to combat iron deficiency. However, iron fortification requires a compromise between highly bioavailable iron compounds versus the sensory stability (e.g. off-taste and undesirable color changes) of the fortified food vehicle. Various encapsulation approaches using proteins, polysaccharides, lipids and surfactants have been attempted, but with limited success in stability during cooking. Microencapsulation of

the highly bioavailable ferrous sulfate, using a pH-sensitive, and heat-stable polymer may address these issues.

Iron overload is another important public health issue. It is well established that homozygous C282Y mutation in the *HFE* gene causes hereditary hemochromatosis (HH). HH is characterized by increased cellular iron export due to the impaired signaling pathway to express hepcidin. Polyphenols (PP) have been proven to inhibit non-heme iron absorption by forming insoluble complexes with iron in the intestinal lumen. In patients with HH, decreased iron absorption and lower iron accumulation has been reported when black tea was consumed with a meal. By reducing iron accumulation, these patients would decrease their frequency of required phlebotomy sessions, the standard of care treatment to maintain a healthy iron status in patients with HH.

Research objectives

The aim of this doctoral thesis was to investigate extrinsic and intrinsic factors affecting iron absorption in iron deficiency and iron overload. Specifically, our objectives were to:

1) unravel whether asymptomatic *H. pylori* infection blunts the effect of iron fortification by reducing iron absorption from commonly used iron compounds in preschool children and women of reproductive age (**Manuscript 1**).
2) assess and compare iron absorption, hepcidin concentrations and other indices of iron metabolism in iron-sufficient women of reproductive age by comparing homozygous variants of the SNP rs855791 in the *TMPRSS6* gene (**Manuscript 2**).
3) investigate iron absorption in women of reproductive age, with depleted iron status, from microparticles containing ferrous sulfate at different loadings using a pH-sensitive polymer for encapsulation (**Manuscript 3**).
4) develop a supplement containing polyphenol rich foods, which is capable to complex iron *in vitro,* and to reduce dietary iron absorption in patients with hereditary hemochromatosis (**Manuscript 4**).

Original studies

*Effect of asymptomatic H. pylori infection on iron absorption (**Manuscript 1**):*

This retrospective, pooled analysis of stable iron isotope absorption studies in preschool children (five studies) and women (four studies) aimed to unravel the effect of asymptomatic *H. pylori* infection on iron absorption. To do so, we collected fractional iron absorption (FIA) data from studies conducted in Benin, Senegal and Haiti, in women of reproductive age (n = 80) and preschool children (n = 90). In all studies, a local commonly consumed test meal was fortified with either ferrous sulfate ($FeSO_4$), sodium iron EDTA (NaFeEDTA) or ferrous fumarate (FeFum) and FIA was assessed by measuring erythrocyte incorporation of the stable iron isotope 14 days after administration.

The prevalence of *H. pylori* seropositivity among women was 51.3% and 54.4% in children. Iron absorption, controlled for age, hemoglobin (Hb), serum ferritin (SF), C-reactive protein (CRP), iron compound and test meal did not differ between *H. pylori* positive versus negative women nor in children. The geometric mean FIA of *H. pylori* infected women was: 9.73% and 8.52% in non-infected. In *H. pylori* infected children, FIA was 9.77%, while it was 8.94% in non-infected. Therefore, in asymptomatic women and children, the seropositivity for *H. pylori* is not associated with iron absorption.

*How a TMPRSS6 polymorphism affects iron absorption (**Manuscript 2**):*

We performed an iron absorption study in Taiwanese women carrying the TT alleles (n = 45) and the CC alleles (n = 35) of the homozygous variant of the rs855791 polymorphism in the *TMPRSS6* gene. We aimed to describe differences in iron absorption, hepcidin concentration, and other iron status indices between these two groups. We measured iron absorption as erythrocyte iron incorporation of stable iron isotopes 14 days after administration of ^{57}Fe and ^{58}Fe in two identical rice-based meals fortified with $FeSO_4$. The meals were administered on two alternate days (day one and three).

Even though the two groups had comparable serum ferritin concentrations, serum iron (SFe) concentration and transferrin saturation (TSat) were significantly lower in the women carrying the TT variants than in the women carrying the CC variant. Also, the normalized hepcidin (Hep) values to SFe and TSat were higher in the women carrying the TT variant. The FIA in the two groups were statistically similar (TT = 7.96% and CC = 6.50%), however, when FIA was

controlled for genetic variant, SF, TSat, soluble transferrin receptor (sTfR), Hep, CRP and menstrual blood loss the model predicted significantly lower iron absorption in women carrying the TT variant compared to the CC variant.

Comparing the intra-individual variation of FIA on study day one and day three, we assessed a higher variation in women carrying the TT (correlation coefficient within individuum: r = 0.67) variant compared to women with the CC variant (correlation coefficient within individuum: r = 0.86). Furthermore, the regulation of FIA in the TT variant was less well predicted by iron stores and the model explained much less variability of the fractional iron absorption ($R^2_{adjusted}$ = 0.375), compared to the same model in CC variant ($R^2_{adjusted}$ = 0.669). When the FIA data were normalized to depleted iron stores (SF = 15 µg/L), using the variant specific regression, the FIA was significantly lower in the TT (18.5%) variant compared to CC (26.6%). Hence, our study suggests that women carrying the TT variant of the rs855791 polymorphism are less able to upregulate iron absorption with low iron stores.

*Evaluation of a novel microparticle for food iron fortification (**Manuscript 3**):*

A novel formulation of encapsulated ferrous sulfate in a pH-sensitive and heat stable-polymer (butylmethacrylate-co-(2-dimethylaminoethyl)methacrylate-co-methylmethacrylate: BMC), was developed using a two-step modified inverse emulsion process. In the first step $FeSO_4$ was embedded in hyaluronic acid (HA), the second step included the encapsulation of this seed with BMC, forming the Fe-HA-BMC microparticle. In study 1, we measured the iron absorption from these microparticles using a randomized, single-blind, cross-over design, in iron depleted women of reproductive age (n = 20). The aim was to measure fractional iron absorption of these microparticles and to assess the effect of cooking. The Fe-HA-BMCs labeled with stable iron isotopes were used to fortify a maize porridge, which was administered to fasting subjects. The shift in enrichment ratio of the administered stable iron isotopes in the erythrocytes was measured 14 days later. The FIA from both, cooked and uncooked Fe-HA-BMCs were comparable (1.45% and 1.41%, respectively), both were significantly lower than FIA from free $FeSO_4$ (3.36%). In sensory stability tests, the Fe-HA-BMCs showed good protection against color changes in a polyphenol rich food matrix (banana milk).

To overcome the low iron bioavailability detected in study 1, and increased particle loading, a new process involving spray drying and a spinning disk atomizer was developed. With this process, particles with a 5- and 30-fold higher iron loading were designed (3.19%-Fe-HA-BMC,

and 18.29%-Fe-HA-BMC), and were used to fortify a wheat bread. The subsequent study 2 aimed to assess the iron absorption of these microparticles, the effect of iron loading, the effect of co-delivery of encapsulated vitamin A (VitA-BMC) and free folic acid (FA), and the effect of the microparticle components HA and BMC. The study included iron depleted women of reproductive age (n = 24). Fortified wheat bread test meals were administered in a partially randomized, single-blind, cross over design. FIA from the 18.29%-Fe-HA-BMC (17.0%) was comparable to FIA from FeSO4 (19.2%), but the 3.19% Fe-HA-BMC had significantly lower FIA (13.7%). However, both microparticles showed poor sensory performance in banana milk, leading to undesirable color changes. Co-delivery of the 3.19%-Fe-HA-BMC with VitA-BMC (FIA: 12.7%) or VitA-BMC and free FA (FIA: 14.3%) did not change FIA. The components of the iron microparticles did not affect FIA when administered separately with free $FeSO_4$; HA: 20.7%; BMC: 16.6%; HA-BMC: 16.3%. Also, the FIA from the Fe-HA seed (15.1%) was comparable to the FIA from the 3.19%-Fe-HA-BMC.

Hence, the highest loaded microparticles had the best iron absorption, but poor performance in terms of protection from sensory changes. The co-fortification with encapsulated vitamin A and free folic acid did not affect iron absorption.

*The effect of polyphenols on iron absorption in patients with hereditary hemochromatosis (**Manuscript 4**):*

In the first part of this study, we aimed to formulate a natural polyphenol rich food supplement capable to form insoluble complexes with iron. To do so, we screened twelve polyphenol rich food powders using *in vitro* digestion. A homogenous mixture of black tea powder, cocoa powder and a grape juice extract (named as polyphenol supplement; PPS), precipitated iron by 79%, when 2 g of the powder was added to 500 g of an iron solution (20 µg Fe/g).

Secondly, in the partially randomized, single-blind, cross over study we aimed to assess iron absorption from a test meal (Meal-PPS vs. Meal-Placebo), and a test drink (Drink-PPS vs. Drink-Placebo), both consumed once with the PPS and once with the placebo supplement (maltodextrin). We enrolled otherwise healthy adults with HH, homozygous in the C282Y mutation (n_{female} = 7, n_{male} = 7) from Porto, Portugal (n = 9) and in Zurich, Switzerland (n = 5). FIA from both the meal (Meal-PPS: 3.01%) and drink (Drink-PPS: 10.3%) was significantly lower when provided with the PPS than when provided with the placebo supplement (Meal-Placebo:

5.21% and Drink-Placebo: 16.9%). The intake of the PPS led to a 42% and 39% significant reduction of fractional iron absorption in meals and drinks, respectively. This reduction of total absorbed iron from the test meal with the PPS would correspond to a reduction of ~230 mg Fe/year or one unit of blood (450 ml) to be phlebotomized, when extrapolated to a period of one year.

Conclusions

Our data suggest that iron absorption from fortified meals is not impaired by the presence of asymptomatic *H. pylori* infection in women and children. The reported association of increased risk for iron depletion with *H. pylori* infection was in our study population not attributed due to impaired iron absorption.

Our data on iron absorption in homozygous variants of the rs855791 SNP in the *TMPRSS6* gene suggest an impaired negative feedback loop on hepcidin synthesis by circulating iron. Women of reproductive age carrying both T alleles, are less able to upregulate iron absorption at low iron status than women carrying both C alleles. Thus, women with the TT variant may have an increased risk of suffering from iron deficiency.

The further developed Fe-HA-BMP microparticles with the highest Fe loading had the highest iron absorption, and showed promising properties for future developments. However, their poor sensory performance in a polyphenol rich food matrix requires careful evaluation of a suitable food vehicle for fortification. Nevertheless, based on data of *in vitro* and *in vivo* (mice and human) testing, the compound might be a platform to deliver iron as well as other micronutrients.

A natural polyphenol rich food supplement has successfully shown to complex soluble iron *in vitro* and to inhibit iron absorption in adults with HH. The PPS induced a 40% reduction in iron absorption, regardless of the food matrix. The polyphenol rich supplement might be a promising approach to reduce iron accumulation and the frequency of required phlebotomy treatments in patients with hemochromatosis as well as other iron overload diseases.

Overall, this thesis has filled gaps in understanding various intrinsic and extrinsic factors in iron deficiency and iron overload and their effect on iron absorption. We report no association of asymptomatic in *Helicobacter pylori* infection and iron absorption. Further we report that the homozygous T variant of the rs855791 SNP in the *TMPRSS6* gene negatively affects iron absorption. Further we describe how an encapsulation of ferrous sulfate in a pH-sensitive polymer affects iron absorption, and the beneficial effect of polyphenols in blunting iron absorption in patients with HH.

ZUSAMMENFASSUNG

Hintergrund

Eisen ist ein essenzielles Element für fast alle biologischen Systeme. Im Menschen ist es am Sauerstofftransport, der DNA-Synthese, dem Elektronentransfer und Funktionen in eisenhaltigen Enzymen beteiligt. Aufgrund der hohen Reaktivität von Eisen, durch das reaktive Sauerstoffspezies gebildet werden, ist sein Stoffwechsel im Menschen stark reguliert. Das hepatische Hormon Hepcidin ist der Hauptregulator des Eisenstoffwechsels. Hepcidin reguliert den zellulären Eisenexport aus Enterozyten oder Makrophagen in den Kreislauf.

Die Transkription des *HAMP*-Gens, welches Hepcidin kodiert, wird durch den Eisenstatus, Entzündungsstatus und die Erythropoese reguliert. Die Matriptase-2 (kodiert von *TMPRSS6*), ist ein negativer Feedbackregulationsmechanismus des BMP-SMAD-Signalweges, der die Hepcidin-Expression reguliert. Mutationen in *TMPRSS6*, die zu einem vollständigen Funktionsverlust führen, verursachen eine refraktäre Eisenmangelanämie, die durch erhöhte Hepcidinkonzentrationen gekennzeichnet ist. Genomweite Assoziationsstudien und Querschnittsstudien haben den Einzelnukleotid-Polymorphismus (SNP), rs855791 (2321 C>T), mit Erythrozytenparametern, Hepcidin und Eisenstatus assoziiert. Ob diese Assoziation auf eine gestörte orale Eisenabsorption zurückzuführen ist, muss noch geklärt werden. Die Häufigkeit des T-Allels beträgt in Grossbritannien 0.41, bei Han-Chinesen 0.54, bei Gambiern 0.10 und bei Peruanern 0.58.

Die Ätiologie des Eisenmangels und der Eisenmangelanämie ist komplex und hat viele Determinanten. Die Infektion mit *Helicobacter pylori* kann ein Faktor sein. Aktuelle Erkenntnisse weisen auf ein erhöhtes Risiko für Eisenmangel bei Personen mit einer *H. pylori* infektion hin. Die Hälfte der Weltbevölkerung ist mit *H. pylori* infiziert, wobei es grosse regionale Unterschiede und eine höhere Prävalenz in Ländern mit niedrigem und mittlerem Einkommen gibt. Die Mehrheit der infizierten Personen weisst jedoch einen typisch asymptomatischen Krankheitsverlauf auf. Ob die asymptomatische Infektion mit *H. pylori* mit einer verminderten Eisenabsorption einhergeht ist jedoch unbekannt und in der Fachliteratur wurden von unterschiedlichen Ergebnissen berichtet.

Die Anreicherung von Lebensmitteln kann eine sehr kosteneffektive Strategie zur Bekämpfung von Eisenmangel sein. Die Eisenanreicherung erfordert jedoch einen Kompromiss zwischen

hoch bioverfügbaren Eisenverbindungen und der sensorischen Stabilität (z. B. Fehlgeschmack und unerwünschte Farbveränderungen) des angereicherten Lebensmittels. Es wurden verschiedene Verkapselungsansätze unter Verwendung von Proteinen, Polysacchariden, Lipiden und Tensiden versucht, jedoch mit begrenztem Erfolg hinsichtlich der Stabilität beim Kochen. Die Mikroverkapselung des hoch bioverfügbaren Eisen(II)-sulfat unter Verwendung eines pH-sensitiven und hitzestabilen Polymers könnte diese Probleme lösen.

Eisenüberladung ist ein weiteres wichtiges Problem der öffentlichen Gesundheit. Es ist bekannt, dass die homozygote C282Y-Mutation im HFE-Gen die hereditäre Hämochromatose (HH) verursacht. HH ist durch einen erhöhten zellulären Eisenexport aufgrund des gestörten Signalweges der Hepcidinexpression gekennzeichnet. Polyphenole (PP) hemmen nachweislich die Absorption von Nicht-Häm-Eisen, indem sie unlösliche Komplexe mit Eisen im Darmlumen bilden. Bei Patienten mit HH wurde über eine verringerte Eisenabsorption und eine geringere Eisenanreicherung berichtet, wenn schwarzer Tee zu einer Mahlzeit konsumiert wurde. Durch eine Verringerung der Eisenakkumulation würde sich bei diesen Patienten die Häufigkeit der erforderlichen Aderlasssitzungen verringern. Aderlass ist die Standardbehandlung zur Aufrechterhaltung eines gesunden Eisenstatus.

Forschungsziele

Das Ziel dieser Doktorarbeit bestand darin, extrinsische und intrinsische Faktoren, welche die Eisenabsorption bei Eisenmangel und -überladung beeinflussen, zu untersuchen. Konkret waren unsere Ziele:

1) herauszufinden, ob eine asymptomatische *H. pylori*-Infektion die Wirkung von Lebensmitteln, die mit üblichen Eisenverbindungen angereichert wurden, durch eine verringerte Eisenabsorption bei Vorschulkindern und Frauen im gebärfähigen Alter reduziert (**Manuskript 1**).
2) die Eisenabsorption, Hepcidin und anderen Indizes des Eisenstoffwechsels bei Frauen im reproduktiven Alter, welche die homozygoten Varianten des SNP rs855791 im *TMPRSS6*-Gen tragen, zu bewerten und zu vergleichen (**Manuskript 2**).
3) die Eisenabsorption von Eisen aus Mikropartikeln, welche Eisen(II)-sulfat in unterschiedlichen Dosierungen enthalten und ein pH-sensitives Polymer zur Verkapselung verwenden, in Frauen im reproduktiven Alter mit tiefem Eisenstatus zu untersuchen (**Manuskript 3**).
4) ein Nahrungsergänzungsmittel zu entwickeln, das aus polyphenolreichen Lebensmitteln besteht, welches in der Lage ist, Eisen *in vitro* zu komplexieren und die Eisenabsorption bei Patienten mit HH aus einer Mahlzeit zu reduzieren (**Manuskript 4**).

Eigenständige Studien

*Einfluss einer asymptomatischen H. pylori-Infektion auf die Eisenabsorption (**Manuskript 1**):*

Diese retrospektive gepoolte Analyse von Eisenaufnahmestudien mit stabilen Eisenisotopen in Vorschulkindern (fünf Studien) und Frauen (vier Studien) hatte zum Ziel, den Effekt einer asymptomatischen *H. pylori*-Infektion auf die Eisenabsorption aufzuzeigen. Zu diesem Zweck haben wir Daten der prozentuale Eisenabsorption (FIA) aus Studien gesammelt, welche in Benin, Senegal und Haiti in Frauen im reproduktiven Alter (n = 84) und Vorschulkindern (n = 91) durchgeführt wurden. In allen Studien wurde eine lokal übliche Testmahlzeit entweder mit Eisen(II)-sulfat ($FeSO_4$), Natrium-Eisen(III)-EDTA (NaFeEDTA) oder Eisen(II)-fumarat (FeFum) angereichert und die FIA durch Messung der Inkorporierung der stabilen Eisenisotope in die Erythrozyten 14 Tage nach der Verabreichung bestimmt.

Die positive Seroprävalenz von *H. pylori* betrug bei Frauen 51.3% und bei Kindern 54.4%. Die Eisenaufnahme, welche bezüglich Alter, Hämoglobin (Hb), Serumferritin (SF), C-reaktives Protein (CRP), Eisenverbindung und Testmahlzeit kontrolliert wurde, unterschied sich weder bei *H. pylori*-positiven noch bei *H. pylori*-negativen Frauen oder Kindern. Das geometrische Mittel der prozentualen Eisenabsorption in mit *H. pylori* infizierten Frauen war 9.73% und 8.52% in nicht infizierten. Bei mit *H. pylori* infizierten Kindern betrug die prozentuale Eisenabsorption 9.77% und 8.94% bei nicht infizierten. Daher ist bei Frauen und Kindern die seropositiv für *H. pylori* sind, eine asymptomatische *H. pylori* Infektion nicht mit der Eisenaufnahme assoziiert.

*Wie ein TMPRSS6-Polymorphismus die Eisenabsorption beeinflusst (**Manuskript 2**):*

Wir führten eine Eisenabsorptionsstudie in taiwanesischen Frauen, die die TT Allele (n = 45) oder die CC Allele (n = 35) der homozygoten Variante des rs855791-Polymorphismus im TMPRSS6-Gen tragen, durch. Unser Ziel war es, Unterschiede in der Eisenabsorption, der Hepcidinkonzentration und anderen Eisenstatusindizes zwischen diesen beiden Gruppen zu beschreiben. Wir bestimmten die Eisenabsorption als Eiseneinbau in Erythrozyten von stabilen Eisenisotopen 14 Tage nach der Verabreichung von ^{57}Fe und ^{58}Fe in zwei identischen,

mit $FeSO_4$ angereicherten Mahlzeiten auf Reisbasis. Die Mahlzeiten wurden an zwei alternierenden Tagen (Tag eins und drei) verabreicht.

Obwohl die beiden Gruppen vergleichbare SF Konzentrationen aufwiesen, waren die Serumeisen (SFe) Konzentration und die Transferrinsättigung (TSat) bei den Frauen mit der TT-Variante signifikant niedriger als bei den Frauen mit der CC-Variante. Auch die zu SFe und TSat normalisierten Hepcidin (Hep)-Werte waren bei den Frauen, die die TT-Variante trugen, höher. Die FIA der beiden Gruppen waren statistisch ähnlich (TT = 7.96% und CC = 6.50%). Wenn jedoch die FIA mit der genetische Variante, SF, TSat, löslicher Transferrin Rezeptor (sTfR), Hep, CRP und den menstrualen Blutverlust kontrolliert wurde, wies das Modell eine signifikant niedrigere Eisenabsorption bei Frauen mit der TT-Variante im Vergleich zur CC-Variante aus.

Beim Vergleich der intra-individuellen Variation der FIA vom ersten und dritten Studientag, wurde eine höhere Variation bei Frauen mit der TT-Variante (Korrelationskoeffizient in Individuum: r = 0.67) im Vergleich zu Frauen mit der CC-Variante (Korrelationskoeffizient in Individuum: r = 0.86) festgestellt. Ausserdem wurde die Regulation der FIA in der TT-Variante weniger gut durch die Eisenspeicher vorhergesagt und das Modell erklärte viel weniger Variabilität in der prozentualen Eisenabsorption ($R^2_{adjusted} = 0.375$) verglichen mit dem gleichen Modell in der CC-Variante ($R^2_{adjusted} = 0.669$). Wenn die FIA-Daten mit Hilfe der Regression spezifisch für die Varianten auf einen tiefen Eisenspeicher (SF = 15 µg/L) normalisiert wurden, war die prozentual Eisenabsorption der TT-Variante (18.5%) im Vergleich zur CC-Variante (26.6%) signifikant niedriger. Daher legt unsere Studie nahe, dass Frauen, die die TT-Variante des rs855791-Polymorphismus tragen, eine geringere Fähigkeit besitzen die Eisenabsorption bei niedrigen Eisenspeichern hoch zu regulieren.

*Evaluation eines neuartigen Mikropartikels zur Fortifikation von Lebensmitteln mit Eisen (**Manuskript 3**):*

Eine neuartige Formulierung um Eisen(II)-sulfat in einem pH-sensitiven und hitzestabilen Polymer (Butylmethacrylat-co-(2-dimethylaminoethyl)methacrylat-co-methylmethacrylat: BMC) zu verkapseln wurde mit Hilfe eines zweistufigen modifizierten inversen Emulsionsverfahrens entwickelt. Im ersten Schritt wurde $FeSO_4$ in Hyaluronsäure (HA) eingebettet, der zweite Schritt beinhaltete die Verkapselung dieses Kernes mit BMC, wodurch

die Fe-HA-BMC Mikropartikel entstanden. In Studie 1 haben wir die Eisenaufnahme aus diesen Mikropartikeln in einer randomisierten, einfach verblindeten, Cross-Over-Studie bei eisenarmen Frauen im reproduktiven Alter (n = 20) gemessen. Das Ziel war es, die prozentuale Eisenabsorption von den Mikropartikeln zu messen und den Effekt des Kochens zu ermitteln. Die Fe-HA-BMC Mikropartikel wurden mit stabilen Eisenisotopen markiert und zur Anreicherung eines Maisbreis verwendet, der nüchternen Probandinnen verabreicht wurde. Die Verschiebung des Anreicherungsverhältnisses der verabreichten stabilen Eisenisotope in den Erythrozyten wurde 14 Tage danach gemessen. Die prozentuale Eisenabsorption von gekochten und ungekochten Fe-HA-BMCs war vergleichbar (1.45% bzw. 1.41%) und beide waren signifikant niedriger als bei freiem $FeSO_4$ (3.36%). Tests zur sensorischen Stabilität zeigten, dass die Fe-HA-BMCs einen guten Schutz vor Farbveränderung in einer polyphenolreichen Lebensmittelmatrix (Bananen Milch) bieten.

Um die in Studie 1 festgestellte geringe Bioverfügbarkeit von Eisen zu überwinden und die Eisenladung der Partikel zu erhöhen, wurde ein neues Verfahren mit Sprühtrocknung und einem Schleuderscheibenzerstäuber entwickelt. Mit diesem Verfahren wurden Partikel mit einer 5- und 30-fach höheren Eisenbeladung entwickelt (3.19%-Fe-HA-BMC und 18.29%-Fe-HA-BMC), welche zur Anreicherung eines Weizenbrotes verwendet wurden. Die anschliessende Studie 2 hatte zum Ziel, die Eisenabsorption dieser neuen Mikropartikeln, den Effekt der Eisenbeladung, den Effekt der gleichzeitigen Gabe von eingekapseltem Vitamin A (VitA-BMC) und freier Folsäure (FA) sowie den Effekt der Mikropartikelkomponenten HA und BMC zu untersuchen. Die Studie schloss Frauen (n = 24) mit tiefem Eisenspeicher im gebärfähigen Alter ein. Angereicherte Weizenbrot-Testmahlzeiten wurden in einer teilrandomisierten, einfach verblindeten Cross-Over-Studie verabreicht. Die prozentuale Eisenabsorption von 18.29%-Fe-HA-BMC (FIA: 17.0%) war vergleichbar mit der FIA von $FeSO_4$ (FIA: 19.2%), aber die FIA von den 3.19%-Fe-HA-BMC war signifikant niedriger. Allerdings zeigten beide Mikropartikel eine schlechte sensorische Qualität in Bananenmilch, was sich in einer unerwünschten Farbveränderungen zeigte. Die gleichzeitige Gabe des 3.19%-Fe-HA-BMC mit VitA-BMC (FIA: 12.7%) oder VitA-BMC und freiem FA (FIA: 14.3%) veränderte die prozentuale Eisenaufnahme nicht. Die Komponenten der Eisenmikropartikel hatten keinen Einfluss auf die prozentuale Eisenaufnahme, wenn diese separat mit freiem $FeSO_4$ verabreicht wurden: HA (FIA: 20.7%), BMC (FIA: 16.6%) und HA-BMC (FIA: 16.3%). Auch die Eisenaufnahme vom Fe-HA Kern (FIA: 15.1 %) war vergleichbar zur FIA vom 3.19%-Fe-HA-BMC. Daher hatte

die Eisenabsorption aus den am höchsten beladenen Mikropartikeln die beste Eisenabsorption, zeigten aber einen schlechten Schutz vor sensorischen Veränderungen. Die Co-Anreicherung mit eingekapseltem Vitamin A und freier Folsäure hatte keinen Einfluss auf die Eisenabsorption.

*Die Wirkung von Polyphenolen auf die Eisenabsorption bei Patienten mit hereditärer Hämochromatose (**Manuskript 4**):*

Im ersten Teil dieser Studie war es unser Ziel, ein polyphenolreiches Nahrungsergänzungsmittel zu entwickeln, das in der Lage ist, unlösliche Komplexe mit Eisen zu bilden. Dazu untersuchten wir zwölf polyphenolreiche Lebensmittelpulver mittels *in-vitro*-Verdauung. Eine homogene polyphenolreiche Mischung aus Schwarzteepulver, Kakaopulver und einem Traubensaftextrakt (bezeichnet als Polyphenol Supplement; PPS), fällte Eisen zu 79% aus, wenn 2 g dieses Pulvers zu 500 g einer Eisenlösung (20 µg Fe/g) hinzugefügt wurden.

Im zweiten Teil wollten wir in einer teilrandomisierten, einfach verblindeten Cross-Over-Studie die Eisenabsorption aus einer Testmahlzeit (Meal-PPS vs. Meal-Placebo) und einem Testgetränk (Drink-PPS vs. Drink-Placebo), welche beide jeweils mit dem PPS oder einem Placebosupplement (Maltodextrin) eingenommen wurden, beurteilen. Wir schlossen ansonsten gesunde Erwachsene mit HH, homozygot für die C282Y-Mutation ($n_{female} = 7$, $n_{male} = 7$) aus Porto, Portugal (n = 9) und Zürich, Schweiz (n = 5) in die Studie ein. Die prozentuale Eisenaufnahme sowohl von der Mahlzeit (Meal-PPS: 3.01%) als auch vom Getränk (Drink-PPS: 10.3%) war signifikant niedriger, wenn das PPS verabreicht wurde, als wenn es mit dem Placebosupplement verabreicht wurde. Die Einnahme des PPS führte zu einer signifikanten Reduktion der prozentualen Eisenabsorption in Mahlzeiten und Getränken um 42% bzw. 39%. Diese Reduktion des gesamten absorbierten Eisens aus der Testmahlzeit mit dem PPS würde, auf ein Jahr hochgerechnet, einer Reduktion von ca. 230 mg Fe/Jahr oder einer Aderlasssitzung (ca. 450 ml Blut) entsprechen.

Schlussfolgerungen

Unsere Daten deuten darauf hin, dass die Eisenabsorption aus angereicherten Mahlzeiten nicht durch das Vorhandensein einer asymptomatischen *H. pylori*-Infektion bei Frauen und Kindern beeinträchtigt wird. Die berichtete Assoziation eines erhöhten Risikos für Eisenmangel bei einer *H. pylori*-Infektion lässt sich in unserer Studienpopulation nicht auf eine geminderte Eisenabsorption zurückführen.

Unsere Daten zur Eisenabsorption bei homozygoten Varianten des rs855791 SNP im *TMPRSS6*-Gen deuten auf eine beeinträchtigte negative Rückkopplungsschleife in der Hepcidin-Synthese durch das zirkulierende Eisen hin. Frauen im reproduktiven Alter, die beide T-Allele tragen, haben eine geringere Fähigkeit die Eisenabsorption bei niedrigem Eisenstatus hochzufahren als Frauen, die beide C-Allele tragen. Somit haben Frauen mit der TT Variante möglicherweise ein erhöhtes Risiko an Eisenmangel zu leiden.

Die weiterentwickelten Fe-HA-BMP Mikropartikel mit der höchsten Fe-Beladung, hatten die höchste Eisenabsorption und zeigen vielversprechende Eigenschaften für zukünftige Entwicklungen. Allerdings erfordert die schlechte sensorische Qualität in einer polyphenolreichen Lebensmittelmatrix eine sorgfältige Auswahl eines geeigneten Lebensmittels zur Anreicherung mit dem verkapselten Eisen. Nichtsdestotrotz könnte dieser Ansatz, basierend auf Daten aus *in-vitro*- und *in-vivo*-Tests (Mäuse und Menschen), eine vielversprechende Plattform für die Verabreichung von Eisen und auch anderen Mikronährstoffen sein.

Ein natürliches polyphenolreiches Nahrungsergänzungsmittel hat erfolgreich gezeigt, dass es lösliches Eisen *in-vitro* komplexiert und die Eisenabsorption bei Erwachsenen mit HH signifikant hemmt. Dies führte zu einer 40%igen Reduzierung der Eisenabsorption, was unabhängig von der Lebensmittelmatrix ist. Die polyphenolreiche Nahrungsergänzung könnte ein vielversprechender Ansatz sein, um die Eisenakkumulation und die Häufigkeit der erforderlichen Aderlassbehandlungen bei Patienten mit HH sowie anderen Eisenüberlastungserkrankungen zu reduzieren.

Insgesamt hat diese Arbeit Wissenslücken zum Verständnis verschiedener intrinsischen und extrinsischen Faktoren von Eisenmangel und Eisenüberladung und ihre Wirkung auf die Eisenabsorption gefüllt. Wir stellten keine Assoziation zwischen einer asymptomatischen

Infektion mit *Helicobacter pylori* und der Eisenabsorption fest. Ausserdem zeigten wir, dass die homozygote T-Variante des rs855791 SNP im *TMPRSS6*-Gen die Eisenabsorption negativ beeinflusst. Weiterhin beschrieben wir, wie eine Verkapselung von Eisen(II)-sulfat in einem pH-sensitiven Polymer die Eisenabsorption beeinflusst und die positive Wirkung von Polyphenolen zur Abschwächung der Eisenabsorption bei Patienten mit HH.

INTRODUCTION

Iron deficiency (ID) is the most common micronutrient deficiency, and affects 1/3rd of the world's population,[1] and accounts for ~50% of all anemia cases worldwide.[1,2] The etiology of ID, iron deficiency anemia (IDA), and anemia is a complex network, in which chronic diseases, malnutrition[3] and genetic disorders are primary determinants.[4] This thesis focuses on two of these: 1) The infection with *Helicobacter pylori* (*H. pylori*) has been associated with increased risk for iron deficiency, iron deficiency anemia and anemia;[5] and 2) Genome wide association studies, and cross-sectional studies have associated the common polymorphism rs855791 in the *TMPRSS6* gene with iron status and erythrocytic traits.[6-12]

Iron fortification can be a cost-effective strategy to combat ID, and requires relatively small changes in consumer behaviors and habits to increase the dietary iron intake of a population.[13-15] To address the continuing struggle of the compromise between highly bioavailable iron compounds versus sensory stability of the fortified food, various approaches for encapsulating iron have been made with limited success in feasability.[16]

The iron overload disease - hereditary hemochromatosis (HH) is characterized by excessive iron absorption from the diet.[17] HH can lead to its deposition in body tissues and organs, causing liver cirrhosis, hepatocellular carcinoma, diabetes, arthropathy, and heart disease.[17,18] Among the Caucasian population, mainly of Nordic or Celtic ancestry, it belongs to the most common genetic disease.[19-23]

The standard treatment to maintain a healthy iron status in HH is regular phlebotomies. This treatment has shown to reduce morbidity and mortality in patients with HH,[24,25] however, it can cause side effects and is inconvenient for many patients.[26] Polyphenols are inhibitors of non-heme iron absorption[27] and are present in many beverages (e.g. tea, and red wine), and foods (e.g. spices, berries, and chocolate).[28] Various studies in healthy subjects and HH patients have reported the inhibitory effect of polyphenol rich foods on non-heme iron absorption.[29-31]

The overall aim of this thesis was to investigate extrinsic and intrinsic factors affecting iron absorption in iron deficiency and iron overload. More precisely our aims were to: 1) Identify the association of asymptomatic *H. pylori* infection on non-heme iron absorption from commonly used iron compounds for food fortification; 2) Assess and compare iron absorption,

hepcidin and iron status in women which are homozygous carriers of the common variants in rs855791 in the *TMPRSS6* gene; 3) Measure iron absorption from a microparticle encapsulating ferrous sulfate using a pH-sensitive polymer for encapsulation; and 4) Develop a natural polyphenol rich supplement, which complexes iron *in vitro* and inhibits iron absorption in patients with HH.

The thesis is structured in three parts. First, the **Literature Review** provides an overview of current knowledge on: 1) iron homeostasis, metabolism and bioavailability (**Chapter 1**); 2) iron deficiency and iron deficiency anemia, their etiology and strategies to overcome these burdens (**Chapter** Error! Reference source not found.); 3) iron overload and the potential of p olyphenols to reduce iron absorption (**Chapter 3**); and 4) an overview of approaches and methods to evaluate iron bioavailability (**Chapter** Error! Reference source not found.). The s econd part is a collection of four scientific papers and includes the original research results. Specifically, **Manuscript 1** describes the association of asymptomatic *H. pylori* infection with iron absorption from iron fortificants. The manuscript includes a retrospective pooled analysis of five iron absorption studies in preschool children and four studies in women of childbearing age. **Manuscript 2** evaluates the effect of a common single nucleotide polymorphism (rs855791; 2207T>C) in the *TMPRSS6* gene on iron absorption from a test meal, comparing women of childbearing age carrying the homozygous variants (CC versus TT). In **Manuscript 3** a novel approach in iron encapsulation using a pH sensitive and heat stable polymer as an encapsulant of ferrous sulfate was developed. Hence, we assessed the iron absorption from these microparticles in two iron absorption studies testing fortified maize porridge and wheat bread in iron depleted women. **Manuscript 4** reports the *in vitro* development of a natural polyphenol supplement, which is able to reduce iron solubility from an iron solution. The subsequent iron absorption study in patients with HH assessed the inhibitory effect of the polyphenol supplement on iron absorption from a meal and a drink. The last section of this thesis (**General Discussion and Conclusion**) provides an interpretation of the original research, their contextualization and recommendations for future research.

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LITERATURE REVIEW

1 Iron metabolism, homeostasis and bioavailability

After oxygen, silicon, and aluminum, iron is the fourth most abundant chemical element in the upper continental crust.[1] It is essential for almost all living organisms, from bacteria to mammals, and is involved in oxygen transport, DNA synthesis and electron transport.[2] Despite its high abundance, most often it is a limiting growth factor, due to the formation of insoluble iron oxides when in contact with oxygen. Iron exists in the oxidation states ranging from -2 to +6, whereas in biological systems the ferrous (+2), ferric (+3) and ferryl (+4) states are predominant.[3] In humans, iron has several essential functions, such as oxygen carrier by hemoglobin of the red blood cells, transfer of electrons, and functions in iron-containing enzymes. In body tissues, iron metabolism is tightly regulated, due to its high reactivity in the Haber-Weiss-Fenton sequence forming hydroxyl radicals.[4] Hydroxyl radical are able to attack proteins, nucleic acids, carbohydrates, and initiate lipid peroxidation.[5] Therefore, in humans iron is mainly bound to proteins, such as hemoproteins (hemoglobin, myoglobin, peroxidases, catalases, and electron transport proteins), iron sulfur proteins, or other iron-containing proteins such as transferrin, lactoferrin and ferritin.[6]

The total body iron content in humans is around 40 mg/kg in women, and around 50 mg/kg body weight in men. It is distributed unevenly between three pools: functional iron (as myoglobin, and heme in the erythroid marrow and circulating within the red blood cells); storage (ferritin in the mononuclear phagocytic system, and hepatocytes); and a transport pool (as transferrin and non-transferrin bound).[6] Around 30 mg Fe/kg circulates as hemoglobin within the erythrocytes, 4 mg/kg in myoglobin and 2 mg/kg in tissues, hemeproteins and iron-sulfur proteins. The storage pool contains around 10-12 mg/kg in men and 5 mg/kg in women, where iron is stored as ferritin and hemosiderin in the liver, spleen, bone marrow and muscle. Only a very small fraction, around 3 mg, circulates as transferrin in the plasma or other extracellular fluids.[6] In mammalian iron homeostasis, no active iron excretion pathway has been identified, and most of the iron turnover consists of recycled iron from senescent erythrocytes by the macrophages. Iron losses through bleeding (e.g. menstruation), desquamation from skin, gastrointestinal and urinary tracts (1-2 mg/day), have to be replaced by dietary iron absorption in the duodenum.[7] Increased amounts are needed to cover requirements for growth. Therefore, iron requirements vary by sex and age

group; in adult men 1.05 mg Fe/day are sufficient to cover basal losses and in adult women 1.46 mg Fe/day cover basal and menstrual losses (**Table 1**).[8]

Table 1: Iron requirements for growth, median basal losses, menstrual losses and total absolute requirements in infants, children, adolescents, adult males and females. Taken from WHO (2004).[8]

Group	Age (years)	Mean body weight (kg)	Required intakes for growth (mg/day)	Median basal iron losses (mg/day)	Menstrual losses[a] (mg/day)[b]	Total absolute requirements[c] (mg/day)[b]
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		0.01		1.05 (1.37)
Females	11–14[d]	46	0.55	0.65		1.20 (1.40)
	11–14	46	0.55	0.65	0.48 (1.90)	1.68 (3.27)
	15–17	56	0.35	0.79	0.48 (1.90)	1.62 (3.10)
	18+	62		0.87	0.48 (1.90)	1.46 (2.94)
Postmenopausal		62		0.87		0.87 (1.13)
Lactating		62		1.15		1.15 (1.50)

[a]Effect of the normal variation in hemoglobin concentration not included in this figure.
[b]Numbers are median and in brackets the 95th percentile.
[b]Total absolute requirements = requirement for growth + basal losses + menstrual losses.
[c]Pre-menarche.

Iron in the diet occurs in heme or non-heme forms. Both are absorbed in the proximal duodenum. However, the mechanisms of heme iron absorption are poorly understood. There are two possible pathways for heme uptake by the enterocytes: either by receptor mediated endocytosis, or by the heme carrier protein 1 (HCP1) via direct uptake into the cytoplasm.[9] However, the role of HCP1 has not yet been fully confirmed.[10] Within the cell, iron is released through the heme oxygenase-1 (HO-1), and subsequently exported via ferroportin 1 (FPN1).[11]

Non-heme iron is mostly in the form of Fe^{3+} which has to be reduced to Fe^{2+} by the duodenal cytochrome b reductase (DCYTB)[12] for transport across the apical brush-border membrane of the enterocytes by the divalent metal-ion transporter 1 (DMT1).[13] Non-heme iron uptake is topic of Chapter 1.1.1. If iron is needed by the body, it is exported from the enterocytes across the basolateral membrane by FPN1. If it is not required, it is stored within the cell as ferritin.[14] When iron is released into the blood stream, it binds to transferrin (TF) in the plasma, and is

distributed to the bone marrow (hemoglobin production), the liver (iron storage) or other tissues.[15] Macrophages, are responsible for iron recycling, a process initiated by phagocytosis of senescent erythrocytes and concluded by iron re-entering the circulation after release from macrophages to TF.[6] The liver-derived peptide hormone hepcidin regulates iron export from cells via its interaction with ferroportin, leading to its degradation.[16] Hepcidin is synthesized by the hepatocytes in response to body iron stores in a homeostatic feedback loop.[17] When body iron is replete, hepcidin concentrations are increased, therefore, reducing the plasma iron supply. In contrast, in deficiency hepcidin synthesis is suppressed allowing an increased absorption of dietary iron and replenishment of iron stores (**Figure 1**).[18]

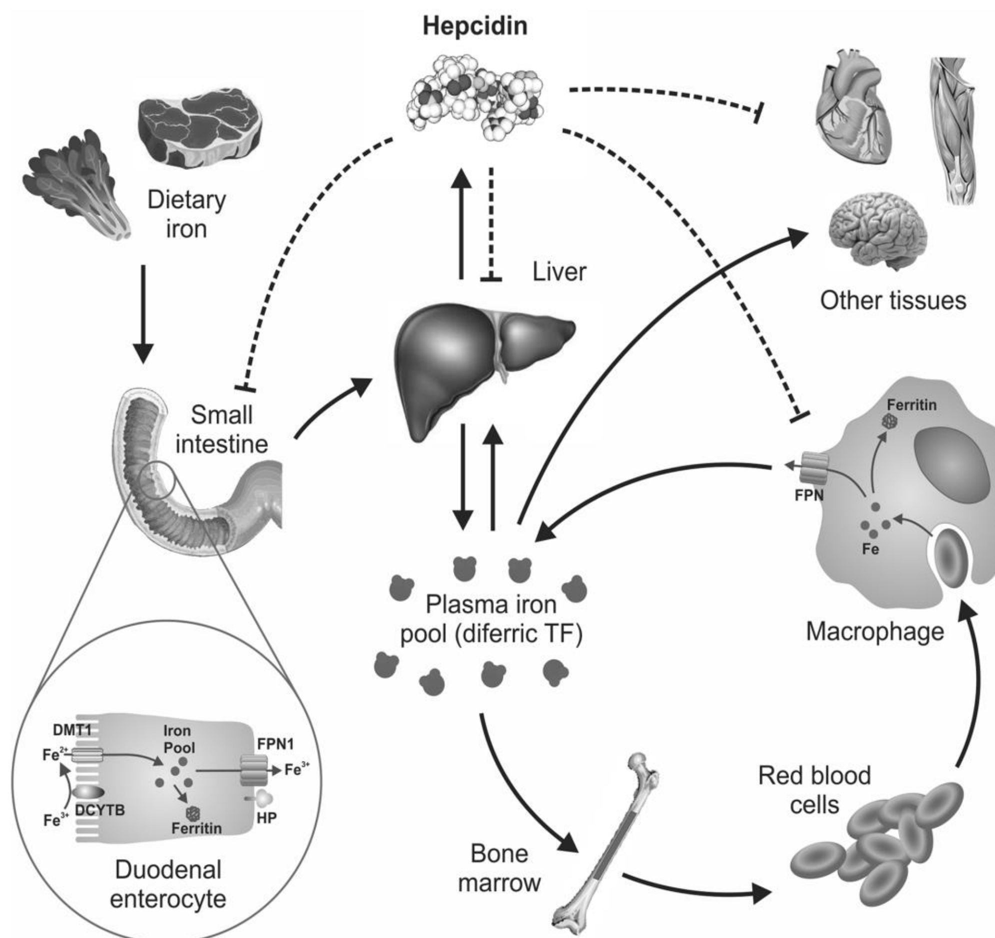


Figure 1: Iron homeostasis in humans. Taken from Anderson et al. 2017.[19]

The essential role of iron for humans is seen in the diverse clinical manifestations of deficiency and overload. Iron deficiency (ID) is the most common nutritional disorder, and is present in

low-, middle-, and high-income countries, and the most prominent cause of anemia (Chapter 2).[20-22] The other extreme – iron overload, has detrimental health consequences leading to liver and heart complications, diabetes, or joint problems.[20] It is caused by hereditary hemochromatosis, iron-loading anemias such as thalassemia, parenteral iron overload, or chronic liver disease (Chapter 3).[23] Therefore, iron's tight regulation of homeostasis and absorption is essential for human health.

1.1 Systemic iron homeostasis

As introduced in Figure 1, the concertmasters of systemic iron homeostasis are the enterocytes (dietary iron uptake) and macrophages (iron recycling), whereas hepcidin is the conductor of its orchestra. These will be thoroughly introduced in this chapter.

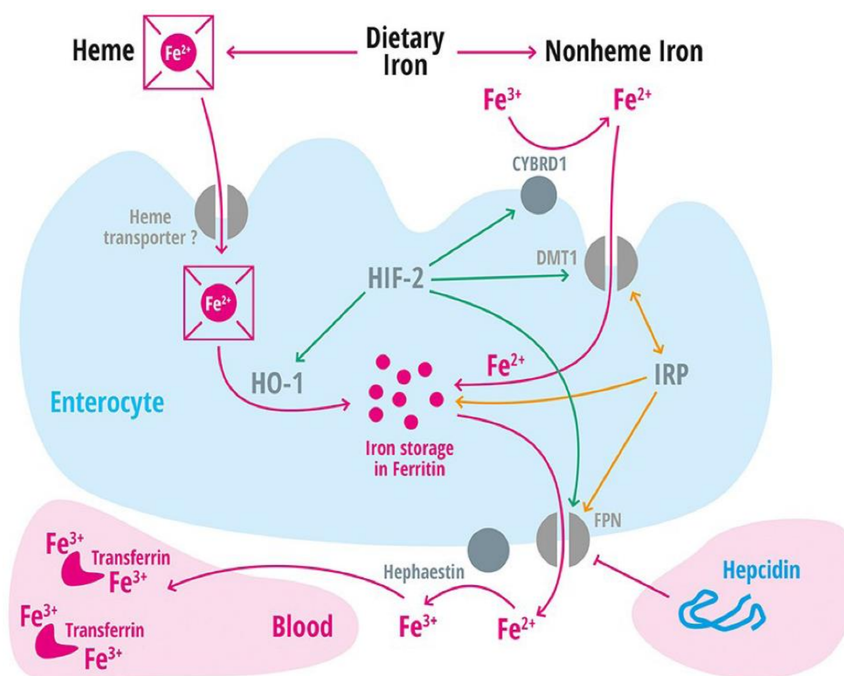


Figure 2: Iron uptake by the duodenal enterocytes. Taken from Steinbicker et al. 2013.[24]

1.1.1 Dietary iron uptake

Dietary iron uptake takes place in the proximal duodenum. From now on, the focus will only be laid on non-heme iron. Non-heme iron, is mainly present in the diet in its oxidized ferric (Fe^{3+}) form. To be transported into the enterocytes it needs to be reduced by the membrane

associated ferric reductase DCYTB, or by ascorbic acid from the diet. DMT1 transports Fe^{2+} via the apical membrane, driven by an H^+ electrochemical gradient.[25] Within the enterocyte iron is stored as ferritin or exported into the blood stream by FPN1. At the basolateral membrane, hephaestin – a multicopper oxidase – is needed to oxidise Fe^{2+} to Fe^{3+} to be incorporated onto the plasma protein transferrin.[26] Diferric transferrin circulates in the blood stream and supplies iron to most body cells. Hence, transferrin-bound iron (TF-Fe2) is an indicator of systemic iron homeostasis, and its saturation is determined by dietary absorption, recycling from macrophages and utilization for erythropoiesis.[27] Within the enterocytes the hypoxia inducible factor 2 (HIF-2) regulates the mRNA expression of DCYTB, DMT1, FPN, and HO-1.[24] The iron regulatory proteins (IRPs) control cellular iron metabolism post-transcriptionally by binding to mRNAs of DMT1, ferritin and FPN (**Figure 2**). Cellular iron regulation is further described in Chapter 1.2.2.

1.1.2 Iron recycling

The major part of the daily iron needs (90%) are generally covered by recycled iron from macrophages and the other 10% are absorbed from the diet.[27] In a steady state of hematopoiesis, around 10^{10} new red blood cells are produced per hour within the erythroblastic islands,[28] and of all cells erythrocytes have the highest concentration of iron (approx. 1 mg Fe/ml). Therefore, ~0.8% of all erythrocytes are recycled daily.[29] After approximately 120 days of circulating in the human blood stream, senescent erythrocytes are phagocytosed by macrophages mostly found in the bone marrow, liver and spleen.[28] The erythrocytes undergo proteolysis in the phagolysosome, hemoglobin is broken down, and heme is exported by the heme transporter HRG1 into the cytosol[30]. From there the iron is released from its protoporphyrin ring by heme oxygenase[31] and either stored as ferritin or exported into the plasma by ferroportin.[32]

1.1.3 Hepcidin expression and its regulation

Hepcidin – the conductor of the orchestra “iron homeostasis”, is a small 25 amino acid peptide hormone (~2.7 kD), synthesized predominantly in the liver.[33] It inhibits the entry of iron into the plasma from enterocytes, macrophages and hepatocytes by its interaction with ferroportin.

Hepcidin is encoded by the *HAMP* gene and mainly produced in the hepatocytes, but also to a much lesser extent in the heart and spinal cord.[33] It is produced at a very high rate, approximately 10 mg per day,[34] and its half-life is just a few minutes (~2.3 minutes),[35] therefore, the hepcidin production rate and it's circulating concentrations can rapidly change. It is presumed that the major clearance of hepcidin is through glomerular filtration in the kidneys, and that some is reabsorbed and degraded by proteolysis in in the proximal tubules.[18] A second pathway is the degradation of hepcidin with ferroportin by lysosomes, but the contribution of this pathway to its total clearance is unknown.[36]

Hepcidin binding to ferroportin causes the ubiquitination of the receptor[37] and the internalization as well as the degradation by a lysosome of the receptor-ligand complex.[38] It is also possible that hepcidin controls iron export form cells by gating ferroportin without inducing its endocytosis, which would allow a rapid control of iron concentrations in the plasma.[39,40] The affinity of hepcidin to FPN may be increased by the binding of Fe^{2+} to ferroportin. This may allow the selective degradation of ferroportin expressed on iron loaded cells, whereas the FPN on iron depleted cells would remain.[41]

As illustrated in **Figure 3,** there are three pathways currently known that regulate hepcidin transcription. They are: I) systemic iron availability via the bone morphogenetic protein-SMAD (BMP-SMAD) pathway (Figure 3A and B); II) erythropoiesis (Figure 3C);and III) inflammation via the interleukin 6 (IL-6)/Janus kinase (JAK) signal transducer and activator of transition (STAT) signaling axis (Figure 3D). These pathways will be described in the following subchapters. Other physiologic conditions, such as hypoxia, growth and pregnancy also require iron, and hepcidin production is suppressed. The hepatocyte growth factor (HGF) and epidermal growth factor (EGF) are responsible to suppress HAMP expression in mouse models.[42] The sex hormone testosterone reduces hepcidin, and increases serum iron and transferrin saturation in mice,[43-45] however, the effects of estrogen are unclear.[40]

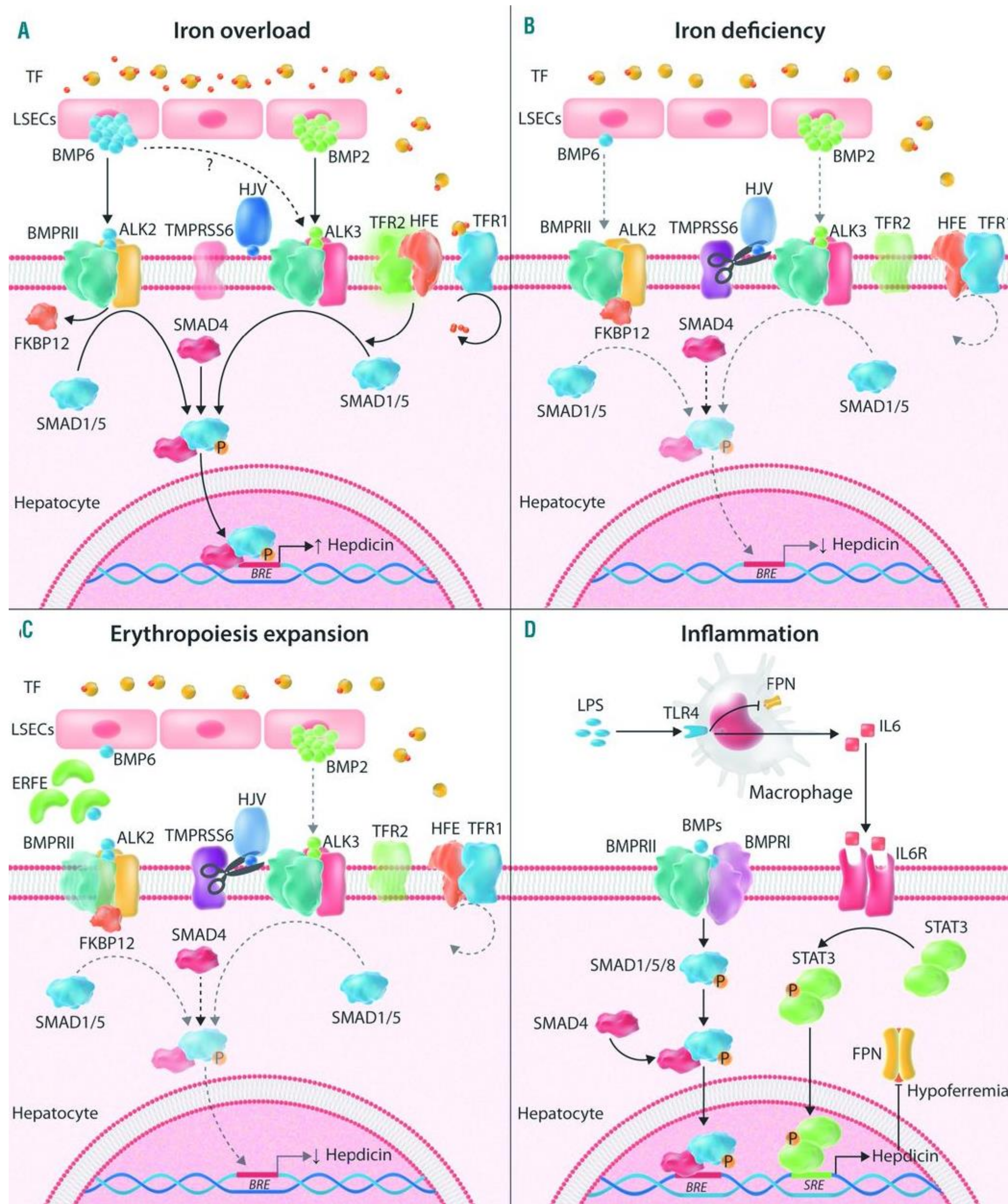


Figure 3: Pathways involved in the regulation of hepcidin transcription, in iron overload (A), iron deficiency (B), erythropoiesis (C) and inflammation (D). **A**: In iron overload, the diferric transferrin (TF-Fe2) displaces HFE from transferrin receptor (TFR) 1 and enables iron uptake and stabilizes TFR2 and activin receptor-like kinase (ALK) 3. Bone morphogenic protein (BMP) 2 is produced by the liver endothelial cell (LSEC), and binds o to BMP receptor type II, phosphorylating the ALK 3. Hemojuvelin (HJV) is a BMP co-receptor. Iron also increases the production of BMP6 by LSEC, which activates ALK2 and ALK3. The phosphorylation of ALK3 activates the SMAD1/5/8 cascade and induces SMAD4 to translocate to the nucleus. SMAD4 binds to the hepcidin promoter BMP responsive elements (BRE). **B**: In iron deficiency hepcidin expression is suppressed. Low serum Fe and TF-Fe2 suppress the BMP2-ALK3 pathway. The TFR2 is instable due to the lack of TF-Fe2, therefore HFE binds to TFR1. TMPRSS6 is stabilized and cleaves HJV, which inactivates the BMP2-ALK3 pathway. The BMP6-ALK2 pathway is also inactive, by the binding of FKBP12. **C**: In erythropoiesis low serum Fe and TF-Fe2 suppress the BMP2-ALK3 pathway. Erythroid cells release erythroferrone (ERFE) which sequesters BMP6 and other BMP, resulting in hepcidin inhibition. **D**: In inflammation, indicated by the production of interleukin 6 (IL6) by macrophages through lipopolysaccharides (LPS) and toll-like receptor 4 (TLR4). IL-6 binds to its receptor (IL-6R), and stimulates the janus kinase 2 (JAK2) an activator of transcription 3 (STAT3) signaling. The phosphorylated STAT3 complex translocate to the nucleus and bind to the hepcidin promoter STAT3 responsive element (SRE). Taken from Camaschella et al. 2020.[46]

1.1.3.1 Iron status

It is believed that circulating transferrin-bound iron is sensed by the transferrin receptor (TFR) 1, 2 and the human hemochromatosis protein (HFE) on the extracellular site of the hepatocytes (Figure 3). TF-Fe2 displaces HFE from TFR1 and forms a complex with hemojuvelin (HJV) and TFR2. In addition, HFE prevents the ubiquitination and proteasomal degradation of the activin receptor-like kinase 3 (ALK3), which also activates the BMP/SMAD signaling.[47] The TFR2-HFE-HJV complex promotes BMP/SMAD signaling which activates the expression of hepcidin. BMPs belong to the TGF-β superfamily – these signal molecules bind to complexes of type I and type II serine and threonine kinase receptors, forming signaling complexes that phosphorylate and activate the downstream SMAD transcription factors.[48,49] BMP6 binds to serine threonine kinase type 1 (ALK1 and ALK2)[50] and type 2 (BMPR2 and ACTR2A)[51] to activate the phosphorylation of SMAD1/5/8, which binds to SMAD4. The activated SMAD complexes translocate to the nucleus and modulate gene transcription.[52,53] *In vitro*, many BMPs are able to induce hepcidin expression[54-57] – however, animal studies have shown that the BMP6 mRNA levels correlate with body iron stores.[58] In humans, mutations in the BMP6 propeptide have been linked to iron loading.[59] All these studies provide evidence that hepcidin regulation by the BMP/SMAD pathway is reliant on HFE, HJV, BMP6, ALK1, ALK2, ALK3, BMPR2, ACTR2A, SMAD1/5/8, and SMAD4.

Additional regulators of iron homeostasis via the BMP-SMAD pathway are: BMP binding endothelial regulator (BMPER),[60] endofin,[61] SMAD6 and SMAD7,[62,63] matriptase-2, and neogenin. Neogenin may facilitate the HJV-BMP induction of hepcidin by binding to HJV and ALK3.[64,65] The Matriptase-2 (transmembrane protease serine 6, expressed by *TMPRSS6*), is a liver transmembrane serine protease, and a negative regulator of the BMP-SMAD pathway.[66,67] The matriptase-2 cleaves the HJV, and reduces the expression of hepcidin via the BMP/SMAD pathway.[68] Mutations in *TMPRSS6* cause elevated hepcidin levels, and patients have reduced serum iron concentrations and altered erythrocyte indices (mean corpuscular volume and mean corpuscular hemoglobin).[69,70] Due to the high hepcidin levels these patients are refractory to oral and parenteral iron substitution, leading to iron refractory iron deficiency anemia (IRIDA).[66,70] Chapter 2.3.2 (*TMPRSS6* mutations) will thoroughly focus on genetic mutations of the matriptase-2 and its effect on iron homeostasis and iron disorders.

1.1.3.2 Erythropoiesis

Erythropoietic activity and therefore, the demand for iron is increased at high altitude, severe blood loss, or after red blood cell (RBC) destruction (hemolysis). Thus, a rapid suppression of hepcidin transcription is required to make iron available from the diet and stores.

Erythropoietin (EPO), is the primary stimulator for proliferation and differentiation of erythroid progenitor cells in the bone marrow and is mainly secreted by the kidney. It has been suggested that EPO may be a direct regulator of hepcidin expression.[71] However, functional bone marrow is required for the suppression of hepcidin under these conditions.[72,73] A erythroid regulator – erythroferrone (ERFE) – has been identified as a hepcidin regulator (Figure 3).[74] ERFE secretion is increased in response to EPO. As shown in a mouse model, ERFE deficiency leads to a lack of the suppressing response on hepcidin expression after hemorrhage or EPO injection.[74] It has been shown that ERFE sequesters BMP6 and other BMP to inhibit the BMP/SMAD signaling and therefore hepcidin.[75,76] Its effect on hepcidin suppression may only be modest, as *Erfe*-knockout mice are not anemic. However, in mice with β-thalassemia, ERFE contributes to iron loading.[77] Therefore, the role of ERFE in humans requires more clarification.

1.1.3.3 Inflammation

As already described by Locke et al.[78] in 1932, infection causes a decrease in plasma iron concentrations.[78] This hypoferremic state serves as the host's defense against the growth of extracellular pathogens, chronic inflammation and infection, causing anemia of inflammation, which is typically characterized as low serum iron, transferrin saturation, and reticulocyte counts and normal to elevated ferritin concentrations.[79-81] The binding of interleukin 6 (IL-6) to its IL-6 receptor (IL-6R) activates the JAK tyrosine kinase, which enables the phosphorylation of STAT3 (Figure 3). Phosphorylated STAT3 is able to translocate to the nucleus and binds to the *HAMP* promoter region inducing its transcription.[82-84]

1.2 Iron metabolism in erythroid cells

1.2.1 Cellular iron uptake, utilization, storage and export

The circulating diferric transferrin in the plasma binds to transferrin receptor 1, which is followed by endocytosis of the transferrin-bound iron (**Figure 4**). Within the acidic environment of the endosomes, iron is released and reduced to Fe^{2+} by STEAP3.[85] Ferrous iron

is then transported into the cytosol via DMT1.[13,86] The Apo-TF/TFR1 complex returns to the cell surface and releases the transferrin to the plasma. When iron overload saturates the TF iron binding capacity, non-transferrin bound iron (NTBI) is imported into hepatocytes and pancreatic acinar cells via the zinc transporter protein 14 (ZIP14).[87] This process is responsible for liver iron deposition in hemochromatosis.[88] *In vitro*, erythroblasts also acquire iron by pinocytosis of ferritin released from macrophages.[89] However, *in vivo* this process is dependent on the transferrin – soluble transferrin receptor (sTfR) interaction.[90]

Once within the cell, the iron enters the Labile Iron Pool (LIP) (Figure 4), and from there it is utilized mainly by mitochondria or stored within ferritin. Mitoferrin-1 (MFRN1/2) is the main iron importer into mitochondria.[91] Within the mitochondria heme proteins and iron sulfur (Fe-S) clusters are synthesized. In erythroblasts the heme is mainly used for hemoglobin synthesis, and iron sulfur clusters are prosthetic groups of essential proteins/enzymes (e.g. IRP1).[92] The "kiss-and-run" mechanism may also transfer iron directly from endosomes to mitochondria via direct inter-organelle contacts.[93] The poly-(rC)-binding protein 1 (PCBP1) is an iron chaperone that delivers iron to ferritin. This is a reservoir and iron provider when needed, meanwhile excess iron is stored and protected to prevent the formation of dangerous reactive oxygen species (ROS).[92] The heteropolymer ferritin has 24 ubiquitous subunits of heavy (FTH) and light (FTL) ferritin chains forming a nanocage, which can hold up to 4500 iron atoms.[10] In iron deficiency, iron is mobilized from ferritin by autophagosomes, a process called ferritinophagy, and is controlled by the nuclear receptor coactivator 4 (NCOA4).[92] NCOA4 deficient mice do not perform ferritinophagy, therefore, they accumulate ferritin. Because they have limited iron recycling, they are more prone to suffer from iron deficiency anemia.[94]

Iron export from all cells occurs via the transporter ferroportin (Figure 4). The iron chaperone protein PCBP2 delivers iron from the LIP to ferroportin.[92] In macrophages PCBP2 also binds to heme-oxygenase 1, connecting heme catabolism and iron recycling.[95] Ferroportin expression is controlled at multiple levels. At the transcriptional level, the ferroportin repressor (BACH, BTB, and CNC homology protein) and activator NRF2, control the transcription of the *SLC40A1* gene.[96] At the translational level, during iron depletion, the protein synthesis is suppressed by binding of the 5' untranslated region (UTR) iron responsive element (IRE) within the *SLC40A1*mRNA to iron regulatory proteins (IRPs).[92] Posttranslational, hepcidin regulates

ferroportin by inducing its internalization and degradation. Additionally, hepcidin also inhibits the iron export by occluding the transporter.[97]

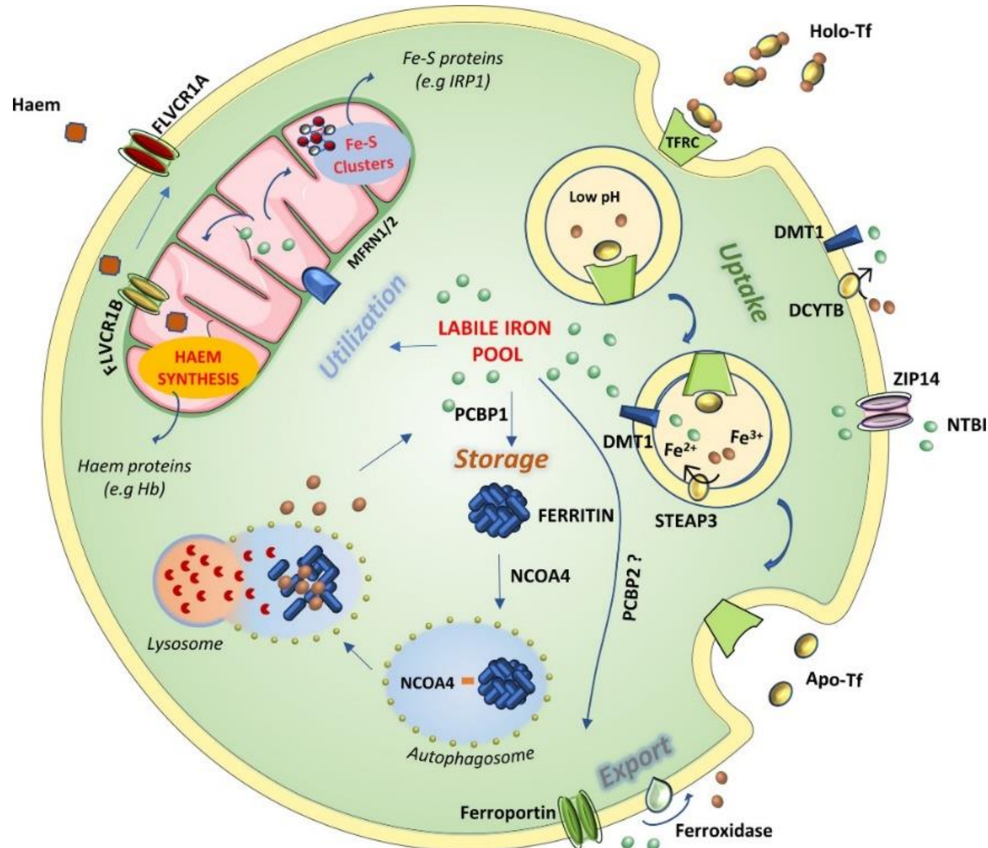


Figure 4: Intracellular iron uptake, utilization, storage and export. Taken from Camaschella et al. 2018.[92]

1.2.2 Cellular iron regulation

Cellular iron homeostasis is regulated on the posttranscriptional level by the RNA-binding proteins iron regulatory proteins (IRPs) 1 and 2. The IRPs bind to cis-regulatory hairpin structures – the so called iron-responsive elements (IREs) in the untranslated regions (UTRs) of the mRNAs of: Ferritin-H &-L (iron storage); ALAS2 (utilization); ferroportin (iron export); HIF2α (erythropoiesis); mitochondrial aconitase (involved in the TCA cycle); TFR1 (iron uptake); DMT1 (iron uptake); and CDC14A (involved in the cell cycle).[98]

In iron deficient cells, the IRPs bind to the IRE located on the 5' UTRs of ferritin, FPN, ALAS2, HIF2α, and aconitase mRNA, and inhibit its translation. IRE binding to the 3' UTR of TFR, mRNA inhibits its degradation, and binding to the 3' UTR of DMT1 mRNA stimulates its expression.[99]

Further, the FTH of ferritin is targeted by NCOA4 to induce its degradation.[100,101] Consequently the LIP is increased due to higher iron uptake, ferritin degradation and decreased iron export.

In iron replete cells the binding of IRP to IREs is suppressed, and the translation of ferritin and FPN mRNA is active. The IRP1 assembles with Fe-S to a cluster and induces the aconitase activity (TCA cycle),[102] while IRP2 undergoes proteasomal degradation.[103,104] The mRNA translation of ferritin, ALAS2, FPN, HIF2α, and aconitase are active, whilst the stability of TFR mRNA is decreased. Due to the accumulation of ferritin NCOA4 is also destabilized and degraded. The DMT1 mRNA contains a 3' UTR IRE, however, its action and mechanism is not yet clear.[10,98]

1.2.3 Crosstalk between cellular and systemic iron homeostasis

The two control systems of IRE/IRP and hepcidin/ferroportin interactions maintain cellular and systemic iron homeostasis. Most likely there is a third coordination level connecting these two systems.[27] Three of these interactions have already been described in literature: I) ferroportin expression; II) HIF2α; and III) TFR.

Ferroportin expression is regulated posttranslational via hepcidin, depending on systemic iron status, whilst the FPN mRNA synthesis is regulated via the 5' UTR IRE. Both of these mechanisms are required to maintain iron export.[105]

HIF2α mRNA expression is regulated by IRPs,[106] it increases the transcription of DMT1, at the apical surface of the duodenal enterocytes.[107]

Hepcidin expression is regulated by the connection of TFR1 and TFR2 with HFE which initiates the BMP-SMAD pathway. However, IRP activity also promotes TFR1 expression via the 3' UTR IRE.[98]

1.3 Iron absorption, utilization and bioavailability

Bioavailability combines absorption and utilization. Absorption is the uptake of a mineral or trace element from the lumen into the blood stream, and utilization describes the incorporation of the nutrient for normal physiological functioning. Therefore, iron bioavailability refers to the amount of iron that is present in the food, which is then absorbed in the duodenum and incorporated into the erythrocytes.[108] Iron utilization is relatively constant in healthy adults (80%)[109,110] Therefore, differences in iron bioavailability are most

likely due to differences in iron absorption. In this thesis, the term iron absorption will be used and refers to the combination of iron absorption and utilization.

Iron absorption is regulated at external (dietary components), and internal (iron status, other nutrient status, inflammation, genetic disorder) levels, until it reaches its purpose in the body. The diet contains heme or non-heme iron, and other dietary components, such as vitamin C or phytic acid, which enhance or inhibit iron absorption (Chapter 1.3.1). Iron uptake in the duodenum is affected by physiological factors which have already been described in Chapter 1.1, such as iron status, inflammation, infection. Vitamin A or riboflavin status also affects iron absorption. Studies have shown that when riboflavin deficiency is corrected, the response to an iron supplement is improved.[111] Genetic disorders, such as hemochromatosis, thalassemia or other single nucleotide polymorphisms (SNPs), also affect iron absorption and will be the topic of Chapter 3.

1.3.1 Dietary factors affecting iron absorption

Dietary iron can be in the heme form or in the non-heme form. Non-heme iron comes from two sources: plants and animal tissues, whilst heme iron is exclusively found in animal sources. It is estimated that heme iron contributes to up to 40% of total iron intake in omnivores.[112,113] Non-heme iron is usually much less absorbed than heme iron. All non-heme iron enters the common iron pool in the digestive tract where its absorption depends on physiological factors of the individual, as well as dietary iron absorption enhancers and inhibitors. These dietary effects are discussed in the sections below.

1.3.1.1 Enhancers of non-heme iron absorption

Organic acids - ascorbic & citric acid

Ascorbic acid – native or added – shows a definite dose-dependent enhancing effect on iron absorption.[114] The enhancing effect is due to two actions: Ascorbic acid acts as an electron donor in the reduction of ferric to ferrous iron; and it is a potent to iron chelator.[115] Ascorbic acid is also able to overcome the negative effects of phytate,[116] polyphenols (PP),[117] milk proteins and calcium.[118]

Addition of ascorbic acid to overcome the negative effects of inhibitory factors, requires a molar ratio of 2:1 for meals with low to medium levels of inhibitors, and 4:1 for meals with high amounts of inhibitors.[119] Unfortunately, ascorbic acid is very instable during food processing (heat) and storage (oxygen), it potentially causes sensory changes when fortified, and has a relatively high cost. Therefore, it is not widely used as an enhancer in iron fortified staple foods.[120]

Citric acid is also a reducing agent, and stabilizes iron in the ferrous form. The addition of citric acid increases iron absorption in adults.[121-123]

Muscle tissue

Iron absorption studies have reported an enhancing effect of meat, fish or poultry on non-heme iron absorption.[124] Evidence indicates that protein fractions and other components of the muscle tissue are involved. It is suggested that the “meat factor” is attributed to a multitude of small peptides, rather than a single peptide fraction.[125,126] Likewise ascorbic acid – cysteine containing peptides are able to reduce and chelate iron.[127]

Iron chelators – EDTA

The iron chelator ethylenediaminetetraacetic acid (EDTA) binds metal cations through its four negatively charged carboxylic groups and the two amine groups –this binding is pH dependent. At low pH, ferric iron binds to EDTA, in this way iron is protected from phytate and PP-binding in the stomach. As the pH rises in the duodenum, ferric iron is released from the EDTA complex and enters the iron pool for uptake via the enterocytes.[128] The food fortificant NaFeEDTA is therefore, an ideal iron compound to fortify high phytate staple foods, such as cereal flours (Chapter 2.4.3).

Nondigestible carbohydrates –GOS

Galacto-oligosaccharides (GOS), a prebiotic, have shown to increase iron absorption from fortified meals containing ferrous fumarate (FeFum) and NaFeEDTA, but not ferrous sulfate ($FeSO_4$) or ferric pyrophosphate (FePP) in adults and infants.[129-131] The mechanism behind these interactions remain unclear, three possibilities have been discussed: I) it may be that the addition of GOS increases gastric residence time, enabling greater iron dissolution;[132] II) each GOS molecule contains a reducing sugar, which may have a chelating or reducing effect on iron;[133] and III) increased colonic iron absorption due to the production of SCFA from prebiotics by the gut bacteria, which generates a lower pH and the formation of soluble iron complexes.[132]

1.3.1.2 Inhibitors of non-heme iron absorption

Phytate

Phytate is mainly present in cereals, legumes and seeds, it serves as the storage form of phosphorous in plant tissues. Phytate is made of an inositol ring with six phosphate ester groups, and has a high potential to bind six divalent cations (e.g. Zn^{2+}, Fe^{2+}, and Ca^{2+}). Once bound to phytate the mineral is unavailable for intestinal absorption.[134] The inhibitory effect of phytate on iron starts at small concentrations of 2-10 mg/ phytate per meal, and increases in a dose dependent manner.[116,135] Food processing and preparation (milling, heat, soaking, germination and fermentation) have shown to decrease the phytate concentration to a certain extent.[136] The addition of exogenous phytase before meal consumption has also shown to improve iron absorption.[135,137] A common estimate to evaluate the effect on iron absorption is the molar ratio of phytate to iron. A ratio of <1:1 or even <0.4:1 is suggested to improve iron absorption from meals without any iron absorption enhancers, or <6:1 in a meal containing vitamin C and meat.[136]

Polyphenols

PP are secondary metabolites that are naturally present in almost all plant tissues, including food products of plant origin. More than 8000 different polyphenolic structures have been identified, they are characterized by the presence of multiple phenolic groups.[138] Human studies with stable iron isotopes show that the absorption of non-heme iron is reduced by various dietary PP.[138-141] Chapter 3.4.2 will thoroughly discuss the nature of PP and their interaction with iron.

Calcium

Calcium affects heme and non-heme iron absorption negatively,[142-144] most likely during the initial uptake into the enterocytes.[145,146] The inhibitory effect is dose dependent when calcium doses of 75-300 mg are added to a bread roll meal, and also doses of 165 mg calcium from milk products inhibit iron absorption.[143]

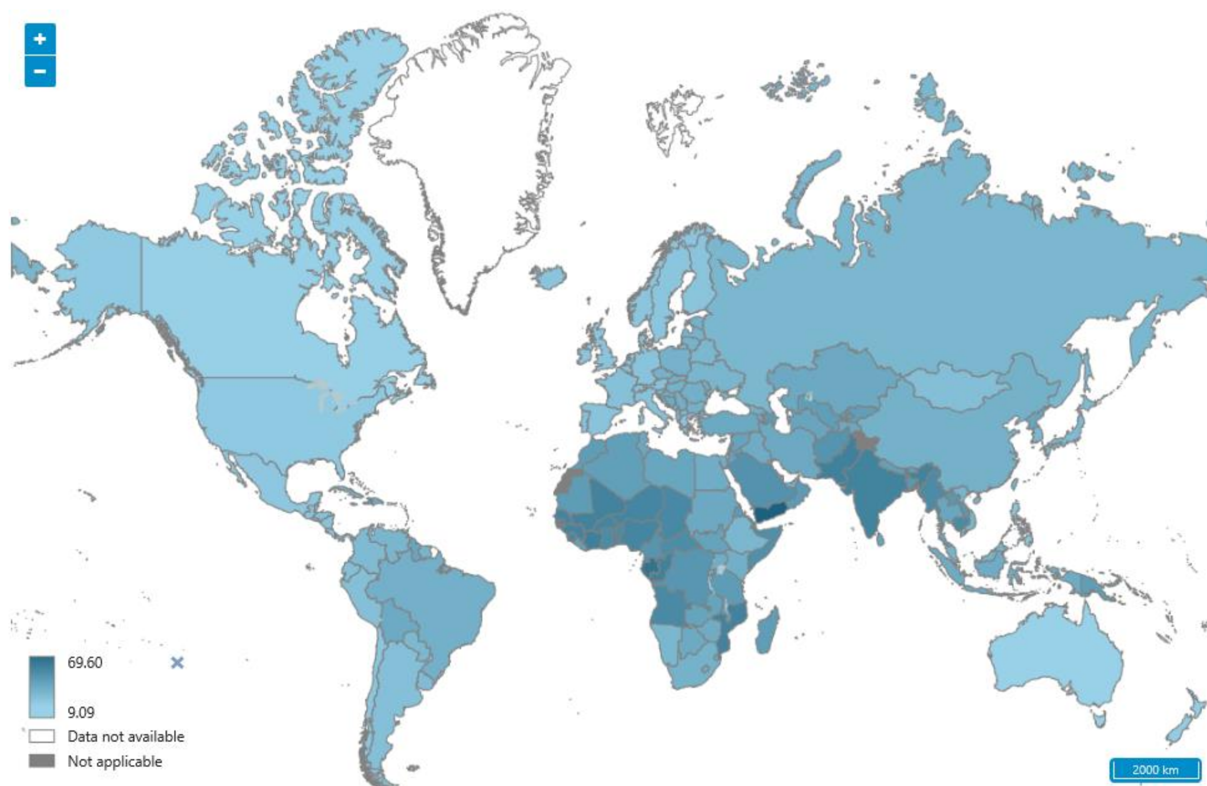
Proteins

Milk, egg and soy proteins, as well as albumin have shown to inhibit iron absorption.[147-150]

2 Iron deficiency and Iron deficiency anemia

2.1 Prevalence, consequences and definitions

Iron deficiency (ID) affects 1/3rd of the world's population and is the most common micronutrient deficiency worldwide.[22] And in 2016 there were over 1.2 billion cases of iron deficiency anemia (IDA) worldwide.[151] As iron demands are highest during growth (infants, children, adolescents), in women of childbearing age, and during pregnancy, these population groups are the most vulnerable to suffer from ID.[25] Poorly bioavailable iron is the most prominent cause for an iron imbalance.[20] It affects a large number of children and women in low- and lower-middle-income countries, and it is the only nutrient deficiency also present in upper-middle- and high-income countries (**Figure 5**).[152] The prevalence of anemia is an indirect indicator measure for the prevalence of ID. Approximately 50% of all anemia cases worldwide, can be accounted to iron deficiency.[22,153]



The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted and dashed lines on maps represent approximate border lines for which there may not yet be full agreement.

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Figure 5: Prevalence of anemia in women of reproductive age (%), last updated in 2017. Taken from WHO (2020).[154]

Table 2: Hemoglobin (g/L) cutoff levels to diagnose anemia at sea level. Taken from WHO (2011).[155]

Group	Non Anemia	Mild[a] Anemia	Moderate Anemia	Severe Anemia
Children 6 - 59 months of age	> 110	100 - 109	70 - 99	< 70
Children 5 - 11 years of age	> 115	110 – 114	80 - 109	< 80
Children 12 - 14 years of age	> 120	110 – 119	80 - 109	< 80
Non-pregnant women (15 years of age and above)	> 110	110 - 119	80 - 109	< 80
Pregnant women	> 110	100 - 109	70 - 99	< 70
Men (15 years of age and above)	> 130	110 - 129	80 - 109	< 80

[a]Mild is a misnomer: iron deficiency is already advanced by the time anemia is detected. The deficiency has consequences even when no anemia is clinically apparent.

The characteristic of anemia is a decreased quantity of erythrocytes, which is most often combined with reduced hemoglobin concentrations.[153] Anemia is associated with increased morbidity, weakness, fatigue, decreased physical work capacity, shortness of breath, and palpitations.[156] IDA most often correlates with decreased mental, motor and emotional development in children,[153] preterm birth, low birth weight, and maternal and child mortality.[157] Each year, around 0.8 million of deaths (1.5% of the total) can be attributed to iron deficiency, according to WHO mortality data. With regards to the loss of healthy life – measured as disability-adjusted life years (DALY), 25 million DALYs are lost due to IDA, which is 2.4% of the global total.[158]

Table 3: Serum ferritin (µg/L) cutoffs for the definition of iron deficiency and iron overload. Taken from WHO (2011).[159]

Group	Depleted iron stores	Depleted iron stores in the presence of infection	Severe risk of iron overload
Male & female < 5 years old	< 12	< 30	-
Male ≥ 5 years old	< 15	-	> 200
Female ≥ 5 years old	< 15	-	> 150

Iron deficiency can be classified in three stages of severity, as proposed by Bothwell *et al.*.[156] The first stage is storage iron depletion (or low iron stores as in **Figure 6**), which is characterized by low serum ferritin, and normal values for transferrin saturation, RBC protoporphyrin, serum transferrin receptor and hemoglobin. At this stage, the stores are empty, however, there are no erythropoietic consequences. At the second stage – iron-deficient erythropoiesis (or absolute iron deficiency non-anemic, Figure 6) – typical

characteristics are; low SF and TSAT; high RBC protoporphyrin and sTfR; and normal Hb. At the third, and last stage (absolute iron deficiency anemia, Figure 6) the reduced circulating red blood cell mass is measurable, and in addition to all above described parameters, the Hb concentration is low. The WHO cutoffs for serum ferritin and hemoglobin are described in **Table 2** and **Table 3.**

	Iron repletion	Low iron stores	Absolute iron deficiency (non-anaemic)	Absolute iron deficiency (anaemia)	Functional iron deficiency	Functional iron deficiency with absolute iron deficiency	Iron-refractory iron deficiency anaemia due to *TMPRSS6* variants
Body iron stores (Adequate / Inadequate)	Body iron stores and iron available for erythropoiesis				Body iron stores; Iron available for erythropoiesis		Body iron stores and iron available for erythropoiesis
No systemic inflammation					Systemic inflammation		No systemic inflammation
Symptoms	Nil	Asymptomatic or midly symptomatic (eg, fatigue); possible reduced physical or cognitive function; underlying condition could be evident (eg, bleeding or nutrition)	Asymptomatic or symptomatic: fatigue, poor concentration, dizziness, tinnitus, headache, pica, or restless legs	Likely symptomatic, decompensation if severe or poor medical reserves	Symptoms of underlying condition; symptoms of anaemia	Symptoms of underlying condition; symptoms of anaemia	Symptomatic iron deficiency anaemia
Haemoglobin	Normal	Normal	Normal or low–normal	Reduced (anaemic)	Mild to moderate anaemia	Mild to moderate anaemia	Reduced (anaemic)
Mean cell volume and mean cell haemoglobin concentration	Normal	Normal	Normal or reduced	Reduced	Normal or mild reduction	Reduced	Reduced
Ferritin	>30–60 µg/L	15–30 µg/L	<15–30 µg/L	<15–30 µg/L	Normal or increased depending on inflammation and body iron stores	<70–100 µg/L depending on degree of inflammation	Typically 20–50 µg/L
Transferrin saturation	>20%	Usually >20%	<20%	<15%	Usually <20%	<20%	<20%, usually <5%
Reticulocyte haemoglobin content*	Normal	Normal	Low	Low	Low	Low	Low
Soluble transferrin receptor*	Normal	Normal	Increased	Increased	Normal	Normal or increased	Increased
Hepcidin*	Normal	Low–normal	Low	Very low	Increased relative to transferrin saturation	Normal or reduced	High relative to transferrin saturation
Bone marrow stainable iron	Normal	Detectable or absent	Absent	Absent	Detectable	Absent	Absent or trace

Figure 6: Characteristics of biomarkers for the diagnosis of absolute iron deficiency, functional iron deficiency and refractory iron deficiency anemia. * thresholds for reticulocyte hemoglobin content, soluble transferrin receptor and for non-standardized hepcidin vary between type of analyzer and manufactures. Taken from Pasricha et al. (2021).[160]

Iron deficiency can be separated by two characteristics: absolute and functional iron deficiency. Absolute iron deficiency is described above and is characterized by low body iron stores, whereby the total available iron is inadequate. It occurs when iron intake or absorption does not cover the physiological needs, due to growth, pregnancy, malabsorption or blood loss. Functional iron deficiency is characterized by normal blood iron stores and inadequate mobilization of iron stores (especially from macrophages). This is the predominant mechanism for anemia of inflammation, which is most common in patients with acute and chronic infection, cancer, chronic kidney disease and autoimmune disease, recent surgery and heart failure. Functional and absolute iron deficiency can coexist, and functional iron deficiency might add on to absolute iron deficiency through increased impairment of iron uptake.[160] IRIDA has already been introduced in Chapter 1.1.3.1 and will also be topic of Chapter 2.3.2.2. A list of characteristics of biomarkers of absolute, functional iron deficiency and IRIDA are described in **Figure 6**.

2.3 Etiology of anemia, iron deficiency anemia and iron deficiency

The etiology of anemia is complex. The primary immediate determinants are:

- chronic disease/exposure and response to infectious diseases, such as: helminth infections, schistosomiasis, malaria, HIV, tuberculosis, and low-grade inflammation.
- micronutrient deficiencies, related to: malnutrition, growth, physiological state, sex, age and race.
- genetic disorders/haemoglobinopathies, such as: thalassemia and structural hemoglobin variants (sickle cell disease).

These immediate determinants lead to decreased or ineffective erythrocyte production or to its increased loss, resulting in anemia.[161]

The most common cause of anemia related to micronutrient deficiency is iron deficiency. Other concurrent or separate deficiencies of copper and zinc or the vitamins A, B_2, B_6, B_9, B_{12}, C D, and E are also reasons for anemia.[161] Iron deficiency is caused through: I) inadequate intake due to poor dietary habits, or low access to bioavailable dietary iron; II) malabsorption due to low nutrient bioavailable diet (rich in absorption inhibitors), or diseases such as celiac disease, chronic *Helicobacter pylori* gastritis (Chapter 2.3.1), autoimmune atrophic gastritis, surgical procedures in stomach or upper small bowel, and IRIDA(Chapter 2.3.2); III) higher needs during pregnancy and growth (premature infants, early childhood and adolescence); and IV) increased losses due to menstruation (menorrhagia), bleeding from the gastrointestinal and genitourinary tract, parasitic infections, intravascular hemolysis, blood donation.[20,161]

Genetic conditions or hemoglobinopathies, such as thalassemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, or sickle cell disease are causes of high risk of anemia, organ damage, infection and related morbidity and mortality.[161] Loss of function mutations in the *TMPRSS6* gene cause IRIDA. Patients with IRIDA are typically characterized by the inability to absorb oral iron due to increased hepcidin concentrations.[66] Other polymorphisms in the *TMPRSS6* gene have also been associated with erythrocyte markers and iron stores in several genome wide association studies (GWAS).[162-169]

The following two Chapters 2.3.1 and 2.3.2, will focus on two of the above named factors, *Helicobacter pylori* infection and mutations and polymorphisms in the *TMPRSS6* gene.

2.3.1 *Helicobacter pylori* infection and its association with iron deficiency

2.3.1.1 Epidemiology and risk factors

Helicobacter pylori is a spiral-shaped flagellated Gram-negative bacterium that colonizes the human stomach of estimated more than half of the human population.[170] However, there is large regional variation in the prevalence of *H. pylori* infection, as shown in **Figure 7**. Regions with highest prevalence are: Africa (70.1%); South America (69.4%); and Western Asia (66.6%). Whereas lowest reported regions were: Oceania (24.4%); North America (37.7%); and Western Europe (34.3%).[171] Since the beginning of the 21st century, the prevalence has been declining in high income countries, whereas in low- and middle-income countries the prevalence has plateaued at a high level.[172] These differences in prevalence reflect the level of urbanization, sanitation, hygiene, access to clean water and different socioeconomic status, and there are differences in prevalence even within the same country.[171]

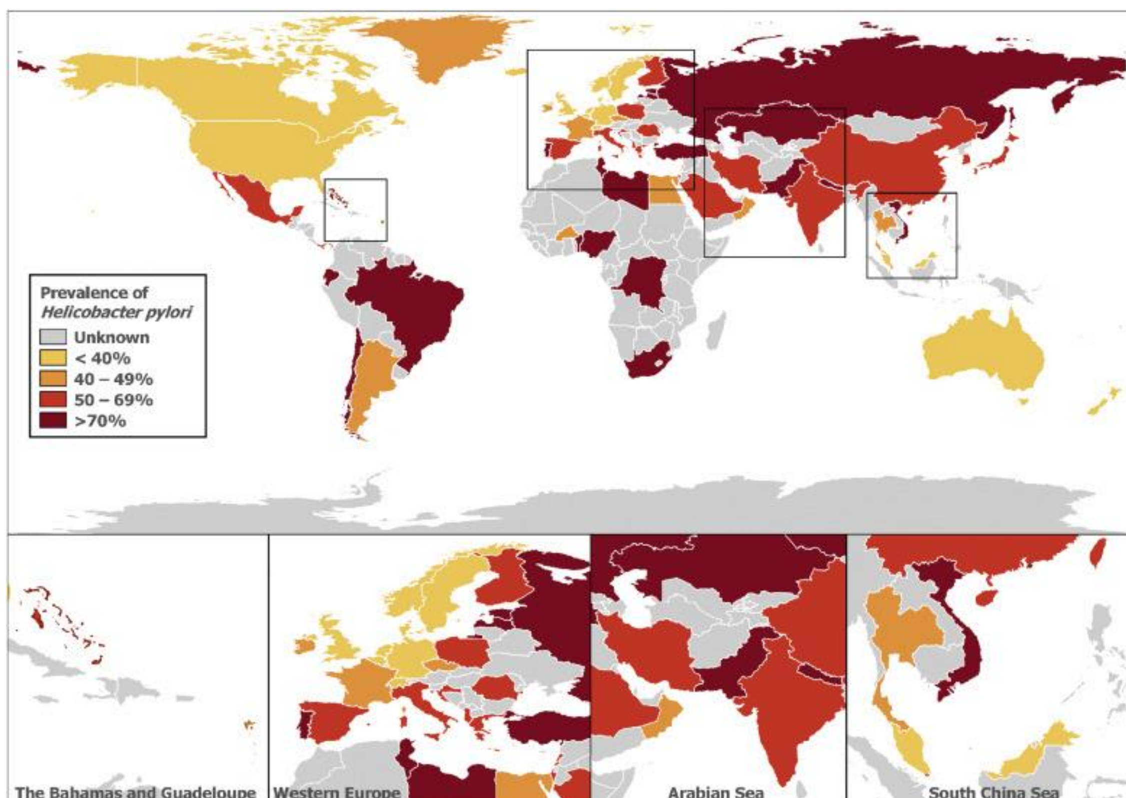

Figure 7: Global Prevalence of *H. pylori* infection. Taken from Hooi et al. (2017).[171]

The main transmission routes are from person to person by oral-oral or fecal-oral routes. Transmission mainly happens from parents to their children, but could also be possible between unrelated people living nearby.[173,174] It is well established that most infections are acquired during childhood, and infections persist lifelong if untreated.[175] Risk factors associated with *H. pylori* infection are of multiple origins. These include: 1) familial and living conditions, such as occupation, low income, low education level, infected family members, poor access to health care, and poor sanitation and hygiene;[172] 2) lifestyle habits, such as alcohol consumption, smoking, but also intake of contaminated foods (e.g. raw milk and vegetables) are other risk factors of *H. pylori* infection;[172] 3) environmental factors may also play an important role, such as living in a rural area, access to sanitary water, and contact with animals (e.g. sheep, cow, dog);[176] 4) intrinsic factors, such as low gut microbiota diversity has been associated with intrafamilial infections and can be a risk factor;[176] and 5) whether breastfeeding is a risk factor or not is unclear and equivocal findings have been reported. However, infants with infected mothers are fed a breast milk with higher levels of anti-*H. pylori* IgA and are at lower risk for infection.[177]

2.3.1.2 *H. pylori* infection and colonization

Despite harsh conditions in the human stomach, *H. pylori* is the only bacterium able to survive and colonize in the gastric mucus.[175] Numerous stress factors of the stomach are: nutrient limitations, pH fluctuations or oxidative attack.[178] *H. pylori* is highly adapted to this environment and grows at pH between 6 to 8.[178,179] These adaptions include: flagella-mediated motility, urease-driven chemotaxis and neutralization of gastric pH,[175,180] iron acquisition,[181] counteraction of antimicrobial nitric oxide production by arginase RocF, and adhesion of the bacteria to gastric epithelial cells using several outer membrane proteins.[182] Urease is the most abundant protein of *H. pylori*, it hydrolyzes urea to ammonia and carbon dioxide, which serves as a buffer in the periplasm and allows the survival at acidic pH.[175]

Adherence to host epithelial cells is essential for its survival, to prevent moving back into the lumen.[183] It achieves the adherence through several related families of outer membrane proteins and proteins of a type IV secretion system (T4SS).[183,184] These adhesion interactions between bacteria and the host are important in the pathogenic process, such as the translocation of the cytotoxin-associated gene A (CagA) – an oncoprotein – into the host gastric epithelial cells.[183,184]

2.3.1.3 Pathogenesis and why only some infections result in disease

Once the *H. pylori* bacteria have anchored to host epithelial cells, they will persist in the stomach. The majority of the hosts (80%) are likely to remain asymptomatic.[185-187] The remaining 20% can develop gastritis, peptic ulcer disease (PUD), mucosa associated lymphoid tissue (MALT) lymphoma and/or gastric cancer (GC) during their lifetimes.[185-187] This bacterium is considered as a carcinogenic agent, and is responsible for ~90% of the gastric cancer cases.[172] However, four factors have been suggested to affect the risk of developing the named diseases:[175]

I) Depending on the virulence of the *H. pylori* strain, more CagA proteins are translocated, and these are strongly associated with the named diseases.[184,187] T4SS and CagA are both encoded in the *cag*-pathogenicity island (*cag* PAI). This island is present in highly virulent *H. pylori* (type-I), and absent in the less virulent (type-II) strains.[182] Further virulence factors described, are either surface exposed, secreted or translocated include: vacuolating cytotoxin A (VacA), γ-glutamyl transpeptidase (GGT), high temperature requirement A (HtrA, a serine protease) and cholesterol glycosyl-transferase (CGT).[182]

II) The type and extent of the host immune response to the infection: these virulence factors may damage the epithelial cells of the host and stimulate the production of proinflammatory cytokines. A high intensity of inflammation increases the risk of developing a *H. pylori* related diseases. A polymorphism in the human cytokine gene (*IL-1β*) and the IL-1 receptor antagonist (*IL-1RN*) can lead to increased levels of IL-1β,[188,189] and inflammation.[188-190] The extent of the T-helper cell mediated immune response also modulates the pathogenesis of *H. pylori* associated diseases. If the immune response activates predominantly Th-1, the severity of gastritis and cancer risk increases, whereas in a Th-1 suppressed response there is low risk.[175,191]

III) The location of the *H. pylori* colonization: *H. pylori* induced gastritis has either a corpus predominant, or antral predominant pattern. It is unknown why some people develop the one pattern or the other. Colonization in the antrum the antral-predominant gastritis. Due to hormonal changes in somatostatin induced by *H. pylori*, the acid production of the healthy parietal cells in the gastric corpus is increased. Leading to an increased risk of duodenal ulceration.[175,192] Whereas the corpus predominant gastritis is associated with reduced or

normal gastric acid production and leads to increased risk of gastric ulceration and adenocarcinoma.[175,193]

And last IV) modulating cofactors: such as smoking, alcohol intake and diet.[175] Salt consumption is associated with increased risk of gastric cancer, in particular when infected with *CagA*-positive *H. pylori* strains.[194] In contrast, consumption of vitamin C rich sources (fruits and white vegetables), are inversely associated with gastric cancer risk.[195]

2.3.1.4 *H. pylori* infection and its association with iron deficiency, anemia and iron deficiency anemia

Many disorders of the upper gastrointestinal tract are linked with the presence of *H. pylori*.[196] Epidemiological studies have also reported a correlation of the presence of this bacterium with clinical effects outside the stomach. These diseases are associated with chronic low-grade systemic inflammation; the induction of a molecular mimicry mechanism, as the expression of proteins that mimic host peptides; and the interference of the absorption of nutrients and drugs.[197,198] *H. pylori* also plays an active role in iron deficiency anemia, idiopathic thrombocytopenic purpura and vitamin B_{12} deficiency. Moreover, evidence of the association of hepatobiliary, pancreatic, cardiovascular and neurodegenerative diseases with *H. pylori* are under investigation.[199]

Three meta-analyses of observational studies including pediatric and adult patients show an association between *H. pylori* infection and IDA, ID and anemia.[200-202] Four meta-analyses of randomized controlled trials show a more pronounced increase in SF and Hb when oral iron is given with an anti-*H. pylori* treatment, than without.[200,202-204] The most recent systematic review and meta-analysis indicates an increased risk (odds ratio and 95% confidence interval) for IDA: 1.72 (1.23-2.42); for ID 1.33 (1.15-1.54); and anemia 1.15 (1.00-1.32), as well as an increased SF (but not Hb) when an anti-*H. pylori* treatment is given with oral iron compared to iron therapy alone.[200]

The biological mechanisms behind this relationship are not fully understood and several pathways may be involved separately or in combination.[201] Chronic gastritis due to *H. pylori* can alter the physiology of the stomach by reducing gastric acid secretion and gastric ascorbic acid levels.[205,206] The excretion of hydrochloric acid is crucial for the effective solubilization and absorption of non-heme iron.[207] *H. pylori* may induce hypochlorhydria by increasing

gastric IL-1β and tumor necrosis factor (TNF)-α,[208,209] which induces parietal cell apoptosis, decreases cell histamine release and inhibits acid secretion.[210,211] Sarker *et al.*[212] reports a reduced acid output in *H. pylori* infected children, and eradication of *H. pylori* was associated with an increase in gastric acid secretion[212] This finding correlates with the gastric acid output of *H. pylori* infected Gambian,[213] and Chilean children.[214]

Ascorbic acid, is an important promotor of non-heme iron absorption (Chapter 1.3.1.1). Baysoy *et al.*[215] reported an association of decreased gastric ascorbic acid levels with *H. pylori* gastritis. An infection with a *CagA*-positive strain induced a greater decrease in gastric ascorbic acid levels than *CagA*-negative strains.[215]

It has been shown *in vivo* that IL-1β is also involved in the upregulation of hepcidin.[216,217] Two studies in *H. pylori* infected patients with IDA reported a decrease in serum hepcidin levels after *H. pylori* eradication therapy.[218,219] However, this decrease is more likely to be related to the anemia status than to *H. pylori* infection, as a decrease of hepcidin was also reported in the group receiving iron supplement only without *H. pylori* eradication therapy.[219]

Another possible mechanism is that the *H. pylori* competes for iron with the host. Like many other bacteria *H. pylori* requires iron as a growth factor,[220,221] and it expresses a protein that resembles ferritin which is most likely used for storing the excess sequestered iron by *H. pylori*.[222]

Another explanation for IDA in *H. pylori* infected subjects is the involvement of lactoferrin. Human lactoferrin is secreted in the gastric mucosa which seems to be controlled by signal transmission from *H. pylori* close to these glands.[222] The lactoferrin levels in the gastric mucosa of *H. pylori* positive patients with IDA is significantly higher,[222] whether this is associated with IDA or inflammation is unclear.[223] However, the expression of a highly specific human lactoferrin-binding protein has been identified in *H. pylori*, which may be involved in the absorption of iron.[224]

Iron losses may also be increased due to bleeding from *H. pylori* induced gastritis.[225,226] However, bleeding is most likely not the main actor, because most studies report no lesions at the time of endoscopy in *H. pylori* infected patients with IDA, negative fecal occult blood, and an eradication therapy is associated with a resolution of IDA.[227-233]

Whether iron absorption is impaired in *H. pylori* infection is unclear and equivocal results have been reported in literature. Lopez de Romana *et al.*[234] reported that iron absorption in asymptomatic adults from a flour-based test meal fortified with radiolabeled ferrous sulfate or ferrous fumarate is smaller in *H. pylori* positive subjects, regardless of the iron compound.[234] No differences in iron absorption were reported in a study in Bangladeshi children,[212] and in Haitian women and children.[235] In Bangladesh, iron absorption was measured from an infant cereal test meal using ferrous sulfate or ferrous fumarate in iron deficient anemic *H. pylori* positive versus negative children. Before and after *H. pylori* eradication therapy, no difference in iron absorption was detected.[212] In Haitian children and women, no difference in iron absorption from a wheat bread meal fortified with ferrous fumarate or NaFeEDTA was measured. Although the results in the Haitian study are based on imbalanced group sizes of *H. pylori* positive versus negative subjects.[235] Ciacci *et al.*[236] reported iron absorption as serum iron response after an oral supplemental iron dose in adults. A significant reduction in serum iron was found among *H. pylori* positive adults compared to negative controls. Further, after the eradication therapy, iron absorption was comparable between the two groups.[236] The association of iron absorption and asymptomatic *H. pylori* infection will be topic of Manuscript 1 of this thesis.

Whether the infection with a *CagA*-positive strain is associated with iron stores, is not yet defined, and studies have reported conflicting results.[201,215,236-238] The previously mentioned study by Ciacci *et al.*[236] reports no difference between *CagA*-positive infected versus *CagA*-negative infected adults.[236]

2.3.2 *TMPRSS6* mutations

The *TMPRSS6* gene is located on chromosome 22,[164] is expressed primarily in the liver,[239] and encodes the matriptase-2, or also called transmembrane protease serine 6 (**Figure 8** and **Figure 9**).[66] Loss-of function mutations in this gene lead to an increased hepcidin expression iron deficiency and the condition termed IRIDA.[66,240,241] Its role in the BMP/SMAD pathway, and its association with iron status and red blood cell markers is discussed in this chapter.

2.3.2.1 The matriptase-2 in the BMP/SMAD pathway

Matriptase-2 is a negative regulator of the BMP-SMAD pathway.[66,242] *TMPRSS6* deficient mice have increased hepcidin transcription and show a phenotype of severe IDA.[243] Further they have reduced ferroportin levels on the basolateral membrane of duodenal enterocytes, leading to iron retention in enterocytes.[67] The negative regulation of hepcidin expression by matriptase-2 is mediated via the proteolysis of the membrane receptor hemojuvelin.[68] HJV is a co-receptor for BMP-2, -4 and -6.[54,244,245] Its cleavage blocks the subsequent phosphorylation of SMAD-1, -5 and -8, their heteromeric complex formation with SMAD4, and translocation to the nucleus to stimulate *HAMP* expression.[246]

The regulation of matriptase-2 occurs at the level of protein stabilization and degradation rather than by changing the translational or transcriptional mechanisms. Studies in iron deficient animals showed no change in mRNA of *TMPRSS6* in the liver.[247,248] Studies in mice showed that the *TMPRSS6* mRNA is neither regulated by iron nor BMP-mediated signaling.[248] Another study in ID rats detected an increase in the matriptase.2, suggesting that the stability of matriptase-2 is regulated by iron itself, and hepcidin expression in acute iron depletion is mediated by an increase in matriptase-2 levels.[247]

2.3.2.2 Iron-refractory iron deficiency anemia

Complete loss-of function of the matriptase-2 due to mutations in the *TMPRSS6* causes IRIDA, which is an inherited disorder of systemic iron balance and impairs iron absorption and utilization.[66,70] Clinical features of these patients are usually lifelong and appear already in childhood, and are: severe microcytosis (mean corpuscular volume, MCV: 45-65 fL); anemia (Hb: 6-9 g/dl); hypoferremia with low TSat (< 5%); minimal response to oral iron supplementation and oral iron challenge; incomplete and transient response to parenteral iron and autosomal recessive transmission. Although anemia in adulthood often ameliorates,

hypoferremia persists.[70] Despite these, IRIDA patients have normal or borderline-low ferritin and high hepcidin levels.[249] In systemic iron deficiency, hepcidin concentrations are normally reduced,[250,251] but in patients with IRIDA hepcidin levels are normally above the reference range. These inappropriate high hepcidin levels can explain the: 1) ID due to impaired iron absorption of dietary iron; 2) the failure of oral iron therapies; and 3) the incomplete utilization of parenteral iron, which requires macrophage processing before usage in erythropoiesis.[70]

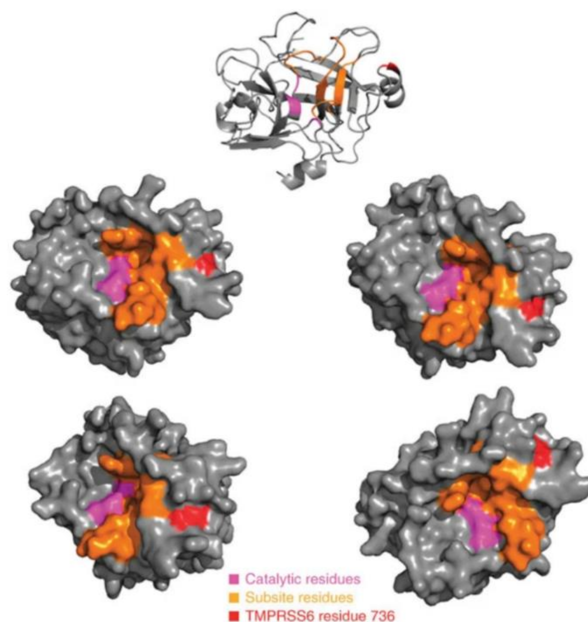

Figure 8: Molecular model of the matriptase2, and it's cleavage domain, showing the binding site (orange), the catalytic residue (magenta) and the location of the V736A amino acid substitution caused by SNP rs855791 (red). Taken from Chambers et al. (2009).[164]

2.3.2.3 Mutations and polymorphisms in the *TMPRSS6*

At least 45 different TMPRSS6 mutations have been reported in literature describing individuals with the IRIDA phenotype, which are mostly unique to families or relatives.[70] These loss-of normal splicing function mutations include: 20 missense mutations; 5 nonsense mutations; 10 frameshift mutations; 1 large in-frame deletion; and 9 intronic mutations predicted to disrupt normal splicing.[66,240,241,252-266] Some of these mutations cause truncated or aberrantly spliced TMPRSS6 transcripts, others have a missense substitution in the catalytic domain (Figure 9) and others have missense substitutions in other domains, which also impairs

its protease function. For example, matriptase-2 trafficking to the plasma membrane is impaired by a missense mutations in the second LDLRA (low-density lipoprotein receptor class A) domain,[253] and an impaired activation of the protease is caused by a missense mutation in the SEA (sea urchin sperm protein, enteropeptidase, agrin) domain (Figure 9).[246]

Besides these rare mutations, several common variants have been described in various global populations, these are single nucleotide polymorphisms within the *TMPRSS6*, and their allele frequency vary among ethnics.[267] GWAS have correlated several of these SNPs to red blood cell traits and iron status,[162-169] a few of these are given in **Table 4**. Out of these, the rs855791 has shown to have the strongest associations[162,165,167,169] and the second strongest were reported with the rs4820268.[162,169]

A recent systematic review by Timmer et al.[268] has found three SNPs (rs855791, rs4820268 and rs5756504) to be associated with erythrocyte parameters based on GWAS studies.[268] Two of these (rs855791 and rs4820268) have been estimated to have a benign clinical relevance for microcytic anemia, based on information from ClinVar.[269,270]

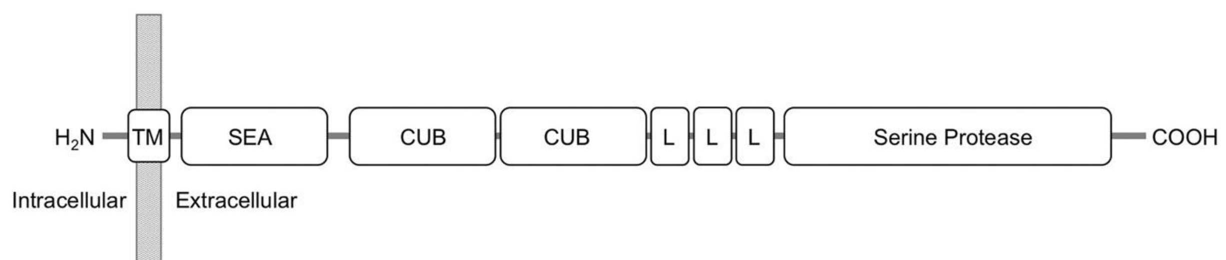

Figure 9: The domains of the matriptase-2 in a schematic representation. Shown are: the transmembrane (TM), and its large extracellular region: the SEA (sea urchin sperm protein, enteropeptidase, agrin) domain, two CUB (C1r/C1s, urchin embryonic growth factor, bone morphogenetic protein 1) domains, three LDLRA (low-density lipoprotein receptor class A) domains (L), and the serine protease domain, a C-terminal catalytic domain containing a triad of serine, histidine and aspartic acid residue. Taken from Heeney et al. (2014)[70]

Table 4: SNPs within the *TMPRSS6*, associated with erythrocyte parameters and iron status in GWAS.

SNP	Associated trait	Reference
rs228904	MCH	Ding et al.[165]
	MCHC	Ding et al.[165]
rs228918	Hb	Chambers et al.[164]
rs228919	Hb	Chambers et al.[164]
rs228921	Hb	Chambers et al.[164]
rs855791	SFe	Tanaka et al.[169], Benyamin et al.[162] and[163]
	TSat	Benyamin et al.[162]
	Hb	Ganesh et al.[166], Chambers et al.[164], Ding et al.[165], Benyamin et al.[162]
	MCV	McLachlan et al.[167], Ding et al.[165], Benyamin et al.[162]
	MCH	McLachlan et al.[167], Ding et al.[165]
	MCHC	Ding et al.[165]
rs855788	MCV	Ding et al.[165]
	MCH	Ding et al.[165]
rs877908	Hb	McLachlan et al.[167]
rs1421312	MCV	Ding et al.[165]
	MCH	Ding et al.[165]
rs2160906	MCH	Ding et al.[165]
	MCHC	Ding et al.[165]
rs2235320	SFe	Tanaka et al.[169]
rs2255324	MCV	Ding et al.[165]
	MCH	Ding et al.[165]
rs2413450	Hct	Ganesh et al.[166]
	Hb	Chambers et al.[164]
	MCV	Ganesh et al.[166]
	MCH	Ganesh et al.[166]
	MCHC	McLachlan et al.[167]
rs4820268	SFe	Tanaka et al.[169], Benyamin et al.[163]
	TSat	Benyamin et al.[163]
	Hb	Chambers et al.[164], Ding et al.[165]
	MCV	Ding et al.[165]
	MCH	Ding et al.[165]
	MCHC	Ding et al.[165]
rs5756504	Serum Fe	Tanaka et al.[169]
	Hb	Soranzo et al.[168]
	MCV	Ding et al.[165]
	MCH	Soranzo et al.[168], Ding et al.[165]
rs5756520	Hb	Chambers et al.[164]

Hb: hemoglobin; Hct: hematocrit; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; SFe: serum iron; TSat: transferrin saturation

The SNP rs855791 will be topic of Manuscript 2 of this thesis. It encodes an alanine to valine substitution at the 736 position (p.Ala736Val, or at DNA base level: c.2207T>C).[270] In this SNP, the altered amino acid is located close to the catalytic and specificity site of the serine protease (Figure 8), which suggested that the rs855791 may be a causal variant.[164] The matriptase-2 variant carrying a thymine at position 2207, expressing a valine, was found to be less effective in suppressing hepcidin levels in vitro, and was associated with serum hepcidin in a large Italian population.[69] Two following studies, however, suggest that the effect of the rs855791 on iron and erythrocyte parameters are at least partly independent on hepcidin levels.[271,272] The *TMPRSS6* variant in these two studies was associated with ferritin, serum iron TIBC, hepcidin normalized to ferritin, and hepcidin normalized to transferrin saturation, but not with serum hepcidin itself.[271,272]

In patients with chronic iron deficiency and/or anemia (cID/A), these normalized hepcidin concentrations have been suggested to identify cID related to *TMPRSS6* mutations. A study by Heeney et al.[273] reports that the normalized hepcidin to serum iron and normalized to transferrin saturation rations are predictors of *TMPRSS6* mutations in this patient group. In which the ratio of serum iron to hepcidin was superior to predict *TMPRSS6* mutation status. Based on their findings, an algorithm based on the measurement of ferritin or hepcidin was developped, in combination with SFe and Hb, which is able to predict biallelic mutations in *TMPRSS6* in patients with cID/A.[273]

In a Chinese (Han and Zhuang) study population, carriers of the polymorphisms in rs855791 and rs4820268 are reported to have an increased risk of anemia and iron deficiency.[274] Further, a Taiwanese case-control study reported a lower prevalence of the rs855791 C homozygotes in the IDA group compared to the healthy control group. In the same study, women with menorrhagia carrying the CC polymorphism had lower risk for IDA compared to women with one or two T alleles.[275] Another publication in a Chinese Han population indicates, that the iron-lowering alleles of both of the above-named SNPs are significantly associated with ferritin, Hb, as well as a reduced risk for iron overload and type 2 diabetes.[276]

A series of studies in Danish blood donors by Sørensen et al.,[277-279] on genetic factors influencing iron status and hemoglobin, revealed a significant association of the rs855791 with iron stores (among male donors)[279] and Hb (among both sexes) in two studies.[278] In contrast,

the first publication of this series does not show a clear association of the rs855791 and rs4820268 with ferritin.[277]

Table 5: Allele frequencies of a selection of SNPs in the *TMPRSS6* gene in different populations. Listed are the nucleobases and their mutation, in brackets its nucleobase pair. Data extracted from 1000 Genome Browser.[267]

	rs855791	**rs2413450**	**rs4820268**	**rs5756504**
British in England and Scotland	A (T) = 0.4121 G (C) = 0.5879	T (A) = 0.4011 C (G) = 0.5989	G (C) = 0.4011 A (T) = 0.5989	C (G) = 0.5879 T (A) = 0.4121
Finnish in Finland	A (T) = 0.3131 G (C) = 0.6869	T (A) = 0.3939 C (G) = 0.6061	G (C) = 0.3939 A (T) = 0.6061	C (G) = 0.5657 T (A) = 0.4343
Toscana in Italy	A (T) = 0.4486 G (C) = 0.5514	T (A) = 0.4766 C (G) = 0.5234	G (C) = 0.4813 A (T) = 0.5187	C (G) = 0.6355 T (A) = 0.3645
Iberian Population in Spain	A (T) = 0.3505 G (C) = 0.6495	T (A) = 0.3551 C (G) = 0.6449	G (C) = 0.3551 A (T) = 0.6449	C (G) = 0.5374 T (A) = 0.4626
Gambian in Western Division of the Gambia	A (T) = 0.1018 G (C) = 0.8982	T (A) = 0.1770 C (G) = 0.8230	G (C) = 0.2699 A (T) = 0.7301	C (G) = 0.3540 T (A) = 0.6460
Luhya in Webuye, Kenya	A (T) = 0.0707 G (C) = 0.9293	T (A) = 0.1414 C (G) = 0.8586	G (C) = 0.3434 A (T) = 0.6566	C (G) = 0.2828 T (A) = 0.7172
Punjabi from Lahore, Pakistan	A (T) = 0.5208 G (C) = 0.4792	T (A) = 0.5365 C (G) = 0.4635	G (C) = 0.5573 A (T) = 0.4427	C (G) = 0.6875 T (A) = 0.3125
Han Chinese in Bejing, China	A (T) = 0.5388 G (C) = 0.4612	T (A) = 0.5437 C (G) = 0.4563	G (C) = 0.5437 A (T) = 0.4563	C (G) = 0.5388 T (A) = 0.4612
Japanese in Tokyo, Japan	A (T) = 0.6250 G (C) = 0.3750	T (A) = 0.6346 C (G) = 0.3654	G (C) = 0.6394 A (T) = 0.3606	C (G) = 0.6346 T (A) = 0.3654
Mexican Ancestry from Los Angeles, USA	A (T) = 0.5313 G (C) = 0.4688	T (A) = 0.5391 C (G) = 0.4609	G (C) = 0.5547 A (T) = 0.4453	C (G) = 0.8125 T (A) = 0.1875
Peruvians from Lima, Peru	A (T) = 0.5765 G (C) = 0.4235	T (A) = 0.5588 C (G) = 0.4412	G (C) = 0.5588 A (T) = 0.4412	C (G) = 0.8412 T (A) = 0.1588

A: Adenine; C: Cytosine; G: Guanine; T: Thymine

Among male donors, each change in amino acids from CC to TC to TT, has an additive effect on a decrease in iron stores and an increased risk of becoming iron deficient.[279] Similarly, the changes in amino acids showed an additive negative effect on Hb levels among both men and women.[278] A study in Australian blood donors has shown an association of the SNP rs4820268 with low ferritin levels in female donors, but not in male, and no such association was found with the rs855791.[280] In US American first time female blood donors, carrying the TT variant was associated with a drop in Hb and ferritin after multiple blood donations, suggesting this genotype has an impaired capacity to replenish iron stores following donation.[281] A recently published small study in middle eastern blood donors reported an association of the *TMPRSS6* rs855791 with hepcidin and the ratio of hepcidin to ferritin.[282]

As reported in separate publications, the T allele frequency of the SNP rs855791 has a population frequency of ≈0.5 in Caucasians,[162,164] ≈0.6 in Japanese[283] and ≈0.2-0.1 in African Americans.[162,164] These are comparable to the allele frequencies reported in the 1000 Genomes Browser, **Table 5** shows a selection of SNPs and populations extracted from this database.[267]

2.3.2.4 Other genes associated to hematological parameters and iron status

This chapter gives a brief (and most likely) incomplete list of SNPs that have been repeatedly associated with hematological parameters or iron status. There is a large number of SNPs and genes that are associated with these traits, which reflects the interplay and the complex network of multiple genes determining hematological parameters and iron status.

The systematic review by Timmer et al.[268] summarizes that a total of 217 SNPs are located in or near 14 different genes, which have been associated with one or more hematological parameters in more than one study. These genes are: *ABO*, *CCND3*, *HBD*, *HBS1L*, *HFE*, *HIST1H1T*, *HIST1H2AC*, *KCTD17*, *MPST*, *MYB*, *SLC17A1*, *SLC17A2*, *SLC17A3* and *TMPRSS6*. Six of these genes are known to be involved in erythropoiesis. These are: 1) *ABO* in the blood group system;[284] 2) *TMPRSS6* is associated with IRIDA;[66] 3) *HFE*, known to be associated with hemochromatosis;[258,285-288] 4) *MYB* gene encodes a protein involved in hematopoiesis;[289] 5) the *HBS1L* is associated with sickle-cell disease;[290] and 6) *HBD* is associated with beta-thalassemia.[291]

Several SNPs in the transferrin gene *TF* have been associated with iron status markers. The SNPs rs1799852 and rs2280673 have been associated with transferrin, the SNPs rs1830084 and rs3811647 with transferrin saturation.[163] The later SNP (rs3811647) was associated with increased transferrin and TIBC in a large Chinese study population.[274] The rs76718398 SNP in the *TF* gene has been associated with ID in US American women.[292] But a stable iron isotope study in iron deficient women comparing both heterozygous variants did not find a difference in iron absorption.[293]

The rs7385804 polymorphism in the *TFR2* gene, has been associated with a decrease in serum iron, in the same Chinese study population as described above.[274]

Variants of the *BTBD9* with SNPs rs9296249,[277] rs9357271[282] and rs3923809,[280] were found to be associated with serum ferritin.[277,280,282] This polymorphism has previously been described also to be associated with restless leg syndrome.[294]

The *BMP2* rs235756 was associated with ferritin in Australian male blood donors, this SNP may lead to altered BMP2 mRNA regulation and a decreased function of its encoded protein.[280,295]

The SNPs rs1800562 (carrying the A-allele) and rs179945 (carrying the G-allele) in the *HFE* gene have also been associated with Hb[278] and iron stores.[279] These SNPs also known as C282Y, and H63D are well documented polymorphisms that cause hemochromatosis.[296] Hemochromatosis, and iron overload will be topic of Chapter 3 and Manuscript 4 in this thesis.

2.4 Strategies to combat iron deficiency

This chapter will give an overview on the different approaches to combat iron deficiency in populations, which may be applied alone or in combination. These are: dietary modification and diversification combined with education, supplementation, fortification, and biofortification. A special focus in this thesis will be given on iron fortification, and iron encapsulation approaches.

The aim of dietary diversification/modification is to improve the availability, access and utilization of foods which are rich in highly bioavailable iron (and other micronutrients) in all communities throughout the year.[297] It involves changes in food production, selection, and preparation practices.[297] However, this intervention has the highest cost per capita, in International dollar (I$) 1148, and the average costs per disability-adjusted life-year (DALY) saved is I$ 103.[298] Despite these costs, it may be more culturally accepted and more sustainable than supplementation, fortification or biofortification.[297] Iron supplementation, preferably in the form of ferrous sulfate or ferrous gluconate can be administered to infants and pregnant women, to treat and prevent ID and IDA.[25,299] However, compliance may be low due to nausea and epigastric discomfort/pain, which may develop in some individuals.[25] Therefore, lower doses, and alternate dosing has shown to reduce adverse side-effects and even increase fractional iron absorption.[300-302] Exclusive iron supplementation has the highest costs per DALY saved (I$ 179), and may not be an approach for long-term use.[298] Biofortification is "the process of breeding nutrients into staple food crops".[303] There are three approaches: 1) selective breeding of cultivars with specifically selected plant genotypes; 2) genetic engineering, using recombinant DNA techniques and molecular breeding; and 3) change in agronomic practices via the application of fertilizers (through soil or foliar application).[304] The success of biofortification depends on consumer acceptance, which may be lowered if the phenotype of the staple food is changed during biofortification.

Fortification of commonly consumed foods with micronutrients is a medium- to long-term public health strategy to increase the nutrient intake of a population.[305] Ideal food vehicles are staple foods and condiments (for example cereals, fats, oils, sugar, salt and sauces), as these are purchased by populations with lower income too.[305] According to the world bank, "…probably no other technology (than food fortification) available today offers as large an opportunity to improve lives and accelerate development at such a low cost and in such short

a time".[306] A major advantage of food fortification over other strategies, is that it requires a relatively small change in consumer behaviors and habits.[307] A disadvantage of food fortification is, that it requires access to centrally processed foods, which is most likely a problem for those located far away with poor access to urban areas. Hence, food fortification programs require clear guidelines, including quality standards, safety and surveillance protocols.[308] A consequence of indiscriminate food fortification is over- or under-fortification leading to imbalanced nutrient intake of individuals, therefore, any changes in fortification policies must be considered within their impact on all segments of the population.[309]

The selection of the food vehicle is the key to a successful fortification program.[309] A food vehicle for a fortification program does need to meet several requirements: easily accessible; regularly consumed in amounts that do not fluctuate from day to day and seasons; affordable to the population at risk; resistant to sensory changes; should not adversely affect nutrient stability and bioavailability.[309] Suitable food vehicles for fortification are: wheat and maize products (flour, bread, pasta, corn grits, corn meal, corn porridges) milled rice, breakfast cereals, infant formula and cereals, milk and other dairy products, margarines, vegetable oils, and condiments (salt, sugar, soy and fish sauce, curry powder).[309]

Food fortification as salt iodization was first implemented in the 1920's in Switzerland and the United States of America. The first micronutrient fortification as a public health program was iodized salt introduced to prevent goiter and cretinism. Currently there are salt iodization programs in around 140 countries worldwide and iodine deficiency has been nearly eliminated globally.[305] Whereas for vitamin A, the most suitable vehicles for fortification are oil, margarine, and other hydrogenated oil products, and packaging in opaque containers is critical to protect from degradation.[305] Oil stabilizes retinol and delays its oxidation, and facilitates the absorption of vitamin A.[310] Also in the 1920's, Denmark initiated vitamin A fortification of margarine to imitate the nutritional value of butter.[311] In Guatemala, a sugar fortification program with retinyl palmitate has been running since 1974, and it has virtually eliminated vitamin A deficiency as a public health problem.[312,313] Sugar is a successful vehicle for fortification in middle-income countries/regions where sugar consumption is high, whereas in regions and countries with low-income, national fortification programs of cooking oils are implemented.[305]

2.4.2 Iron fortification

Iron is the most complex micronutrient to fortify, because iron compounds that have the highest bioavailability are water soluble (Chapter 2.4.3) and lead to undesirable organoleptic changes in taste, color and texture of the food vehicle. The overall objective when selecting an iron compound for fortification is to maintain the greatest bioavailability and at the same time it should not cause any sensory changes.[314] It is challenging to demonstrate the cost-effectiveness of iron fortification programs, as brain development, poor pregnancy outcomes, decreased cognitive performance and anemia all have various etiologies and the hypothetical effect size on such outcomes of a fortification program is likely to be small.

2.4.2.1 Organoleptic changes in iron fortified foods

The most common sensory problems caused by iron fortificants is increased rancidity and color changes. Iron has a strong oxidative capacity and accelerates oxidation of unsaturated lipids in iron fortified foods.[315] Rancidity diminishes the quality of a food product in terms of shelf life, taste, odor, appearance, and nutritional value.[316]

The color change is usually a green or bluish discoloration in cereals, while chocolate and cocoa products can turn greyish, and table salt becomes yellow or red/brown. These sensory changes are not always predictable and vary depending on storage time and condition (humidity), the water content, composition of the food, and the iron compound.[317] The mechanism how iron induces off-colors is not well understood and may vary between foods. The oxidation of PP leads to browning,[318] and iron is able to react with PP containing ortho-dihydroxyl groups (e.g. gallic acid, chlorogenic acid, caffeic acid and catechin).[319]

2.4.2.2 Iron fortified food vehicles from cereals to salt

The first iron fortified foods were cereals, fish sauce and sugar.[320-322] The major proportion of iron fortified food nowadays is cereal flours, but can be high in phytate.[305] Different strategies for each individual country are needed, as differences in climate, wheat flour quality, processing methods, storage conditions, as well as different use of flour, influence the stability and bioavailability of wheat flour and iron. Ferrous sulfate is the compound of first choice for wheat flours with a fast turnover, or for wheat flour pasta. Ferrous sulfate as well as ferrous fumarate, cause rancidity during long-term storage.[317] The SUSTAIN Task Force recommends the use of ferrous sulfate whenever possible, followed by ferrous fumarate and electrolytic

iron (but in double iron concentration).[323] NaFeEDTA is the preferable compound in high-phytate flours, but it is the most expensive compound and it might interfere with the bread fermentation process,[317,324,325] and its ADI is limited to 1.9 mg/kg body weight per day.[326] **Table 6** gives an overview on the WHO recommendations on nutrient concentrations and iron compounds to be added to wheat flour depending on their extraction, and flour availability.[327] Evidence of the effectiveness of iron fortification programs are not consistent. A systematic review including thirteen wheat flour fortification studies, concluded that flour fortification has little effect on reducing anemia prevalence, whereas evidence is consistent in reducing the prevalence of low ferritin.[328] Another large systematic review on 60 iron fortification trials, concluded an improvement in hemoglobin, ferritin and iron nutrition after consuming iron fortified foods.[329] However, the most recent Cochrane review including nine randomized controlled trials, concluded that wheat flour fortification may have little or no effect on anemia and contributes little or not to reduce iron deficiency.[330]

Table 6: Overview of the WHO recommendation on iron fortification of wheat flour, considering the iron compound, wheat flour extraction, and the estimated average flour availability per capita. Taken from WHO (2019).[327]

Flour extraction	Compound	Fe concentration added (ppm) to wheat flour by estimated average availability per capita (g/day)[a]			
		<75[b] g/day	75-149 g/day	150-300 g/day	>300 g/day
Low	NaFeEDTA	40	40	20	15
	Ferrous sulfate	60	60	30	20
	Ferrous fumarate	60	60	30	20
	Electrolytic iron	NR[c]	NR	60	40
High	NaFeEDTA	40	40	60	40

[a]Considering only wheat flour as main fortification vehicle in a public health program.
[b]Estimated per capita availability of <75 g/day does not allow sufficient level of iron to cover the needs for women of childbearing age, other fortified food vehicles and interventions should be considered.
[c]NR = Not recommended, high levels of electrolytic iron negatively affects sensory properties of wheat flour.

Salt is an inexpensive ideal food vehicle to fortify, first attempts for double-fortified salt (DFS) started in 1969, and it took decades to develop a stable form combining iodine and iron.[331] The current formulation contains encapsulated ferrous fumarate. The granulation process consists of fluidizied ferrous fumarate, which is sprayed to salt sized particles with a granulation solution containing hydroxy propyl methyl cellulose (HPMC), sodium hexametaphosphate, and titanium dioxide (TiO2) to mask the color. These granules are then

coated with a suspension of titanium oxide and soy stearine to form microencapsulated ferrous fumarate.[332] The use of encapsulated ferrous fumarate does not change the organoleptic properties of the salt and food.[332] In 2014, the Food Safety Standards Authority of India approved this DFS formulation and the Nutrition Initiative recommends to include DFS in all programs addressing dual deficiency of iron and iodine.[332] A systematic review and meta-analysis on double fortified salt (DFS) reported a significant increase in hemoglobin concentrations, and reduced risk ratios (RR) for anemia and IDA using data of twelve efficacy studies. However, data of two effectiveness studies of DFS usage in India reported a low coverage of DFS among households.[333]

2.4.3 Iron compounds for food fortification

A variety of iron compounds are available, however, they differ in their solubility, bioavailability and potential to develop undesirable sensory changes.[314] They can be divided into three groups: 1) water soluble; 2) poorly water soluble but soluble in dilute acid; and 3) water insoluble and poorly soluble in dilute acid.[119,317] To choose the optimal iron compound for a fortification, the following aspects have to be considered: the relative bioavailability (RBV) of an iron compound (assessed in relation to ferrous sulfate); the effect on sensory changes to the food vehicle; and the costs of a compound. An overview of these compounds, in terms of RBV and costs are given in **Table 7**.

2.4.3.1 Water soluble iron compounds

Water soluble iron compounds are highly soluble in gastric juice, therefore, they have the highest RBV of all iron fortificants. Unfortunately these compounds also possess the ability to lead to adverse organoleptic changes of the foods, especially color and flavor changes.[317] During storage these iron compounds can cause oxidation of fatty acids and vitamins, leading to rancidity and degradation of vitamins.[317] In bouillon cubes and fruit drinks soluble iron compounds can cause a metallic taste, whereas in soy sauce, fish sauce and tea infusions it may cause precipitates. In cocoa, infant cereals, salt and tortillas they cause unacceptable color changes.[119] Therefore, these water soluble iron compounds are suitable for dry food vehicles with a fast turnover.[317] Ferrous sulfate is used to fortify bread, infant formula and pasta, and wheat flour.[119] Sodium iron EDTA has the advantage that it prevents iron to bind to iron absorption inhibitors (mainly phytic acid). The iron absorption from NaFeEDTA can be two- to threefold higher than from ferrous sulfate.[334] Ferrous bisglycinate is a commercially

developed product, it has been claimed to protect iron from dietary inhibitors, and a fourfold iron absorption in humans.[335]

Table 7: Iron content, Relative bioavailability and cost of iron compounds used in food fortification. Taken from WHO (2006).[317]

Compound	Iron content (%)	Relative bioavailability[a]	Relative cost[b] (per mg iron)
Water soluble			
Ferrous sulfate $7H_2O$	20	100	1.0
Ferrous sulfate, dried	33	100	1.0
Ferrous gluconate	12	89	6.7
Ferrous lactate	19	67	7.5
Ferrous bisglycinate	20	>100[c]	17.6
Ferric ammonium citrate	17	51	4.4
Sodium iron EDTA	13	>100[c]	16.7
Poorly water soluble, soluble in dilute acid			
Ferrous fumarate	33	100	2.2
Ferrous succinate	33	92	9.7
Ferric saccharate	10	74	8.1
Water insoluble, poorly soluble in dilute acid			
Ferric orthophosphate	29	25-32	4.0
Ferric pyrophosphate	25	21-74	4.7
Elemental iron	-	-	-
H-reduced	96	13-148[d]	0.5
Atomized	96	(24)	0.4
CO-reduced	97	(12-32)	<1.0
Electrolytic	97	75	0.8
Carbonyl	99	5-20	2.2
Encapsulated forms			
Ferrous sulfate	16	100	10.8
Ferrous fumarate	16	100	17.4

EDTA, ethylenediamineteraacetate; H-reduced, hydrogen reduced; CO-reduced, carbon monoxide reduced.

[a]Relative to hydrated ferrous sulfate (FeSO4.7H2O), in adult humans. Values in parenthesis are derived from studies in rats.

[b]Relative to dried ferrous sulfate. Per mg of iron, the cost of hydrated and dry ferrous sulfate is similar.

[c]Absorption is two-three times better than that from ferrous sulfate if the phytate content of food vehicle is high.

[d]The high value refers to a very small particle size which has only been used in experimental studies.

2.4.3.2 Poorly water soluble iron compounds, soluble in diluted acid

These compounds cause less organoleptic changes, and they are reasonably absorbed, depending on the gastric acid production in healthy adults and adolescents.[119,317] Infants may secrete less acid, which makes these compounds less suitable for the fortification of infant food products.[317] Ferrous fumarate has the advantage to cause fewer sensory changes, however, it is less bioavailable from bread meals in infants, than ferrous sulfate.[336]

2.4.3.3 Water insoluble iron compounds, poorly soluble in diluted acid

These compounds are the least well absorbed, however, these are widely used in the food industry as they cause less organoleptic changes of the foods. Their absorption is difficult to predict and depends on size, shape and surface area of the particle, meal composition and food processing.[119,337,338] This group of compounds can be divided into two subgroups, the iron phosphate compounds and the elemental iron powders.[119] Ferric phosphate, orthophosphate and pyrophosphate have a modest relative iron bioavailability, and are used to fortify rice, infant cereals and chocolate drink powders.[317] By reducing the particle size of ferric pyrophosphate, its solubility and bioavailability increases.[339] Elemental iron powders are widely used to fortify cereals. Their bioavailability is strongly dependent on the manufacturing process, which characterizes size, shape and particle surface area, and even between batches there may be different dissolution characteristics.[119]

2.4.4 Encapsulation of iron

Encapsulation is defined as the process of entrapping active agents into particles.[340] These alternative delivery systems include water insoluble iron compounds as micronized particles or colloids. An overview of these delivery systems and their characteristics is given in **Table 8** and the following two chapters. It is important to find a good balance between decreasing the sensory off flavors and increasing the bioavailability of iron from fortified foods. An illustration of these conflicting requirements is given in **Figure 10**. Ideally, with the encapsulation approach one would operate in the top right corner of this graph. To produce a functioning and applicable delivery system, several requirements need to be fulfilled.

Safety of all ingredients needs to be ensured, as people from different age groups will eat the fortified food.[341]

Secondly, the delivery system needs to be robust under a variety of circumstances, such as during production, storage, transport and utilization. These are changes in temperature (freezing, chilling, cooking, pasteurization), changes in pH, light and oxygen exposure, interactions with other food components and mechanical impacts.[341] However, these delivery systems need to dissolve rapidly in the digestive system, in order to release the encapsulated iron for absorption.[342]

Another important characteristic of the delivery system is the compatibility with the food matrix. It should not alter appearance, flavor, smell, texture, or mouthfeel of the food item.[341]

The last requirement for a successful system is that it needs to be economically feasible. Otherwise producers will hesitate to implement it into their end product, and it needs to be affordable to the target population.[341]

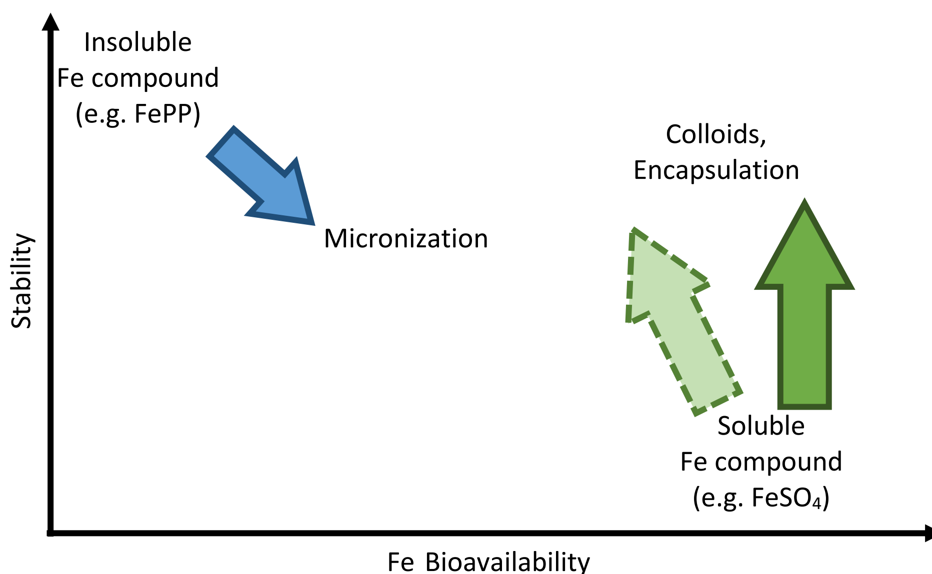

Figure 10 Iron bioavailability and stability of iron compounds in food products are conflicting partners. Adapted from Zuidam (2012).[343]

2.4.4.1 Technologies and approaches in iron delivery systems

Spray-drying is the atomization of dispersion of an iron containing carrier solution in a hot chamber, which results in a powder after water evaporation. It operates at a high throughput, is inexpensive, and therefore, a popular encapsulation technique.[341] However, spray-drying of ferrous fumarate with HPMC, TiO_2, could only partially prevent iodine degradation when stored in dual fortified salt.[344]

Table 8: Characteristics of various iron delivery systems suitable for food fortification. Taken from Zuidam (2012)[343]

Technology	Load (%)	Particle size (µm)	Water-resistant	Estimated price range
Spray-drying	10-50	10-400	No	Low
Spray-cooling	10-50	5-200	Yes	Low
Fluid bed coating	10-50	5-5000	Yes	Middle
Extrusion	5-40	200-8000	Yes, if modified	Middle
Modified microspheres	20-40	1-800	Yes, if modified	High
Liposomes	0.5-8	0.03-7	Yes	Middle
Aerosol solvent extraction	10-60	0.1-25	Yes	Middle
Colloidal systems	various	<1	Yes	Middle/High

The % of load is not the same as the % of iron, encapsulated iron is in the form of iron compounds.

Spray-cooling is comparable to spray drying, except that instead of water evaporation lipid crystallization is used. A dispersion of iron in molten lipids is atomized into a chilled air chamber, and after solidification of the lipids, fine particles remain. Lipids with melting points between 40°C and 70°C are used, such as hydrogenated plant oils.[341] Spray-cooled ferric pyrophosphate with hydrogenated palm oil containing 1% lecithin has a lower relative bioavailability (43%) than non-encapsulated ferric pyrophosphate.[345] Co-encapsulation of ferric pyrophosphate with potassium iodine and retinylpalmitate, has lead to a 40% and 30% degradation of iodine and retinylpalmitate.[346,347] Another approach was made with ferrous sulfate and potassium iodate in hydrogenated palm oil with 1% lecithin, which also lead to a 25% degradation of iodine.[348]

In fluid bed coating an iron powder is lifted by an air stream while a coating is applied. Suitable coatings are: lipids, maltodextrin or ethylcellulose. The water soluble coatings (maltodextrin) are less suitable as they are moisture sensitive.[341] With this method, ferrous sulfate can be coated with three fat layers made from stearic acid (1st and 3rd layer) and palm oil (2nd layer). A triple coating leads to a higher leakage temperature compared to a single coating.[349] Using fluid bed coating to form granules containing ferrous fumarate, HPMC, TiO_2 and sodium hecametaphosphate (SHMP), and subsequent spraying of soy stearine and TiO_2, has led to no ferrous oxidation and low dissolution of iron at pH 4.[343,350]

Extrusion is a thermomechanical process, in which a melt is pressed through a horizontal tunnel with double or twin counter-rotating screws at elevated temperatures, and is then

pressed through one or more holes.[341] One approach was being made using denatured proteins to encapsulate iron particles by extrusion, using calcium stearate, sodium caseinate and TiO_2. To make this encapsulate partially water soluble, maltodextrin can be added to the caseinate.[351] To produce larger particles similar to salt crystals or rice grains, cold (40°C) or hot extrusion (80-98°C), using a binder material (e.g. durum wheat flour), these extrudates could also further be coated using HPMC, soy stearine or other hydrophobic polymers.[352,353]

Microspheres consist of a biopolymer network and are usually made from calcium-alginate, gelatin, agarose, agar, or potassium-κ-carrageenan. Methods used are extrusion, dropping droplets into a gelling bath or via emulsion.[340] These microbeads are usually very porous and are less able to protect the food product from the water soluble iron compounds.[341] Approaches are being made using iron lactate in calcium-alginate microsphere prepared by phase inversion.[354] Another approach is made combining an iron-alginate core with a calcium alginate outer layer. These microspheres are claimed to be stable during storage and release iron at pH 2.[355] Ferrous sulfate can be encapsulated using sodium alginate, however, these particles are not stable in water.[356]

Liposomes are vesicles enclosed by a bilayer membrane made of phospholipids or cholesterol.[341] The usage of liposomes in foods is limited due to instability during storage, low encapsulation yield, leakage of water soluble agents, and relatively high costs. Nevertheless, some iron-liposomes are commercially available and could be used to fortify dairy products. For example, Biofer™ by Lipotech S.A. (Argentina) use ferrous sulfate in soy lecithin liposomes,[357] and has been used to fortify milk and yoghurt.[341] LipoFer® from Lipofoods S.L. (Spain) contains ferric pyrophosphate within liposomes, however, reported results on *in vivo* bioavailability are equivocal.[358,359]

Colloids of ferric pyrophosphate are made by salt precipitation. Sunactive® (Taiyo Kagaku, Japan) is the commercially available product and contains iron pyrophosphate colloidal particles stabilized by enzymatically modified lecithin. Despite their high costs, they are a good bioaccessible iron source as they dissolve well at $pH<3$.[341]

Nanoscale delivery systems have the potential to deliver lipophilic bioactive agents, such as nutrients, nutraceuticals and vitamins. They can be formulated as microemulsions, liposomes, nanoemulsions, emulsions, multiple emulsions, microclusters, colloidosomes, microgels, filled microgels, and biopolymer nanoparticles. However, they must be carefully formulated to

ensure safety, economic viability, and effectiveness to be implemented within the food industry.[341]

The development and evaluation of a novel delivery system of the highly bioavailable ferrous sulfate in a pH-sensitive polymer is described in Manuscript 3 of this thesis.

3 Iron overload

Systemic iron overload can be divided into three groups: 1) inherited causes of iron overload; 2) various causes of secondary iron overload; and 3) a small group of miscellaneous disorders.[360]

Firstly, inherited causes of iron overload, described as hereditary hemochromatosis, occur through to two distinct mechanisms: hepcidin deficiency and ferroportin disease.[361] Hepcidin deficiency includes the HH types 1 (also referred as *HFE*-related hemochromatosis), 2A, 2B (type 2 is also referred as juvenile hereditary hemochromatosis) and 3 (also referred as *TfR2*-related hereditary hemochromatosis), which implicate mutations in the genes: *HFE*, *HJV*, *HAMP* and *TfR2*, respectively. The HH type 4 (also referred as ferroportin-related iron overload) is caused by a ferroportin disease, which has two mechanisms. Mutations in the *SLC40A1* gene cause losses of the ferroportin activity,[362] or a resistance to hepcidin is developed by mutations in ferroportin.[363] The *HFE*-related hemochromatosis is the most prevalent form of iron overload (85-90%), mainly caused by homozygosity in the C282Y mutation of the *HFE* gene, a minority of *HFE*-related hemochromatosis are heterozygotes in C282Y in combination with a H63D or S65C mutation.[360] The other 10-15% are non-*HFE* related and include mutations in the genes named above.[364] Within the scope of this thesis, the main focus will be given on homozygous mutation of the C282Y, which will be topic of this Chapter and Manuscript 4.

The second group of systemic iron overload suffers from excessive iron absorption underlying other metabolic defects than *HFE*, and therefore, non-*HFE* hemochromatosis.[365] These are mainly characterized by ineffective erythropoiesis – the so-called iron-loading anemias, by parenteral iron overload, and chronic liver disease.[360] Iron-loading anemias are: thalassemia syndromes, sideroblastic anemia, chronic hemolytic anemia, pyruvate kinase deficiency and pyriodoxine -responsive anemia.[20] Parenteral iron overload is always iatrogenic, due to transfusional iron overload or parenteral iron injections.[360] Chronic liver diseases causing iron overload are: porphyria cutanea tarda, hepatitis (B and C), alcoholic liver disease, nonalcoholic fatty liver disease and following a portocaval shunt.[20]

The third group includes neonatal iron overload, which is a form of congenital alloiumune hepatitis followed by iron deposition.[366] Aceruloplasminemia is caused by homozygous mutations in the ceruloplasmin gene, and characterized by low plasma iron and transferrin

saturation despite systemic iron overload.[367] Mutations in *TF* cause congenital atransferrinemia, which is characterized by decreased plasma transferrin, increased non-transferrin bound iron, and decreased hepcidin expression. All these contribute to the development of iron overload.[367] And lastly, iron overload may also be due to mutations in *DMT1* which affects duodenal iron absorption and intracellular iron trafficking causing anemia despite iron overload.[367]

3.1 *HFE*-related hereditary hemochromatosis

It was first described in the mid-1800s by the French physician Armand Trousseau from an autopsy of a patient with a “bronze-like appearance”, with a “granular grayish-yellow liver”.[368] By the end of the 19th century the term hemochromatosis was used to describe the bronze-colored organs and tissues.[369] It wasn’t until 1935 when Joseph Sheldon suggested that this disease was hereditary and was probably caused by excessive iron tissue deposition.[370] In 1950s, bloodletting was introduced as a treatment for hemochromatosis.[371] Simon et al.[372] showed in the 1970s and 80s that it is an autosomal recessive disorder, which is linked to the HLA-A on the short arm region of chromosome 6.[372] Finally, in 1996 Federer et al.[373] identified the mutated gene *HFE*.[373]

3.1.1 Prevalence, origin, and etiology of *HFE*-related hemochromatosis

Most cases of hereditary hemochromatosis are caused by a SNP in the *HFE* gene, which encodes the hemochromatosis protein. Within the *HFE*-related HH, also called HH type 1, the p.Cys282Tyr or C282Y mutation is the most prevalent, with a 1:200-300 prevalence of homozygosity among Caucasians, and a gradient from North-to-South and West-to-East in Europe.[374,375] It is much less prevalent among Hispanic, Asian American, Pacific Islander and persons of African origin.[376] Within Europe, the highest allele frequency is in Ireland (14.2%)[377] followed by western France (9.4%)[378] and Wales (8.3%),[379] with lowest in Hungary (3.4%)[380] in the east and Italy (1.6%)[381] in the south. In Asian, Indian subcontinent, African, Middle Eastern and Australasian populations (including Aboriginal and Vanuatuan Australians and Papuans) the C282Y homozygosity is not found and the prevalence of heterozygosity is very low (0-0.5%).[286]

The spread of the C282Y mutation within Europe suggests a Celtic origin, but also elevated frequencies are observed in Scandinavia.[382] A Celtic origin would also explain the distribution

of HH in some regions where migration from Europe happened over the past 400 years, North America, Australia and New Zealand.[296] In 2010, Olson et al.[383] rejected the hypothesis of an Irish origin of the C282Y mutation due to the equivalent haplotype diversity in Sweden and Ireland. They suggested that the origin of this mutation may even be older and had spread in Europe by earlier seafarers.[383] As this genetic mutation does not affect reproduction and may even be advantageous against iron deficiency or pathogen infections, it had spread throughout populations.[384]

Other polymorphisms causing HFE-related HH are the H63D and S65C polymorphisms. The H63D polymorphism has an average allele frequency of 14%, however, its clinical impact is low, and is considered a 'minor' HFE polymorphism. A meta-analysis including cross-sectional and case control studies in hemochromatosis patients of European ancestry reported that 80.6% of all cases are homozygous for the C282Y polymorphism, and 5.3 % were compound heterozygous for C282Y and H63D. In the control group the C282Y/C282Y frequency was 0.6% and 1.3% for the C282Y/H63D polymorphism. The S65C HFE polymorphism is also associated with iron overload when in combination with the C282Y mutation. This polymorphism is highest in Brittany France and has an allele frequency of ~0.5%.[375]

3.1.2 Symptoms, diagnosis, and disease prevention

Free iron alters between Fe^{3+} and Fe^{2+}, which results in the gain or loss of free electrons and contributes to the formation of reactive oxygen species. These radicals are able to cause damage on lipid membranes, DNA or other cellular organelles.[4]

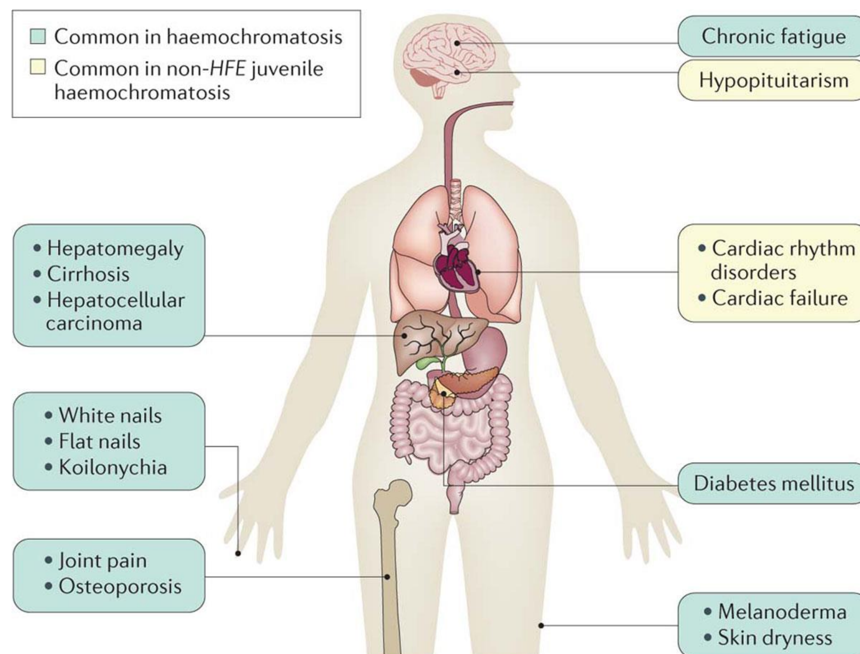

Figure 11: Symptoms of hemochromatosis. Taken from Brissot et al. (2018).[367]

At the stage of increased transferrin saturation (>45%, as in patients with *HFE* hemochromatosis) [385] NTBI is formed, mostly bound to low molecular weight molecules, such as citrate and acetate.[386] A type of NTBI – labile iron – is involved in the production of ROS, and excess of cellular iron alters mitochondrial function in the liver.[387,388] Cellular NTBI uptake occurs despite iron overload,[389] is independent on TFR1,[390] and involves the zinc transporter ZIP14 for iron uptake into the hepatocytes and the ZIP8 for iron uptake into the cardiomyocytes.[87,88] Therefore, hepatocytes, cells of the pancreas and heart are prone to take up NTBI,[391,392] which explains the most common complications of hemochromatosis affecting: the liver (fibrosis, cirrhosis, liver failure, and hepatocellular carcinoma); the heart (congestive cardiac failure and cardiac arrhythmias); and the pancreas (diabetes mellitus).[20] Other complications are: fatigue, malaise, lethargy, weakness, weight loss, joint problems, increased pigmentation, porphyria cutanea tarda, hypothyroidism, hypogonadism, amenorrhea, and the disruption of the hypothalamic-pituitary axis (**Figure 11**).[20] These clinical symptoms related to *HFE*-hemochromatosis usually occur in middle aged patients.[23] However, in the modern-day clinical practices, these symptoms are rarely seen due to screening and awareness of the disease among clinicians and early diagnosis.[384]

In terms of biomarkers for iron overload, TS is the earliest biochemical marker to indicate an iron overload (>45%).[367] Elevated TS may also be a biomarker for malnutrition, protein malabsorption, proteinuria, and hepatocellular failure.[393] Furthermore, in ferroportin disease or hereditary aceruloplasminaemia TS may be normal or even low.[367] Increased ferritin (≥300 µg/L in men and ≥200 µg/L in women) may also not solely indicate iron overload. Other mechanisms for increased ferritin that need to be ruled out are: metabolic syndrome, chronic alcoholism, inflammation and marked cytolysis and excessive iron supplementation.[367]

To diagnose hemochromatosis, the combination of biological and imaging findings replaces the need of a liver biopsy. Imaging using x-ray of the joints can be used to detect chondrocalcinosis or subchondral arthropathy, and a liver MRI can be used to confirm iron excess.[367] Confirming genetic iron overload is simple for *HFE*-related hemochromatosis caused by homozygosity in the C282Y variant. The confirmation of non-*HFE* hemochromatosis requires further genetic testing, such as next-generation sequencing.[367]

Once diagnosed, there is a five-grade classification to assess the body iron excess and extent of organ damage in hemochromatosis related to hepcidin deficiency (not including the ferroportin disease). It is based on the presence/absence of increased TS, increased serum ferritin, impaired quality of life, and sings for an impaired life expectancy.[394]

As soon as an individual has been diagnosed with hemochromatosis, a family screening is recommended to actively prevent the penetrance of the disease. The screening is based on genetic testing. Patients with heterozygosity, are not at risk for developing hemochromatosis, but may pass it on to their offspring. Hemochromatosis type 4 (ferroportin disease) is a dominant genetic disorder, therefore, heterozygous individuals are at risk of developing the disease. Targeted family members for screening are the siblings of an HH patient, and in case of *HFE*-related hemochromatosis also the screening of the offspring is justified due to the high prevalence of the C282Y mutation in the Caucasian population.[367] A conclusion from the HEIRS study, was that population based *HFE* genotyping is not recommended before phenotyping.[395] The implementation of a population-based screening of phenotypes in routine practice might involve the measurement of transferrin saturation in young adults and around 5 years after menopause.[367]

3.3 Clinical management of hereditary hemochromatosis

Iron removal

Phlebotomy – or bloodletting is the standard treatment for all types of hemochromatosis that are associated with hepcidin deficiency.[396] Up to now, there are no evidence-based guidelines about when it should be started, its frequency and therapeutic end point. However, its initiation before the development of cirrhosis and/or diabetes, the morbidity and mortality of HH patients is significantly reduced.[397,398] The aim of the induction phase is to introduce the patient into a mildly iron-deficient stage, which is usually done in the weekly removal of 1 unit of blood (400-500 ml, equivalent to 200-250 mg Fe). In the maintenance phase, a yearly removal of 2 to 4 units of blood are usually sufficient to maintain a serum ferritin concentration between 50 and 100 µg/L. Lower serum ferritin levels should be avoided, as they are associated with an increased hepcidin suppression and increased iron absorption.[384] Patients with HH should never stop monitoring their iron status, and plan their lifelong treatment accordingly regarding their iron status, general condition, and age.[399] This treatment is considered as a simple, safe- and cost-effective.[400] But for some patients it is also perceived as inconvenient due to its effects (problems with needles, tiredness, fainting, and loss of appetite).[401]

Erythrocytapheresis, is the separation of erythrocytes from the blood of a patient and the remaining is returned into circulation. This can also be used to treat hemochromatosis patients,[402] however, it is more expensive and less available than phlebotomy.[367]

Whenever possible, the blood taken from HH patients should be available for transfusion. However, due to abnormal liver functions, diabetes, medication use and legal aspects, they are usually rejected as blood donors.[384] Whether patients with HH are eligible as blood donors depends on the countries policy. For example, Switzerland accepts asymptomatic carriers with normal iron as blood donors, whereas in Portugal they are not accepted.[403]

Iron chelation therapy

Iron chelation therapies are usually applied to threat iron overload related to chronic anemia, special cases of HH, when the efficacy cannot be achieved with phlebotomies, or when they are impossible due to poor vein conditions.[399] However, the use of deferoxamine, administered by either subcutaneous injection or intravenously, is inconvenient and patients often non-compliant due to adverse effects. Deferiprone and deferasirox are oral iron

chelators, whereby the latter one is favored due to the lower dosing requirement and favorable adverse effect profiles.[404]

Other therapies

Hepcidin-based therapies might become an adjunct treatment of HH to phlebotomy. The possible use of mini-hepcidin and derivatives would decrease serum iron and TS and therefore, diminish tissue iron overload.[399] Proton pumps inhibitors (PPIs) have shown to decrease intestinal iron absorption, as serum iron response was 50% reduced when PPIs were administered before or after the intake of a test meal.[405] Further, a daily treatment with PPIs reduced the yearly phlebotomy requirements by 1.33 units in C282Y homozygous HH patients.[406] As previously mentioned in Chapter 3.1.1, a nonrandomized intervention study where black tea was consumed with main meals reported a nonsignificant ~50% reduced iron accumulation over a period of one year.[407] The potential benefit of the inhibitory effect of plant PP will be thoroughly discussed in the following Chapter 3.4.2.

3.4 Disease penetrance and dietary factors

Although the prevalence of C282Y homozygosity is high among Caucasian populations, only a minority of these develop the disease and accumulate excess iron. It is estimated that 25-60% of the C282Y homozygous individuals develop hemochromatosis.[408,409] Modifying genes and polymorphisms have also been described, such as the SNP rs884409 in the *CYBRD1* gene,[410] the polymorphism p.D519G in *GNPAT*.[411] Further polymorphisms in *HAMP*, *HJV*, *TFR2*, *SLC40A1*, *TMPRSS6*, and BMP6 have also been described.[367] Penetrance of the disease is usually higher in males than females, most likely due to menstruation, pregnancy, and lactation. In addition, the hormone testosterone has an inhibitory effect on hepcidin transcription.[43-45] Another important host factor on the penetrance of hemochromatosis is heavy alcohol abuse. Patients with hemochromatosis who drink >60 g alcohol per day are nine-times more likely to develop liver cirrhosis than those who drink <60 g per day.[412]

Whether dietary iron intake contributes to the penetrance of hemochromatosis is not fully clear and equivocal results have been reported in literature. A systematic review by Moretti et al.[413] summarizes that, despite limited evidence, a dietary modulation may reduce the speed of re-accumulation of iron in HH patients who are in the maintenance phase and undergo regular phlebotomy.[413]

Iron absorption studies in HH subjects have reported a 2-10 times higher iron bioavailability of iron from test meals than in non-HH individuals.[413,414] Further, iron bioavailability in HH individuals is high relative to their iron stores, in particular for heme iron,[415] and at high iron stores (>300 µg SF/L) dietary iron bioavailability stabilizes at around 15-35%, which is comparable to non-HH iron deficient subjects.[413,414,416] Like in non-HH subjects, iron bioavailability is affected by the food matrix. Two studies in idiopathic hereditary hemochromatosis subjects reported an increase in iron absorption from iron given without a food matrix than when given with a food matrix. Bezwoda et al.[416] report an iron absorption of 74.2% from iron given with ascorbate with no food matrix (74.2%) compared to 36.4% and 37.1% when iron was given with a whole wheat flour meal or a lamb meat meal.[416] A subsequent iron absorption study by Bezwoda et al.[417] reported a 20-25% iron absorption from a maize porridge meal (given with ascorbic acid) and 72% iron absorption from iron given with water and ascorbic acid.[417]

Two longitudinal studies in HH subjects describe the effect of iron fortified wheat flour,[418] and regular tea drinking with main meals[407] on serum ferritin increase and period length between phlebotomies. In 1995, Sweden had withdrawn their iron fortification program of wheat flour. A cohort of HH subjects were followed over a time period of 4 years before and after the fortification program had stopped. The study reported a difference of net 0.65 mg absorbed Fe per day during the fortification period compared to without iron fortified wheat flour, which would correspond to 240 mg Fe per year or ~0.5 L of blood to be phlebotomized.[413,418] Further, the interval between phlebotomy was prolonged from an average of 59 (±15) days to 69 (±17) days.[418] Regular black tea drinking with main meals over a period of one year has led to a smaller increase in serum ferritin compared to a control group who was allowed any drink with their meals.[407] After the intervention period, the tea-drinking group had ~400 mg less accumulated iron (approximately 50% less compared to the control group), which would correspond to ~0.7 L of phlebotomized blood.[407,413]

Cross-sectional studies have reported equivocal results. Two studies from the UK[419,420] and Netherlands[421] reported an association between heme iron intake and iron status (ferritin). The association between heme iron intake and serum ferritin was twice as strong among C282Y homozygous women than wild types, which indicates a significant gene-diet interaction.[419,420] A higher heme iron intake was associated with significantly higher serum

ferritin, particularly in postmenopausal women, homozygous in C282Y, or compound heterozygous in C282Y and H63D mutation consuming relatively high amounts of heme iron.[421] In contrast to these studies Gordeuk et al.[422] did not find a significant relationship of serum ferritin with dietary heme content, non-heme iron content, and the intake of supplemental iron in individuals homozygous in C282Y.[422]

3.4.1 The effect of dietary bioactive components

Dietary bioactive compounds can influence intestinal transit, modify nutrient absorption and excretion, and possess detoxifying as well as antioxidant properties.[423] They further act as inhibitors or enhancers of dietary non-heme iron absorption (reviewed in Chapter 1.3.1).

Inhibitors of non-heme iron absorption (listed in Chapter 1.3.1.2) may be beneficial in reducing dietary iron absorption in patients with HH. Phytates have shown to alleviate the iron induced oxidative stress and liver injury in a mouse model of iron overload,[424] therefore, they may be beneficial in reducing iron bioavailability in HH patients predisposed with liver damage.[425] An epidemiological study of extrinsic factors on the phenotypic expression of HFE in Danish men concluded that alcohol and meat consumption has an elevating effect on serum iron and ferritin, whereas milk consumption has a lowering effect on serum ferritin, and blood donation has a lowering effect on iron status markers.[426]

Vitamin C is commonly known as an enhancer of non-heme iron absorption, however, the impact of vitamin C on iron status is unknown and evidence from long-term studies is limited and contradictory. Two studies in pre-school children and one study in young women with a plant-based diet reported a significant improvement of iron status after daily consumption of 164 mg vitamin C for 16 weeks.[427-429] In contrast to these studies, Garcia et al.[430] and Hunt et al.[431] showed no improvement in serum ferritin after a vitamin C supplementation period with either 1500m g/day for 5 weeks or 50 mg/day for 24 weeks, respectively, in young women with a typical western diet. A retrospective study in Australian men and women reported a significant protective effect of non-citrus fruit consumption on iron stores (as serum ferritin), independently on *HFE* genotype. Neither citrus fruit consumption nor vitamin C supplementation increased iron stores, but the consumption of 14 or more portions of non-citrus fruits per week correlated with a 20% reduction in serum ferritin.[432]

For vitamin A, a fat-soluble antioxidant, it is unconfirmed whether it enhances iron absorption or not.[433,434] However, experimental studies showed that vitamin A deficiency is related to the upregulation of hepatic *HAMP* expression in vivo and induces *FPN* expression in vitro.[435] [426] In agreement with that, serum levels of vitamin A positively correlated with iron status in humans.[436] In contrast, studies in HH have reported a vitamin A deficiency in these patients,[437,438] and after the induction phase of phlebotomy vitamin A serum levels have restored.[438] However, whether vitamin A supplementation may be beneficial in preventing iron overload in HH patients has not yet been reported.

Vitamin E is an important lipid-soluble antioxidant and protects lipid membranes from injuries mediated by free radicals.[439,440] In animal models, iron overload has been shown to reduce vitamin E levels,[441] and consistently HH patients have lover vitamin E levels than controls.[442,443] When HH patients reach the maintenance phase of phlebotomy, vitamin E levels were comparable to controls.[442] The positive effect of vitamin E supplementation in iron overload on the prevention of lipid peroxidation were successfully confirmed in animal models, and may also be beneficial for HH patients.[444,445]

The effect of PP on the inhibition of non-heme iron absorption, its antioxidative properties, influence on miRNA expression, and its potential positive health impact in patients with hereditary hemochromatosis is topic of Chapter 3.4.2.

3.4.2 Polyphenols and iron

Phenolic compounds are able to form complexes with proteins,[446,447] polysaccharides[448] and metallic ions.[449] They interfere with iron absorption making iron less available for absorption in the intestinal lumen.[138] Therefore, they may be beneficial in the management of hereditary hemochromatosis by attenuating iron accumulation and reducing the required numbers of phlebotomies. This chapter describes the nature of PP, their potential health benefits and interactions with iron.

3.4.2.1 Polyphenols – classification and structures

PP are secondary plant metabolites, characterized by the presence of large quantities of phenol structural units. They represent the most numerous group of substances and more than 8000 different structures have been identified in various plant species.[450] They are important for the plant's growth, and protection from herbivores and microbial infections.[451]

Further they are responsible for the attraction of pollinators and seed-dispersing animals, and serve as UV protectants.[452,453] The properties of PP are: 1) water soluble; 2) have a molecular mass ranging from 500 to 3000-4000 Da; 3) structures of a 1000 Da relative mass and possess around 12-16 phenolic groups and five to seven aromatic rings.[454] They can be classified into four main classes (**Figure 12**): phenolic acids (**Figure 13)**, flavonoids (**Figure 14**) , and the less common stilbenes (Figure 13**)** and lignans (Figure 13**)**.[455,456]

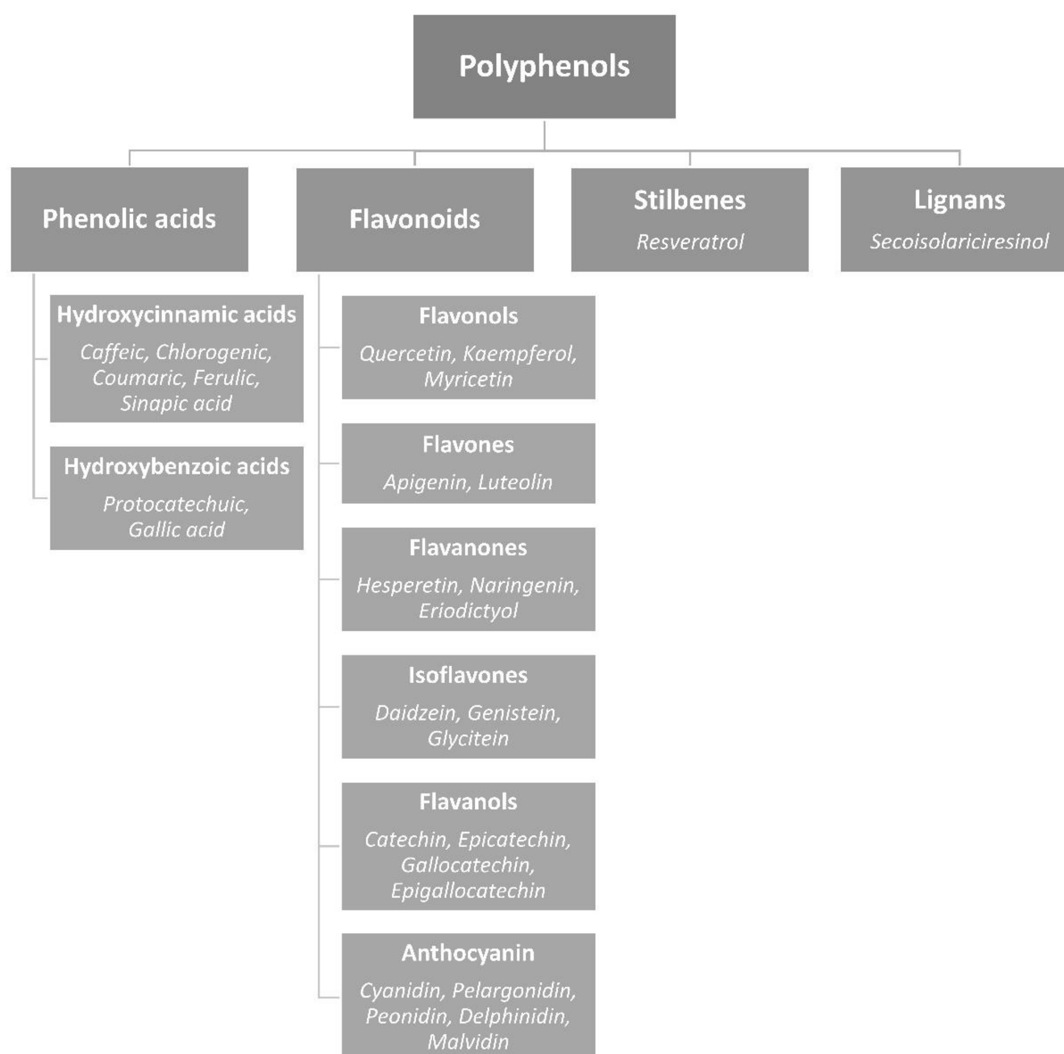

Figure 12: Classification of polyphenols based on phenol ring and their structural elements with some examples of polyphenol compounds. Adapted from Manach et al. (2004)[455] and Scalberg et al. (2000).[456]

Phenolic acids

Phenolic acids can be divided into two subclasses: 1) derivatives of hydroxybenzoic acid, and 2) derivatives of hydroxycinnamic acid (Figure 12 and Figure 13), with the latter group being more common in foods.[455] The content of hydroxybenzoic acids in foods is generally low, and is mainly present in red fruits, black radish and onions.[455] Gallic acid is an important source of PP in tea, which contains up to 4.5 g/kg fresh weight.[457] Hydrobenzoic acids are components of complex structures, and these esterified acids, such as gallotannins or ellagitannins, are present in mangoes, red fruits and berries.[458] Hydrocinnamic acids consist of *p*-coumaric, caffeic, ferulic and sinapic acids, and these are rarely in their free form, and mainly found as glycosylated derivatives or esters of quinic, shikimic and tartaric acid.[455] Caffeic and quinic acid form chlorogenic acid, which is the most abundant phenolic acid present in fruits and coffee. One cup of coffee (200 ml) contains 70-350 mg of chlorogenic acid.[455] Caffeic acid is mainly present in fruits and decreases during the course of ripening.[455] Ferulic acid is the most represented phenolic acid in cereal grains, wheat grains contain around 0.8 – 2 g/kg dry weight, which represents up to 90% of total PP.[459,460]

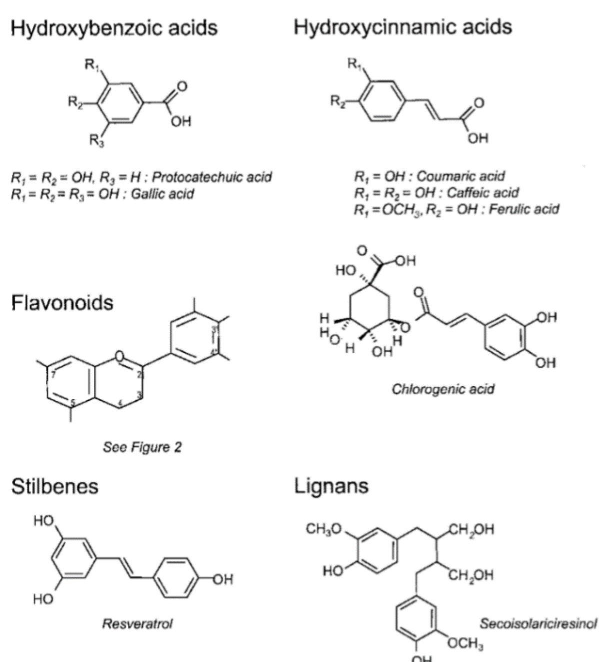

Figure 13: Chemical structures of polyphenol classes. Taken from Manach et al. (2004)[455]

Flavonoids

Flavonoids are the most abundant PP group in our diet.[456] This group is characterized by the basic chemical structure of two aromatic rings (ring A and B), which are bound together by a three-carbon chain which forms an oxygenated heterocycle (ring C) (Figure 14). Depending on their oxidation state, they are divided into six subclasses: flavonols, flavones, flavonones, isoflavones, flavanols, and anthocyanins (Figure 12 and Figure 14).[455,456]

The widest represented group with quercetin and kaempferol are flavanols, which are richest in onions, kale, leeks, broccoli and blueberry. Flavonols are often glycosylated to glucose or rhamnose. Those flavonol glycosides are present in red wine tea, fruits and vegetables.[455] As their biosynthesis is simulated by light, remarkable differences in concentrations exist between pieces of fruit from the same tree,[461] and even within a lettuce or cabbage, the outer layers may contain ≥10 times more glycosides than the inner layers.[462]

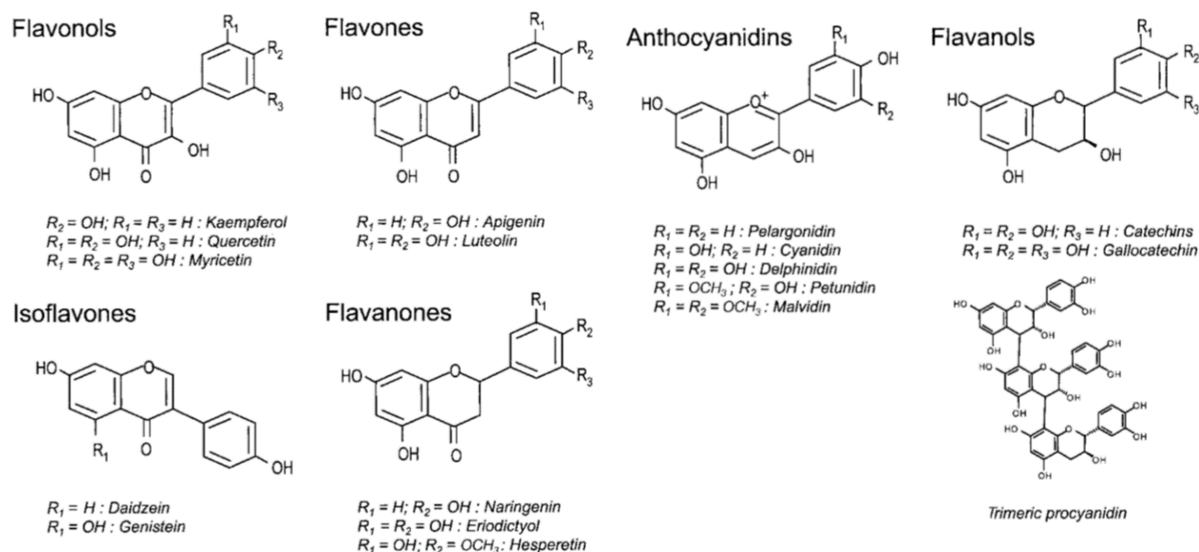

Figure 14: Chemical structures of the flavonoids subclasses. Adapted from Manach et al. (2004).[455]

Flavones are a lot less common, they are mainly represented as glycosides of luteolin and apigenin. Their main edible sources are parsley and celery,[455] whereas polymethoxylated flavones are mainly present in the skin of citrus fruits.[463]

Flavanones are mainly represented in citrus fruits, but also in tomatoes and aromatic plants such as mint. Especially the solid parts of citrus fruits have high contents of flavanones; naringenin is mainly found in grapefruits, hespertin in oranges and eriodictyol in lemons.[455]

Isoflavones are found almost exclusively in leguminous plants as genistein, daidzein and glycitein. Because of their structural similarity with estrogens, they are classified as phytoestrogens. They possess pseudohormonal properties, such as the ability to bind to estrogen receptors.[464] However, they are heat sensitive, during the production of soy milk they are hydrolyzed to glycosides,[465], or fermentation results in hydrolysis of glycosides to aglycones.[466,467]

Flavanols are mainly represented as catechins or as proanthocyanidins (also known as condensed tannins), which are dimers, oligomers or polymers of catechins.[455] Catechin and epicatechin are mainly occurring in fruits, while gallocatechin, epigallocatechin and epigallocatechin gallate are represented in seeds of leguminous plants, grapes, tea and chocolate.[468,469] In contrast to other classes of flavonoids, they occur in nature as aglycones, meaning they are not glycosylated in foods.[455] An infusion of green tea can contain up to 200 mg of catechins,[470] up to 30% of the plant's dry leaf is accounted to catechins.[471] During the fermentation to black tea these flavanols are condensed to theaflavins (dimers) and thearubigins (polymers).[455] Condensed tannins in grapes, peaches, kakis, apples pears, berries, wine, cider, tea or beer are responsible for their astringent character and for the bitter taste of chocolate,[472] which occurs through a complex formation with salivary proteins when consumed. Ripening of fruits often reduces their astringent character due to polymerization of tannins.[455]

The last group of flavonoids – the anthocyanins – are responsible for the red, blue, and purple colors of fruits, vegetables and flowers.[473] Cyanidin is the most common anthocyanidin. Their conent in food is proportional to its color intensity, and increases with ripening.[455]

Stilbenes

Stilbenes are composed of two phenyl moieties which are connected by a two-carbon methylene bridge (Figure 13), and their dietary occurrence is generally low.[450] The most prominent stilbene is resveratrol, present in grapes and red wine, which has received great

attention for its anticarcinogenic properties.[474,475] Health benefits of resveratrol, are topic of Chapter 3.3.2.3.

Lignans

Lignans are formed of two phenylpropane units (Figure 13). The richest dietary source of lignans are linseeds and they contain secoisolariciresinol and matairesinol. They are metabolized to enterolactone and enterodiol by the colonic microbiota, and are have agonists and antagonists of estrogen.[456,476]

3.4.2.2 Dietary sources and intake of polyphenols

PP are omnipresent in all plant foods: vegetables, cereals, legumes, fruits, nuts, spices, as well as in beverages: wine, cider, beer, cocoa, and fruit juices.[451] The analytical determination of polyphenolic concentrations is complex, due to their large variation in nature.

PP vary greatly between cultivars of the same species, environmental conditions, genetic factors, germination, degree of ripeness, processing and storage.[451,455,461,462] The online database Phenol-Explorer has a systematic collection of more than 60’000 content values found in more than 1300 scientific publications, and summarizes content values for 502 different PP in 452 different foods.[477-479] A list of the 100 richest dietary sources of PP was published by Pérez-Jiménez et al.,[480] and a summary of the top 30 and selected foods are given in **Table 9**. The complete list of ranking by PP concentration includes food items from the food groups: seasoning (n = 22), fruits (n = 20), seeds (n = 16), vegetables (n = 16), nonalcoholic beverages (n = 11), cereals (n = 10), cocoa products (n = 4), alcoholic beverages (n = 3) and oils (n = 2). The concentrations range from more than 15’000 mg per 100 g in cloves to 7.8 mg per 100 ml in rosé wine. When listed according to total PP content per serving, in the top 10 berries are largely represented. There are: eight fruits (seven berries and one other fruit: sweet cherry), one vegetable (globe artichoke heads) and one beverage (filtered coffee). Spices and herbs are very rich in PP, however, their importance in the diet diminishes due to their low serving sizes. They are not listed in this ranking due to the lack of available data for serving size. The following 10 ranks are occupied by three fruits and one fruit juice (plum, red raspberry and apple and its juice), two seeds (flaxseed and chestnut), dark chocolate, black and green tea and one cereal (whole grain rye bread).[480]

Table 9: Ranking of the 30 richest foods in polyphenol concentration including the ranking of coffee, tea, red wine, and black grape. The estimated serving size of the food item, the total polyphenol content per serving, and its ranking according to total polyphenol per serving. Table adapted from Pérez-Jiménez et al.[480]

Food	PP concentration [a] (mg/100 g or 100 ml)	Ranking of PP by concentration	Serving size [b] (g)	Total PP per serving [c]	Ranking of PP content per serving
Cloves	15188	1	-[d]	-	-
Peppermint, dried	11960	2	-	-	-
Star anise	5460	3	-	-	-
Cocoa powder	3448	4	3	103	24
Mexican oregano, dried	2319	5	-	-	-
Celery seed	2094	6	-	-	-
Black chokeberry	1756	7	145[e]	1595	2
Dark chocolate	1664	8	17	283	14
Flaxseed	1528[c]	9	20[f]	306[g]	13
Black elderberry	1359	10	145[e]	1956	1
Chestnut	1215	11	19	230	15
Common sage, dried	1207	12	-	-	-
Rosemary, dried	1018	13	-	-	-
Spearmint, dried	956	14	-	-	-
Common thyme, dried	878	15	-	-	-
Lowbush blueberry	836	16	145[e]	395	7
Blackcurrant	758	17	145[e]	1092	3
Capers	654	18	-	-	-
Black olive	569	19	15	85	28
Highbush blueberry	560	20	145[e]	806	4
Hazelnut	495	21	28[f]	138	21
Pecan nut	493	22	15	69	33
Soy flour	466	23	20[f]	93	26
Plum	377	24	85	320	11
Green olive	346	25	15	52	39
Sweet basil, dried	322	26	-	-	-
Curry, powder	285	27	-	-	-
Sweet cherry	274	28	145[e]	394	8
Globe artichoke heads	260	29	168	436	5
Blackberry	260	30	144[e]	374	10
Coffee, filter	214	36	190	408	6
Black grape	169	41	54	91	27
Black tea	102	52	195	197	16
Red wine	101	53	125	126	22
Green tea	89	54	195	173	17

PP, polyphenol

[a] Sum of the content of individual PP as determined by chromatography and of proanthocyanidin oligomers as determined by direct-phase high performance liquid chromatography (HPLC).

[b] From the Food Standards Agency, UK (Food Standards Agency, 2002), except for values marked with a superscript.

[c] Sum of individual PP determined by reverse-phase HPLC and proanthocyanidins oligomers determined by direct-phase HPLC.

[d] not listed by Pérez-Jiménez et al.[480]

[e] From Wu et al.[481]

[f] From Cassidy et al.[466]

[g] PP content determined by chromatography after hydrolysis of the glycosides and esters.

In Manuscript 4 of this thesis, the focus will be on the inhibitory effect of black tea powder, cocoa powder and grape juice extract (*Vitis vinifera*) on iron absorption. Therefore, in this and the following section, the focus lays on these PP rich food sources. Phenol-explorer lists 29 different PP for grapes (*Vitis vinifera*), and their mean total content is 88.14 mg/100 g. For black tea infusion, 42 different PP structures are listed, with a mean total content of 101.4 mg/100 ml. Cocoa powder has 17 characterized PP structures, and these sum up to a mean content of 549.1 mg/100 g fresh weight.[477-479] **Figure 15** gives an overview of the PP subclasses represented in these PP rich foods and their % contribution to the total PP content.

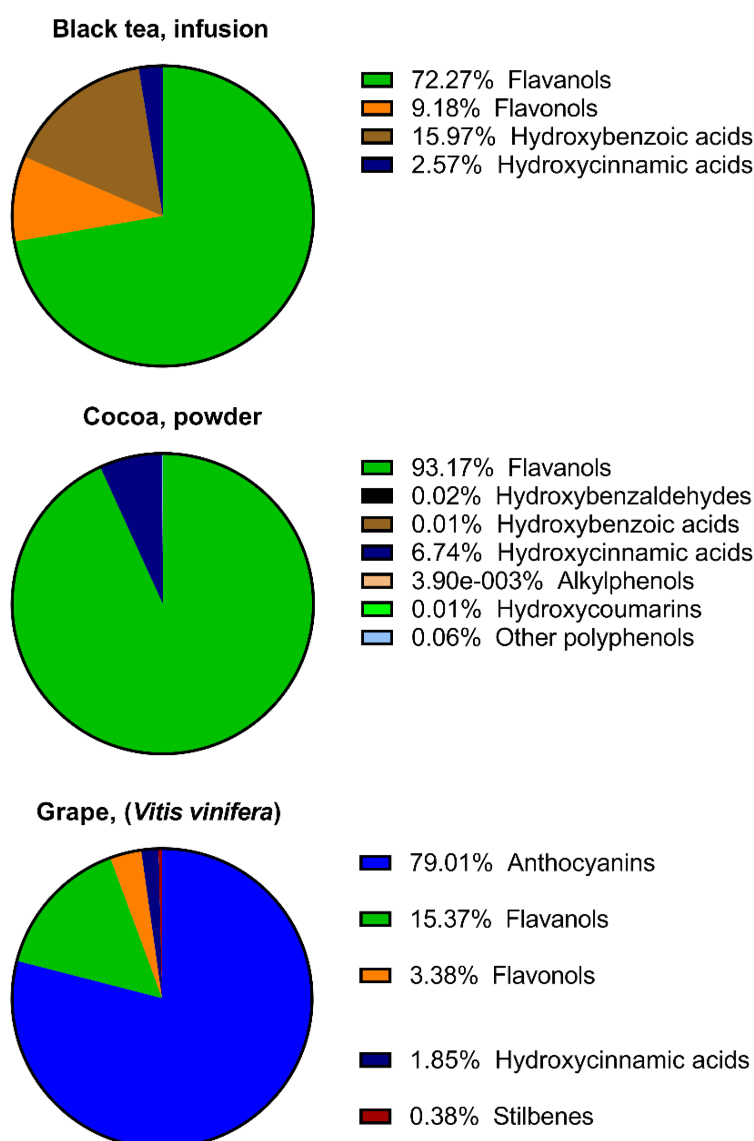

Figure 15: The polyphenol subclasses represented in black tea (infusion), cocoa (powder), and red wine (*Vitis vinifera*), according to their mean content measured by chromatography. Data extracted from phenol-explorer,[477-479] and figure from personal collection.

General dietary intakes of PP are difficult to assess, due to their high variation in structures and concentrations in foods. Different habitual dietary intakes and preferences vary greatly, as well as the assessment methods used. The following section gives an overview of cross-sectional studies assessing daily PP intakes in different study populations.

North, Middle and South America

The Brazilian National Dietary Survey conducted in 2008/9 reports a median PP intake of 346.3 mg per day, however, there are wide variations. After adjustment for energy intake, the median PP intake among the Brazilian population was 204 mg/1000 kcal/d. The most consumed PP class among the Brazilian population is hydroxycinnamic acids (299.9 mg/d), followed by flavanones (86.5 mg/d) and flavones (12.8 mg/d).[482] Among female teachers in Mexico, the total PP intake was 694 mg/d, whereas 62% of these are hydroxycinnamic acids, 17% flavanols and 9% are flavanones. The main dietary sources were nonalcoholic beverages (coffee 47% and orange juice 5%) and fruits (apples 7%, orange and mandarins 5%).[483] In the United States of America, among the NHANES cycles from 2013-2016, the estimated mean usual PP intake was 1656.6 mg/d, and energy adjusted intake was 884.1 mg/ 1000 kcal/d. The four main represented subclasses of the consumed PP among US adults are: hydroxycinnamic acids (sourcing from coffee, corn and broccoli), followed by flavanols (from tea, beans and apples), flavonols (from tea, spinach and broccoli) and lignans (from broccoli, cabbage, and strawberry). The trend analysis from 2007 until 2016 showed no significant difference over time in energy adjusted dietary PP intake.[484]

Europe

The European Prospective Investigation into Cancer and Nutrition (EPIC) study, includes cohorts from 10 European countries. Stratified by European regions/ population groups, the UK health-conscious group had the highest PP intake (1521 mg/d), and their main contributor to PP were flavonoids (909 mg/d). The non-Mediterranean countries had a mean daily intake of 1284 mg, and phenolic acids are their main contributor (700 mg/d). The Mediterranean countries had a daily intake of 1011 mg total PP, with a balanced intake of phenolic acids (503 mg) and flavonoids (449 mg). This group had the highest intake of stilbenes (3.1 mg/d). The highest daily intake of lignans was in the UK health-conscious group (9.1 mg/d).[485] The polish arm of the HAPIEE study reported a mean intake of 1741 mg/d of total PP, the energy-adjusted intake was 854 mg/ 1000 kcal/d. The main consumed PP subgroups were:

hydroxycinnamic acids (706 mg/d) from coffee (75%), vegetable oil (8%), and apples (5%); and flavanols (637 mg/d) from tea (60%), chocolate (25%) and apples (7%).[486]

Middle East and Asia

Data from a cross-sectional study in Tehran, Iran observed a mean PP intake of 1780 mg/d, of which the majority are flavonoids (1652 mg/d).[487] In elderly Japanese, the mean PP intake was 1492 mg/day, whereas 43% of these were attributed to coffee, the other major contributor was 27% green tea.[488] In the Takayama cohort (males & females, ≥ 35 y of age), a mean intake of 759 mg/ 1000 kcal/ day was reported.[489] Among Chinese university students, the mean daily intake was 1378 mg/day, of which the majority was attributed to apples (32%) and orange juice (19%).[490]

3.4.2.3 Bioavailability, efficacy and health benefits of polyphenol

PP bioavailability

The bioavailability of PP is highly variable. Also, the most common PP in our diet are not equivalently leading to high concentrations of active metabolites in the targeted tissues. A review by Manach et al. summarized 97 bioavailability studies of various PP classes. The authors reported a wide variability of PP bioavailability. Comparing plasma concentrations of their metabolites, gallic acid showed to be the best absorbed polyphenolic compound, followed by isoflavones (daidzin, daidzein, genistin, genistein, glycitin). However, isoflavones are not abundant in the Waestern diet, as their main source is from soybeans and soy products. Flavonols, catechins, flavanones, and quercetin glucosides show moderate bioavailability, whereas the poorest bioavailability show proanthocyanidins, galloylated tea catechins, and anthocyanins. Differences in the absorption kinetics have also been measured. Gallic acid, quercetin glucosides, catechins, free hydroxycinnamic acids and anthocyanins are absorbed in the stomach and small intestine, and their maximum metabolite plasma concentration is reached ~1.5 h after ingestion. Other PP compounds need the release of their aglycones by the microflora, such as rutin, hesperidin, naringin, and their maximum plasma concentrations are reached after ~5.5 h of ingestion.[491]

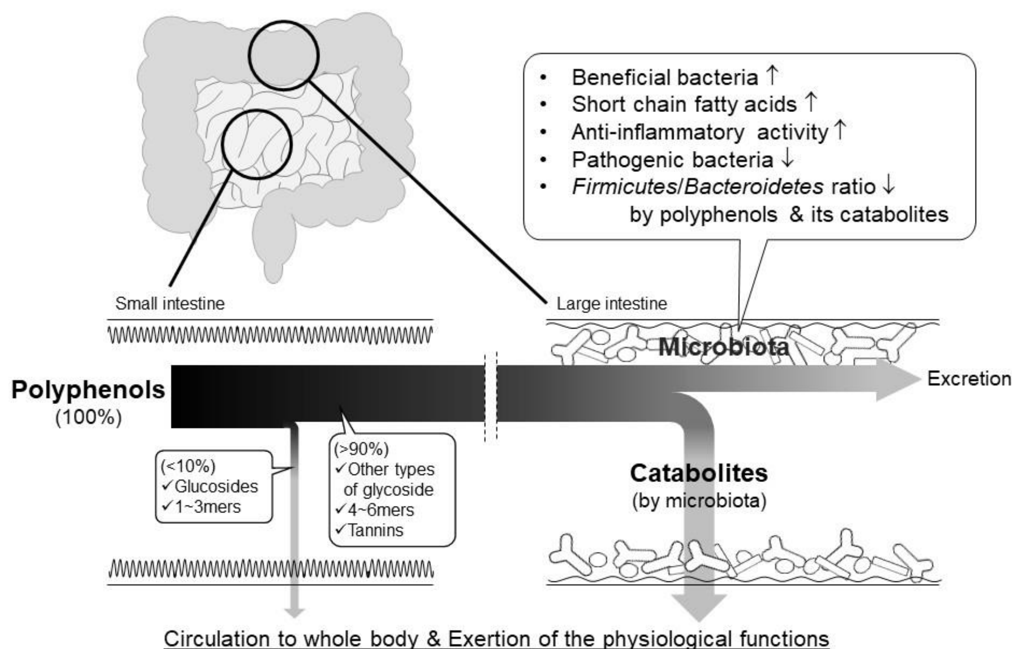

Figure 16: Pathways of polyphenol absorption, bioconversion and physiological actions of non-absorbable polyphenols in the large intestinal tract. Taken from Kawabata et al. (2019).[492]

It is estimated that around 10% of the dietary PP cross the epithelial cells in the small intestine (**Figure 16**). These are mainly monomeric PP and oligomeric tannins.[493-495] The majority of PP reach the large intestine, where they are catabolized by the intestinal bacteria, or excreted (Figure 16). Gallotannins and ellagitannins generate gallic acid, ellagic acid and other catabolites, of which some are absorbed into the blood stream and some are excreted into the feces. However, the physiological function of these catabolites may be weaker than from their original PP, such as quercetin catabolites, which show one-half to one-eighth lower anti-oxidative activity than quercetin.[496] It has been reviewed that PP also exhibit prebiotic action on intestinal bacteria.[497-500] The growth of beneficial bacteria may be partially associated with the antimicrobial activity against pathogenic bacteria of some polyphenolic compounds. Various *in vitro* studies reported a reduction in growth of pathogenic bacteria with various polyphenolic compounds.[492] For example, the growth of *Salmonella typhimurium*, *Listeria monocytogenes* and *Staphylococcus aureus*, but not *Lactobacillus rhamnosus,* were inhibited by phenolic extracts from berries.[501] Flavonoids and phenolic acids may promote the growth of *Bifidobacterium spp.*, and *Akkermansia muciniphila*, which are short chain fatty acid (SCFA) producing bacteria.[492] In vitro and animal studies have reported an increase in the beneficial

SCFA production from rutin,[502] a grapefruit extract (containing hesperidin and naringin),[503] and apple PP (epicatechin, procyanidins, chlorogenic acid).[504]

Health benefits of polyphenols

Starting in the 1980's, studies showed that a vegetarian diet can lower blood pressure. [505] The 1990's population-based studies showed that dietary flavonoids were associated with a reduced risk of cardiovascular disease (CVD),[506] and that PP from red wine could inhibit the oxidation of low density lipoproteins (LDL).[507] Initially it was perceived that the PP act as simple antioxidants and are linked to disease prevention due to the strong *in vitro* antioxidative activity of various PP.[508] However, well controlled intervention studies have failed to show this antioxidative action *in vivo*.[509] There is increased evidence that the metabolites of dietary PP act as signaling molecules and are able to induce the expression of protective enzymes. Metabolites of flavonoids enhance nitric oxide production, which in turn improves endothelial function and may lower blood pressure. Data from 25 randomized controlled trials indicate that some flavonoid-rich foods (in particular cocoa) are able to improve endothelial function by increasing flow-mediated dilation by ~20-30%.[508,510,511] Population studies showed that a long-term flavonoid intake (from black tea and cocoa) results in the reduction of systolic and diastolic blood pressure by 2-3 mmHg,[512-514] which would translate to a 10% reduced risk of a cardiovascular (CV) event.[508]

In 2014, a high-flavanols cocoa extract, has received the approval from the European Food Safety Authoroty (EFSA) for the health claim: "cocoa flavanols help maintain the elasticity of blood vessels, which contributes to normal blood flow". This is for a daily consumption of 200 mg of cocoa flavanols, which would correspond to 1 g of the high-flavanol cocoa extract.[515] A Cochrane review, published in 2017, summarized that there is moderate-quality evidence from literature that chocolate and cocoa products – rich in flavanols – have a short-term blood pressure lowering effect, (by 2 mmHg) in healthy adults.[516] Human intervention studies, animal models, and *in vitro* studies have examined an improving effect of PP on insulin resistance, which was mainly attributed to (–)-epicatechin and (–)-epicatechin-rich foods (as cocoa), and anthocyanins.[517]

A limited number of randomized controlled trials (RCTs) suggest a favorable effect of green and black tea (*Camellia sinensis*) drinking on the prevention of CVD. A Cochrane review by Hartley et al.[518] from 2013 summarized the results from four RCTs with black tea,[513,519-521] and

stated a significant reduction in LDL cholesterol, systolic and diastolic blood pressure. Seven RCTs [522-528] with green tea showed a significant reduction in total cholesterol, LDL cholesterol, as well as systolic and diastolic blood pressure.[518] Whether green tea consumption is beneficial to decrease the risk ratio for cancer has very limited evidence. Conflicting results have been reported depending on study designs. A decrease or no difference in RR has been reported in case-control studies, whereas cohort studies have reported an increased RR for cancer.[529]

In vitro and in animal models, the consumption of red wine, has shown to inhibit platelet aggregation.[530-533] Case-control and cohort studies have reported a lower risk of myocardial infarction with moderate wine consumption.[534,535] Resveratrol, which is present in red wine and grapes has been shown to possess anticarcinogenic properties and its structure may be of interest for the development of new drugs. But due to its low concentration in red wine (0.3-2 mg/L) its physiologic health benefit is very unlikely.[536,537] Supplementation with resveratrol (250-500 mg/d) has shown to affect brain function by increasing cerebral blood flow and reduce fatigue levels. However, the overall cognitive function was not improved.[538,539] Animal models have reported a blood glucose lowering effect of resveratrol.[540] Jeyaraman et al.[541] reviewed three RCTs[542-544] that addressed the effect of oral resveratrol supplementation on the treatment of adults with type 2 diabetes. The authors concluded that current evidence is insufficient to evaluate the safety and efficacy of resveratrol supplementation. More recent results report neutral effects on fasting blood glucose levels, insulin resistance and glycosylated hemoglobin A1c in adults with type 2 diabetes.[541]

Overall, to date the protective role of PP against degenerative diseases has been supported by many *in vitro* and animal studies.[536] However, its translation to human health remains restricted due to limited knowledge on bioavailability. To date only a few human studies have investigated PP bioavailability to support their bio-efficacy.[545] Nevertheless, PP-rich dietary habits seem to promote health benefits and may be valuable to prevent chronic diseases. It has been suggested that an inverse correlation of PP intake with the risk of a CV event and mortality can be attributed to a daily flavonoid intake of ≥500 mg/day. Unfortunately, reported studies are very heterogenous, and there are several limitations. Therefore, future human studies should address the harmonization of: 1) dietary assessment methods; 2)

standardized and validated analysis of PP in foods; 3) an extended food database; and 4) the validation of specific PP intake biomarkers (metabolites).[546]

3.4.2.4 The polyphenol – iron interaction

Polyphenol – iron complexes

Protonated phenolic groups are not good ligands for metal cations, however, the deprotonated form has an oxygen center which possesses a high charge density.[547] The deprotonated forms of catechol and gallol are referred to as catecholate and gallate groups, these groups coordinate with Fe^{3+} to a complex in a 3:1 ratio (**Figure 17**).[548] These complexes with a 3:1 ratio of PP-Fe have very large stability constants, but their formation depends on pH.[549,550] The chelation process might also occur with other metal cations, such as Cu^{2+}, Al^{3+}, and Zn^{2+}.[449,547]

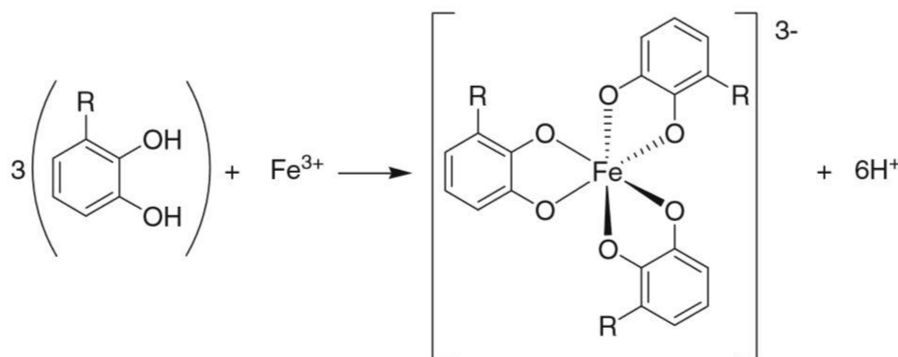

Figure 17: Expected octahedral coordination geometry of general polyphenol – iron complexes. Gallols, R = OH; catechols, R = H. The coordination requires deprotonation of the polyphenol ligands. Taken from Perron et al. (2009).[548]

The pH and PP-Fe complex formation

The deprotonation of PP and complex formation with iron are favored at high pH, most PP have pK_a values between 9 and 10.[551] However, if iron is present, deprotonation also occurs at physiological pH between 5 and 8.[547] The complex formation of iron and tannic acid is highest at pH above 5. Purawatt et al.[552] concluded that at pH 2, iron is associated to smaller molecules (< 1 kDa), from pH 5 and higher, iron forms a complex with tannic acid (~25 kDa). At pH 7 iron appears with molecules above 500 kDa, concluding that all iron is complexed by tannic acid.[552]

Chemical structures of polyphenols and PP-Fe complex formation

Monodentate PP (e.g. phenol) have only one donor atom (one hydroxyl group) that binds to the central iron atom, and these complexes are rather weak. On the other hand, bidentate PP, possessing two hydroxyl groups (catechol groups), bind iron at two sites and are very strong ligands.[547] They form PP-Fe complexes with a ratio of 1:1, 2:1, and 3:1, and the complex ratio is affected by the structure of the PP, the pH of the solution and the concentration of the PP and Fe.[551]

Flavonols (e.g. quercetin and myricetin) bind iron with their galloyl or catechol group at position A, B (3-position) and C (5-position) (**Figure 18**). The binding affinity depends on pH. The sites B and C in quercetin have much less affinity to iron than the catechol group at position A at pH 7.[547] At lower pH, the favored binding site of iron is the hydroxyl group at the 3-position (B), with a molar ratio of 2:1.[549] Catechin has a similar binding behavior of iron at low pH, like quercetin.[553] Catechins complex iron at pH 7, at a molar ratio of 3:1, whereas gallocatechins have a binding ratio of 2:1.[554] Glycosylated PP (e.g. rutin) have a carbohydrate conjugate most often at the 3-position, therefore, this binging site is lost.[547]

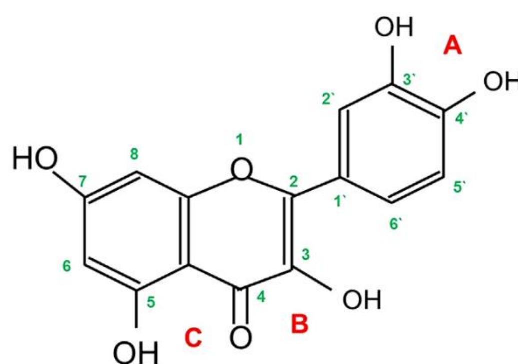

Figure 18: The possible iron binding sites of quercetin (PP subclass flavonols). Taken from Petry et al. (2014).[551]

Data from human iron absorption studies conducted by Hurrell et al.[140] and Brune et al.[138] suggest that PPs with an *ortho*-dihydroxy-(chatechol) or a trihydroxy-benzene (galloyl) group, are most potent at inhibiting iron absorption. For example, proanthocyanidins and hydrolysable tannins possess these groups (Figure 14).[138,140]

Other food components and PP-Fe complex formation

Ascorbic acid present in the diet protects iron from the complex formation with PPs, and also EDTA has the same properties although to a lesser extent.[551] Studies have shown that only 50 mg of ascorbic acid is needed to overcome the negative effect of 100 mg tannic acid.[117] An *in vitro* study has shown that ascorbic acid is able to prevent the complex formation of iron with tannic acid.[555] Cercamondi et al.[556] performed an iron absorption study with a dephytinized sorghum meal, which has a high PP content (167 mg gallic acid equivalents (GAE)). The addition of external vitamin C (40 mg) has led to an almost triple increase in fractional iron absorption from ferrous sulfate than when given without. The replacement of ferrous sulfate with sodium iron EDTA showed a 1.7 times increase in fractional iron absorption (FIA).[556]

The ionic state of iron and PP-Fe complex formation

It is generally accepted that PP prefer to stabilize Fe^{3+} over Fe^{2+}. Catechol derivatives and other flavonoids promote the oxidation of iron from Fe^{2+} to Fe^{3+} to form thermodynamically stable complexes with a large stability constant and low reduction potential.[557-560] Perron et al.[561] have shown that galloyl groups are much faster at oxidizing ferrous to ferric iron, and they indicated that the oxidation rate correlates with the PP-Fe complex stability.[561] In contrast to these oxidizing PP, non-flavonoids and tannic acid have been reported to be able to reduce ferric to ferrous iron and possess the ability to form PP-Fe complexes with ferrous iron.[557,562] Nevertheless, these complexes are much weaker.[548]

***In vitro* studies – mechanistic**

It has recently been suggested that the complex formation of iron with PP not solely leads to its inhibitory property. The intestinal iron uptake may even be enhanced by PP, but they block the basolateral iron export.[563,564] Kim et al.[563] performed Caco-2 cell studies looking at the effect of high doses of grape seed extract (GSE) on iron absorption. *In vitro*, the apical iron uptake was significantly increased in Caco-2 cells, which they attributed to the reducing effect of the epigallocatechin-3-gallate in GSE. However, the basolateral membrane export was reduced and as a consequence the iron concentration in the enterocytes was high.[563] The same research group revealed the same study results with heme iron, although with a blunted effect.[565] In a subsequent study, with GSE and green tea extract (GE), the basolateral iron exit was decreased by increasing GSE and GE, however, this effect was overcome by the addition of ascorbic acid.[566]

In vivo studies in rat models demonstrated that iron absorption is decreased by the following mechanism: quercetin increases the apical iron uptake into the enterocytes and prevents the basolateral iron export. [564] A long-term administration (10 days) of quercetin (50 mg/kg/daily) in rats, resulted in a decrease of DMT1, Dcytb and FPN mRNA which leads to iron depletion. In addition, one intraperitoneal administration of 50 mg/kg quercetin was sufficient to reduce iron parameters and increase liver hepcidin expression.[564]

Regarding these findings, if PP increase the apical iron uptake, this is only the case for monomeric PP. However, in foods, PP occur in more complex structures, such as glycosides, and these would still be expected to form insoluble PP-Fe complexes.

Iron absorption studies

The first study to describe the inhibitory effect of tea on iron absorption was published in 1975 by Disler and colleagues.[139] They reported an inhibition of non-heme iron absorption up to 80%.

A series of iron absorption studies conducted by Gillooly et al.[121] investigated the effect of various vegetables on iron absorption. Wheat germ, eggplant, butter beans, spinach, brown lentils, beetroot greens and green lentils showed poor iron absorption. On the other hand, the iron absorption from carrot, potato, beetroot, pumpkin, broccoli, cauliflower, tomato, cabbage, turnip and sauerkraut was moderate to good.[121]

Brune et al.[138] performed an iron absorption study in 1989 comparing the effect of gallic acid, chlorogenic acid, catechin and tannic acid. Gallic acid reduced iron absorption from a non-inhibitory wheat bread by 50%, compared to a 30% reduction with chlorogenic acid, and no effect with catechin. Tannic acid reduced iron absorption in a dose dependent manner, 5 mg reduced iron absorption by 20%, 25 mg by 67% and 100 mg by 88%. The authors concluded that the major determinant of the inhibitory effect of PP may be attributed to the galloyl groups, whereas catechol groups were of minor importance. The same inhibitory dose-dependent manner was also observed when spinach (38% reduction), coffee (61% reduction), tea (69% reduction) and oregano (69% reduction) was given with the same meal.[138] A rosemary extract has been described to have a moderate inhibitory effect on iron absorption.[567]

In 1995, Cook et al. published an iron absorption study in humans, showing that red wine has a greater inhibitory effect on iron absorption than white wine, and the reduction of alcohol has led to an even greater decrease in iron absorption (up to ~30%).[568] A subsequent study by the same researchers reported the inhibitory effect of beverages on non-heme iron absorption. The effects of black tea, herb teas, cocoa, white and red wine on iron absorption are summarized in **Figure 19**. These studies reported that 200 mg (GAE) of total PP per meal (from black tea or herb tea) inhibited iron absorption by 60-80%, and 116 mg (GAE) PP from cocoa reduced iron abortion by 70%. The greater their measured total PP content the greater was their ability to reduce non-heme iron absorption.[140,568]

In general, when comparing beverages, black tea PP seem to be the most potent iron absorption inhibitors, most likely due to their higher concentrations of PP containing galloyl groups.[551] The inhibitory effect of black tea can be reduced by ~50% by a time lag of 1 h between meal and tea drinking.[569] The inhibitory effect of PP on iron absorption are independently of the iron compound. The consumption of tisane Kinkéliba with a fortified bread meal (either ferrous sulfate or ferrous fumarate) has led to a 56 and 50% reduction in iron absorption in women of reproductive age, respectively.[570]

The addition of chili, but not turmeric, inhibited iron absorption from a rice based meal. Despite the fact that chili contains less total PP than turmeric (25 mg GAE and 50 mg GAE, respectively), it reduced iron absorption by 38%. This effect may be explained by their different content of quercetin, which is a lot higher in chili.[571] Yod Kratin is a typical Southeastasian vegetable, and its leaves contain a significant amount of iron binding phenolic groups. It has been shown that even one portion size is sufficient to reduce iron absorption by 90%, in contrast the addition of ascorbic acid was able to diminish this effect.[572]

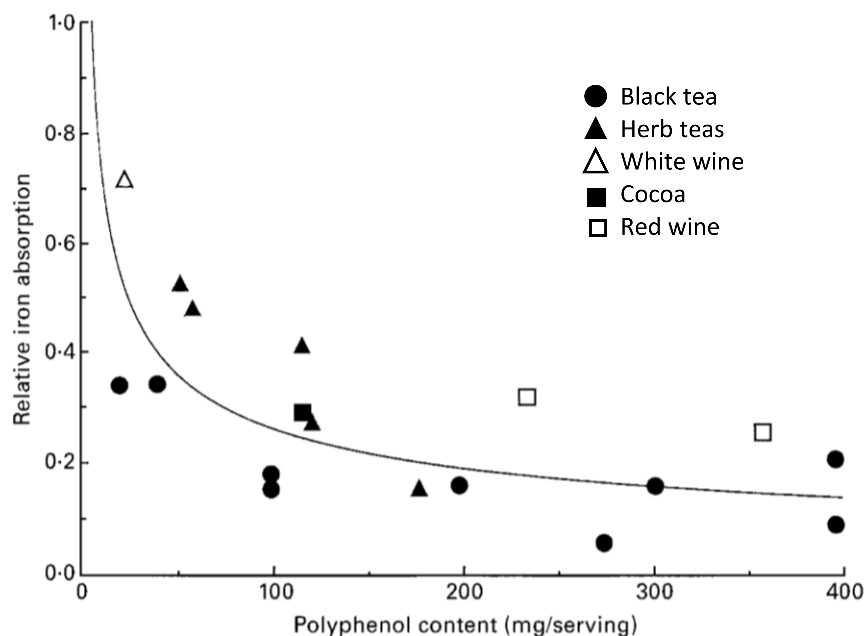

Figure 19: Relative iron absorption from a bread and beverage meal according to the polyphenol content (measured as GAE) of the beverage. Relative iron absorption is defined as iron absorption (%dose) from a bread meal consumed together with a beverage relative to iron absorption in the same subject from a bread meal consumed with water. Adapted from Hurrell et al. (1999)[140] and Cook et al. (1995).[568]

Overall, the effects of PP on iron absorption have been well described in various clinical studies. However, their impact on iron status in populations is not well investigated. A review of cross-sectional and case control studies concluded that tea consumption in the Western population does not affect iron status. However, in populations with marginal iron status, there is a negative association of tea consumption with iron status.[573] In contrast, for people with iron overload, the iron absorption inhibition may be beneficial and will be discussed in the following section.

The potential benefit of polyphenols in iron overload diseases

An early iron absorption study in thalassemia major and intermedia subjects, published by Alarcon et al.[574] in 1979, reported a reduction in iron absorption of 41 to 95% by the administration of tea.[574]

Kaltwasser and colleagues[407] performed an iron absorption study in patients with hereditary hemochromatosis, as well as a longitudinal study. The results of their longitudinal study with regular tea drinking is reported in a previous section of this thesis (Chapter 3.4). They report a 70% reduction in iron absorption when a meal was consumed with 250 ml black tea (6.9%)

instead of water (22.1%). As previously reported, regular tea drinking with all main meals reduced the frequency of required phlebotomies. [407]

Sylibin is a flavonoid derived from the milk thistle (*Silibum marianum*), and reduces serum iron response in hereditary hemochromatosis patients. After the consumption of a meal with 140 mg silybin the serum iron response was reduced to the same extent as 200 ml black tea when compared to water.[575]

The iron absorption lowering effect of quercetin in rats has been discussed in the previous section. It also has been successfully tested in a double-blind RCT including 84 patients with β-thalassemia major, who received 500 mg/d quercetin. After 12 weeks, the intervention group had lower CRP, iron, ferritin, TSat, and increased transferrin, compared to baseline and the control group.[576]

An *in vitro* study with a proanthocyanidin extract from grape seeds, chelated iron to the same extent as deferoxamine.[577] Procyanidin – a member of the proanthocyanidin class of flavonoids – was tested in 20 patients with hereditary hemochromatosis and 20 dysmetabolic iron overload syndrome patients. The administration of one supplement containing 50 mg proanthocyanidins did not affect serum iron response after the ingestion of an iron rich meal in neither groups.[578]

These conflicting reports on the effectiveness of PP to chelate iron could be attributed to their tremendous variety of different chemical structures and their potential to chelate iron. Other potential factors interfering with their effectiveness might be their intrinsic bioavailability, the variation in experimental designs (timing and dosage) or the different methods used to measure iron absorption. Nevertheless, a PP-enriched diet might be beneficial for patients suffering from iron overload, such as hereditary hemochromatosis or β-thalassemia.[425]

4 Evaluating iron bioavailability

Poor iron bioavailability is a crucial contributor to nutritional iron deficiency worldwide. The majority of the world's population consumes sufficient iron to cover their physiological requirements, but in often absorption is limited due to low intake of heme iron and a plant-based diet rich in phytates and polyphenols (Chapter 1.3.1).[20] Several methods have been developed to assess iron bioavailability, aiming to improve iron nutrition. Algorithms provide semi-quantitative information and *in vitro* methods generate ideas and develop hypotheses. But solely human studies can form the basis for decisions in food fortification policies, plant breeding programs or in the development process for a new food product.[579]

4.1 Algorithms and other approaches

Based on dietary data, algorithms have been developed to estimate iron absorption in humans. The first approach was suggested by Monsen et al.[580] in 1978 and is based on the form of iron (heme or nonheme), ascorbic acid, and animal tissue (meat fish or poultry).[580] This algorithm is used by the FAO/WHO to calculate the recommended nutrient intakes (RNI) based on three levels of iron bioavailability: 5%, 10% and 15%. For a diet low in vitamin C and animal proteins, bioavailability is estimated to be 5%,; for a diet rich in cereals including sources of vitamin C, the bioavailability is estimated to be 10%; and for diet rich in vitamin C and animal proteins, an iron bioavailability of 15% is estimated.[299] In 2000, Hallberg and Hulthén published a more comprehensive algorithm,[581] which includes all dietary factors that affect non-heme iron absorption. In addition, they also proposed an adjustment for iron status to calculate iron bioavailability.[581] In 2013, a similar algorithm was published by Armah et al.,[582] which is based on the US diet. Both of these algorithms include many factors of iron bioavailability, but they rely on dietary intake data. This is their main limitation due to the difficulty in obtaining accurate semiquantitative intake data of iron and its major absorption enhancers and inhibitors. Another limitation is the applicability of the results of these algorithms from one study population to others. And lastly, these algorithms tend to underestimate the actual iron bioavailability.[583]

To calculate iron bioavailability at a population level, Dainty et al.[584] have developed a model based on iron requirements, daily iron intake, and the distribution of serum ferritin concentrations in a population.[584] A subsequent study by the same researchers, included data from three studies conducted in the UK and Ireland into the same model. Their model predicts

iron absorption at serum ferritin concentrations of 15, 30, and 60 mg/L, which would be: 22.3%, 16.3% and 11.6% in men; 27.2%, 17.2%, and 10.6% in premenopausal; and 18.4%, 12.7%, and 10.5%, in postmenopausal women, respectively. This model can be adapted to any population, providing representative data on iron intake and iron status. Hence, with this model, the work of risk managers and public health professionals would be facilitated to formulate new dietary recommendations.[585]

4.2 *In vitro* methods

4.2.1 Iron solubility and dialyzability

Iron solubility and dialyzability are tools to screen foods and iron compounds to predict their availability of non-heme iron for absorption. The process involves a two-stage simulated digestion. Food mixtures are homogenized and digested for 2 h at 37°C to pepsin at pH 2 (to simulate an adult stomach, pH 4 is used to simulate the stomach of infants), this first step simulates the gastric digestion. In the second stage (intestinal digestion), the pH is adjusted to intestinal levels (pH 5.5), pancreatin and bile salts are added. To assess iron solubility at the beginning of the intestinal digestion, the process can be stopped at this stage by centrifugation. To assess dialyzability of iron, a semipermeable membrane (with a cutoff of 6000–8000 of molecular weight) is added at this stage and the digestion is continued for 2 h at 37°C. The quantity of iron that diffuses across the membrane is then analyzed.[20] In most cases, it predicts the direction of iron solubility response, however, not the magnitude. Another limitation of this method is the assumption that iron which is ready for absorption is bound to small-molecular weight complexes. Iron bound to large molecular complexes are retained by the membrane, which would underestimate the result. On the other hand, small PP-Fe complexes may pass through and iron solubility may be overestimated.[579]

4.2.2 Iron uptake – Caco-2 cells

Caco-2 cell models include iron transport and signaling mechanisms and are useful to explore iron absorption.[579] These cell models are cell lines isolated from an adenocarcinoma in a 72-y-old Caucasian man. They have a fast proliferation rate and their development to small intestinal enterocytes can be induced under the right conditions.[20] These cell monolayers are combined with a simulated *in vitro* digestion to measure iron bioavailability.[586] However, its methodology can vary between laboratories, depending on the prior *in vitro* digestion,

including dialyzability or not, preparation methods to grow the cell lines, the passage number and the analytical method used to measure iron uptake.[579]

There are two methods available to determine iron uptake into Caco-2 cells, the first is the direct measurement of radio-labeled iron uptake. The second (indirect) method, measures cell ferritin concentrations, which is proportional to cellular iron uptake.[587] The measurement of ferritin is the biomarker for cellular iron uptake, but in cellular response to inflammation and oxidative stress it is also upregulated. But there are no data that have demonstrated the induction of inflammation or oxidative stress by food. This physiological response in mucosal cells must be considered when using ferritin expression as the biomarker to assess cellular iron uptake.[579] Nevertheless, cellular iron uptake does not reflect iron bioavailability, since the absorbed iron from the enterocytes may be stored as ferritin and lost upon cell death.[587]

A combination of both methods, *in vitro* digestion and Caco-2 cells, are useful to differentiate the soluble iron from the iron that is available for the uptake by the enterocytes. For example, ferrous ascorbate and ferric citrate seem to have similar solubility and dialyzability, but they differ in their bioavailability.[579]

4.3 *In vivo* - animal models

The only standardized method, with a widespread use by the Association of Official Analytical Chemists (AOAC) is called the Rat Hemoglobin Repletion Assay.[588,589] In this model, a tested diet is given to IDA weanling rats, and iron absorption is calculated based on the increase in red blood cell mass.[20] This method has most often been used to evaluate iron bioavailability of compounds that are considered as food fortificants. Its results are comparable to iron compound bioavailability in humans.[590] However, rats do not respond the same way to inhibitors and enhancers of iron absorption as humans. Hence, for some research questions this might not be an appropriate model.[591]

4.4 Human iron absorption studies

4.4.1 Serum iron response

The serum iron response – or iron tolerance measurement – is a method that can only be applied for doses of iron of 10 mg or higher. It involves the intake of an iron compound and the subsequent determination of serum iron response at hourly intervals for six hours.[20] The

area under the curve is then used as a measure of iron absorption,[592,593] or compartmental modelling.[594] The serum iron response is most often repeated with a well-absorbed iron compound (eg. $FeSO_4$) and results are reported as relative bioavailability.[20]

4.4.2 Iron isotopes

To understand the effect of food and food fortification on iron bioavailability, data from isotopic studies are the fundamental basis.[20] By using isotopic iron as a label, its bioavailability can be distinguished from iron of other sources.[595]

The first use of stable isotopes as labels were settled in 1935, with using ^{13}C, followed by stable isotopes of hydrogen, nitrogen and oxygen.[595] Of the essential minerals (iron, copper, calcium, magnesium and zinc), studies initially used their radioisotopes to study their metabolism.[596] However, of these, also stable isotopes are available to use as labels in human studies, unless they are monoisotopic (eg. fluorine, sodium, phosphorus, manganese, iodine, cobalt, aluminum and arsenic).[595]

Isotopes are of the same element, with the same number of protons but differ in number of neutrons, which changes their atomic mass. Iron isotopes are either radioisotopes, or stable isotopes, the later one occurs in a fixed abundance in nature (**Table 10**).[597]

Critical aspects of an iron isotopic label are: I) the labels should have the same metabolic fate as the iron from the food or supplement which is given with the label; and II) the amount of label given should not affect the test meal/supplement composition which is examined.[597]

Table 10: Atomic mass and natural abundance of stable iron isotopes, adapted from[597] and.[598,599]

Stable isotope	Atomic mass (g/mol)	Protons (n)	Neutrons (n)	Natural abundance (%)
^{54}Fe	53.940	26	28	5.845
^{56}Fe	55.935	26	30	91.754
^{57}Fe	56.935	26	31	2.1191
^{58}Fe	57.933	26	32	0.2819

Starting in 1939, radioisotopes of iron were used to study anemia in animals,[600] followed by human studies in 1942 assessing iron absorption in pregnancy, anemia and hemochromatosis.[601] The advantages of radioisotopes are that they can be added in trace amounts due to their easy detection, further only little sample preparation is required prior

to analysis.[601] Their major disadvantage is the exposure of the subjects to radioactive emissions due to their unstable nuclei. The iron radioisotopes that have been used in nutrition research are ^{55}Fe and ^{59}Fe, of which the first one has a half-life of 2.94 years, whereas the latter one only 45 days.[597] Within this thesis, only stable iron isotopes were used to assess iron bioavailability in all Manuscripts. The main focus from now on is spent on the stable iron isotopes, however, it has been demonstrated that both approaches yield highly comparable results.[602] The only difference is the amount of label that needs to be used with stable iron isotopes, which is in the milligram range.[20]

Stable iron isotopes are non-radioactive and are safe for nutritional studies in vulnerable population groups of infants, children, and pregnant women.[597] There are four stable isotopes of iron naturally occurring in foods and nature, their abundance and atomic mass are given in Table 10.[598,599] The three stable iron isotopes (also referred as minor isotopes) with the lowest abundance (^{54}Fe, ^{57}Fe, and ^{58}Fe), are used in nutritional studies as iron labels. All Manuscripts in this thesis use these labels to assess iron bioavailability from a test meal under different circumstances.

There are three methods that can be applied to assess iron bioavailability in humans: I) fecal recovery; II) plasma appearance; and III) erythrocyte iron incorporation (Chapter 4.4.2.1).

The first approach involves the administration an of oral stable iron isotope and the difference between the administered dose and excreted dose is used to estimate iron absorption.[597] To obtain this difference, fecal collections for up to 7-10 days post-oral dosing is required.[603] The advantage of this method is, that it is non-invasive as no blood sample collection is required. However, the study procedures are very time consuming for both, subjects and investigator. And in case of an incomplete collection, the results will lead to an overestimation of iron absorption.[597]

The second approach assesses plasma iron turnover by collecting multiple samples at regular time intervals after oral iron isotope administration.[603] This method was used by Zimmermann et al.[604] to assess changes in iron isotopes appearance in whole blood, collected in seven samples during 6 h after administration to compare to the plasma hepcidin response.[604] A recent iron absorption study by Husmann et al.[605] measured kinetics of serum iron appearance after administration of ferrous fumarate with and without GOS, collecting eleven samples during 24 h after administration.[605]

4.4.2.1 Erythrocyte incorporation of stable iron isotopes

The last approach – erythrocyte iron incorporation – is used in all studies in this thesis. The erythrocyte incorporation measure combines iron absorption and utilization. It assumes that a fixed fraction of the absorbed label is incorporated into the erythrocytes. In adults this fraction is 80%,[110] in children 90%[606] and in infants 75%[607] of the label is incorporated into the erythrocytes after a time period of 14 days.[110]

In some physiological states (for example in pregnant women, premature infants or patients with a disease affecting iron homeostasis), the iron incorporation rate can be highly variable. Therefore, in some cases it may be beneficial to measure iron utilization to be able to interpret iron bioavailability. Iron utilization is assessed by the administration of an intravenous (IV) dose of iron isotopes, most often as ferrous citrate.[597]

Once the iron is incorporated, the enrichment ratio will remain constant for the duration of the lifespan of an erythrocyte in adults, in average 120 d. After approximately one year, the stable iron isotopes reach an equilibrium to body iron. When this stage is reached, the enrichment ratio is proportional to iron losses, which in turn is proportional to the dietary iron absorption. This approach can also be an accurate method to assess long-term iron balance to evaluate iron intervention programs.[608]

Assumptions to assess erythrocyte iron incorporation

To assess iron bioavailability using the erythrocyte incorporation approach, further assumptions are made: a fixed fraction of absorbed iron is incorporated into erythrocytes; the hemoglobin in the erythrocytes contain a constant fraction of iron (3.47 mg Fe/g Hb); once the iron is incorporated, there is no net exchange with plasma iron over the lifespan of the erythrocyte; and an accurate estimate to calculate the blood volume of the study population is available.[597]

Blood volume can be assessed by three approaches: 1) the direct measurement of intravenous dosing of either a radioisotope (labelled to either erythrocytes or albumin) or an intravenously administered dye;[609] 2) calculating based on the hematocrit and measured Hb mass, which can be quantified by the carbon monoxide rebreathing technique;[610] and 3) calculating based on combinations of body weight, length/height, surface area, lean body mass and age.[597] Different equations have been developed for children[611] or healthy adults.[612,613] The total

circulating iron can then be derived with the calculated blood volume and analyzed Hb concentration of the study subject. In a next step, the amount of stable iron isotope that has been incorporated into the erythrocytes is calculated based on the analyzed shift in enrichment ratio of the stable iron isotope (either ^{54}Fe, ^{57}Fe, or ^{58}Fe:^{56}Fe) from baseline to the time-point (14 d) after dosing.[597]

Other key factors

The administration of stable iron isotopes needs to be within the milligram range, which may change the total iron content of a meal.[614] In general, ^{58}Fe is the lowest abundant isotope, and therefore a much lower dose to reach an acceptable signal to noise ratio, but it is also the most expensive. Using large doses of stable iron isotopes is of no concern when studying iron fortified foods, in which the labels (as labelled iron compound, or labelled fortification compound) are extrinsically added to the test meal. When studying the iron bioavailability from dietary iron (from foods or biofortified food items), the contribution of the extrinsically labelled iron should not contribute more than 20-30% to the total iron content of the test meal. The results of extrinsically labeled biofortified foods are comparable to intrinsically labeled biofortified foods, as all non-heme iron enter a common iron pool.[615-617] Intrinsic labelling can be difficult and expensive, e.g. for biofortified plants, this would require the growth of a plant in an enriched source of iron.[597] In order to administer a detectable amount of label with biofortified foods, the administration of multiple meals with the same label would not significantly increase the native non-heme iron content.[618-620]

Variability in iron absorption and normalizing approaches

Iron absorption in individuals is highly variable and depends on iron status (Chapter 1.1), dietary components (Chapter 1.3), inflammation and infection (Chapter 1.1 and 2.3.1) and genetic factors (Chapter 2.3.2), which all potentially predict iron absorption in humans.

To allow for comparison between study subjects, two approaches can be applied. The first one involves an additional reference dose of 3 mg ferrous sulfate with ascorbic acid at a 2:1 molar ratio.[621] The absorption of the reference dose are then standardized to usually 40% iron absorption and the iron absorption of the other experimental test meals are then normalized based on this factor.[597] As an alternative, the correction developed by Cook et al.[622] is applied, and typically corrects iron absorption data to reference values of serum ferritin: 40 µg/L.[622] The equation is based on the linear negative correlation of log serum ferritin and log iron

absorption; $\log(FIA_C) = \log(FIA_O) + a * \log(SF_C/SF_O)$. Thereby FIA_C is the corrected fractional iron absorption, FIA_O is the observed value, SF_C is the corrected serum ferritin concentration, and SF_O the observed, and a is the slope of the regression line (-1). The correction of fractional iron absorption data according to Cook et al.[622] was applied in Manuscript 1, and an adaption of it (using the study specific regression slope) in Manuscript 2.

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MANUSCRIPT 1

ASYMPTOMATIC *HELICOBACTER PYLORI* INFECTION IN PRESCHOOL CHILDREN AND YOUNG WOMEN DOES NOT PREDICT IRON BIOAVAILABILITY FROM IRON FORTIFIED FOODS

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Abstract

Helicobacter pylori infection is common in low-income countries. It has been associated with iron deficiency and reduced efficacy of iron supplementation. Whether *H. pylori* infection affects iron absorption from fortified and biofortified foods is unclear. Our objective was to assess whether asymptomatic *H. pylori* infection predicts dietary iron bioavailability in women and children, two main target groups of iron fortification programs. We did a pooled analysis of studies in women of reproductive age and preschool children that were conducted in Benin, Senegal and Haiti using stable iron isotope tracers to measure erythrocyte iron incorporation. We used mixed models to assess whether asymptomatic *H. pylori* infection predicted fractional iron absorption from ferrous sulfate, ferrous fumarate or NaFeEDTA, controlling for age, hemoglobin, iron status (serum ferritin), inflammation (C-reactive protein), and test meal. The analysis included 213 iron bioavailability measurements from 80 women and 235 measurements from 90 children; 51.3% of women and 54.4% of children were seropositive for *H. pylori*. In both women and children, hemoglobin (Hb), serum ferritin (SF), and C-reactive protein (CRP) did not differ between the seropositive and seronegative groups. Geometric mean (95% CI) fractional iron absorption (%), adjusted for SF, was 8.97% (7.64, 10.54) and 6.06% (4.80, 7.67) in *H. pylori* positive and negative women ($p = 0.274$), and 9.02% (7.68, 10.59) and 7.44% (6.01, 9.20) in *H. pylori* positive and negative children ($p = 0.479$). Our data suggest asymptomatic *H. pylori* infection does not predict fractional iron absorption from iron fortificants given to preschool children or young women in low-income settings.

Introduction

Helicobacter pylori (*H. pylori*) is a common colonizer of the human gastric mucus.[1] It is estimated that ≈50% of the global population may be infected,[2] and prevalence is higher in low income countries, ranging from 65% in adults from Thailand[3] and Ethiopia[4] to 85% in mothers from Bangladesh.[5] Infection with *H. pylori* usually begins during childhood and may have lifelong persistence if not treated; in Bangladesh prevalence range from 47% in children below the age of 2,[5] 60% in children less than 5 years-old,[6] up to 93% prevalence in children below the age of 15.[7] Although infection with *H. pylori* is a major risk factor for chronic gastritis, peptic ulcer disease, and gastric cancer, the majority of cases remain asymptomatic.[2]

A systematic review of studies conducted in adults and children, concluded that *H. pylori* infection increases the risk for low iron status. The meta-analysis reported a 1.33 odds ratio for iron deficiency (ID) among seropositive individuals, a 1.15 odds ratio for anemia, and a 1.72 odds ratio for iron deficiency anemia (IDA).[8] A large cross-sectional study in China (n = 17791, mean age 45 – 18 years old) reported a significantly higher prevalence of anemia in *H. pylori* infected individuals.[9] The clinical outcome of an *H. pylori* infection remains complex as an antral mucosa infection leads to increased gastric acid secretion. In contrast, infection of the corpus mucosa leads to decreased acid secretion, which is observed in the majority of the infected patients.[10,11] Further, gastric ascorbic acid secretion was reported to be significantly lower in *H. pylori* infected versus uninfected children.[12] Several studies have associated *H. pylori* infection with depleted iron stores; in symptomatic adults: Atrophic gastritis is associated with IDA, and lower hemoglobin (Hb) levels.[13-15] In symptomatic children, lower serum ferritin (SF), hemoglobin, serum iron and transferrin were associated with *H. pylori* infected children with hypochlorhydria.[16,17]

Studies examining iron absorption during *H. pylori* infection have produced equivocal results. Two studies in adults with *H. pylori* infection reported impaired iron absorption from a test meal or an iron dose.[18,19] In contrast, in a study in Bangladeshi children, there was no significant difference in iron absorption before and after treatment of *H. pylori* infection, although the *H. pylori* positive children had impaired gastric acid production.[20] A subsequent study in Bangladeshi children reported no significant associations between *H. pylori* infection, iron deficiency, iron-deficiency anemia and iron absorption.[21]

IDA is a major global public health problem in children and young women.[22] Iron fortification can be a cost-effective approach to prevent IDA,[23-25] and fortification programs have been introduced in many low-income countries.[26] Whether *H. pylori* contributes to the high prevalence of IDA in children and young women and/or blunts the efficacy of iron fortification programs remains unclear. Therefore, our study aim was to assess whether asymptomatic *H. pylori* infection is associated with iron bioavailability from commonly used iron food fortificants in preschool children and women of reproductive age. Our hypothesis was that seropositive *H. pylori* infection would be an independent predictor of lower iron bioavailability from iron fortificants in both of these age groups.

Materials and Methods

Subjects and Study Design

This was a retrospective pooled analysis of stable iron isotope absorption studies done in women and preschool children that were carried out in Benin,[27-29] Haiti,[30] and Senegal (N. Ndiaye, unpublished results) between 2010 and 2015. All studies had ethical approval from locally independent ethics committees and from ETH Zurich, Switzerland. Written informed consent was obtained from the subjects in the adult studies or the caregiver of the children in the pediatric studies. The studies were registered at clinicaltrials.gov (NCT01108939, NCT01321099, NCT01634932, NCT02096250, and NCT02437955).

All studies followed the same general design and used standardized methods to assess *H. pylori* infection and iron bioavailability. Subjects were recruited, screened for eligibility and infection with *H. pylori* was assessed at baseline. Inclusion criteria for all studies were: (1) Apparently healthy with no history of a diagnosed gastrointestinal disorder, or another chronic disease; (2) had not received a blood transfusion or had substantial blood loss within six months before the start of the study; (3) did not consume vitamin or mineral supplements within two weeks before the study start; (4) for women, not pregnant or lactating, and (5) for women, bodyweight < 65 kg. Included participants then consumed iron fortified test meals labelled with stable iron isotopes (^{54}Fe, ^{57}Fe, ^{58}Fe, as ferrous sulfate, ferrous fumarate or ferrous sodium EDTA). All test meals were administered in a single-blind, randomized cross-over design. After a study-specific incorporation period (minimum of 14 days), a venous blood sample was taken to measure incorporation of the stable iron isotopes into erythrocytes.

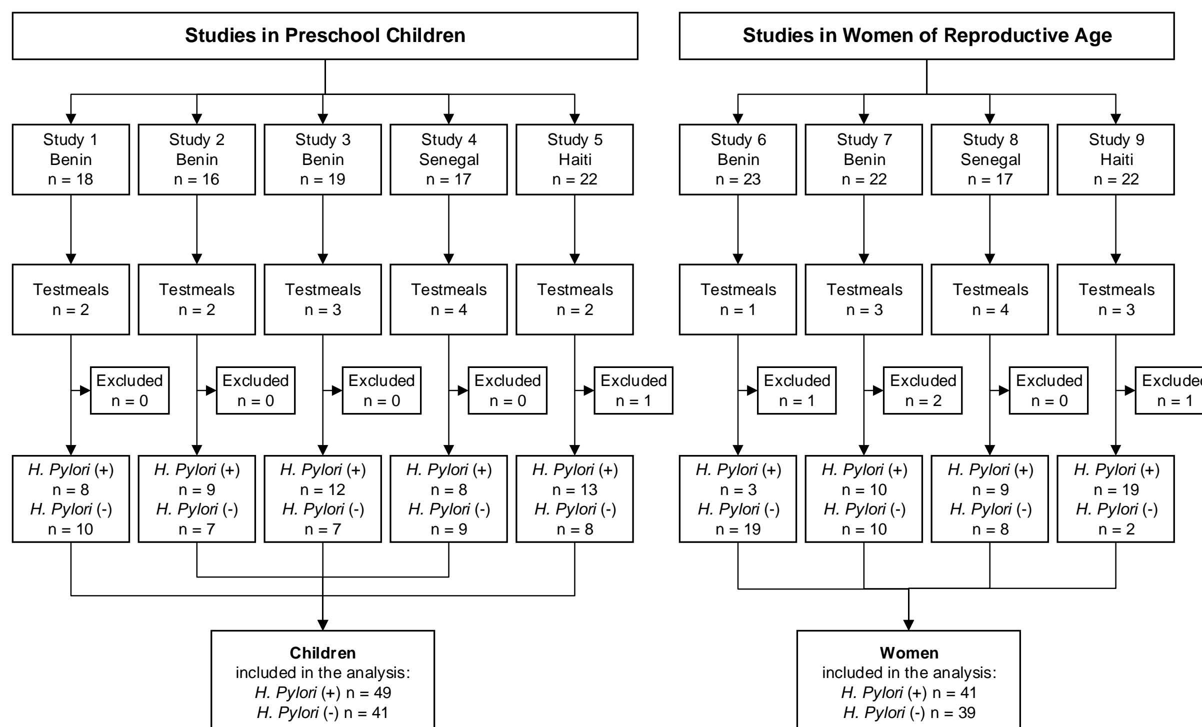

Figure 1: Study flow chart: Five iron absorption studies in preschool children and four iron absorption studies in women of reproductive age were conducted at different sites. Five participants were excluded from the analysis because *Helicobacter pylori* status was not assessed.

Table 1 gives an overview of the iron compounds and test meals of all studies, and **Figure 1** the flow of participants. All of the studies, except the studies conducted in Senegal, have been previously described in detail. Studies 1 to 3 were done in children (n = 52) aged 1.5 to 3 years, at the local state hospital in Natitingou in Benin. All children were afebrile, had a negative malaria smear, had a bodyweight > 8.3 kg and weight-for-age z-score (WAZ) > -3. The children were divided into three studies, and all groups received a millet-based porridge with different iron compounds and absorption enhancers (ascorbic acid or phytase) (Table 1).[28] Study 6 was done in Beninese women (n = 23), at the local state hospital in Natitingou and Toucountouna in Benin, aged 16 to 40 years without severe anemia (Hb > 8 g/dL), who received a labelled sorghum-based test meal (Table 1) during afebrile malaria. Following successful treatment of malaria and inflammation, participants received a second labeled test meal. The analysis in the current manuscript only includes the iron bioavailability data from the test meal that was administered after malaria treatment.[27] One participant from this study was excluded due to missing sample. Study 7 was done in Beninese women (n = 22), at the local state hospital in Natitingou, aged 17 to 35 years, who had a negative malaria smear and depleted iron stores (SF < 25 µg/L). They were given a millet-based test meal (Table 1).[29] Two participants from this

study were excluded due to missing samples. Studies 4 and 8 were done in Senegal, at the laboratory of nutrition at the University of Cheikh Anta Diop in Dakar. It included non-anemic (Hb > 11 g/dL) women (study 8, n = 17) and their preschool children (study 4, n = 17), aged 3 to 6 years; they received wheat bread test meals with a herbal tea (polyphenol-rich tea, inhibitor of iron absorption) or water, fortified with either ferrous sulfate or ferrous fumarate. One mother and one child from this study were excluded due to missing samples. Studies 5 and 9 were done in Haiti at the Ministry of Health in Port au Prince. It included mothers (study 9, n = 22) without severe anemia (Hb > 10 g/dL) and their preschool child (study 4, n = 22), aged 2.5 to 5 years, with weight-for-height (WHZ) and height-for-age z-score (HAZ) > -2; they were given a wheat bread-based test meal.[30] One participant of each study was excluded due to missing sample.

All studies used isotopically enriched elemental iron ^{54}Fe, ^{57}Fe, and ^{58}Fe, which were purchased from Chemgas (Boulogne, France). The isotopically labeled iron compounds were produced as described in each study.[27-30]

Laboratory Analysis

At recruitment, hemoglobin, serum ferritin, C-reactive protein (CRP), and anthropometrics were measured as previously described.[27-30] Anemia in women was defined as Hb < 120 g/L; in children ≥ 5 year-old, Hb < 115 g/L, and in children < 5 year-old, Hb < 110 g/L. ID in women was defined as SF < 15 µg/L; in children ≥ 5 y, SF < 15 µg/L; in children < 5 y: SF < 12 µg/L. IDA in women and in children was defined as both Hb and SF below these cutoffs.[31] *H. pylori* infection was assessed from a baseline serum sample using a qualitative rapid immunochromatographic assay (rapid anti-*H. pylori* test, Rapid Labs Ltd., Essex, UK), to detect the presence of IgG antibodies anti-*H. pylori* in serum or plasma. This test has 86.7% relative sensitivity and 91% relative specificity, and an overall agreement of 89.8% compared to an ELISA assay.[32] The test was carried out according to the instructions of the manufacturer: One drop of thawed serum was placed on the sample well, and then one drop of sample diluent was added, the result was read after 15 minutes; the test was judged invalid if the control line did not appear. Iron bioavailability (erythrocyte iron incorporation) was calculated based on the shift in isotope ratios and the estimated amount of iron circulating in the body, with the use of the participants' blood volume.[33] The calculations are based on the principles of isotope

dilution, considering that iron isotopic labels were not monoisotopic.[34-36] Details have been previously described.[27-30]

Statistical Analysis

To detect an inter-subject difference of 30% with a β of 0.8 and α of 0.05, a sample size of 33 per group was calculated to be sufficient. Data were analyzed in IBM SPSS statistics (version 23). For children under five years of age WHO Anthro (v3.2.2, 2011; WHO) and above five years WHO Anthro plus (v1.0.4, 2007; WHO) was used to calculate age-specific HAZ, WAZ, and WHZ. Adjustment of serum ferritin for inflammation (CRP) was performed as previously described.[37] Iron bioavailability was also standardized to an SF concentration of 40 µg/L, as described.[38] Statistical tests were performed on both unadjusted and adjusted iron bioavailability data. Data were tested for normal distribution by using the Kolmogorov-Smirnov test. If the data were not normally distributed, log-transformed data were used for analysis. Normally distributed data are presented as mean ± SD (WAZ, HAZ, WHZ) and not normally distributed data as geometric mean and 95% confidence interval (age, weight, height, Hb, SF, CRP, fractional iron absorption). Independent samples t-tests were performed to detect differences in iron status, and anthropometric measures between *H. pylori* positive versus negative groups. To detect differences in fractional iron absorption, a linear mixed model (LMM) analysis was performed with subject ID as random intercept, fractional iron absorption as dependent factor, *H. pylori* infection, the iron compound, the food matrix of the test meal and whether the meal was given with an enhancer or inhibitor of iron absorption as fixed factors. The same model was repeated with the SF corrected fractional iron absorption data. To describe predictors of fractional iron absorption in these populations, a backward linear regression was performed separately for children and women to find the minimal adequate model. Fractional iron absorption was set as dependent variable and independent variables were: *H. pylori* infection, the food matrix of the test meal, whether the test meal was given with an enhancer of inhibitor of iron absorption, the iron compound, gender (in the children's model), age, Hb, the SF adjusted for CRP and the subject ID. Then an LMM analysis was performed with subjects' ID defined as random intercept, fractional iron absorption as the dependent variable, and the variables from the minimal adequate model set as fixed factors. p values < 0.05 were considered as statistically significant.

Table 1: Overview of the studies included in this analysis: Study population, sample size, test meals and iron compounds given.

Study	Location	Age Group	N	Test Meal Matrix	Fe/Meal (mg)	Study Arms (Fe Compound, Inhibitor or Enhancer)
1	Benin	Preschool children	18	Pearl millet porridge	6	1. FeSO4 2. FeSO4 and NaFeEDTA[a]
2	Benin	Preschool children	16	Pearl millet porridge	6	1. $FeSO_4$ 2. $FeSO_4$ and Ascorbic Acid
3	Benin	Preschool children	18	Pearl millet porridge	6	1. $FeSO_4$ 2. $FeSO_4$ and phytase 3. $FeSO_4$ and phytase and Ascorbic Acid
4	Senegal	Preschool children	17	Wheat bread	2	1. Fe Fumarate 2. $FeSO_4$ 3. Fe Fumarate and tea infusion 4- $FeSO_4$ and tea infusion
5	Haiti	Preschool children	21	Wheat bread	2	1. Fe Fumarate 2. NaFeEDTA
6	Benin	Women	23	Fermented sorghum porridge	3	1. NaFeEDTA
7	Benin	Women	20	Pearl millet paste	4	1. Regular millet: $FeSO_4$ 2. Biofortified millet: $FeSO_4$ 3. Post-harvest fortified millet: $FeSO_4$
8	Senegal	Women	17	Wheat bread	4	1. Fe Fumarate 2. $FeSO_4$ 3. Fe Fumarate and tea infusion 4. $FeSO_4$ and tea infusion
9	Haiti	Women	21	Wheat bread	4	1. Fe Fumarate 2. NaFeEDTA 3. NaFeEDTA and Fe fumarate[b]

[a]3 mg Fe as FeSO4 mixed with 3 mg Fe as NaFeEDTA.
[b]2 mg Fe as NaFeEDTA mixed with 2 mg Fe fumarate.

Results

This analysis included data from 84 women and 91 children (**Table 2**); four women and one child were excluded from the analysis because *H. pylori* status could not be determined. Data on iron bioavailability from 11 different test meals were included for women and data from 13 different test meals for children; the analysis included a total of 213 iron bioavailability measures in women and 232 measures in children.

Table 2: Subject characteristics: Age, anthropometrics, hemoglobin (Hb), serum ferritin (SF), and C-reactive protein (CRP) concentrations, of all subjects grouped by *H. pylori* infection status and age group.[a]

		Children			Women	
***H. pylori* Status**	**Positive**	**Negative**	***p***	**Positive**	**Negative**	***p***
n	49	41	-	41	39	-
Age y	2.8 (2.6, 3.1)[b]	2.9 (2.6, 3.2)	0.882	25.7 (23.4, 28.3)	21.8 (20.2, 23.6)	0.007
Weight, kg	12.3 (11.7, 13.0)	12.4 (11.7, 13.2)	0.975	55.1 (53.3, 57.0)	55.0 (52.7, 57.4)	0.997
Height, cm	91 (89, 94)	92 (89, 95)	0.972	160 (158, 162)	162 (159, 164)	0.447
WAZ	−0.94 ± 0.78[c]	−0.93 ± 1.08	0.759	n.a.	-	
HAZ	−0.88 ± 1.32	−0.89 ± 1.63	0.818	n.a.	-	
WHZ	−0.68 ± 0.87	−0.67 ± 1.10	0.938	n.a.	-	
Hb g/L	109 (107, 112)	114 (110, 117)	0.052	126 (121, 132)	125 (121, 130)	0.776
SF µg/L	32.6 (26.7, 39.8)	31.7 (25.1, 40.0)	0.855	30.1 (22.1, 41.0)	30.0 (22.9, 39.4)	0.989
SF adjusted[d] µg/L	15.8 (12.9, 19.4)	16.6 (13.3, 20.7)	0.744	18.2 (13.8, 24.1)	19.1 (14.6, 25.1)	0.797
CRP mg/L	1.20 (0.71, 2.03)	0.82 (0.49, 1.37)	0.499	0.67 (0.43, 1.05)	0.60 (0.41, 0.88)	0.696
CRP > 5 mg/L (n)	12	8	-	2	3	-

[a]Differences between *H. pylori* positive versus negative were assessed by unpaired t-test. WAZ, weight-for-age z score; HAZ, height-for-age z score; WHZ, weight-for-height z score; n.a., not applicable; Hb, hemoglobin; SF, serum ferritin; CRP, C-reactive protein.
[2]All such values are geometric means (95% CIs).
[3]All such values are means ± SD.
[4]Serum ferritin adjusted for C-reactive protein.[37]

The prevalence of *H. pylori* infection among women and children was 51.3% and 54.4%, respectively (Table 2). The age of the women ranged from 16 to 43 years-old, the age of the children ranged from 18 to 74 months. *H. pylori* infected women were significantly older than non-infected women ($p < 0.05$) (Table 2). All anthropometric measures (weight, height, and all z-scores in children) did not differ significantly between *H. pylori* infected and non-infected children nor women (Table 2). Iron status (Hb, SF and SF adjusted for inflammation) and inflammation (CRP) did not differ between the infected and non-infected groups. Among the *H. pylori* positive women, 27% (n = 11) were anemic, 22% (n = 9) had ID and 15% (n = 6) had IDA, adjusted for inflammation prevalence of ID was 41% (n = 17) and of IDA 22% (n = 9).

Among the *H. pylori* negative women, 31% (n = 12) were anemic, 23% (n = 9) had ID and 21% (n = 8) had IDA, when adjusting for inflammation prevalence of ID was 38% (n = 15) and IDA 26% (n = 10). Among the *H. pylori* positive children, 42% (n = 21) were anemic, 8% (n = 4) had ID and 6% (n = 3) had IDA, and 32% (n = 16) had ID and 20% (n = 10) had IDA when adjusting for inflammation. Among the *H. pylori* negative children, 27% (n = 11) were anemic, 12% (n = 5) had ID and 5% (n = 2) had IDA, and 27% (n = 11) had ID and 15% (n = 6) had IDA when adjusting for inflammation.

Factors predicting fractional iron absorption in women and children are shown in **Table 3**. In children, the adjusted R^2 was 0.218, predictors were: Food matrix of the test meal ($p < 0.001$); Fe compound ($p < 0.001$); whether the meal contained an iron absorption enhancer ($p < 0.001$) or inhibitor ($p < 0.001$); and the age ($p = 0.019$) (estimates and standard errors are listed in Table 3). Removed variables were: *H. pylori* infection, gender, Hb concentration and SF (adjusted for inflammation) concentration. In women the adjusted R^2 was 0.254, variables in the minimal model were: Food matrix ($p < 0.001$); presence of iron absorption inhibitor ($p < 0.001$); SF (adjusted for inflammation) ($p < 0.001$); and Hb concentration ($p = 0.273$). Removed variables were: *H. pylori* infection, Fe compound, and age.

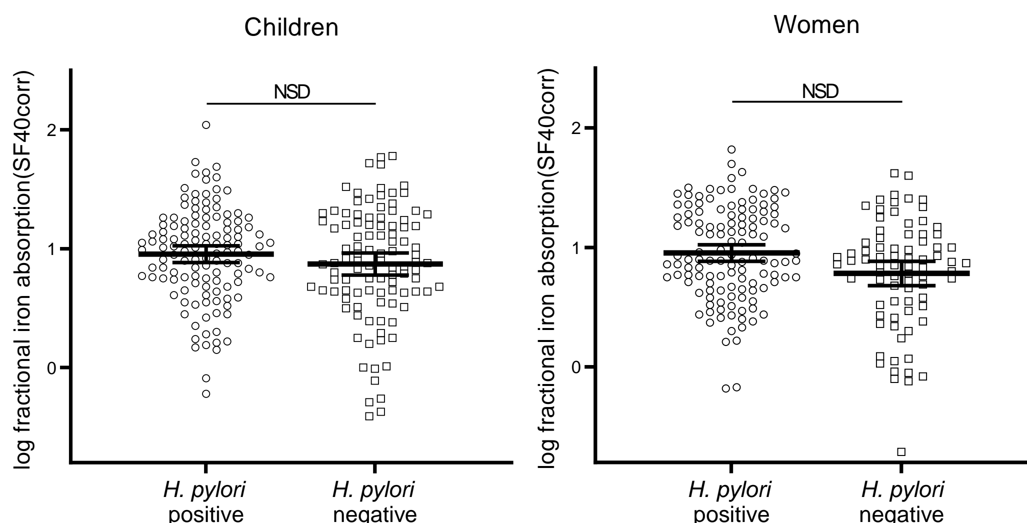

Figure 2: Log fractional iron absorption adjusted for SF of 40 µg/L, of *H. pylori* positive and negative children (n = 90) and women (n = 80), each dot represents a test meal. *H. pylori* positive children: n = 128, negative: n = 107, women n = 126 and n = 87 respectively. The line indicates the mean and 95% CI. There were no significant differences (NSD) between *H. pylori* positive versus negative women nor children, assessed by LMM (dependent variable: Fractional iron absorption adjusted for serum ferritin; fixed factors: *H. pylori* infection, iron compound, test meal food matrix and iron absorption enhancer or inhibitor; random factor: Subject ID number).

Figure 2 shows the fractional iron absorption data from the women and children, by group, adjusted for SF. Geometric mean (95% CI) iron absorption (%). In the *H. pylori* infected and non-infected women fractional iron absorption was 9.73% (8.24, 11.48) and 8.52% (6.99, 10.39), respectively, and did not predict absorption ($p = 0.231$) in a model including iron compound ($p = 0.179$), test meal matrix ($p = 0.154$), and whether the test meal contained an iron absorption inhibitor ($p < 0.001$). After adjusting for differences in SF, fractional iron absorption was 8.97% (7.64, 10.54) and 6.06% (4.80, 7.76), respectively. In a model including iron compound ($p = 0.167$), test meal matrix ($p < 0.001$), or whether the test meal contained an iron absorption inhibitor ($p < 0.001$), *H. pylori* infection did not result in significant prediction ($p = 0.274$), as shown in Figure 2. In children, fractional iron absorption in the *H. pylori* infected group was 9.77% (8.39, 11.4) and 8.94% (7.57, 10.6) in the non-infected group. *H. pylori* status was not a significant predictor ($p = 0.669$), when the model was controlled for the iron compound ($p < 0.001$), test meal matrix ($p = 0.002$), or whether the test meal contained an iron absorption enhancer ($p < 0.001$) or inhibitor ($p < 0.001$). After adjusting for differences in SF, fractional iron absorption was 9.02% (7.68, 10.59) and 7.44% (6.01, 9.20), respectively. When controlled for an iron compound, test meal matrix, or whether the test meal contained an iron absorption enhancer or inhibitor (for all, $p < 0.001$), *H. pylori* infection was not a significant predictor ($p = 0.479$), also shown in Figure 2.

Discussion

Our findings suggest that asymptomatic *H. pylori* infection in preschool children and young women does not have a significant effect on fractional iron absorption from iron compounds commonly used as food fortificants. Two previous studies assessed iron absorption in humans with *H. pylori* infection using iron isotope techniques, and have produced equivocal results.[19,20] This study is consistent with the previous study in Bangladeshi children.[20] In 2–5 year-old children (thirteen with *H. pylori* infection and twelve uninfected) with IDA, iron absorption from ferrous sulfate and ferrous fumarate from infant cereal was measured before and after a 14-day course of eradication treatment. There was no significant difference in iron absorption comparing *H. pylori* non-infected to *H. pylori* infected children before treatment and eradication therapy did not affect iron absorption from ferrous sulfate or ferrous fumarate.[20] The authors concluded that although gastric acid output was impaired in *H. pylori*-

infected children and that treatment of *H. pylori* infection improved gastric acid output, it did not significantly influence iron absorption.

Table 3: The minimal adequate model predicting fractional iron absorption of preschool children (n = 90) and women of reproductive age (n = 80) in relation to *H. pylori* infection, food matrix, iron compound, whether the test meal contained an iron absorption enhancer or inhibitor, gender, age, hemoglobin and serum ferritin adjusted for inflammation.[a]

	Children[b]			**Women**[c]		
Variables	***b***	**SE**	***p***	***b***	**SE**	***p***
Intercept	1.62	0.21	0.000	−0.22	1.69	0.898
H. pylori infection	removed from the model[d]			removed from the model		
Food Matrix (all pairwise)						
wheat bread-millet paste		n.a.		0.34	0.09	0.000
wheat bread-fermented sorghum		n.a.		−0.21	0.09	0.016
millet paste-fermented sorghum		n.a.		−0.55	0.10	0.000
millet-porridge-wheat bread	0.41	0.11	0.000		n.a.	
Fe compound (all pairwise)				removed from the model		
Sulfate-Fumarate	0.17	0.05	0.001			
Sulfate-EDTA	0.05	0.08	0.557			
Sulfate-Sulfate and EDTA	0.15	0.06	0.024			
Fumarate-EDTA	−0.13	0.07	0.055			
Fumarate-Sulfate and EDTA	−0.02	0.08	0.762			
EDTA-Sulfate and EDTA	0.10	0.10	0.320			
Fe absorption enhancer					n.a.	
Ascorbic Acid-none	0.19	0.07	0.005			
Phytase-none	0.29	0.06	0.000			
Ascorbic Acid and Phytase-none	0.36	0.06	0.000			
Fe absorption inhibitor						
Tea-none	−0.42	0.05	0.000	−0.33	0.05	0.000
Gender	removed from the model				n.a.	
Age	−0.83	0.34	0.019	removed from the model		
Hemoglobin		removed		0.93	0.85	0.273
Serum ferritin adjusted for CRP		removed		−0.52	0.13	0.000

[a]The minimal adequate model assessed by backward regression. Estimates (*b*) and standard errors (SE) assessed by linear mixed model: Random factor: Subject ID number; dependent variable: Fractional iron absorption; fixed factors in children's model: Food matrix, Fe compound, Fe absorption enhancer, Fe absorption inhibitor, and age; fixed factors in women's model: Food matrix, Fe absorption inhibitor, hemoglobin and serum ferritin adjusted for CRP (C-reactive protein).
[b]Minimal adequate regression model of children: R^2 = 0.239; adjusted R^2 = 0.218.
[c]Minimal adequate regression model of women: R^2 = 0.272; adjusted R^2 = 0.254.
[d]Removed variable by the backward regression to assess the minimal adequate model.

However, these study results differ from those in the study by Lopez de Romana et al.[19] in which the effect of *H. pylori* infection (assessed using the ^{13}C urea breath test) on iron absorption was compared in iron-sufficient asymptomatic adults, 24 who were *H. pylori*-positive and 26 who were *H. pylori*-negative. They consumed wheat flour-based test meals fortified with radiolabeled ferrous sulfate or ferrous fumarate. The *H. pylori*-negative subjects absorbed significantly more iron from ferrous sulfate (10.5% vs. 4.4%) and ferrous fumarate (0.6% vs. 0.4%). Iron absorption was not significantly different between groups after they

received a proton pump inhibitor. Compared to the women in this study, the adults in that study had better iron status (mean serum ferritin, ≈45 µg/L versus 30 µg/L in this study) and received a much larger dose (55 mg) given with the test meal, compared to the smaller dosages provided in the test meals in this study (3–6 mg). Further, the diagnosis of *H. pylori* infection was made with different methods.

In a study by Ciacci et al.[18] in adults (n = 55) who were *H. pylori* positive or negative, serum iron levels were measured before and 2 hours after oral supplementation of 1 mg ferrous iron per kg bodyweight. *H. pylori* positive subjects were then administered antibiotic therapy, and the oral iron absorption test was repeated. They reported that *H. pylori* positive subjects before treatment had a smaller increase in serum iron compared to *H. pylori* negative subjects, and after *H. pylori* eradication in the *H. pylori* positive subjects, their serum iron increase was similar to those of non-infected subjects, suggesting that *H. pylori* infection impairs oral iron uptake.[18] Sarker et al.[21] randomized *H. pylori* infected children 2–5 years of age with IDA to receive 2-week anti-*H. pylori* therapy plus 90-day oral ferrous sulfate, 2-week anti-*H. pylori* therapy alone, 90-day oral iron alone, or placebo; non-infected children with IDA received iron treatment as a negative control. *H. pylori* infection did not inhibit the response to iron, suggesting it is not a cause of iron deficiency or a reason for treatment failure of iron supplementation in this setting.[21] However, other studies have suggested that *H. pylori* infected children show a blunted response to oral iron.[39] A recent systematic review reported that, in observational studies, compared to uninfected persons, *H. pylori* infected individuals are at greater risk for iron deficiency and iron deficiency anemia. Also, prospective trials comparing *H. pylori* eradication therapy plus iron supplementation, as compared with iron supplementation alone, showed greater increases in serum ferritin with combined therapy.[8]

Several mechanisms have been suggested as a potential cause of iron deficiency and/or low iron absorption during *H. pylori* infection. Chronic gastritis, due to *H. pylori*, can alter the physiology of the stomach by reducing gastric acid secretion and gastric ascorbic acid levels. Both of which are essential for the absorption of dietary iron.[11-17] *H. pylori* requires iron for its growth,[40,41] it expresses proteins associated with iron metabolism,[41] and it is suggested that it may disrupt host hepcidin regulation.[42] A decrease in serum hepcidin levels has been reported after an *H. pylori* eradication therapy, in two studies,[43,44] however one of them concluded that the decrease is more related to anemia status than to *H. pylori* infection as a decrease of

hepcidin was also reported in the group receiving iron supplement only without *H. pylori* eradication therapy.[43] Finally, iron losses may increase due to occult bleeding from *H. pylori* gastritis.[42,45] Our finding that asymptomatic *H. pylori* infection does not impair fractional iron absorption from a meal, suggests that if asymptomatic *H. pylori* infection increases the risk for iron deficiency,[8] it may do this through increasing gastrointestinal blood loss, rather than reducing dietary iron absorption.

The model looking at predictors of iron absorption from fortified test meals confirms earlier studies which showed that food composition, or the addition of ascorbic acid, phytase or a polyphenol-rich tea, affect iron absorption in both women and children.[23,46] Iron status is a commonly known predictor of iron absorption,[38,47] and the current study data in women confirm this. In contrast, the current data in preschool children suggest that iron status does not predict iron absorption; however, this can potentially be explained by the narrow range of SF values in this group.

Both the severity and location of *H. pylori* related gastritis might be determinants of gastric function and acidity, and iron absorption. Gastritis affecting the antral mucosa increases the release of gastrin and secretion of acid. In contrast, when gastritis is predominant in the corpus this impairs parietal cell acid secretion, leading to hypochlorhydria, this form of infection is seen in most infected subjects.[10] *H. pylori* positive children with hypochlorhydria (pH > 4) were hypoferremia compared to *H. pylori* positive children with gastric pH ≤ 4.[16] When gastritis affects both the antrum and corpus, the acid output may remain normal.[10] Altogether, these data suggest that *H. pylori* gastritis may alter gastric physiology to favor either an increase or a decrease in absorption of dietary iron, and this may confound studies, such as the present study, looking at differences in iron absorption-based only on serology. However, the location of gastric inflammation caused by *H. pylori* can be determined only with endoscopy, which we did not perform in our subjects.

Our study has several strengths. It is the first pooled analysis to date assessing the association of asymptomatic *H. pylori* infection with fractional iron absorption quantified using iron stable isotopes. We studied two risk groups for iron deficiency, young women and preschool children, residing in three low-income countries. We were able to assess potential effects from a diverse range of test meals and three commonly used iron fortificants. We used linear mixed model analysis to assess whether *H. pylori* infection predicted fractional iron absorption,

controlling for age, hemoglobin, iron, inflammation status, and test meal matrix, all measured using standardized methods across the studies. However, there are several limitations to this study. It was retrospective, and assessment of *H. pylori* infection was performed using serology, which may produce false positives results, because (despite an eradicated infection) IgG titers may remain in the serum and decline only over several months.[48] However, none of the subjects in this study had received recent *H. pylori* eradication therapy. A more precise assessment method of a current *H. pylori* infection would have been a ^{13}C urea breath test. Also, the assessment of gastric acid output before and after *H. pylori* eradication would have been valuable to distinguish between antral and corpus infection. Despite these limitations, this study provides important new data on the link between *H. pylori* and iron absorption, and suggests, in asymptomatic women and preschool children in low-income settings, *H. pylori* status is not a major predictor of fractional iron absorption from iron fortificants.

Author Contributions

The authors' responsibilities were as follows: D.M., N.F.N., C.I.C., I.H.-A., and M.B.Z.: Designed the studies; D.M., N.F.N, C.I.C. and I.H.-A.: Conducted the studies; N.F.N., C.I.C., I.H.-A. and S.B.: Conducted the laboratory analysis; S.B. and D.M.: analyzed the data; S.B. and M.B.Z.: Wrote the first draft of the manuscript; S.B., D.M. and M.B.Z.: Had primary responsibility for the final content; and all authors: Read and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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MANUSCRIPT 2

THE TMPRSS6 VARIANT (SNP RS855791) AFFECTS IRON METABOLISM AND ORAL IRON ABSORPTION – A STABLE IRON ISOTOPE STUDY IN TAIWANESE WOMEN

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Abstract

Genome wide studies have associated *TMPRSS6* rs855791 (2321 C>T) with iron status and hepcidin. It is unclear whether this polymorphism affects iron absorption. In nonanemic Taiwanese women (n = 79, 44 TT variant, 35 CC variant), we administered standardized rice-based test meals containing 4 mg of labeled ^{57}Fe or ^{58}Fe as FeSO4 on alternate days. Fractional iron absorption was measured by erythrocyte incorporation of the tracers 14 days after administration. Compared to the CC variant, in the TT variant serum iron and transferrin saturation were lower ($P = 0.001$; $P < 0.001$, respectively) and serum hepcidin/transferrin saturation and serum hepcidin/serum iron ratios were higher ($P = 0.042$; $P = 0.088$, respectively). Serum hepcidin did not differ between groups ($P = 0.862$). Geometric mean (95% CI) fractional iron absorption, corrected to a serum ferritin of 15 µg/L, was 26.6% (24.0, 29.5) in the CC variant and 18.5% (16.2, 21.1) in the TT variant ($P = 0.002$). Overall, predictors of iron absorption were: serum ferritin ($P < 0.001$); genetic variant ($P = 0.032$); and hepcidin ($P < 0.001$). In the models by variant, in the CC variant the model explained 67-71% of variability in absorption and serum ferritin was the only significant predictor ($P < 0.001$); in the TT variant, the model explained only 35-43% of variability, and hemoglobin ($P = 0.032$), soluble transferrin receptor ($P = 0.004$) and hepcidin ($P < 0.001$) were significant predictors. Women with the *TMPRSS6* rs855791 (2321 C>T) polymorphism show altered iron homeostasis which affects oral iron absorption and may increase their risk for iron deficiency. The trial was registered at www.clinicaltrials.gov as NCT03317873, and funded by the Kaohsiung Chang-Gung Memorial Hospital, Kaohsiung, Taiwan, (Grant: CMRPG8F0721) and ETH Zurich, Switzerland.

Introduction

In absence of a physiological iron excretion mechanism, long-term iron balance in humans is determined by dietary iron absorption. Systemically, iron absorption is controlled by hepcidin, a peptide hormone synthesized in hepatocytes[1] that regulates iron export from cells via its interaction with ferroportin.[2] Hepcidin (Hep) is synthesized in response to increasing body iron in a homeostatic feedback loop, involving iron sensing of iron-saturated transferrin by transferrin receptors (Tfr1 and Tfr2) and associated proteins (HFE, hemojuvelin) initiating a cascade involving bone morphogenic protein (BMP) receptor activation.[3,4]

The *TMPRSS6* gene encodes the transmembrane serine protease matriptase-2, which interacts with hemojuvelin, modulating the hepcidin activation pathway.[5] Consistently with this regulatory model, nonsense mutations in *TMPRSS6* cause iron refractory iron deficiency anemia (IRIDA), due to inappropriately elevated hepcidin levels.[6] The ratio of serum hepcidin/transferrin saturation (TS) may be useful to differentiate subjects with IRIDA from subjects with chronic iron deficiency (ID),[7] consistent with a disrupted feedback loop between TS and hepcidin.

Common genetic variants of *TMPRSS6*, are associated with erythrocyte parameters in human genome wide association studies.[8-11] The single nucleotide polymorphism (SNP) rs855791 (2321 C>T) of *TMPRSS6* has a population frequency of ≈0.5 in Caucasians,[10,12] ≈0.6 in Japanese[13] and ≈0.2-0.1 in African Americans.[10,12] It causes a nonsynonymous substitution near the catalytic and active site of the protease,[10] with a strong association with iron status, erythrocyte parameters,[8-10,12,14-17] Hepcidin[18] and ratios of Hep to iron indices.[19,20] T-allele variants in the rs855791 are associated with an increased risk for ID and iron deficiency anemia (IDA).[16,17] In a case-control study in Taiwan, homozygotes for the SNP rs855791 CC had a lower prevalence of IDA, compared to subjects with the CT or TT variant.[21] In European populations, variants (TT) in rs855791 are associated with lower TS and serum ferritin (SF), higher Hep, and higher ratios of hepcidin to iron indices.[18-20] In first time blood donors, the TT variant was associated with larger decreases in SF and hemoglobin (Hb) after multiple donations, suggesting an impaired capacity to replenish stores following donation.[22]

ID is considered the most prevalent nutritional deficiency worldwide and one of the leading causes of anemia among non-pregnant and pregnant women.[23] While iron status and dietary composition are the main determinants of iron absorption, individual factors other than iron

status have been estimated to account for ≈50% of the variance in iron absorption.[24] Furthermore, a strong familial tendency in iron absorption has been reported in mother child pairs using stable iron isotopes;[25,26] this could be due to genetic, epigenetic or shared environmental mechanisms.

The genetic determinants of iron status and hepcidin metabolism in humans, including the effect of mutations in *TMPRSS6*, are poorly understood. The study aim was to compare iron absorption, hepcidin and other indices of iron metabolism in iron-sufficient Taiwanese women carrying the TT or the CC variant of the rs855791 SNP in the *TMPRSS6* gene. We hypothesized that the TT variant would be associated with higher serum hepcidin concentrations, higher ratios of hepcidin to iron indices, and lower iron absorption at comparable iron status.

Methods

Subjects

The study was performed at the Kaohsiung Chang Gung Memorial Hospital (K-CGMH) in Taiwan, between February 2018 and February 2019. The flow of study participants is shown **Figure 1**. We invited for screening apparently healthy females, with no known history of thalassemia or anemia, aged between 20 to 45 years, assessed medical history and measured body weight and height, complete blood count, SF and the rs855791 genotype. Inclusion criteria are described in the Online Supplementary Methods. All participants that were homozygous in the rs855791 (TT or CC), and fulfilled all inclusion criteria were recalled one week before the first test meal administration, where we assessed Hb, SF, C-reactive protein (CRP), and menstrual blood losses. Study inclusion criteria were: 1) Hb > 120 g/L; 2) SF 30 – 120 µg/L and 3) CRP < 5 mg/L. The ethical committees of ETH Zurich in Switzerland and the Chang Gung Memorial Foundation Institutional Review Board in Taiwan approved the study. All participants provided written informed consent, and the study was registered at clinicaltrials.gov (NCT03317873).

On study days one and three (D1, D3), we administered two standardized rice test meals to fasting participants, labelled with 4 mg iron (^{57}Fe, and ^{58}Fe) as labelled ferrous sulfate ($FeSO_4$). Detailed description of the test meal administration and preparation of stable iron isotopes, can be found in the Online Supplementary Methods.

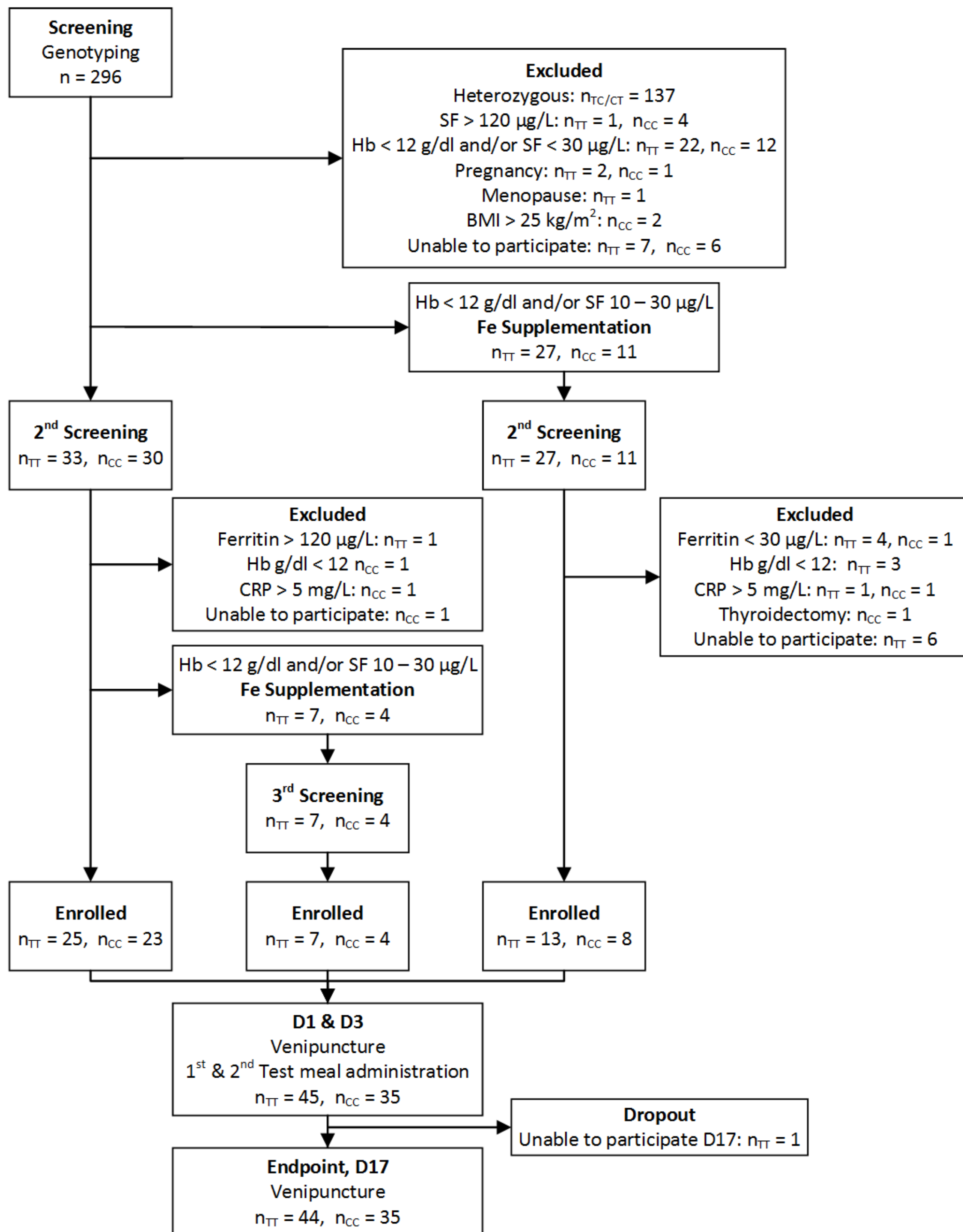

Figure 1: Study flow chart.

Laboratory analyses

We determined fractional iron absorption (FIA) based on the shift in the enrichment ratio of stable iron isotopes into the erythrocytes on D17. We performed the analyses by inductively coupled plasma mass spectrometry (MC-ICP-MS, Neptune; Thermo Finnigan) as previously

described.[27] We calculated the amounts of ^{57}Fe, and ^{58}Fe isotopic labels in blood on D17 on the

basis of the shift in iron isotope ratios and on the estimated amount of iron circulating in the body.[28] We corrected the FIA for SF to the cutoff for ID (15 µg/L),[29] and to 50 µg/L as a level representing sufficient iron stores with a modification of the Cook *et al.* formula,[30] as described in the Online Supplementary Methods. Procedures such as the assessment of menstrual blood loss, and laboratory measurements such as genotyping, measurement of erythrocyte parameters,

CRP, acute phase protein alpha-1-acid glycoprotein (AGP), SF, serum iron (SFe), total iron binding capacity (TIBC), Hep, and soluble transferrin receptor (sTfR) are also described in the Online Supplementary Methods.

Sample size calculation

We based the sample size calculation on a design with two repeated measurements with a compound symmetry covariance structure. Based on previous studies from the Human Nutrition Laboratory, using log transformed data, we assumed an intra-individual correlation of 0.7, and a standard deviation of 0.235. A difference of 30% in iron absorption was considered relevant. Therefore, we planned to recruit 40 subjects per variant, with 80% power and $\alpha = 0.05$, it allows 2 dropouts per group. Due to the imbalanced distribution of the minor allele in the Taiwanese population, and difficulties enrolling the planned number of CC subjects, we made a protocol amendment to include 35 CC and 45 TT subjects. This unbalanced distribution results in an estimated power of 75%.

Data and Statistical analysis

We used IBM SPSS statistics (Version 24) for statistical analysis. After testing for normality, we used log-transformed data further analysis if not normally distributed. Normally distributed data is presented as means ± standard deviation (SD), transformed normal data as geometric mean with the 95% confidence interval (95%CI), non-normal data as median and the interquartile range (IQR). Means or medians of red cell parameters, are based on the concentrations measured on D1. Means, medians, or geometric means of CRP, AGP, SF, SFe, TIBC, TS, sTfR, BIS, Hep, Hep/TS, Hep/SF, and FIA are based on concentrations measured on D1 and D3. We tested between group differences for normally distributed variables with

independent samples T-Test and for not normally distributed variables using Mann-Whitney U Test; differences in CRP, AGP, SF, SFe, TIBC, TS, sTfR, BIS, Hep, Hep/TS, Hep/SF, and FIA by linear mixed models (LMM), with subjects' code as random intercept, the corresponding variable as dependent variable and genotype as fixed effect. We assessed Pearson's correlations and differences between the coefficients with the Fishers r to z transformation. We assessed predictors of iron absorption with LMM using subjects' code as random intercept, FIA as dependent variable, and genotype, Hb, SF, TS, sTfR, Hep and PBAC as fixed factors. We performed a backward linear regression to assess a minimal adequate model, and we fitted the variables in a LMM. Statistical significance was defined as $P < .05$.

Results

Subjects

We screened 296 women and identified 93 women carrying the TT variant and 66 with the CC variant, while 137 were excluded as heterozygotes (Figure 1). Of the identified subjects, 33 women with the TT variant and 30 with CC variant met all inclusion criteria. Thirty-four women with TT variants and 15 with CC variants received iron supplements. After the iron supplementation period, 20 subjects with the TT and 12 subjects with the CC variant were included into the study. Finally, 35 subjects with CC and 45 with the TT variant fulfilled all study inclusion criteria and were enrolled (Figure 1). One woman with the TT variant left the study after study D3, thus, 79 women completed the study.

Iron indices

Serum ferritin concentrations were balanced between the two variants, while SFe was lower in the TT compared to the CC variant ($P = 0.001$; **Table 1**). Similarly, TS was lower ($P < 0.001$), and TIBC higher ($P = 0.086$) in the TT variant (Table 1**)**. While Hep did not differ between groups ($P = 0.862$), the Hep/TS ratio ($P = 0.042$) and the Hep/SFe ratio ($P = 0.088$) were 28% and 25% higher in the TT variant, respectively (Table 1). None of the subjects had systemic inflammation, during the study period (Table 1). The menstrual blood loss scores (PBAC) was higher in the TT variant ($P = 0.015$, Table 1).

Table 1: Subject characteristics of Taiwanese women with the homozygous CC and TT variants of the rs855791 in TMPRSS6.

	CC	TT	*P*
n	35	45	-
Age, y [a]	34 ± 6	36 ± 7	0.436[d]
Weight, kg [a]	54.6 ± 4.8	54.2 ± 5.2	0.717[d]
Height, cm [a]	160 ± 4	160 ± 5	0.780[d]
CRP, mg/L [b]	0.271 (0.213, 0.346)	0.388 (0.306, 0.491)	0.125[e]
AGP, g/L [a]	0.426 ± 0.0962	0.455 ± 0.113	0.206[e]
RBC, million/µl [c]	4.51 (4.19-4.59)	4.58 (4.37-4.74)	0.032[f]
Hb, g/dl [a]	13.3 ± 0.6	13.3 ± 0.7	0.762[d]
HCT, % [c]	40.0 (38.4-40.9)	40.3 (38.7-41.5)	0.349[f]
MCV, fl/cell [c]	89.8 (88.3-92.0)	88.4 (86.4-90.6)	0.008[f]
MCH, pg/cell [c]	30.0 (29.2-31.0)	29.6 (28.8-30.0)	0.018[f]
SF, µg/L [b]	45.1 (41.0, 49.7)	47.0 (43.5, 50.9)	0.626[e]
SFe, µg/dL [b]	114.5 (105.2, 124.7)	90.3 (82.6, 98.7)	0.001[e]
TIBC, µg/dL [a]	315.6 ± 29.7	327.3 ± 33.5	0.086[e]
TS, % [b]	36.5 (33.3, 39.9)	27.7 (25.5, 30.2)	<0.001[e]
sTfR, mg/L [b]	4.14 (3.97, 4.32)	4.33 (4.12, 4.55)	0.351[e]
BIS, mg/kg BW [a]	7.13 ± 1.62	7.12 ± 1.80	0.978[e]
Hep, nM [b]	2.10 (1.80, 2.46)	2.06 (1.79, 2.37)	0.862[e]
Hep/SFe, pmol/µg [b]	183.4 (160.3, 209.8)	227.8 (193.3, 268.4)	0.088[e]
Hep/TS, pM/% [b]	57.6 (50.8, 65.4)	74.2 (63.0, 87.4)	0.042[e]
Hep/SF, pmol/µg [b]	46.5 (40.7, 53.2)	43.7 (38.4, 49.8)	0.537[e]
PBAC [a]	126 ± 63	171 ± 99	0.015[d]

Anthropometrics, RBC indices and Hb were assessed on D1, inflammation, iron parameters, plasma hepcidin concentration were assessed on D1 and D3.

AGP, acute phase protein alpha-1-acid glycoprotein; BIS, body iron stores; CRP, C-reactive protein; Hb, hemoglobin; HCT, hematocrit; Hep, plasma hepcidin; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; PBAC, pictorial blood-loss assessment chart; SF, serum ferritin; SFe, serum iron; sTfR, soluble transferrin receptor; TS, transferrin saturation.

[a] Means ± SD.

[b] Geometric means (95% CI).

[c] Medians (IQR).

[d] Differences were assessed by two-sided independent *t* test.

[e] Differences were assessed by fitting linear mixed models with genotype as fixed effect, participants as the random effects, and the corresponding variable as dependent variable.

[f] Differences were assessed by Mann-Whitney U test.

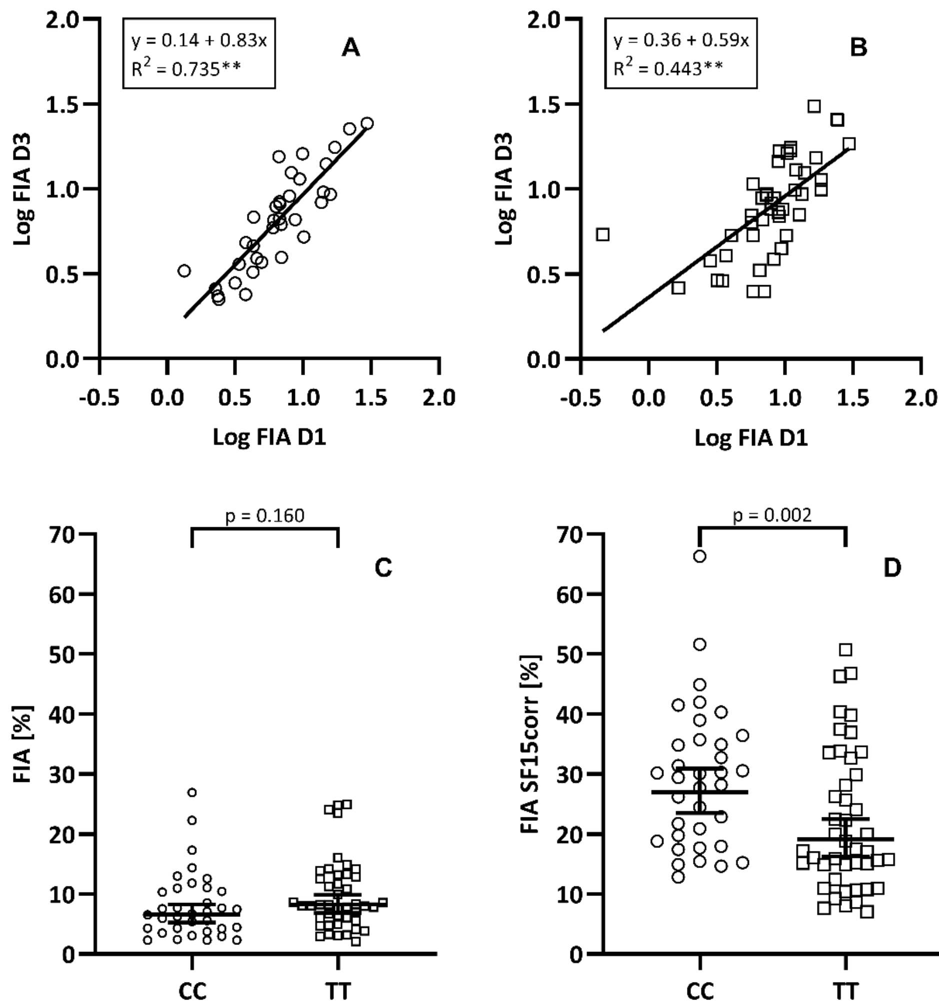

Figure 2: Fractional iron absorption (FIA) in rs855791 variants (A-B) and correlation of the interindividual FIA (C-D). A-B: Each point represents the mean of the FIA on day one and day three from two identical rice meals, the line represents the geometric mean and the bars the 95% CI. (A) the measured FIA, versus (B) the FIA corrected to a serum ferritin concentration of 15 µg/L is shown. Differences between the two variants were assessed by fitting linear mixed models with genotype as fixed effect, participants as the random effects, and FIA or FIA corrected for SF as the dependent variable CC (○, n = 35) versus TT (□, n = 44). **C-D:** FIA measured from identical rice test meals on study day one and three, separated by variant in the TMPRSS6 rs855791, in (C) the CC variant (○, n = 35), and in (D) the TT (□, n = 44). The Pearson's correlation factors are: 0.86, and 0.67 for the CC and TT, respectively (both, ** = P < 0.001).

Fractional iron absorption

The uncorrected FIA on D1 and D3 within variant did not differ (**Table 2; Figure 2**) but the Pearson's correlation between days (D1 and D3) FIA was stronger in the CC (r = 0.86) than in the TT variant (r = 0.67; for both, *P* < 0.001; Figure 2). The mean uncorrected FIA of D1 and D3 of the TT variant, was 7.96% (6.87, 9.22), and in the CC variant was 6.50% (5.54, 7.62) (*P* = 0.160; Figure 2). FIA corrected to a SF concentration of 15 µg/L was significantly lower in the TT 18.5% (16.2, 21.1), compared to the CC variant 26.6% (24.0, 29.5) (*P* = 0.002; Table 2;

Figure 2). When corrected to a SF of 50 µg/L, the TT variant had significantly higher FIA than the CC variant: 7.59% (6.66, 8.66), compared to 5.70% (5.15, 6.31) (*P* = 0.012).

Table 2: Fractional iron absorption from rice meals in CC and TT variants of the rs855791.

	CC variant	TT variant	*P*
FIA_{D1}, %	6.50 (5.14, 8.22) [a]	7.99 (6.39, 9.98) [b]	0.206 [c]
FIA_{D3}, %	6.49 (5.17, 8.15) [a]	7.93 (6.50, 9.68) [b]	0.183 [c]
$FIA_{D1\ \&\ D3}$, %	6.50 (5.54, 7.62)	7.96 (6.87, 9.22)	0.160 [d]
$FIA_{D1\ \&\ D3}$, SF15corr, % [e]	26.6 (24.0, 29.5)	18.5 (16.2, 21.1)	0.002 [d]

Values are the geometric means and the 95% CI.
D1, study day 1; D3, study day 3; FIA, fractional iron absorption; SF15corr, serum ferritin correction to a concentration of 15 µg/L.
[a] differences between study day one and three were assessed by paired *t* test *P* = 0.984.
[b] differences between study day one and three were assessed by paired *t* test *P* = 0.935.
[c] differences between the two variants were assessed by two-sided independent *t* test.
[d] differences between the two variants were assessed by fitting linear mixed models with genotype as fixed effect, participants as the random effects, and the corresponding variable as dependent variable.
[e] correction was done using the formula: $\log(FIA_C) = \log(FIA_O) + a * \log(SF_C/SF_O)$, with $a_{CC} = -1.28$, $a_{TT} = -0.74$.

Correlation of fractional iron absorption, iron indices and hepcidin

The Pearson's correlation between FIA and SF was more pronounced in the CC variant (r = -0.79, *P* < 0.001) than in the TT variant (r = -0.45, *P* = 0.002) and there was a difference in the strength of the correlation between groups (*P* < 0.001, **Figure 3)**. Fractional absorption was correlated with TS only in the CC variant (CC: r = -0.45, *P* = 0.006; TT r = -0.14, *P* = 0.360) and the correlation coefficients tended to differ (*P* = 0.070). The correlation of FIA with Hep was more pronounced (*P* = 0.004) in the CC variant (CC: r = -0.81, *P* < 0.001, TT: r = -0.45, *P* = .002).

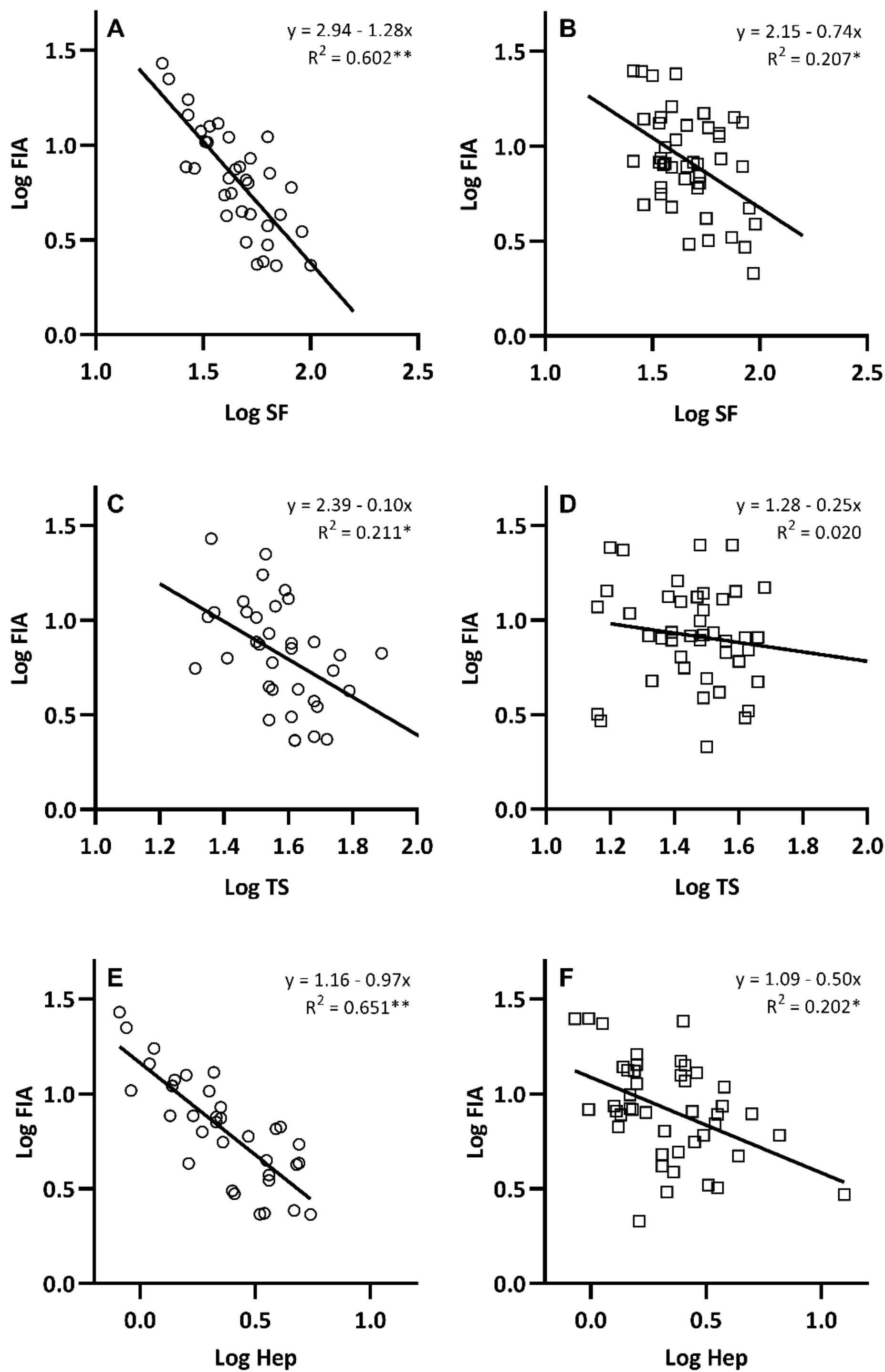

Figure 3: Correlations of FIA. Between FIA and (A, B) serum ferritin; (C, D) transferrin saturation; and (E, F) hepcidin, of the participants separated by the variants in the TMPRSS6 rs855791, CC (○, n = 35) and TT (□, n = 44). Each point represents one participant and their mean of FIA, SF, TS, and Hep measured on study day one and three. Pearson's correlation factors r for FIA to SF correlation are: -0.79 ($P < 0.001$) and -0.45 ($P = 0.002$); for FIA to TS correlation: -0.45 ($P = 0.006$) and -0.14 ($P = 0.360$); for FIA to Hep correlation: -0.81 ($P < 0.001$) and -0.45, ($P = 0.002$), for the CC variants and TT variants respectively. ** = $P < 0.001$, * = $P < 0.05$,

Predictors of fractional iron absorption

Genetic variant (β = -0.346, *P* = 0.032) was a significant predictor of overall FIA along with SF (β = -0.393, *P* < 0.001), and Hep (β = -0.312, *P* < 0.001), ($R^2_{adjusted}$ = 0.468), **Table 3**.

Table 3: Predictors of iron absorption in healthy Taiwanese women (n = 79).

	Overall Model [a]			**Minimal adequate model** [b]		
Variables	β	SE	*P*	β	SE	*P*
Intercept	0.14	0.10	0.159	0.13	0.01	0.197
Variant (CC vs. TT) [c]	-0.35	0.16	0.032	-0.30	0.15	0.045
Hemoglobin	-0.13	0.07	0.085	-0.11	0.07	0.154
Serum Ferritin	-0.39	0.08	<0.001	-0.41	0.07	<0.001
Transferrin Saturation	0.11	0.06	0.063	removed from the model [d]		
Soluble Transferrin Receptor	0.12	0.07	0.083	0.12	0.07	0.105
Plasma Hepcidin	-0.31	0.06	<0.001	-0.29	0.06	<0.001
PBAC	0.07	0.08	0.389	removed from the model [d]		
C-reactive Protein	-0.05	0.07	0.434	removed from the model [d]		

Analyzed by LMM using standardized variables, dependent variable: fractional iron absorption; fixed factors: potential continuous or categorical predictors; random effects: Subjects' code. Shown are standardized β-coefficients standard errors (SE).
Hb, PBAC were assessed on D1, inflammation, iron parameters, plasma hepcidin concentration, and FIA are based on data measured on D1 and D3.
[a] Regression model fit: R^2 = 0.498; $R^2_{adjusted}$ = 0.468.
[b] Assessed by backward linear regression; regression model fit: R^2 = 0.486; $R^2_{adjusted}$ = 0.469.
[c] Nominal variable; 1 = CC, 2 = TT.
[d] Removed variable by the backward regression to assess the minimal adequate model.

Stepwise deletion removed TS, PBAC and CRP from the model ($R^2_{adjusted}$ = 0.469). In the prediction model by variant, in the CC variant only SF was significantly associated with FIA (β = -0.696, *P* < 0.001), explaining 67% of the variability in iron absorption ($R^2_{adjusted}$ = 0.669) (**Table 4**).

Table 4: Potential predictors of iron absorption in variants of the TMPRSS6 rs855791 (n_{CC} = 35, n_{TT} = 44).

	CC variant [a]			**TT variant** [b]		
Variables	β	SE	*P*	β	SE	*P*
Intercept	0.0003	0.08	0.997	-0.02	0.10	0.851
Hemoglobin	0.05	0.09	0.559	-0.25	0.11	0.023
Serum Ferritin	-0.70	0.010	<0.001	-0.20	0.11	0.060
Transferrin Saturation	-0.02	0.07	0.752	0.20	0.08	0.011
Soluble Transferrin Receptor	-0.12	0.08	0.161	0.32	0.10	0.004
Plasma Hepcidin	-0.16	0.08	0.053	-0.35	0.08	<0.001
PBAC	-0.12	0.09	0.229	0.14	0.11	0.211
CRP	0.04	0.08	0.614	-0.05	0.10	0.610

Analyzed by LMM using standardized variables, dependent variable: fractional iron absorption; fixed factors: potential continuous or categorical predictors; random effects: Subjects' code. Shown are standardized β-coefficients with their standard errors.
Hb, PBAC were assessed on D1, inflammation, iron parameters, plasma hepcidin concentration, and FIA are based on data measured on D1 and D3.
[a] Regression model fit of CC variant: R^2 = 0.707; $R^2_{adjusted}$ = 0.669.
[b] Regression model fit of TT variant: R^2 = 0.432; $R^2_{adjusted}$ = 0.375.

In contrast, in the TT variant, Hep (β = -0.353, $P < .001$), sTfR (β = 0.317, $P = 0.004$), Hb (β = -0.252, $P = 0.023$), and TS (β = 0.199, $P = 0.011$) were associated with FIA (Table 4), but with a substantially lower coefficient of determination ($R^2_{adjusted} = 0.375$), explaining 38% of the variability. In the minimal adequate model (**Table 5**), for the CC variant, significant predictors are SF (β = -0.667, $P < 0.001$) and Hep (β = -0.217, $P = 0.002$) ($R^2_{adjusted} = 0.688$). For the TT variant, significant predictors are Hep, (β = -0.411, $P < 0.001$) sTfR, (β = 0.320, $P = 0.003$) and Hb (β = -0.226, $P = 0.038$) ($R^2_{adjusted} = 0.356$, Table 5).

Table 5: The minimal adequate model and predictors of iron absorption in variants of the TMPRSS6 rs855791 ($n_{CC} = 35$, $n_{TT} = 44$).

	CC variant [a]			**TT variant [b]**		
Variables	β	SE	*P*	β	SE	*P*
Intercept	0.0004	0.08	0.996	-0.01	0.11	0.911
Hemoglobin	removed from the model [c]			-0.23	0.11	0.038
Serum Ferritin	-0.67	0.09	<0.001	removed from the model [c]		
Transferrin Saturation	removed from the model [c]			removed from the model [c]		
Soluble Transferrin Receptor	removed from the model [c]			0.32	0.10	0.003
Plasma Hepcidin	-0.22	0.07	0.002	-0.41	0.08	<0.001
PBAC	-0.11	0.09	0.226	removed from the model [c]		
CRP	removed from the model [c]			removed from the model [c]		

The minimal adequate model is assessed by backward linear regression using standardized variables. Parameters shown are analyzed by LMM, dependent variable: fractional iron absorption; fixed factors: potential continuous or categorical predictors; random effects: Subjects' code. Shown are standardized β-coefficients with their standard errors.
Hb, PBAC were assessed on D1, inflammation, iron parameters, plasma hepcidin concentration, and FIA are based on data measured on D1 and D3.
[a] Regression model fit of CC variant: $R^2 = 0.702$; $R^2_{adjusted} = 0.688$.
[b] Regression model fit of TT variant: $R^2 = 0.378$; $R^2_{adjusted} = 0.356$.
[c] Removed variable by the backward regression to assess the minimal adequate model.

Discussion

Our study shows that the *TMPRSS6* rs855791 TT variant is associated with lower iron absorption in an overall model controlling for other iron status indicators. At a standardized serum ferritin concentration of 15 µg/L, iron absorption was significantly lower in the TT variant. The TT variant also had lower transferrin saturation and serum iron and higher Hep/TS ratios, suggesting an altered interplay of serum iron, hepcidin, iron stores and the regulation of dietary absorption compared to the CC variant. Similarly, known predictors of iron absorption explained much less of the variability in iron absorption in the TT variant. To our knowledge, this is the first study comparing dietary iron absorption using stable iron isotopes among the common SNP rs855791 of the *TMPRSS6*, which has been associated with iron status and red blood cell parameters in various genome wide association studies and large cross-sectional studies.[10,12,15-17]

In humans, inter-subject absorption of nonheme iron shows a wide variation in healthy young women. Zimmermann *et al.*[31] reported a variation from 1% to 58% in iron absorption from standardized test meals labelled with 4 mg Fe as stable isotopes.[31] Some of this variation is due to differences in iron status and meal matrix, however, taken together, it has been estimated that iron status and food factors predict only ≈50% of the variance in iron absorption in a population.[24] Cook *et al.*[32] reported a striking positive correlation in body iron in iron-replete mothers and their young children and suggested this close correlation was due to a shared diet and/or possible genetic determinants of iron status such as shared iron-regulatory genes. In Mexican (n = 18), and Senegalese mother-child pairs (n = 17), nonheme-iron absorption measured with stable isotopes exhibited strong and intermediate correlations, respectively.[25,26] A common polymorphism in the transferrin protein (G277S) has been associated with ID in American women,[33] but a stable isotope study comparing 25 iron deficient, nonanemic women who had either a heterozygous G277S/G277G or wild-type G277G/G277G genotype did not find a significant difference in iron absorption.[34] However, the G277S carriers did not show the typical inverse correlation between iron absorption and SF.[34] Similarly, in our study with the *TMPRSS6* rs855791 mutation, in the TT variant, the correlation between iron absorption and SF, and iron absorption and Hep, were only weak and moderate, respectively. In contrast to the CC variant, where both these correlations are strong. Also the models computed by variant show remarkable differences. In the CC variant

serum ferritin alone is significantly associated with FIA, explaining 67% of the variability. In the TT variant, in contrast, several factors identify as being associated with FIA: hepcidin, soluble transferrin receptors, hemoglobin, and transferrin saturation, and their total contribution explain only 38% of the variability in iron absorption.

Our hypothesis that the CC variant would have increased iron absorption was based on a regulatory model of *TMPRSS6* acting as a negative regulator of the hepcidin activation pathway, and we hypothesized the largest effects would be seen in an iron replete population, where hepcidin expression would be activated. However, our findings indicate the effects of the genetic variant are likely most relevant at low iron status (Figure 3); at lower SF, women with the TT variant were less able to upregulate iron absorption, which could increase the risk for ID. Further, the overall model (Table 3) shows that iron status indices, hepcidin, and genotype, but not inflammation and menstrual blood loss are associated with fractional iron absorption. A recent large study in blood donors suggests an impaired capacity in the TT variant to replenish iron stores after repeated blood donations, even if the possibly protective CC variant was not enriched in high intensity donors.[22] It is also possible that cellular mechanisms controlled by iron regulatory proteins are, especially at intermediate serum iron levels, able to compensate for the altered interplay of hepcidin and transferrin saturation in the TT variant by inducing the translation of iron transporters (e.g., DMT1) and transcription factor HIF-2α.[35] Such a compensatory mechanism was suggested in a recent study in women in whom an acute inflammatory stimulus increased Hep but did not affect iron absorption.[36]

Our findings suggest that, at low serum ferritin concentrations, women with the TT variant have lower iron absorption, whereas when iron stores are replete, they may be less able to downregulate iron absorption compared to the CC variant. Our variant-specific FIA correction to serum ferritin uses a similar approach as the original formula of Cook *et al*.[30] used to correct dietary absorption measurements for the individual iron status; that formula employs a slope of -1 between log FIA and log SF. We propose adapted, regression formulas with slopes of -1.28 and -0.74 for CC and TT variants, respectively (Figure 3). In a case control study in Taiwanese women comparing women with IDA to nonanemic controls, the CC variant was less frequent in the IDA group compared to the control group (12% versus 25%); this suggests the CC variant may reduce risk of IDA.[21] This effect is also suggested in our screening data: among the screened subjects, 60% of women with the TT variant had either an hemoglobin below 12

g/dl and/or a serum ferritin below the study inclusion criteria, compared to 42% of women with the CC variant. Also, among women who received iron supplementation because of ID, 80% of the women with the CC variant replenished their iron stores, in contrast to only 59% of women with the TT variant. While this is consistent with the view that women with the TT variant are higher risk for ID and may have a blunted response to iron supplements when body iron stores are low, this hypothesis needs confirmation in larger prospective trials. Further mechanistic studies in monozygotic twins would be particularly informative as they may distinguish potential genetic and epigenetic sources of variability in iron absorption.

Our findings are consistent with previous studies that have shown that serum iron and transferrin saturation are lower, and total iron binding capacity higher in the TT variant.[18-20] The higher Hep/TS ratio reported in women with the TT variant in our study has been previously described in Italian[18] and Dutch populations.[20] In our study, the lack of association between variant and hepcidin suggests a different modulation of iron regulatory signals (transferrin bound iron and iron stores) in the regulation of hepcidin between the two different variants. Consistent with this interpretation, a recent study has suggested that the Hep/TS ratio may be a useful diagnostic marker to differentiate IRIDA patients from those with chronic ID.[7]

A strength of this study is that iron absorption was assessed from an isotopically labelled standardized labeled test meals in a relatively large number of subjects, using erythrocyte incorporation of stable iron isotope labels. Due to its precision, this approach allows, in combination with iron indices, to study regulatory aspects of iron metabolism in humans.[36,37] We performed iron absorption measurements twice in each subject; this increased statistical power and allowed us to make intra-individual comparisons. Our study also has limitations: our assessment of menstrual loss using PBAC is semiquantitative, and while we found no association with iron absorption after correcting for iron status in the overall model, we cannot fully exclude a potential effect of menstrual blood loss on iron absorption. Our proposed genotype-specific slopes of serum ferritin and iron absorption are based on a relatively narrow range of iron status and should be studied in populations with broader iron status distribution. We focused our hypothesis on a single SNP, and we did not study the interplay with other SNPs known to affect iron homeostasis. However, the *HFE* rs1800562 (C282Y) mutation is known to be rare in Taiwanese women.[38] In contrast, the *GNPAT*

rs11558492 has been associated with a high-iron phenotype,[39] and a recent study in Taiwanese women has shown a minor allele frequency of 12%, and a significant higher serum iron response after a supplement.[40] We did not study heterozygotes, despite the fact that effects on iron absorption are conceivable in this group. Furthermore, unknown SNPs associated with rs855791 may explain the observed effects. However, we think this possibility is unlikely, as rs855791 has been repeatedly shown to be associated with iron status, as discussed above.

To summarize, we have shown that in a fully adjusted model of iron absorption, women with the TT variant have lower iron absorption compared to women with the CC variant. This may be associated with higher Hep/TS and Hep/SFe ratios, suggesting impaired negative feedback on hepcidin synthesis by circulating iron. Furthermore, in the TT variant, regulation of iron absorption is less well predicted by iron stores. Thus, our findings suggest women with the TT variant are less able to upregulate iron absorption at low iron status, which may increase their risk of ID.

Author Contributions

Contribution: MBZ, DM, SB, and SNP designed the study; SNP, SCH and CTL conducted the study and collected the samples; SB CZ analyzed the samples and performed the statistical analyses, SB, DM, MBZ, and SNP participated in the data interpretation; SB wrote the first draft of the manuscript; all authors edited the manuscript and approved the final version.

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Conflicts of Interest

Conflict-of-interest disclosure: None of the authors declare a conflict of interest.

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Supplemental Material

Subjects

Inclusion criteria for the study were: 1) homozygous in the SNP rs855791 (wild type (CC) or mutation (TT)); 2) 20-45 years of age ; 3) body mass index (BMI) 18.5 – 25 kg/m^2; 4) body weight < 65 kg; 5) nonanemic, defined as Hb > 120 g/L; 6) iron sufficient, defined as SF > 30 µg/L; 7) no high body iron stores, defined as SF < 120 µg/L; 8) no oligomenorrhea or amenorrhea; 9) not pregnant or lactating; 10) no chronic disease; 11) no blood donation, transfusion or significant blood loss over the previous six months; 12) no use of long-term medication; 13) no use of vitamin/mineral supplements during the study and two weeks prior the first test meal and 14) no night shift work one month prior the study. We invited women who fulfilled all criteria to participate into the study. If the participant met all inclusion criteria, except a SF < 30 µg/L and/or a Hb < 120 g/L, they were assigned to an iron supplement group and consumed daily 27 mg Fe as ferrous fumarate (Multivitamins + Iron Stresstabs®, Pfitzer Inc.) for three months and were then invited for rescreening.

Test meal administration

We planned the test meal administrations on study days one and three (D1, D3) to be within the luteal phase of each subject menstrual cycle (MC); we administered the first test meal on the 14th MC day at the earliest, and the second test meal at the latest on the final day of the MC. On all study days (D1, D3, and D17), participants came to the KCGMH in the morning after an overnight fast (no food intake after 8 pm and no drinks after midnight). Before each test meal administration, we collected a blood sample by venipuncture for determination of Hb, SF, serum iron (SFe), total iron binding capacity (TIBC), Hep, soluble transferrin receptor (sTfR), CRP, and the acute phase protein alpha-1-acid glycoprotein (AGP). On D1, the standardized test meals contained 4 mg iron (^{57}Fe) as labelled ferrous sulfate ($FeSO_4$), on D3 with 4 mg iron (^{58}Fe) as labelled $FeSO_4$. Participants consumed the entire test meal under supervision, and then remained fasting for three hours. On study D17, we collected a blood sample for determination of incorporation of stable iron isotopes into erythrocytes.

Preparation of stable iron isotopes, test meals and label administration

We labelled FeSO4 with isotopically enriched elemental Fe, ^{57}Fe, and ^{58}Fe (Chemgas, Boulogne-Billancourt, France) as previously described.[1] The test meals consisted of a rice meal

(273 g rice) (Rift Valley Nine, Taiwan Rice, Taiwan Costco) with 25 g seaweed sauce (Sea Tangle Seaweed Sauce, Gurume, Gurume Industrial Co., LTD). The labelled $FeSO_4$ solution was added to the meal just before consumption. The subjects consumed 350 ml bottled water with each meal.

Assessment of menstrual blood loss

We estimated menstrual blood losses using the semi-quantitative pictorial blood-loss assessment chart (PBAC), as previously described,[2] and defined menorrhagia as PBAC score > 100.

Laboratory analyses

We extracted DNA from peripheral leukocytes with a DNA extraction kit (QIAamp® DNA Mini kit, QIAGEN). We determined the TMRPSS6 rs855791 C>T polymorphism by sequencing allele specific PCR (TaqMan® SNP Genotyping Assay, ABI) using two forward allele-specific primers which differ by a single nucleotide complementary to the nucleotide of interest, and a common reverse primer in the PCR. We confirmed random samples (10%) by direct sequencing.

We collected venous blood samples using heparinized tubes (for immunoassays, isotopic analyses, hepcidin concentration), EDTA tubes (for erythrocytes and isotopic composition analysis), and non-anticoagulated tubes (for serum iron parameters). We assessed erythrocytes parameters by Sysmex XE-5000 or Sysmex XN-Series (Sysmex Co, Kobe, Japan). At screening, we determined CRP using a CRP kit (Fujifilm Wako Corporation, Osaka, Japan) and SF using by a two-site EIA (ADVIA Centaur, Siemens Healthcare Diagnostics, NY, USA). We measured SFe, and TIBC using a Fe/UIBC kit (Shino Corporation, Kanagawa, Japan). We shipped frozen samples to ETH Zurich for determination of CRP, AGP, and sTfR, Hep, and isotopic ratio. We measured CRP, AGP, and sTfR from D1, D3, and D17 by immunoassay,[3] and calculated body iron stores (BIS, mg/kg),[4] TS using the formula (SFe/TIBC) × 100. We measured Hep concentrations with the c-ELISA DRG Hepcidin 25 (bioactive) HS ELISA (DRG Instruments GmbH, Marburg, Germany).

Fractional iron absorption correction to iron status.

We corrected the FIA for SF with a modification of the Cook *et al.* formula,[7] using the variants specific regression slope (a): $\log(FIA_C) = \log(FIA_O) + a * \log(SF_C/SF_O)$. Where FIAC is the corrected, and FIA_O the observed FIA, SF_C is the corrected, and SF_O is the observed SF. We corrected FIA to the cutoff for ID (15 µg/L),[8] and to 50 µg/L as a level representing sufficient iron stores. We calculated circulating iron in the body based on hemoglobin and blood volume, derived from the participant's height and weight[9] and assuming an 80% incorporation of absorbed iron into erythrocytes.[6]

Sample size calculation

We based the sample size calculation on a design with two repeated measurements with a compound symmetry covariance structure. Based on previous studies from the Human Nutrition Laboratory, using log transformed data, we assumed an intra-individual correlation of 0.7, and a standard deviation of 0.235. A difference of 30% in iron absorption was considered relevant. Therefore, we planned to recruit 40 subjects per variant, with 80% power and $\alpha = 0.05$, it allows 2 dropouts per group. Due to the imbalanced distribution of the minor allele in the Taiwanese population, and difficulties enrolling the planned number of CC subjects, we made a protocol amendment to include 35 CC and 45 TT subjects. This unbalanced distribution results in an estimated power of 75%.

Data and Statistical analysis

We used IBM SPSS statistics (Version 24) for statistical analysis. After testing for normality, we used log-transformed data further analysis if not normally distributed. Normally distributed data is presented as means ± standard deviation (SD), transformed normal data as geometric mean with the 95% confidence interval (95%CI), non-normal data as median and the interquartile range (IQR). Means or medians of red cell parameters, are based on the concentrations measured on D1. Means, medians, or geometric means of CRP, AGP, SF, SFe, TIBC, TS, sTfR, BIS, Hep, Hep/TS, Hep/SF, and FIA are based on concentrations measured on D1 and D3. We tested between group differences for normally distributed variables with independent samples T-Test and for not normally distributed variables using Mann-Whitney U Test; differences in CRP, AGP, SF, SFe, TIBC, TS, sTfR, BIS, Hep, Hep/TS, Hep/SF, and FIA by linear mixed models (LMM), with subjects' code as random intercept, the corresponding

variable as dependent variable and genotype as fixed effect. We assessed Pearson's correlations and differences between the coefficients with the Fishers r to z transformation. We assessed predictors of iron absorption with LMM using subjects' code as random intercept, FIA as dependent variable, and genotype, Hb, SF, TS, sTfR, Hep and PBAC as fixed factors. We performed a backward linear regression to assess a minimal adequate model, and we fitted the variables in a LMM. Statistical significance was defined as $P < .05$.

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MANUSCRIPT 3

A HEAT-STABLE MICROPARTICLE PLATFORM FOR ORAL MICRONUTRIENT DELIVERY

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Abstract

Micronutrient deficiencies affect up to 2 billion people and are the leading cause of cognitive and physical disorders in the developing world. Food fortification is effective in treating micronutrient deficiencies; however, its global implementation has been limited by technical challenges in maintaining micronutrient stability during cooking and storage. We hypothesized that polymer-based encapsulation could address this and facilitate micronutrient
absorption. We identified poly(butylmethacrylate-co-(2-dimethylaminoethyl)methacrylate-co-methylmethacrylate) (1:2:1) (BMC) as a material with proven safety, offering stability in boiling water, rapid dissolution in gastric acid, and the ability to encapsulate distinct micronutrients. We encapsulated 11 micronutrients (iron; iodine; zinc; and vitamins A, B_2, niacin, biotin, folic acid, B_{12}, C, and D) and co-encapsulated up to 4 micronutrients. Encapsulation improved micronutrient stability against heat, light, moisture, and oxidation. Rodent studies confirmed rapid micronutrient release in the stomach and intestinal absorption. Bioavailability of iron from microparticles, compared to free iron, was lower in an initial human study. An organotypic human intestinal model revealed that increased iron loading and decreased polymer content would improve absorption. Using process development approaches capable of kilogram-scale synthesis, we increased iron loading more than 30-fold. Scaled batches tested in a follow-up human study exhibited up to 89% relative iron bioavailability compared to free iron. Collectively, these studies describe a broad approach for clinical translation of a heat-stable ingestible micronutrient delivery platform with the potential to improve micronutrient deficiency in the developing world. These approaches could potentially be applied toward clinical translation of other materials, such as natural polymers, for encapsulation and oral delivery of micronutrients.

Introduction

Micronutrient deficiencies are prevalent across the developing world, affecting 2 billion people[1] and causing cognitive and physical disorders such as anemia, blindness, birth defects, impaired growth in children,[2-7] and around 2 million childhood deaths per year.[8-10] Large-scale human trials have established that micronutrient fortification of foods can effectively treat micronutrient deficiencies,[11-18] but this approach has been limited[19-21] because of poor implementation in certain countries and unaddressed technical challenges related to micronutrient stability during storage and cooking. For example, heat, moisture, and oxidation encountered during cooking can impair absorption of vitamins through degradation[17,18,22-26] or make food unpalatable through chemical changes to minerals.[27] Hence, the development of technologies that address these stability challenges can facilitate implementation of staple food fortification and affect global health by treating worldwide micronutrient deficiencies.

Current micronutrient technologies focus on encapsulation in microparticles (MPs), nanoparticles, agglomerates, and powders using biopolymers and food additives such as proteins, polysaccharides, lipids, and surfactants.[28-33] However, these approaches are limited in addressing the stability challenges encountered during end use of the micronutrients (cooking) and delivery challenges related to micronutrient release and subsequent absorption by the body. We hypothesized that we could address this by using a pH-sensitive polymer that encapsulates both water-soluble and fat-soluble micronutrients; protects the encapsulated micronutrient from high temperature, moisture, and oxidizing agents; and rapidly releases in the stomach to ensure intestinal absorption.

Using an encapsulating matrix, we describe an MP delivery platform that encapsulates 11 micronutrients individually and up to 4 micronutrients in combination; enhances micronutrient stability after exposure to boiling water, light, or oxidizing chemicals found in common foods; and rapidly releases micronutrients upon exposure to simulated gastric fluid (SGF). In vivo studies confirmed rapid micronutrient release in the stomach and subsequent absorption in the small intestine. In humans, we investigated the bioavailability of ferrous sulfate (iron) after ingestion of iron-loaded MPs. Alongside complementary studies in an organotypic intestinal model, we identified MP iron loading and MP polymer content to be absorption-limiting parameters in humans. We then developed and subsequently leveraged large-scale process development approaches to simultaneously increase loading and decrease

polymer content in MPs. In a second human trial, MPs synthesized at scale with higher iron loading and lower polymer content demonstrated non-inferior absorption as compared to non-MP controls. These results indicate that this MP platform can be used to individually encapsulate or co-encapsulate micronutrients in a modular manner, maintain stability over 2 hours in boiling water, and then rapidly release in gastric conditions to successfully deliver micronutrients to humans. Overall, our study details a broad approach from conception to human trials of a highly heat-stable MP platform for oral micronutrient delivery.

Results

Formulation of MPs at laboratory scale

We initially considered more than 50 potential polymers that could simultaneously be stable in boiling water and dissolve rapidly in low pH and closely evaluated 10 candidates (table S1). BMC [poly(butylmethacrylate-co-(2-dimethylaminoethyl)methacrylate-co-methylmethacrylate) (1:2:1)], available commercially as either a U.S. Food and Drug Administration (FDA)–approved inactive ingredient (Eudragit E PO powder) or a self-affirmed generally recognized as safe (GRAS) status material (Eudraguard Protect powder), was selected as the MP encapsulation material as it simultaneously addresses the above challenges (table S1).[34-41] Micronutrients were encapsulated individually in MPs using a one-step (Fig. 1A) or two-step (Fig. 1B) emulsion process, followed by centrifugation to remove unencapsulated micronutrients. In both approaches, BMC was used as the encapsulant; however, the two-step process used either hyaluronic acid (HA) or gelatin as an additional stabilizing excipient included in the first step (Fig. 1B). For the two-step process, MPs sampled during the first step were about 5 µm in diameter (Fig. 1C), whereas after the second step, they exhibited a hierarchical particle-in-particle structure about 200 µm in diameter (Fig. 1, D and E). The two-step process was used for water-soluble micronutrients that could not be encapsulated using the one-step process due to nonhomogeneous suspension of water-soluble micronutrients. This two-step approach was additionally used to enable the co-encapsulation of vitamins A, D, folic acid, and B_{12} (Fig. S1). In contrast, MPs synthesized via the one-step process exhibited homogeneous internal structure and were about 200 µm in diameter (Fig. 1F). Formulation parameters, loadings, and encapsulation efficiencies for each of the laboratory-scale MPs are shown in table S2.

Controlled release of micronutrients in vitro

In vitro release studies confirmed the retention of micronutrients in the encapsulated MPs after exposure to room temperature (RT) water or boiling (100°C) water (Fig. 2). pH-responsive burst release was exhibited when particles were exposed to 37°C SGF at pH 1.5 (Fig. 2). Micronutrient retention during 2 hours in boiling water was used as a baseline index of MP stability under simulated cooking conditions, because micronutrients such as vitamin A undergo chemical degradation when exposed to high temperature or humidity.[16,17] The one-step process was confirmed to achieve retention (>80% at 120 min) in 100°C or RT water and rapid release (>80% at 30 min) in 37°C SGF for most individually encapsulated micronutrients (Fig. 2). The two-step process was developed to further stabilize highly water-soluble micronutrients within the BMC matrix (Fig. 2). More specifically, when the two-step process that included HA as the stabilizing biopolymer was used to encapsulate $FeSO_4$, the payload was largely retained (>90% at 120 min) in 100°C or RT water and rapidly released (>80% at 30 min) in 37°C SGF, whereas $FeSO_4$ formulations synthesized via the one-step process exhibited payload release even in RT water. The role of pH in modulating release kinetics was investigated using vitamin B_{12} as a representative micronutrient, where payload release was achieved more rapidly at lower pH values (Fig. S2). Time-lapse imaging of vitamin A–BMC MPs immersed in SGF exhibited payload release in <1 min (Fig. 2B), as did Fe-HA-BMC MPs (Fig. 2C). Four co-encapsulated vitamins, water-soluble vitamins B_{12} and folic acid introduced in step 1 and fat-soluble vitamins A and D introduced in step 2 (Fig. S1), each maintained payload retention (>80% at 120 min) in 100°C or RT water and rapidly released (60 to 90% at 30 min) the payloads in 37°C SGF (Fig. 2, D to G). Together, these results indicate that the BMC MP platform system can be used to individually encapsulate or co-encapsulate micronutrients in a modular manner, provide retention during 2 hours in boiling water, and enable burst release in 37°C SGF.

Controlled release of micronutrients in vitro

Micronutrient stability under heat, water, ultraviolet light, and oxidizing agents Many micronutrients, such as vitamin A (23–25) and iron,[26] are sensitive to high temperatures, moisture, ultraviolet (UV) light, or oxidizing chemicals, which can lead to degradation or changes in the oxidative states and thus limit absorption after ingestion.[26] Hence, we studied the role that BMC encapsulation plays in improving micronutrient stability against these

challenges for both individually and co-encapsulated formulations. We first investigated protection of the micronutrient payload during 2 hours in boiling water, which exposed the payload to high temperatures and moisture. For the encapsulated fat-soluble micronutrients vitamin A and D, more than 5- and 18-fold enhanced recovery was observed, respectively, as compared to unencapsulated counterparts (Fig. 3A), after exposure to boiling water conditions for 2 hours. Similarly, encapsulation protected water-soluble vitamins C and B_2 during boiling, as both water-soluble vitamin groups exhibited enhanced recovery as compared to unencapsulated controls (Fig. 3A). We next investigated protection of the micronutrient payload after 24 hours of light exposure (280 $\mu W/cm^2$), because both vitamin A[32] and vitamin D[42] are rapidly degraded by UV light in their unencapsulated forms (Fig. 3B). Recovery after light exposure was significantly improved by more than 15- and 3-fold for vitamin A and D, respectively, encapsulated in BMC MPs as compared to unencapsulated controls (P   0.05; Fig. 3B).

Similar to individually encapsulated micronutrients, co-encapsulated micronutrients maintained their biological activity after exposure to boiling water for up to 2 hours (Fig. 3C). Negative oxidizing interactions between micronutrients in fortified products and micronutrients naturally present in food sources readily occur, and these interactions can negatively affect absorption and bioavailability.[18] For example, polyphenols present in food catalyze iron oxidation, resulting in a marked color change, from a highly bioavailable ferrous (Fe^{2+}) state to a ferric state (Fe^{3+})[43] that exhibits poor bioavailability.[26] To examine whether BMC encapsulation prevents interactions between the encapsulated iron and oxidizing chemicals present in food, BMC-encapsulated and unencapsulated iron was added to polyphenol-rich banana milk and the color change was quantified over time. Iron encapsulation in HA-BMC MPs exhibited less color change, and therefore less oxidation, in banana milk as compared to unencapsulated iron (Fig. 3D). These results indicate that the BMC MP matrix can limit interactions between the encapsulated iron and the free polyphenols in food. Last, to demonstrate a maintained capability for pH-controlled release of iron after exposure to high temperature, moisture, and oxygen, iron-loaded MPs that were first boiled for 2 hours and then immersed in SGF were visualized using real-time microscopy, confirming that they maintained their ability to rapidly release their iron payload at low pH (Fig. 3E). After boiling, HA-BMC MPs retained similar morphology (Fig. S3) to pre-boiling (Fig. 1D). Overall,

these results indicate that encapsulation in BMC protects micronutrient payloads during exposure to high temperatures, moisture, UV light, and oxidizing chemicals.

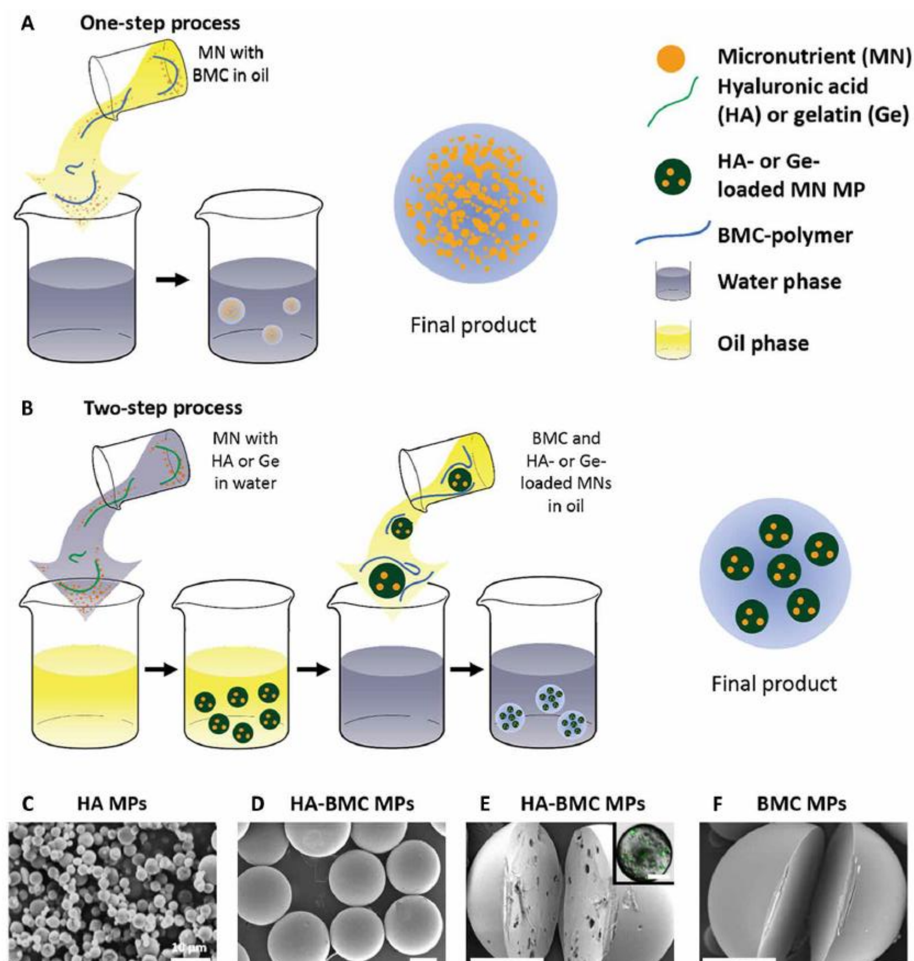

Fig. 1. Particle synthesis and characterization. Schematic representations of the (**A**) one-step and (**B**) two-step processes for formulating MPs. SEM images of (**C**) HA MPs, (**D**) HA-BMC MPs, (**E**) the cross section of an HA-BMC MP (inset, confocal image of an HA-BMC MP with fluorescently labeled HA), and (**F**) the cross section of a BMC MP. Scale bars, 100 µm, unless otherwise noted.

Release of a model payload and absorption of vitamin A in vivo

To confirm BMC MP dissolution in vivo, we used female SKH1-Elite mice to track payload release using the near-infrared fluorescent dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR) encapsulated in BMC. DiR can be differentiated in the encapsulated and released states by investigating the influence of environmental conditions on DiR's fluorescent properties using established imaging techniques.[44] A 14-point spectral fingerprint of a DiR-loaded BMC MP was obtained when the MP was suspended in

water. In contrast, when DiR is released from a BMC MP in SGF, the resulting blue shift exhibits a spectral profile distinct from encapsulated DiR. Hence, the encapsulated and released DiR could be differentiated using their distinct fluorescent fingerprints (Fig. S4). The two fingerprints of the dye in either encapsulated or released form were used to indirectly reflect the dissolution of the BMC MPs in vivo. DiR-loaded BMC MPs were administered orally to mice, and at timed intervals, the animals were euthanized and the complete gastrointestinal tract was excised for ex vivo fluorescence imaging (Fig. 4A). Both the physical state of the dye (encapsulated or released) and the physiological location of the dye in the gastrointestinal tract were visualized (Fig. 4A) and quantified (Fig. 4B). At 15 min, the stomach contained a mixture of encapsulated and released DiR, suggesting that the BMC MPs were partially dissolved and a portion of payload was released but had not yet entered the intestines. At 30 min, DiR signal was predominately detected as both encapsulated dye in the stomach and released dye in the intestines. At 60 min, minimal signal of BMC-encapsulated DiR was detectable, highlighting how the majority of particles released their payload within 1 hour. Furthermore, at 1 hour, the released dye signal was exclusively in the intestines, implying that the released payload had passed through the stomach and into the intestines within 1 hour. These findings confirm rapid release of a model payload from orally administered MP into the murine gastrointestinal tract. To determine whether this rapid release would facilitate absorption of encapsulated micronutrients, we evaluated the absorption of vitamin A in female Wistar rats. Tritium-labeled vitamin A was orally administrated to rats by gavage in both the free form and BMC-encapsulated forms, and blood samples were taken to evaluate vitamin A content over a period of 6 hours (Fig. 4C). Encapsulated vitamin A exhibited statistically indistinguishable absorption relative to free vitamin A (Fig. 4C), highlighting that encapsulation in BMC did not influence absorption.

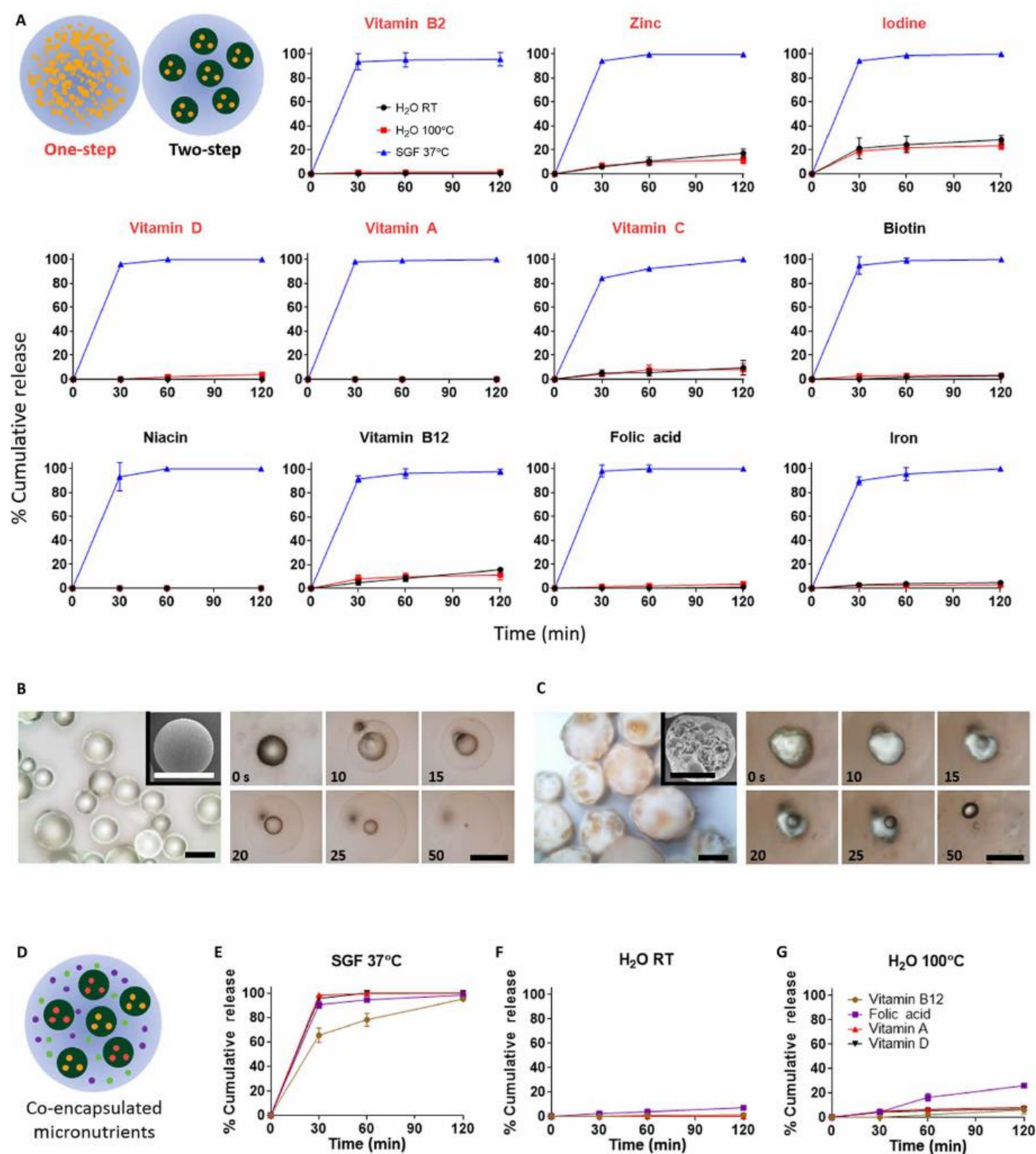

Fig. 2. Controlled release of micronutrients. (**A**) Percent cumulative release of 11 different individually encapsulated micronutrients in 37°C SGF, pH 1.5 (blue lines), and RT water (black lines) or boiling water (red lines). Schematic shows micronutrients encapsulated via one-step process (red text) versus two-step process (black text). (B and C) Representative bright-field images and time-lapse release of (**B**) vitamin A from BMC MPs and (**C**) iron from HA-BMC MPs in SGF (insets, SEMs). (**D**) Schematic of four co-encapsulated micronutrients [folic acid (purple), B12 (brown), vitamin A (red), and vitamin D (black)] and percent cumulative release in (**E**) 37°C SGF, (**F**) RT water, and (**G**) boiling water. Error bars represent SD of the mean (n = 3). Scale bars, 200 µm.

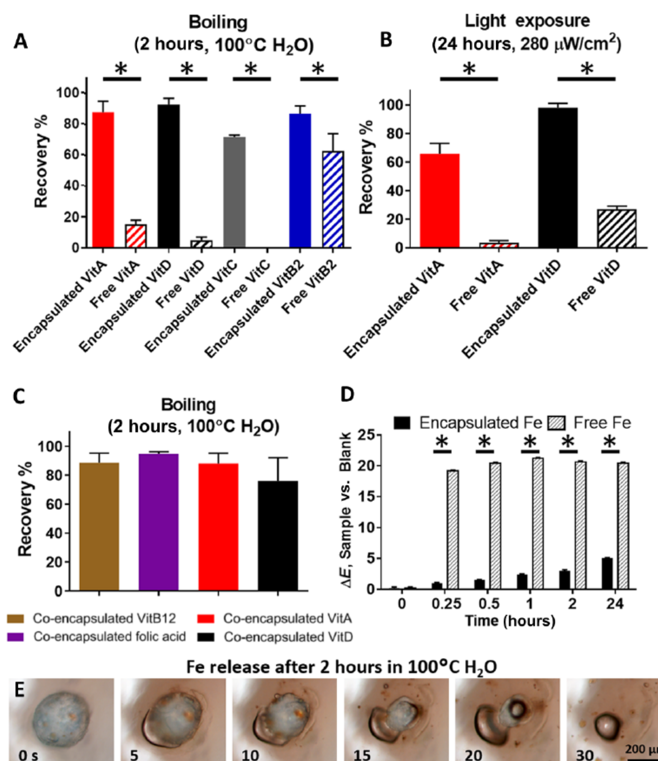

Fig. 3. Protection from heat, light, and chemical interactions. Recovery of individually encapsulated versus unencapsulated (free) micronutrients after exposure to (**A**) boiling water and (**B**) light. (**C**) Recovery of co-encapsulated micronutrients after boiling in water. (**D**) Time history of color change (ΔE), an indication of a chemical reaction between iron and polyphenols present in banana milk, of laboratory-scale Fe-HA-BMC MPs versus unencapsulated (free) iron. (**E**) Time-lapse release of iron from HA-BMC MPs after boiling in water for 2 hours at RT and upon immersion in RT SGF. Error bars represent SD of the mean (n = 3). *P < 0.05 as determined by Student's *t* test.

Bioavailability of iron in humans and iron transport in an in vitro intestinal barrier model

Fe-HA-BMC MPs were investigated for their ability to deliver bioavailable iron in humans. Iron bioavailability was investigated through the consumption of three stable iron isotope–labeled test meals administered in a randomized single-blind, cross-over design to fasting young women (n = 20; mean ± SD, hemoglobin (Hb) = 13.4 ± 0.85 g/liter; and geometric mean [95% confidence interval (CI)], plasma ferritin (PF), 11.6 (9.4, 14.5) μg/liter) (table S3). The test meals were whole-grain maize porridge with vegetable sauce, an iron absorption inhibitory meal[45] with an iron:phytic acid molar ratio of 1:6.5 and negligible (0.4 mg/meal) ascorbic acid content, to which abeled unencapsulated or encapsulated ferrous sulfate was added after cooking. Two test meals contained 4 mg of iron as labeled ferrous sulfate (either ^{54}Fe or ^{57}Fe) in HA-BMC MPs (encapsulated iron), which was added either before or after cooking, to

investigate the effects that cooking (30 min of baking at 100°C) had on iron bioavailability after MP encapsulation. The third test meal, the reference, contained 4 mg of unencapsulated iron (free) as labeled ferrous sulfate (^{58}Fe). The geometric mean (95% CI) of fractional iron absorption (FIA) of the reference meal (free uncooked Fe) was 3.36 (2.29, 4.95)%, whereas the FIA of ferrous sulfate from uncooked Fe-HA-BMC MPs was 1.46 (0.77, 2.79)% (Fig. 5A). Fe-HA-BMC MPs exhibited 44 (22, 88)% relative iron bioavailability (RBV) as compared to unencapsulated ferrous sulfate ($P < 0.01$) (Fig. 5A). Cooking the encapsulated ferrous sulfate had no effect on its bioavailability [FIA, 1.41 (0.91, 2.19)%; RBV, 42 (34, 53)%] (Fig. 5A).

Although the first human study showed that Fe encapsulation in HA-BMC MPs reduces iron bioavailability as compared to unencapsulated iron (Fig. 5A), our platform demonstrated efficacy in delivering bioavailable iron to humans, independent of cooking conditions (Fig. 5A). It has been previously reported that materials that encapsulate micronutrients can interfere with absorption;[15] hence, we investigated the role that HA and BMC independently play in the intestinal absorption of iron. In vitro studies were designed to simulate conditions of iron penetration of the intestinal epithelial cell barrier in humans after oral ingestion of Fe-HA-BMC MPs. A commercially available human intestinal epithelial cell barrier model (EpiIntestinal, MatTek) provided a test platform to investigate the effect the MP constituents have on intestinal iron absorption by systematically varying the relative concentrations of iron, HA, and BMC. The model consisted of primary small intestine epithelial cells obtained from a healthy human donor, dissociated enzymatically, and cultured in customized medium on cell culture inserts within 12-well plates to form a functional, columnar-like three-dimensional epithelial barrier layer.[46]

Oral administration of iron formulations was modeled by adding samples into the apical surface of the intestinal barrier, accessible as the cell culture insert in the upper compartment of the well plate, and, after a 1-hour incubation period, quantifying iron transport as the amount that passed through the tissue barrier and could be determined by analysis of the culture medium in the lower compartment of the well plate. The transport of iron added in combination with HA (Fig. 5B) and/or BMC (Fig. 5C) was expressed as a percentage of the transport of free iron added in the absence of HA or BMC. HA presence exhibited no significant effect on iron transport through the intestinal barrier (Fig. 5B). Moreover, iron was readily transported through the barrier at the Fe:HA ratio used in the MPs tested in this first human

study. In contrast, unencapsulated BMC added to iron at increasing percentages significantly reduced iron transport through the intestinal barrier (Fig. 5C). In particular, iron was poorly transported through the barrier when present at the BMC percentage of 96%, which corresponds to the percent BMC in the MPs tested in human subjects. At the percentage of BMC present in the current MP formulation, iron transport was reduced to 37% compared to free iron. Similarly, iron transport was reduced to 33% of that measured for free iron when the neutralized contents of MPs dissociated by incubation in SGF were added to the intestinal barrier (Fig. 5C). As BMC percentage decreased, the iron transport–inhibiting effects of BMC became negligible, which indicates that formulations containing lower percentage of BMC may not inhibit iron transport across the intestines. By using this organotypic model to determine the conditions that do not inhibit iron transport, we have highlighted the utility of in vitro models for use in screening micronutrient formulations to inform MP design.[47]

Process development and scale-up

The MPs described were conceived and synthesized as laboratory-scale research formulations. Although emulsion-based microencapsulation methods are a staple in many biomaterial and formulation laboratories at the academic level,[28,48] we encountered considerable challenges in increasing the iron loading when encapsulated in BMC. To address this, and to overcome the absorption issues that were encountered in the first human study, we developed new processes to increase the loading of iron in our formulation (Fig. 6A). A commercially available spray dryer and a customized spinning disc atomizer were used to formulate Fe-HA MPs (Fig. 6B) and Fe-HA-BMC MPs (Fig. 6C), respectively, at the kilogram scale. The initial scaled formulation was designed to recreate the 0.6% iron loading used in the first human study. Batches of Fe-HA-BMC MPs produced at the pilot scale (>1 kg) and at the same compositions of those used in the first human study met the same loading, stability, and pH-controlled release criteria as the laboratory-scale formulation tested in humans (Fig. 6D). We further developed processes to increase the loading of iron in BMC particles to 3.19% (Fig. S5A) and 18.29% (Fig. S5B), which additionally decreased BMC amounts (table S4). These scaled and high loaded iron BMC MPs exhibited near-complete release of iron in SGF in less than 5 min (Fig. S5C). As expected, the release of iron in pH 5 buffered water demonstrated a pH dependency (Fig. S5, A and B). For human studies, food-grade iron-loaded BMC particles with acceptable values for residual dichloromethane (DCM), endotoxins, and microbial

bioburden (table S5) were used. These scaled MPs were also examined for their ability to prevent interactions between the encapsulated iron and oxidizing chemicals present in food as described above with polyphenol-rich banana milk. It was demonstrated that the scaled Fe-HA-BMC MPs induced less color change as compared to all free forms of iron, both with and without the other MP constituents (HA, BMC, and HA with BMC) (Fig. S5D).

Bioavailability of iron particles of 5- and 30-fold higher loading in humans

Fe-HA-BMC MPs at more than 5-fold and more than 30-fold higher iron loading, compared to the laboratory-scale batch used in the first human trial, were investigated for their ability to deliver bioavailable iron to humans in a second human study. In this study, a non–iron-inhibiting food matrix (wheat bread) was used to better compare unencapsulated iron and encapsulated iron by solely focusing on absorption, as opposed to both absorption and particle-mediated protection against small molecules that chelate iron. Nine test meals containing identical doses of iron (4 mg Fe) were administered in a partially randomized single-blind, cross-over design to fasting young women [n = 24; Hb, 13.2 ± 0.95 g/liter; and PF, 13.2 (10.5, 16.5) µg/liter] (table S3). Three meals contained iron as labeled ferrous sulfate in 3.19% ^{54}Fe-HA-BMC MPs, 18.29% ^{57}Fe-HA-BMC MPs, and 4 mg of unencapsulated ferrous sulfate (^{58}Fe, reference meal). In all cases, iron was added before baking the bread at 190°C for 20 min. In contrast to the first human study, 18.29% Fe-HA-BMC-MPs [FIA: 17.0 (13.2, 21.9)%] exhibited iron absorption that was not statistically different relative to unencapsulated iron [FIA: 19.2 (15.3, 24.29)%] (Fig. 7). The fivefold higher loaded 3.19% Fe-HA-BMC MPs [FIA: 13.7 (11.1, 16.8)%] exhibited significant lower absorption as compared to both unencapsulated and the highest loaded 18.29% Fe-HA-BMC MPs. Compared to the reference meal, 3.19 and 18.29% Fe-HA-BMC MPs exhibited 71 (62, 82)% and 89 (74, 107)% RBV, respectively.

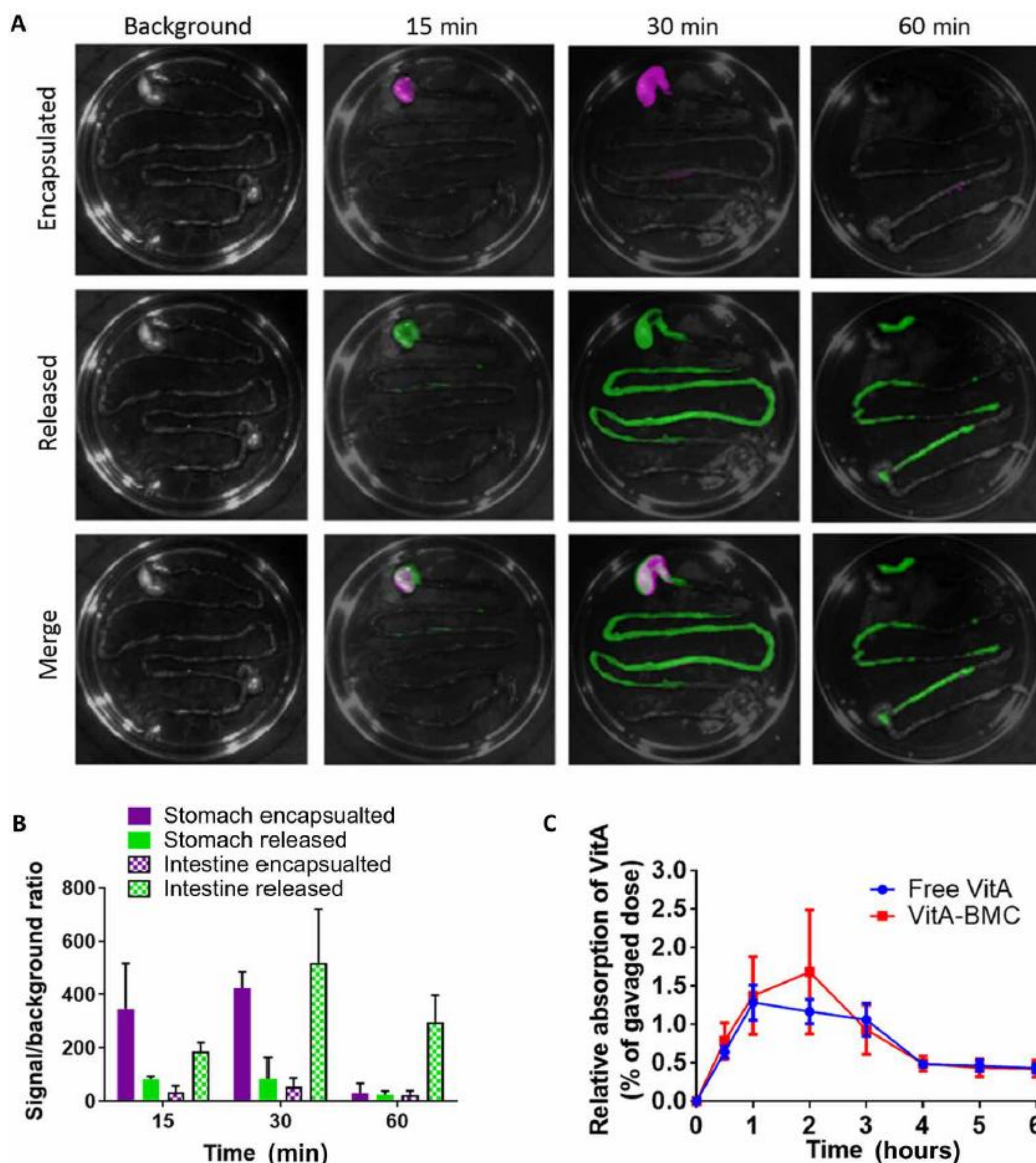

Fig. 4. In vivo release of a model dye and absorption of vitamin A. (**A**) Representative IVIS images (logarithmic scale) of explanted murine gastrointestinal tract harvested after oral administration of dye-loaded BMC MPs showing encapsulated dye (purple) and released dye (green) over 60 min. (**B**) Quantitative analysis of encapsulated dye in the stomach (solid purple bars), released dye in the stomach (solid green bars), encapsulated dye in the intestines (hatched purple bars), and released dye in the intestines (hatched green bars). Error bars represent SD of the mean (n = 3). (**C**) Blood content of radiolabeled vitamin A over a 6-hour period after oral gavage of free vitamin A (blue lines) or VitA-BMC MPs (red lines). Error bars represent SEM (n = 6).

In this same human study, we investigated how competitive absorption, related to the co-delivery of other micronutrients or BMC-encapsulated micronutrients alongside Fe-HA-BMC MPs, can influence absorption of iron from Fe-HA-BMC MPs. Co-delivery of VitA-BMC MPs [FIA: 12.7 (9.29, 17.5)%] or VitA-BMC MPs with free folic acid [FIA: 14.3 (11.2, 18.3)%] did not affect iron absorption (Fig. S6), indicating that competition between co-delivered micronutrients or BMC-encapsulated micronutrients is not a major concern for the combinations studied here. In four additional test meals, we investigated the individual role of each MP component and how co-administering these components in free form influences absorption of iron as compared to formulation Fe-HA-BMC MPs. Our results indicated that absorption from free ferrous sulfate was not significantly affected by either HA [FIA: 20.7 (16.1, 26.7)%], BMC [FIA: 16.6 (12.0, 23.2)%], or HA-BMC [FIA: 16.3 (11.7, 22.8)%]. Similarly, when Fe was encapsulated in HA [FIA: 15.1 (11.3, 20.3)%], iron absorption was not significantly different from the reference meal. Our results indicated that absorption was not significantly affected by either HA or BMC as compared to free iron; however, when HA and BMC were formulated as MPs, a decrease in absorption compared to free iron and free iron with HA was observed (Fig. S7). This phenomenon is unlikely to occur for our highest loaded 18.29% Fe-HA-BMC MPs formulation, because it demonstrated comparable absorption relative to the reference (Fig. 7). Collectively, these results indicate that the absorption-limiting encapsulation that was observed in the first human study can be overcome and addressed through further development and increased loading of iron and decreased BMC content in HA-BMC MPs.

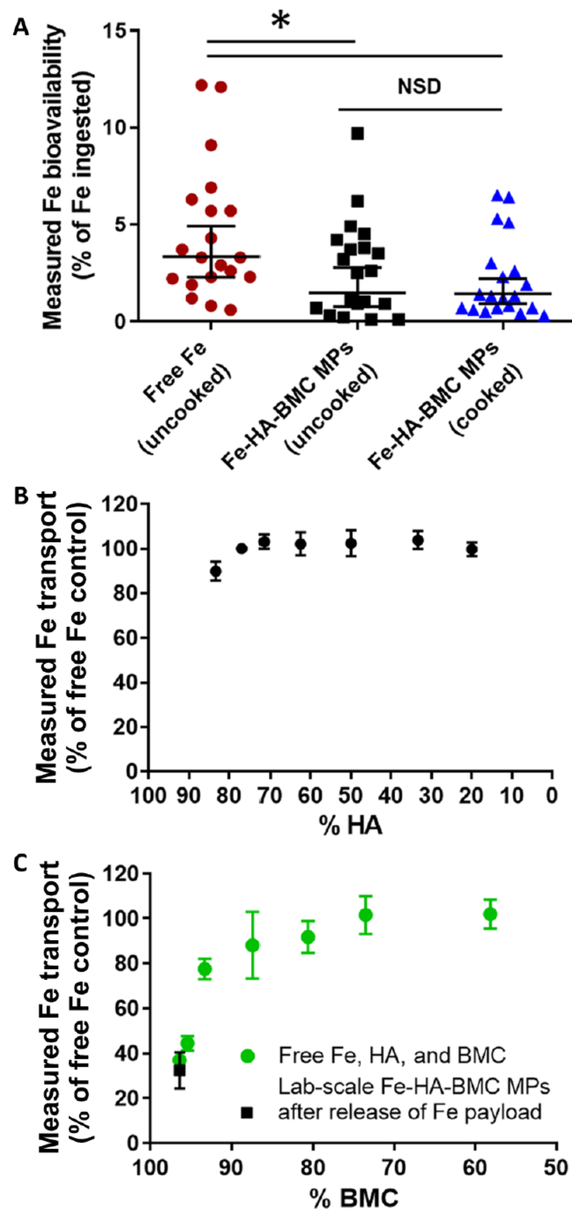

Fig. 5. Bioavailability of iron in humans and iron transport in an in vitro intestinal barrier model. (**A**) Iron bioavailability as assessed by erythrocyte iron incorporation in young women (n = 20) after ingestion of unencapsulated (free) uncooked iron as FeSO4 (red circles), encapsulated uncooked iron (black squares), and encapsulated cooked iron (blue triangles) and expressed as a percentage of the total amount that was ingested. Iron transported across a human in vitro intestinal barrier model after addition of iron in the presence of varying amounts of MP constituents (**B**) HA and (**C**) BMC and expressed as a percentage of transported free iron. Error bars in (A) represent geometric means (n = 20) with 95% CI. *P < 0.05, free Fe and each encapsulated group as determined by post hoc paired Student's t test with Bonferroni correction. Error bars in (B) and (C) represent SD of the mean (n = 3). NSD, no significant difference.

Discussion

In this work, we describe polymer-based MPs that encapsulate 11 micronutrients individually or up to 4 in combination, maintain micronutrient stability during cooking and storage, and rapidly release micronutrient payloads. Modularity of the MP platform affords freedom to control the delivery and thus the dosing of each micronutrient for individual or large-population fortification needs. Studies in mice confirmed rapid micronutrient release in the stomach and absorption of vitamin A. In the first human study, the bioavailability of iron from iron-loaded MPs was verified to be 44% of unencapsulated iron control after oral ingestion. Using an organotypic human intestine model, we discovered that low iron loading and high BMC amount were responsible for the lower bioavailability in this first human study. Hence, process development approaches were leveraged to overcome these limitations of our laboratory-scale MPs. Industrially relevant spray drying and spinning disc atomization processes enabled increased iron loading while reducing the amount of BMC polymer in our iron-loaded MPs. In a subsequent human study, using iron-loaded BMC MPs of more than 5- and 30-fold higher iron loading and 25 and 85% less BMC polymer, we showed that our leading formulation exhibited statistically indistinguishable bioavailability as compared to an unencapsulated control. Specifically, the MP formulation of higher iron loading and lower BMC content used in the second human study address bioavailability issues; however, it should be noted that the highest iron-loaded MP exhibited lower stability in water and higher pH solutions. Hence, further optimization of BMC content and iron loading for both release and absorption may be required because variability of pH values in the stomach of human subjects at the time of ingestion[49] may be a potential source for observed absorption differences. Together, we describe a broad approach encompassing the design and clinical introduction of a controlled release platform for vitamins and minerals that can address unmet technological needs to improve global health. Furthermore, these approaches could readily be applied towards the clinical translation of other materials, such as natural polymers, for the encapsulation and oral delivery of micronutrients.

The basis of our strategy for food fortification focused on formulation of MPs using BMC, a polymer encapsulant that has historically been used for its advantages in encapsulation, enhancing stability, facilitating rapid and controlled release, toxicity, amenability to manufacturing processes, and widespread use in many nutraceuticals and pharmaceutical

products.[34-41,50] BMC was selected after an indepth analysis of more than 50 potential polymers. With regard to enhanced stability, it has been previously demonstrated that the water vapor permeability of BMC[51] is lower than what is commonly used for preservation of fruits and vegetables;[52] thus, it is likely that the known moisture barrier properties of BMC provide protection against humidity and small-molecule diffusion by acting as a physical barrier between the payload and the environment. The UV-protective abilities of BMC can likely be attributed to the increased refractive index due to encapsulation in BMC,[53] which will lower light exposure to encapsulated micronutrients. BMC is soluble at low pH in aqueous solutions

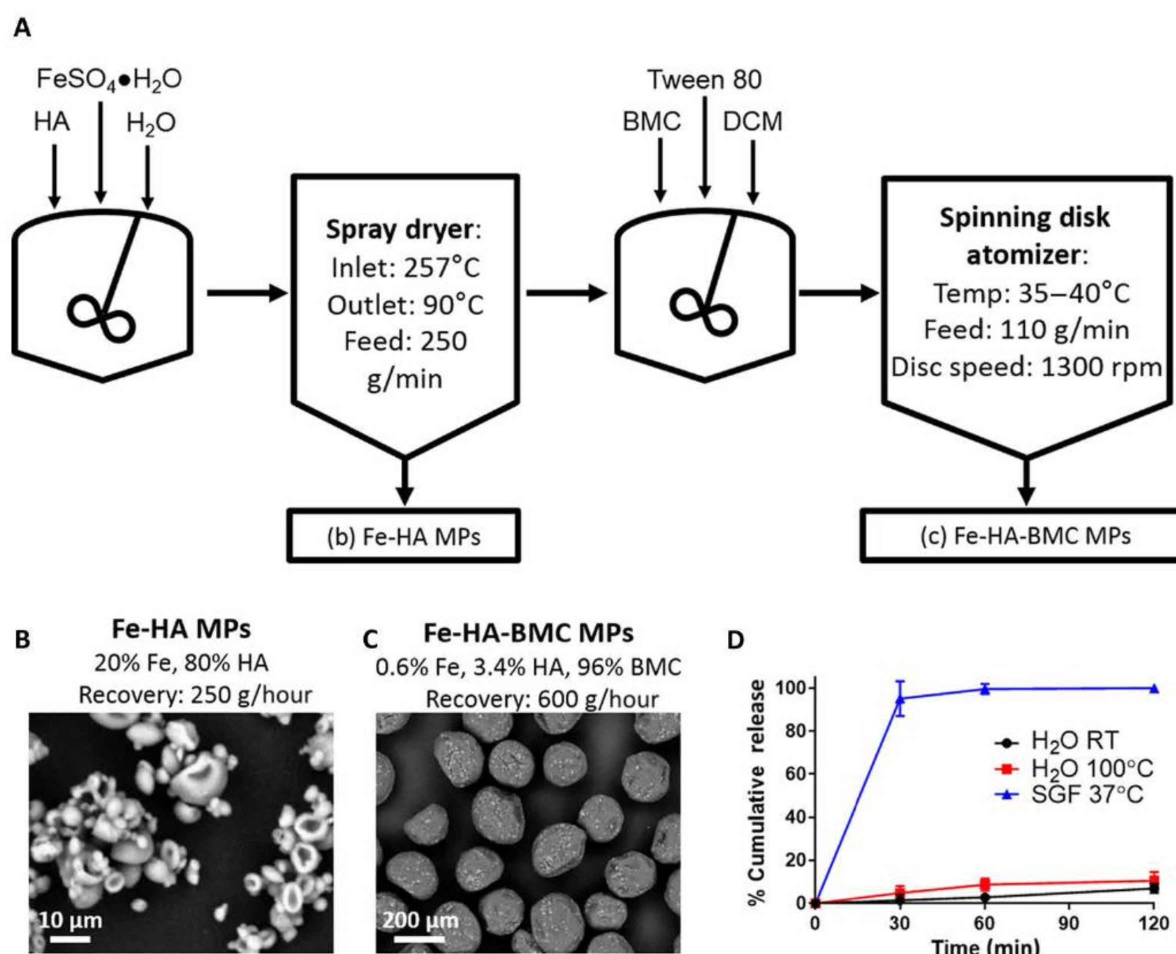

Fig. 6. Process development and scale-up. (**A**) Schematic showing the process for the scaled synthesis of 1 kg of Fe-HA-BMC MPs. SEM images of (**B**) the Fe-HA MP intermediate product and (**C**) the Fe-HA-BMC final product. (**D**) Iron release from scaled Fe-HA-BMC MPs in 37°C SGF, pH 1.5 (blue line), RT water (black line), and boiling water (red line). Error bars represent SD of the mean (n = 3).

and in non-aqueous solvents; hence, the solubility of BMC enabled rapid dissolution and release in stomach conditions, thus providing the versatility to facilitate encapsulation of 11 distinct micronutrients. An additional aspect of our reasoning for selecting BMC involves the existing infrastructure and knowledge base that would enable the translation of a BMC based technology. For example, BMC is already used commercially, has been shown to be compatible with large-scale manufacturing processes, has a history of acceptance by the FDA, and is generally regarded as safe.[54] The choice of solvent, DCM, used in the particle fabrication process at scale and removed afterward, has essential roles in the preparation of other everyday consumer products such as decaffeinated coffee.[55] Recently, the Joint Food and Agriculture Organization of the United Nations and the World Health Organization Expert Committee on Food Additives (JECFA) yielded a positive response for BMC, specifically for micronutrient encapsulation for food fortification.[56] JECFA concluded that the use of BMC is not a safety concern when the food additive is used for micronutrient encapsulation for food fortification at the intended use amounts and recommended an acceptable daily intake of "not specified." Subsequently, the Codex Committee on Food Additives (CCFA) agreed to accept the JECFA recommendations.[57] Hence, concerns about the toxicity of BMC have been previously and extensively evaluated and addressed;[50,54] furthermore, the doses described here and the potential doses that may be used in practice would be unlikely to exceed the well-published limitations for oral exposure.

The human absorption studies described here were performed in a well-controlled setting. Both studies were performed in young healthy women where the prevalence of iron deficiency in the study population was 65 and 58% in human studies 1 and 2, respectively. The meal was specified in terms of mass and volume, the dosing of encapsulated iron was measured precisely, and the cooking conditions were well controlled using standardized procedures. Iron status will likely differ in real-world settings where people consuming the micronutrients will be from different countries where staple foods vary. In addition, the age, health status, and eating habits of individuals will vary in different cultures as well as within households. Hence, further human testing for efficacy, especially in target countries and populations, will be informative for evaluating and developing these micronutrient formulations to ensure that they are suitable in terms of stability, release, and absorption. In previous work, it has been shown that the encapsulating material can inhibit iron bioavailability,[15,18] which agrees with the results from both of our human studies. This

relationship between the encapsulant and the micronutrient is directly tied to absorption and should be further studied for our MP platform in animals and in humans. Since micronutrients such as vitamin A or D are more sensitive to thermal degradation as compared to iron, there exists a unique opportunity to leverage our MP platform for the storage and delivery of highly sensitive micronutrients. Our preliminary storage and in vivo absorption work suggest that delivery of nonmineral micronutrients will be promising; however, further evidence is needed in humans. We also demonstrated successful co-encapsulation of vitamins A, D, folic acid, and B_{12}; future efforts should focus on investigating how co-encapsulated micronutrients with known compatibility issues[58] may interact with each other, as this study was designed to evaluate bioavailability of a single micronutrient (iron). The experimental approaches we have detailed here lay the groundwork to facilitate these additional studies and provide a path toward clinical translation.

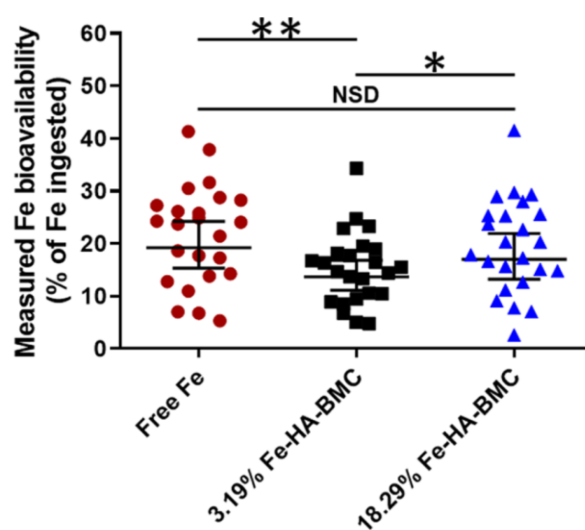

Fig. 7. Bioavailability of iron in humans from higher loaded Fe-HA-BMC MPs. Iron bioavailability as assessed by erythrocyte iron incorporation in young women (n = 24) after ingestion of free iron as $FeSO_4$ (red circles), 3.19% Fe-HA-BMC MPs (black squares), and 18.29% Fe-HA-BMC MPs (blue triangles) and expressed as a percentage of the total amount of iron that was ingested. Error bars represent geometric means (n = 24) and 95% CI. *$P < 0.05$ and **$P < 0.01$. Significant effect of meal on iron absorption determined by linear mixed models, participants as random intercept, meal as repeated fixed factor, and post hoc paired comparisons with Bonferroni correction $P < 0.05$.

In practice, translation of this technology for worldwide use, especially in countries in need of solutions for nutrition deficiencies, will still require scaling beyond the kilogram batch size to metric tons. The spray drying and atomization processes described here are translatable to

current commercial scale processes for micronutrients.[59] Hence, the main cost implication is directly related to the added raw material cost of BMC; future considerations to offset this cost or justify it must be considered on a country or regional level, because the severity of micronutrient deficiencies will be a driving force to balance the technical advantages offered by our encapsulation approach. Beyond manufacturing and BMC, cost considerations include the choice of iron sulfate, because it is one of the lowest cost forms of iron[60] and because iron sulfate enables higher bioavailability as compared to other iron forms.[61] Additional implications will require country-, region-, and/or individual-specific micronutrient needs, and careful attention must be paid to local public and regulatory policies to enable widespread use of this technology. The MP delivery system has been tested in vitro, in vivo in mice, and in humans and has been scaled up using commercially relevant processes. Furthermore, we have demonstrated the modularity and tunability of our platform by identifying and subsequently modifying the MPs to address absorption-limiting design criteria. In summary, a heat-stable pH-responsive polymer-based MP delivery system has been developed that shows promise as a platform for micronutrient delivery to humans.

Materials and Methods

Study design

We hypothesized that a polymer-based MP encapsulation system that maintained micronutrient stability in boiling water for hours and rapidly releases micronutrient payloads in acidic stomach conditions could address challenges with micronutrient delivery. Hence, we studied micronutrient absorption in both rodents and human subjects to evaluate our MP technology. Animal studies were approved by the Institutional Animal Care and Use Committee and were performed at the Massachusetts Institute of Technology (MIT). For humans, two studies were performed using a single-blind, randomized, cross-over design. In study 1, three maize porridge porridge test meals were administered; in study 2, participants consumed nine wheat bread test meals. All test meals were labeled with 4 mg of Fe as $FeSO_4$ using stable iron isotopes (^{54}Fe, ^{57}Fe, or ^{58}Fe). Labeled $FeSO_4$ was prepared by Dr. Paul Lohmann GmbH (Germany) from isotopically ^{54}Fe-, ^{58}Fe-, and ^{57}Fe-enriched elemental iron (Chemgas, Boulogne, France). Particles used in study 1 were produced at MIT, and those used in study 2 were produced at Southwest Research Institute in San Antonio, Texas. Different participants were included in each study. After enrollment, each participant was allocated to

a predefined schedule of test meal sequence and combinations in a randomized balanced block design. Each participant served as their own control. In study 1, the maize porridge test meals contained fortified salt, added either before or after cooking. The fortified salt contained either (i) $FeSO_4$ (reference), (ii) iron-loaded BMC-HAFe (0.6%) added before cooking, or (iii) iron-loaded BMC-HA-Fe (0.6%) added after cooking. In study 2, the wheat bread test meals were fortified before baking. The test meals contained either (i) iron-loaded BMC-HA-Fe (3.19%), (ii) iron-loaded BMC-HA-Fe (18.29%), (iii) iron-loaded HA-Fe (8.75%), (iv) iron-loaded BMCHA- Fe (3.19%) with VitA-BMC (3.4%; 37.65 mg of vitamin A), (v) iron-loaded BMC-HA-Fe (3.19%) with VitA-BMC (3.4%; 37.65 mg of vitamin A) with free folic acid (0.34 mg), (vi) $FeSO_4$, (vii) $FeSO_4$ with HA [25.68 mg to match HA in group (i)], (viii) $FeSO_4$ with BMC [85.19 mg to match BMC in group (i)], or (ix) $FeSO_4$ with BMC [85.19 mg to match BMC in group (i)] with HA [25.68 mg to match HA in group (i)]. Within 1 week, each participant consumed a test meal on three consecutive days, and after a 14-day (study 1) and 19-day (study 2) break, a blood sample was taken for measurement of stable iron isotope incorporation into the erythrocytes. In study 2, this procedure was repeated twice. The total study duration of study 1 was 17 days, and study 2 was 64 days. Participants were recruited among female students at the Swiss Federal Institute of Technology in Zürich and University of Zürich. Inclusion criteria were as follows: apparently healthy, nonpregnant (assessed by a pregnancy test), nonlactating, age between 18 and 40 years, weight <65 kg, body mass index of 18.5 to 25 kg/m^2, depleted iron status defined as a PF concentration of <20 mg/liter), and in the absence of systemic inflammation [defined with a C-reactive protein (CRP) concentration of >5 mg/liter]. Exclusion criteria were as follows: chronic disease or intake of long-term medication (except for oral contraceptives), consumption of mineral and vitamin supplements within the 2 weeks before first test meal administration, and significant blood loss or transfusion, within 4 months before the study initiation. Informed written consent was obtained from all participants. Ethical approval for both studies was provided by the ethical review committee of Cantonal Ethics Commission of Zürich (study 1, KEK-ZH-Nr. 2015-0094; study 2, KEK-ZH-Nr. 2017-01624) and the Committee on the Use of Humans as Experimental Subjects at MIT (study 1, COUHES #1502006932; study 2, COUHES #1801201448/1801201448A001); both trials were registered on ClinicalTrials.gov: study 1 (NCT02353325) and study 2 (NCT03332602). Both studies were powered to detect a nutritionally relevant, 30% within-group difference in iron absorption, based on an SD of 0.35

from log-transformed iron absorption from previous studies by our laboratory,[62] an α level of 5% (two-tailed), and 80% power; 18 subjects were calculated. In study 1, a 10% dropout rate was anticipated; in study 2, because of the longer duration of the study, a 30% dropout rate was anticipated; therefore, 20 and 24 subjects were recruited, respectively.

Formulation of one-step MPs (BMC MPs) and two-step MPs (HA-BMC MPs)

BMC MPs were prepared by a modified oil/water emulsion method.[63] The organic phase for the emulsion consisted of either (i) 1 mg of blank or dye-labeled HA MPs homogeneously dispersed in 1 ml of BMC solution (100 mg/ml) in methylene chloride; (ii) vitamin A (10 mg/ml), vitamin D (2 mg/ml), folic acid–loaded HA MPs (1.3 mg), and B_{12}-loaded HA MPs (1.3 mg) dissolved into BMC solution (100 mg/ml, 1 ml) in methylene chloride to prepare BMC MPs co-encapsulated with four different types of micronutrients; (iii) HA MPs encapsulated with various micronutrients as described in table S2 to synthesize HA-BMC MPs with various micronutrient loads; (iv) free micronutrients as described in table S2 to synthesize BMC MPs with various micronutrient loads; or (v) lipophilic carbocyanine DiOC18(7) dye (1 mg/ml) (DiR, Life Technologies) and BMC (100 mg/ml) in methylene chloride to synthesize fluorescently labeled BMC MPs. The resulting organic phases were then emulsified in polyvinyl alcohol (PVA) solution (20 ml, 10 mg/ml) with a stirring rate at 300 rpm for 10 min. The obtained emulsion was added into 100 ml of deionized water with stirring (500 rpm for 10 min) to solidify the MPs. The obtained MPs were allowed to settle by gravity and thoroughly washed with water. The final dry MPs were obtained by lyophilization.

Micronutrient-MP loading, release, and stability

Vitamins B_2, niacin, folic acid, B_{12}, A, and D were analyzed via high-performance liquid chromatography (Agilent 1100; Agilent Technologies) using a C-18 column (Acclaim Polar Advantage II, 3 µm, 4.6 mm × 150 mm) and were detected by a photodiode detector at 265, 265, 286, 550, 325, and 264 nm, respectively. Iron, biotin, zinc, and vitamin C were analyzed using BioVision colorimetric assay kits, and vitamin biotin was analyzed using a Sigma colorimetric assay kit. Iodine was measured using UV-visible absorbance at 288 nm. DiR-loaded BMC MPs were dissolved in dimethyl sulfoxide, and then the dissolved cargo was quantified using a multimode reader (TECAN Infinite M200 PRO) at 750 nm. Micronutrient-loaded HA MPs were dissolved in water, and micronutrient content was determined as described above for each respective micronutrient. To quantify micronutrient loading in the

co-encapsulated HA-BMC MPs or the BMC MPs, a known mass of MPs was first dissolved in SGF and then analyzed for the total micronutrient mass. The precipitated BMC was removed via centrifugation using Amicon Ultra centrifugal filters (3000 normal molecular weight limit) at 14,000g for 30 min to remove HA and BMC. The dissolved micronutrients were separated and quantified as described above. To quantify vitamin A and D loading, the MPs were dissolved in methylene chloride and the dissolved vitamins A and D were separated and quantified as described above. The release profiles of micronutrients were studied in water at RT, boiling water at 100°C, and SGF at 37°C. At each time point, samples were centrifuged at 4000 rpm for 5 min, and 900 µl of the supernatant was collected for analysis, and then samples were replenished with 900 µl of fresh release medium. For vitamins A and D, the aqueous release medium was brought into contact with a layer of methylene chloride, and then the extracted fat-soluble vitamins within the organic phase were used for analysis. The cumulative release was calculated as the total amount of micronutrient released at a particular time point relative to the amount initially loaded. Dry micronutrients were dispersed in water and then heated at 100°C for 2 hours before being centrifuged at 4000 rpm for 5 min. The stability percentage equals the ratio of stable micronutrient after to the actual loading of the micronutrient in the MPs. For samples in unencapsulated form, they were either dissolved in water or dispersed in water before being heated for 2 hours. The sensory performance was measured in duplicate as the absolute color change in a food matrix after the addition of the Fe microspheres. A banana milk slurry was chosen as polyphenol-rich food matrix. The fortificants were added to 70 g of banana milk at a concentration of 60 ppm Fe in banana milk. The banana milk was prepared fresh: 180 g of fresh banana with 520 g of organic whole milk (3.9% fat, homogenized, pasteurized). Color change was measured in duplicate at baseline (before fortification) and 2 hours after fortification and stirring at 350 rpm. The absolute color change of ΔE was measured and calculated as previously described.[64] $FeSO_4$ and ferric pyrophosphate (FePP; 20% Fe, micronized powder) were used as positive and negative controls.

Dissolution study of DiR-loaded BMC MPs in mice

Female SKH1-Elite mice (Crl:SKH1-hr) were purchased from Charles River Laboratories at 8 to 12 weeks of age. Mice were fed an alfalfa-free balanced diet (Harlan Laboratories, AIN-76A) for 10 days before treatment to reduce food-related autofluorescence. About 200 mg of DiR-

loaded BMC MPs was administered in 100 µl of water via gavage (n = 3). After 15, 30, or 60 min, mice were euthanized using carbon dioxide asphyxiation. The gastrointestinal tract was immediately explanted and imaged using in vivo imaging system (IVIS, PerkinElmer). The fluorescent signals from mice that had ingested DiR-loaded BMC MPs were compared to mice that did not receive MPs. The spectral signatures associated with encapsulated and released DiR were then computationally separated from tissue autofluorescence (identified in the control samples) to determine the location and status of dye release. Quantified signal/background ratios were determined by normalizing the encapsulated or released dye signal, in either the stomach or intestines, to a background in control animals receiving no BMC MPs.

In vivo vitamin A absorption in rats

Tritium-labeled retinyl palmitate (American Radiolabeled Chemicals Inc.) was used to detect the amount of absorbed vitamin A in blood. Radiolabeled VitA-BMC MPs were prepared by the oil/water emulsion method described above. Female Wistar rats (~250 g) were purchased from Charles River Laboratories. The rats were divided into two groups: (i) free vitamin A and (ii) VitA-BMC MPs. In the free group, vitamin A was delivered in a 4% (v/v) ethanol/water mixture to enable solubilization of vitamin A. The VitA-BMC MPs were dispersed in water and vortexed to form a suspension. Each rat was oral gavaged 10 µCi of vitamin A in either its free form or encapsulated MPs in 350 µl of either ethanol/water mixture or water total. Residual vitamin A in the syringe and gavage needle was saved and quantified by scintillation counter to calculate the actual feeding amount of tritium-labeled retinyl palmitate for each rat. At 0.5, 1, 2, 3, 4, 5, and 6 hours, the rats were anesthetized via isoflurane and 200 µl of blood was collected from the lateral tail vein. The radioactivity in the samples was quantified via liquid scintillation counting with a Tri-Carb 2810 TR liquid scintillation counter. To calculate loading of vitamin A in the VitA-BMC MPs, the MPs were first dissolved in 1 ml of DCM, and then 5 µl of the solution was mixed with 10 ml of an Ultima Gold F liquid scintillation cocktail (PerkinElmer Inc.). Blood (200 µl) was dissolved in SOLVABLE (PerkinElmer Inc.) following recommended protocol, and then 1 ml of the dissolved blood was mixed with 10 ml of Hionic-Fluor liquid scintillation cocktail.

MatTek EpiIntestinal transport

EpiIntestinal tissues were purchased from MatTek (Ashland) and used as recommended. For transport experiments, the particle constituents BMC, Fe, and HA were separately prepared and added to achieve final mass percentages as reported. After 1 hour of incubation at 37°C and 5% CO_2, transport iron was analyzed in the bottom transwell chamber using the previously described BioVision colorimetric assay.

Process development and scale-up

The detailed process described here was used to manufacture 1 kg of Fe-HA-BMC MPs as shown in Fig. 6. A Niro Production Minor pilot scale spray dryer was used to first prepare Fe-HA MPs. The feed solution contained 525.5 g of sodium hyaluronate, 1309.5 g of iron sulfate monohydrate, and 77 liters of deionized water. This solution was fed into the dryer at 250 g/min and atomized with a 2-mm two-fluid nozzle. The dryer inlet temperature was set to 257°C, resulting in an outlet temperature of 90°C. MPs (1215 g) were recovered. Fe-HA MPs were encapsulated with BMC using a custom spinning disc atomization system. The feed solution was prepared with 1152 g of BMC and 1.87 g of polysorbate 80 dissolved in 12,000 g of DCM.

Fe-HA MPs (48 g) were added to the DCM solution and placed in a sonication bath for 10 min to form a stable suspension. The suspension was fed at 110 g/min onto a 10.16-cm-diameter stainless steel custom disc spinning at 1300 rpm. The disc was mounted 9.144 m high in a 6.096 m × 6.096 m tower. The room was heated to 35° to 40°C. Particles were collected on antistatic plastic located at the bottom of the tower. MPs (1059 g) were recovered. These processes were modified for batches used in human study 2 by using a ProCepT 4 M8 laboratory spray dryer for the Fe-HA MPs. All new tubing and filters were used with the spray dryer, in addition to cleaning all wetted parts with soapy water and a 70% aqueous isopropanol solution. The inlet temperature for the spray dryer was set to 160°C, resulting in an outlet temperature of about 53°C. Solution was dried at 8 ml/min through a 0.4-mm air-atomized nozzle. The same spinning disc setup was used for encapsulating the Fe-HA MPs within BMC. The tower was mopped and cleaned, followed by treatment with Vesphene IIse. Encapsulated vitamin A for feed studies was also prepared using the same spinning disc system. A disc speed of 1675 rpm was used, as the feed solution was fed to a 10.16 cm spinning disc at about 115 g/min. The material was collected in a powdered Dry-Flo starch.

The excess starch was then sieved from the sample to recover the vitamin A MPs. All samples were placed under vacuum with a slow N2 purge for 1 week to remove residual DCM. Specific feed conditions for the MPs used in human study 2 are listed in table S4.

Statistical analysis

For in vitro and rodent studies, all quantitative measurements were performed on three independent replicates. All values are expressed as means ± SD. Statistical significance was evaluated using a twotailed Student's t test. Statistical analysis of the human studies was done using SPSS version 22 (human study 1) and version 24 (human study 2) (IBM SPSS Statistics). All data were checked for normal distribution before analysis: Age, weight, height, Hb, and CRP were normal, and the data are presented as means and SD. PF and fractional Fe absorption are non-normal and are presented as geometric means and 95% CI. Comparisons between meals were done using the square root–transformed data fitted in a linear mixed model. Meals were entered as a repeated fixed factor (covariance type of scaled identity) and subjects as random factors (intercept). If a significant overall effect of meals was found, post hoc tests within different meals were performed using the Bonferroni correction for multiple comparisons. $P < 0.05$ was considered statistically different.

Author Contributions

A.C.A., X.X., B.N., L.W., P.A.W., J.D.O., D.M., M.B.Z., R.L., and A.J. conceived and designed the research. X.X., J.D.O., and K.J.M. performed electron microscopy on MPs. A.C.A. designed and performed in vitro transport experiments and imaged time-lapse MP release. A.C.A., X.X., Y.Z., W.T., A.M.B., E.R., A.R.D., J.L.S., J.Z., J.C., X. Lu, T.G., S.A., T.D.N., X. Le, and A.S.G. performed in vitro release experiments. A.C.A., X.X., S.B., Y.Z., W.T., E.R., and J.Z. performed in vitro stability experiments. W.T., S.B.W., and K.J.M. designed in vivo mouse experiments. W.T., K.J.M., J.L.S., S.Y.T., and S.R. performed in vivo experiments. A.C.A., C.B.S., J.D.O., and A.J. designed process development approaches. J.D.O. synthesized scaled batches. S.B., D.M., and M.B.Z. designed the human clinical experiments. S.B. performed the human clinical experiments. A.C.A., L.E.F., R.L., S.B., and A.J. analyzed the data and wrote the manuscript.

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Conflicts of Interest

A.J., R.L., X.X., B.N., P.A.W., and L.W. are inventors on patent no. 9,649,279 held/submitted by the MIT and Tokitae LLC that covers micronutrient fortified salts that are thermally stable and release micronutrient payloads in the gastrointestinal tract. A.C.A., A.J., R.L., W.T., and X.X. are inventors on patent application no. 16/239284 submitted by the MIT that covers spray drying micronutrient MP formulations.

Data and materials availability:

All data associated with this study are present in the paper or Supplementary Materials.

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Supplementary Materials

Materials and Methods

Materials

All materials were from Sigma unless otherwise specified. The BMC polymer, poly(butylmethacrylate-co-(2-dimethylaminoethyl)methacrylate-co-methylmethacrylate) (1:2:1), was from Evonik Industries, where Eudragit E PO-powder and Eudraguard Protect-powder were respectively used to formulate small scale and larger scale batches of MPs. The HA was from Lifecore Biomedical. The Ge was from Sigma; The Iron (ferrous sulfate, $FeSO_4 \cdot 7 H2O$) was from Sigma; Iodine (potassium iodate, KIO3) was from Sigma; Zinc (zinc sulfate, $ZnSO_4$) was from Sigma. The Vitamin A (retinyl palmitate) was from Alfa Aesar; Vitamin B_2 (riboflavin) was from Sigma; Niacin (nicotinic acid) was from Sigma; Vitamin B_7 (biotin) was from Sigma; Vitamin B_9 (folic acid) was from Sigma; Vitamin B_{12} (cobalamin) was from Sigma; Vitamin C (ascorbic acid) was from Sigma; Vitamin D_3 (cholecalciferol) was from Sigma. The dichloromethane was from Sigma. The SGF was from Ricca Chemical Company.

Formulation of HA MPs

HA MPs were formulated using a modified inverse emulsion technique.[65] Blank HA MPs were prepared by homogenizing an HA solution (Low molecular weight HA, Mn = 384 kDa, Mw = 803 kDa; 1 wt % in de-ionized water) in mineral oil (30 ml) containing 120 µl of Span80 for 10 min using a Silverson L5M-A laboratory mixer (Silverson Machines, Inc.). To prepare the lab-scale MN encapsulated HA MPs, vitamins folic acid, B_{12} and/or ferrous sulfate heptahydrate were dissolved in the HA aqueous solution (1 wt% in 2 ml of de-ionized water) as described in **table S2**. The resulting solution was then used for the preparation of the emulsion as described above. The aqueous phase of the emulsion was allowed to evaporate for 24 hours at 45°C with constant stirring. The obtained HA MPs were then isolated by centrifugation at 3000 rpm for 5 min and thoroughly washed by hexane and acetone before drying under vacuum overnight. To prepare fluorescently labeled HA MPs, HA derivatives containing aldehyde groups (HA-CHO) were first synthesized using sodium periodate following reported procedures.[66] Since oxidation causes chain cleavage of the HA,[66] high molecular weight HA (Mn = 1096 KDa, Mw = 2698 KDa) was used in this case. The molecular weight of obtained HA-CHO was analyzed by gel permeation chromatography (GPC). The degree of

modification was quantified as 65% by an iodometry method following reported methods.[67] To formulate fluorescent HA MPs, HA-CHO and unmodified HA were mixed with a weight ratio of (1:1) and were then used to prepare the MPs by the inverse emulsion method as described above. For dye labelling, one milligram of the HA-MP containing aldehyde groups was dispersed in a methanol solution of CF405M (fluorescent dye containing aminooxy group, Biotium Inc.). Acetic acid (5 µl) was added to accelerate the reaction. The reaction was then allowed to proceed for 12 hours at room temperature. The dye labelled particles were collected by centrifugation (3000 rpm, 5 min), and thoroughly washed using methanol before drying under vacuum.

Morphological MP characterization

Three different microscopic methods were used to characterize the MP size, morphology, and cross sections; namely, optical microscopy (Olympus MX40), scanning electron microscopy (JEOL 5910 SEM), and confocal microscopy (Zeiss LSM 700 Laser Scanning Confocal). Dry MPs were coated with Pt/Pd before SEM imaging. Dye labeled HA MPs were visualized by the confocal microscope at an excitation wavelength at 405 nm, with a band pass filter of 420-475 nm. Reported mean particle diameters were estimated using ImageJ based on at least 20 counts of the particles from SEM images.

Clinical study: Iron absorption studies, procedure, and laboratory analyses

Study procedure: Study 1 was conducted in March – April in 2016 at the Laboratory of Human Nutrition (HNL) in Zürich. 118 participants attended the screening 1-2 weeks before test meal administration, weight and height were measured, a blood sample for Hb, PF and CRP measurement was collected, and 20 participants meeting the inclusion and exclusion criteria were invited to participate. Test meals A, B and C were administered on 3 consecutive days (study days D1, 2 and 3), the subjects were instructed to consume no solid food after 20.00 h and no fluids after 24.00 h the evening before test meal administrations. They consumed the test meals between 07.00-09.00 h each morning under direct supervision. After consuming the entire maize porridge test meal, the bowl was rinsed twice with 10 ml water and participants drank the rinsing liquid and remained fasting (no food nor drink) for 3 hours after test meal administration. On D17, a venous blood sample was taken for determination of Hb, PF, CRP, and determination of stable iron isotope ratio into the erythrocytes. The test meal consisted of porridge made from 50 g whole maize flour, served with 30 g vegetable sauce

(44% cabbage, 21% carrots, 21% zucchini, 12% onions, 2% oil) and 1 g salt. The amount of iron added to the porridge through fortified salt would correspond to a level of 80 ppm iron in directly fortified maize flour. The maize flour contained 1.52 mg Fe/100 g and 736.8 mg phytic acid /100 g. Each test meal contained 50 mg of maize and an additional 4 mg of fortification iron; thus, total iron and phytic acid content in the test meals was 4.8 mg Fe and 368 mg phytic acid, resulting in an iron to phytic acid ratio of 1:6.5. Ascorbic acid content of the test meals was negligible, 0.4 mg/meal. Thus, the test meal matrix was an inhibitory matrix in terms of iron absorption.[45] Nanopure water (300 ml) was served as a drink with the test meals. The vegetable sauce was prepared in bulk and stored frozen in portions until administration. Maize flour was precooked as follows: on the night before test meal administration, each individual maize portion was mixed with warm 18 MΩ/cm water, preheated in the microwave (1 min, 600 W), and then baked in an oven at 100°C for 60 min. After overnight refrigeration, on the administration day, maize porridge was preheated in the microwave for 1 minute at 600 W, and then cooked for further 30 min in the oven (100°C). The test meals with the cooked iron-loaded BMC-HA MPs were fortified before the microwaving step. The test meals with the non-cooked iron-loaded BMC-HA MPs were cooled down for 10 minutes to just under 50°C before the microspheres were added. The defrosted and preheated vegetable sauce was added just before serving.

Study 2 was conducted between April – July 2018 at the HNL. Prior the test meals, 77 participants attended screening, weight and height were measured, a blood sample for Hb, PF and CRP measurement was collected, 24 eligible participants were invited to participate. The participants were instructed with the same fasting conditions as in study 1. After consuming the entire bread test meal, the participants were instructed to consume all bread crumbs that had fallen into the plate. As in study 1 the participants remained fasting for 3 hours after test meal administration. The 9 test meals were administered in 3 blocks, within the first week, 3 test meals were administered on 3 consecutive days (D1, 2, and 3). On D22 a blood sample was drawn for determination of Hb, PF, CRP, and determination of stable iron isotope ratio into the erythrocytes. The next block of test meals was administered on D22, 23 and 24, and again on D43 a blood sample was drawn, within that week the last block of test meals was administered on D43, 44 and 45. The last blood sample was taken on D64. All bread roll test meals were prepared the afternoon before test meal administration, two doughs were prepared made of 1 kg refined wheat flour each, 5.5 g salt, 14 g dry yeast and 650 g of

nanopure water, the dough was kneaded for 10 min using a kitchen machine. And then weighed into portions of 100 g. 1/3rd of the portion was fortified with the microspheres, and 2/3rd of the portion was used to cover the fortified core. The amount of iron added to the bread was 67 ppm iron in wheat flour. After forming, the bread rolls were fermented for 45 minutes at 30°C and 80% relative humidity, and then baked for 20 minutes at 190°C. They cooled down on a cooling rack and wrapped in paper and stored at RT until consumption the next morning. The bread rolls consisted of 59.9 g wheat flour, 0.3 g of salt and 0.8 g of dry yeast, with the bread roll 300 ml of nanopure water was served as a drink.

Test meal analysis: Sample analyses of the test meals were done in triplicate. Iron concentrations of the maize flour and bread rolls were measured by graphite-furnace atomic absorption spectrophotometry (AA240Z; Varian) after mineralization by microwave digestion (MLS ETHOSplus, MLS). The phytate concentration of the maize flour and the bread roll was measured by spectrophotometry Makower method, in which iron was replaced by cerium in the precipitation step.[68] Ascorbic acid concentration in the vegetable sauce was measured by HPLC (Acquity H-Class UPLC System; Waters AG) after stabilization in 10 % metaphosphoric acid.

Blood analysis: Hb was measured by using a Coulter Counter (Study 1: Beckman Coulter, CA, USA; Study 2: Sysmex XN-350). PF and CRP was measured by using immunoassays (Study 1: Siemens Healthcare IMMULITE 2000; study 2: IMMULITE1000). Anemia was defined as Hb < 12 g/dL, Iron deficiency (ID) as PF < 15 mg/L and ID anemia as Hb < 12 g/dL and PF < 15 mg/L.[69] Whole blood samples collected on D17 (study 1), and in study 2 on D22, 43, and 64 were mineralized by using HNO_3 and microwave digestion followed by separation of the iron from the blood matrix by anion-exchange chromatography and a precipitation step with ammonium hydroxide.[70] All isotopic analyses were performed by using MC-ICP-MS (Neptune; Thermo Finnigan). The amounts of ^{57}Fe, ^{54}Fe and ^{58}Fe isotopic labels in blood 14 d after administration of the test meals were calculated on the basis of the shift in iron isotope ratios and on the estimated amount of iron circulating in the body. Circulating iron in the body was calculated based on hemoglobin and blood volume, derived from the participant's height and weight.[71] Fractional absorption (FIA) was calculated based on the assumption of an 80% incorporation of absorbed iron into the red blood cells. In study 2, the isotopic ratio value of

D22 and 43 served as a new baseline value for the after test meal administrations. Relative bioavailability (RBV) of iron was calculated as follows: $100/FIA_{reference\ meal}*FIA_{test\ meal}$.

Supplemental Figures

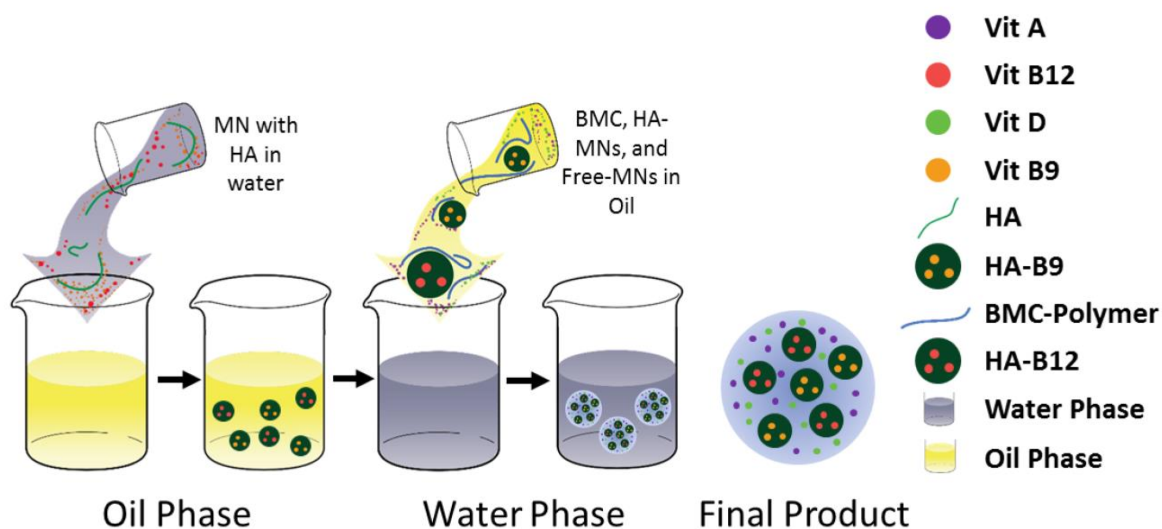

Fig. S1. Laboratory-scale co-encapsulation of micronutrients. Schematic representations of the two-step process for formulating co-encapsulated micronutrient MPs.

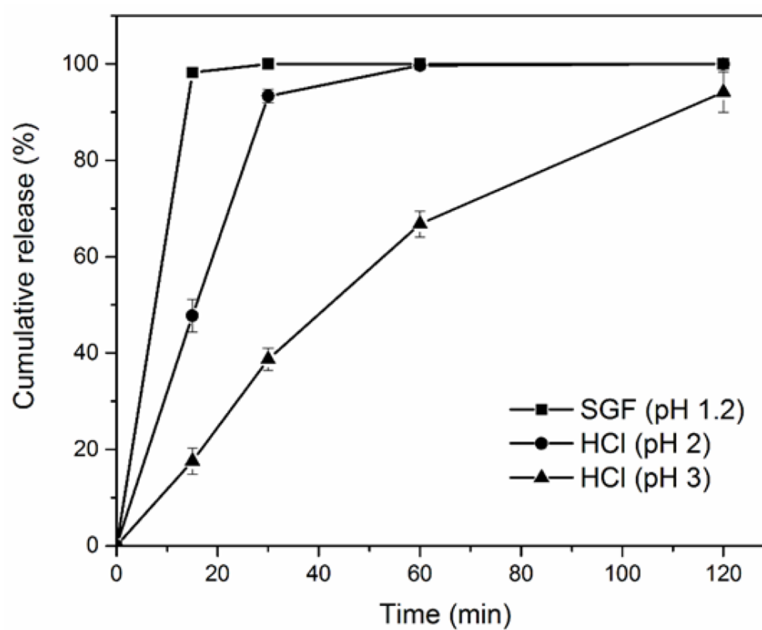

Fig. S2. Vitamin B12 release as a function of pH. Release of vitamin B12 from HA-BMC MPs in SGF (black squares), pH 2 HCl (black circles), and pH 3 HCl (black triangles). Error bars represent SD of the mean (n = 3).

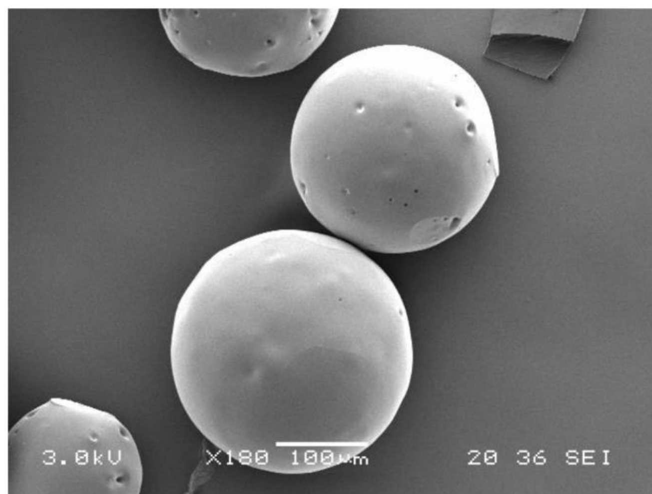

Fig. S3. HA-BMC MP electron micrograph after 2 hours in boiling water. SEM image of HA-BMC MPs after exposure to boiling water for 2 hours.

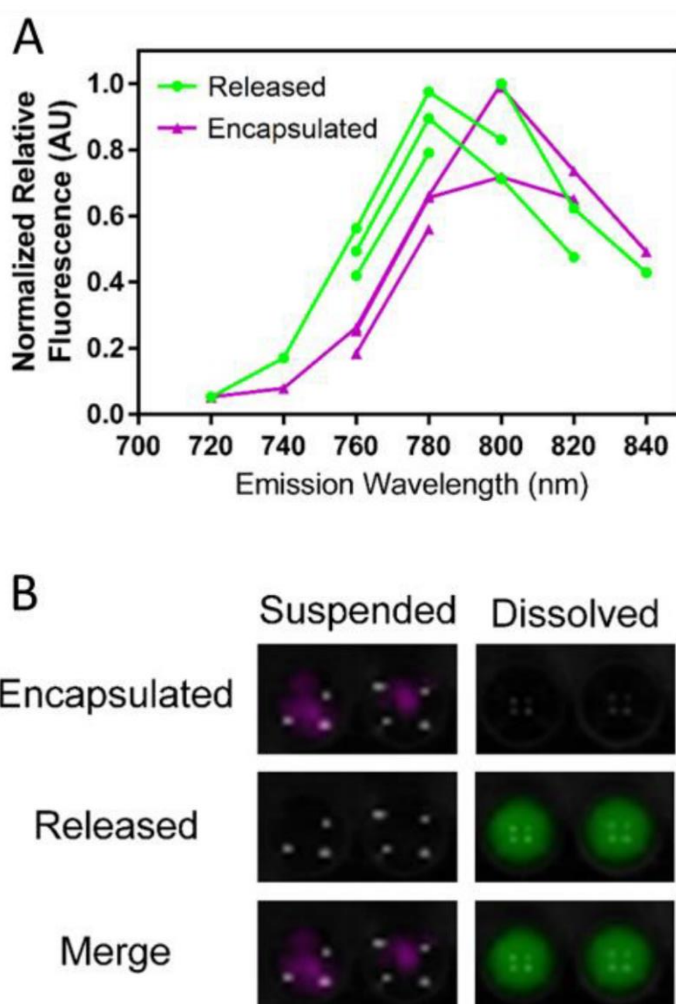

Fig. S4. Spectral fingerprinting of DiR-loaded MPs. (**A**) Spectral fingerprint and (**B**) representative IVIS images of a DiR-loaded BMC MPs when encapsulated (purple) or released in SGF (green).

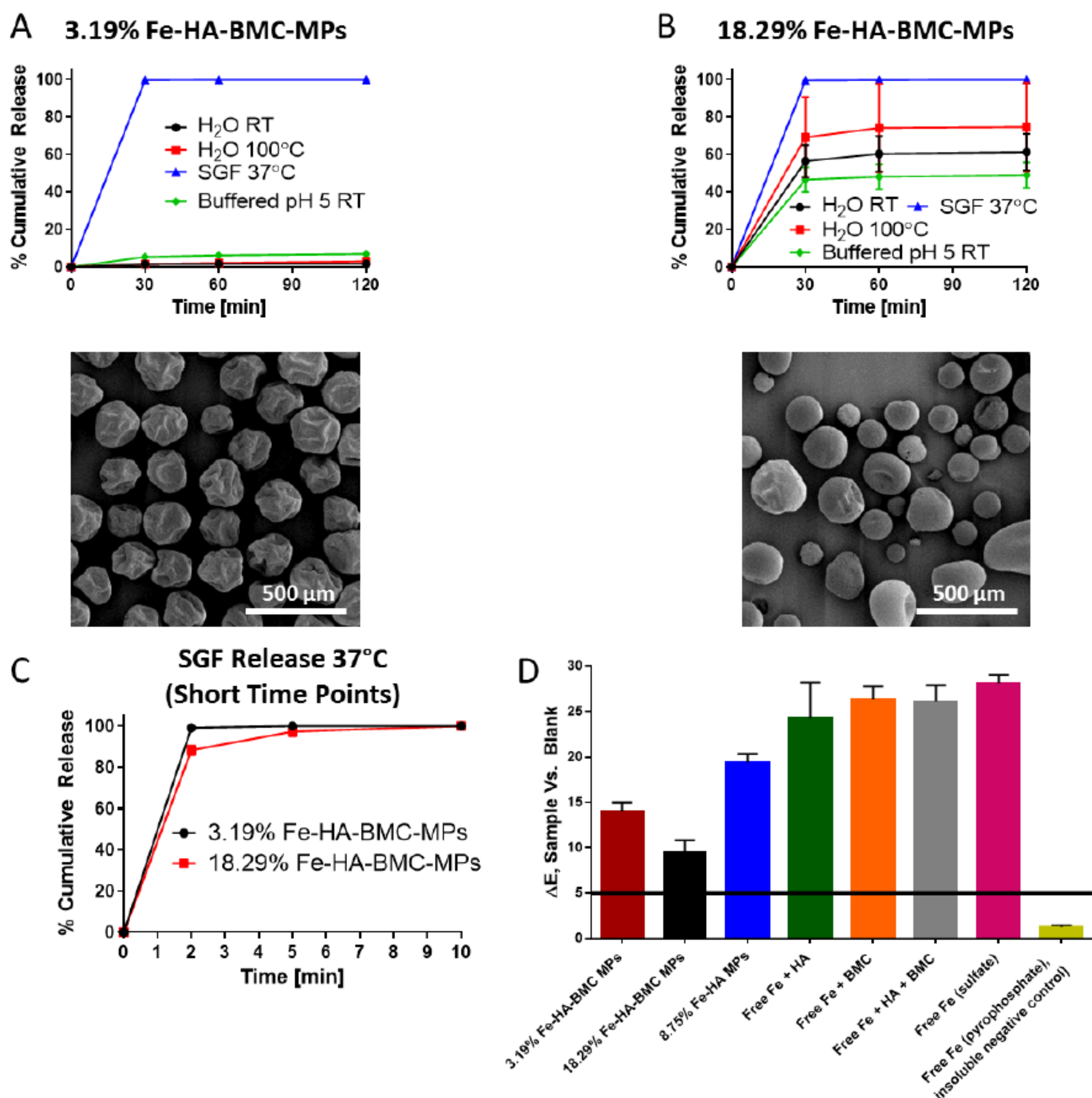

Fig. S5. Release, electron micrographs, and time history of color change for 3.19 and 18.29% Fe-HA-BMC MPs. (**A**) Release of iron from, and SEM image of, 3.19% Fe-HA-BMC MPs. (**B**) Release of iron from, and SEM image of, 18.29% Fe-HA-BMC MPs. (**C**) Release of iron from 3.19% and 18.29% Fe-HA-BMC MPs at short time points in SGF. (**D**) Sensory performance of scaled Fe-HA-BMC MPs and their individual constituents in a food matrix (banana milk), compared to FeSO4 and FePP, at 60 ppm Fe. Absolute color change ΔE±SD is given at 120 min against the non-fortified matrix. Horizontal line represents the threshold below which ΔE cannot be detected. Error bars represent SD of the mean (n = 3).

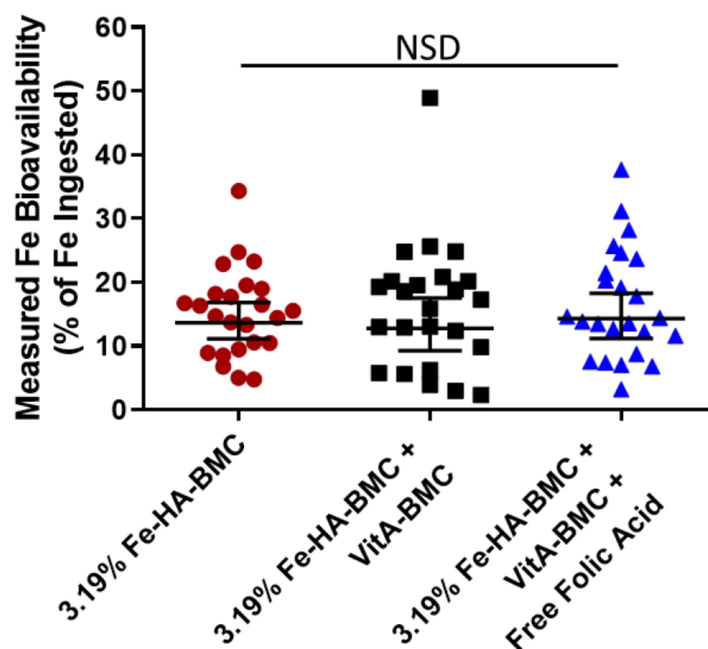

Fig. S6. Evaluation of iron absorption from 3.19% Fe-HA-BMC MPs in humans when co-administered with VitA-BMC MPs and free folic acid. Iron bioavailability as assessed by erythrocyte iron incorporation in young women (n=24) after ingestion of 3.19% Fe-HA-BMC MPs (red circles), 3.19% Fe-HA-BMC MPs with VitA-BMC MPs (black squares), and 3.19% Fe-HA-BMC MPs with VitA-BMC MPs and free folic acid. These values are expressed as a percentage of the total amount of iron that was ingested. Error bars represent geometric means (n = 24) and 95% CI. Significant effect of meal on iron absorption determined by linear mixed models, participants as random intercept, meal as repeated fixed factor, and post-hoc paired comparisons with Bonferroni correction $P < 0.05$. NSD: no significant difference.

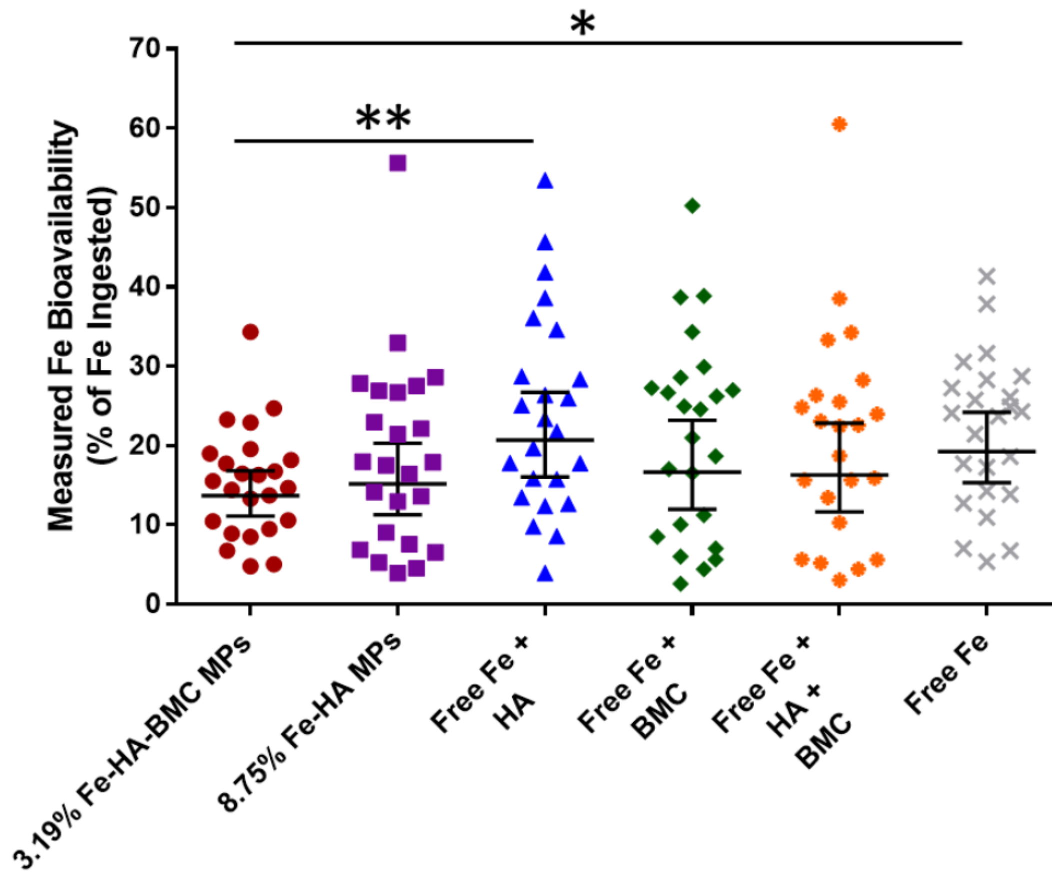

Fig. S7. Comparison of iron absorption from 3.19% Fe-HA-BMC MPs with each MP constituent both individually and in combination. Iron bioavailability as assessed by erythrocyte iron incorporation in young women (n = 24) after ingestion of 3.19% Fe-HA-BMC MPs (red circles), 8.75% Fe-HA MPs, free iron with free HA (purple squares), free iron with free BMC (green diamonds), free iron with free HA and free BMC (orange stars), free iron (grey crossbars). These values are expressed as a percentage of the total amount of iron that was ingested. Error bars represent geometric means (n = 24) and 95% CI. *$P < 0.05$, **$P < 0.01$ Significant effect of meal on iron absorption determined by linear mixed models, participants as random intercept, meal as repeated fixed factor, and post-hoc paired comparisons with Bonferroni correction $P < 0.05$. NSD: no significant difference.

Supplemental Tables

Table S1. Polymers evaluated as potential MP matrix materials. Polymers were evaluated based on the need to address key issues limiting current food fortification technologies, specifically enabling: (i) the encapsulation of both water-soluble and fat-soluble micronutrients, (ii) rapid release in the stomach to ensure intestinal absorption, and (iii) the protection of the encapsulated micronutrients from high temperature, moisture, and oxidizing chemicals during cooking or storage. To assess whether polymers could address these challenges, we selected specific requirements for the chosen polymer. Requirement 1 (Column 2) is that the polymer must be soluble in organic solvents to facilitate formulation with fat-soluble micronutrients. Requirement 2 (Column 3) is that the polymer must be soluble in gastric conditions (below pH 5) to enable burst release in the stomach and subsequently intestinal absorption. Requirement 3 (Column 4) is that the polymer must currently be used in either pharmaceuticals or dietary supplements to enable clinical translation. Requirement 4 (Column 5) is that the polymer must be stable at high temperatures and high humidity to ensure that the encapsulated micronutrients will be protected during cooking and storage. Shaded boxes indicate requirements that have been met by the polymer. Only the pH-responsive basic methacrylate copolymer (BMC), Eudragit E PO, met the requirements for each column and as such was chosen to be the encapsulating matrix. Abbreviations: Methanol: MeOH; Ethanol: EtOH; Acetone: Ace; Dichloromethane: DCM; Isopropanol: IPA.

Polymer (Commercial Product)	**Solvent Solubility**	**Aqueous Solubility**	**GRAS Status/ History of Use**	**Storage Stable at High Temperatures and Humidity**	**Refs**
Requirements	*Solubility in organic solvents required to encapsulate fat-soluble micronutrients*	*pH <5 aqueous solubility required to enable stomach release*	*Use in pharmaceutical or dietary products required for safety and approval*	*Stability required to ensure that micronutrients remain viable after cooking or long-term storage*	
Polyvinyl Acetate Phthalate (Opadry 91 Series, Sureteric)	MeOH, EtOH, EtOH:Ace, MeOH:DCM, IPA:DCM, IPA:H_2O	Aqueous ≥pH 5	Listed in FDA inactive ingredients for approved drug products	Yes	72,73
Cellulose Acetate Phthalate (Cellacefate, Eastman C-A-P)	Ace, Ace:EtOH, Ace:IPA, Ace:MeOH, Ace:DCM, DCM:EtOH	Aqueous ≥pH 6	Listed in FDA inactive ingredients for approved drug products	No: hydrolyze at high temperature and humidity	74-76
Hypromellose Phthalate	EtOH:DCM, EtOH:Ace, EtOH:H_2O	Aqueous ≥pH 5	Listed in FDA inactive ingredients for approved drug products	No: hydrolyze at high temperature and humidity	77,78

Hypromellose Acetate Succinate (AQOAT, AquaSolve)	MeOH, Ace, EtOH:DCM, EtOH:H_2O	Aqueous ≥pH 5-6.5	Listed in FDA inactive ingredients for approved drug products	No: hydrolyze at high temperature and humidity	79,80
Shellac	MeOH, EtOH, IPA	Aqueous >pH 7	Listed in FDA inactive ingredients for approved drug products	No: polymerize at high temperature and humidity	81-85
Chitosan	No	Aqueous <pH 6.5	Self-affirmed GRAS	No: hydroscopic and variations in molecular weight, deacetylation, and purity	86-88
Ethyl methacrylate-methacrylic acid copolymer (1:1 copolymer ratio) (Eudragit L 100-55)	MeOH, EtOH, IPA, Ace	Aqueous >pH 5.5	Listed in FDA inactive ingredients for approved drug products	Yes	50,89,90
Methyl methacrylate-methacrylic acid copolymer (1:1 copolymer ratio) (Eudragit L 100)	MeOH, EtOH, IPA, Ace	Aqueous >pH 6	Listed in FDA inactive ingredients for approved drug products	Yes	50,89,90
Methyl methacrylate-methacrylic acid copolymer (1:2 copolymer ratio) (Eudragit S 100)	MeOH, EtOH, IPA, Ace	Aqueous >pH 7	Listed in FDA inactive ingredients for approved drug products	Yes	50,89,90
Methyl methacrylate-dimethylaminoethyl methacrylate-butyl methacrylate copolymer (1:2:1 copolymer ratio) (Eudragit E PO, Eudraguard)	MeOH, EtOH, IPA, Ace, DCM, ethyl acetate	Aqueous <pH 5	Listed in FDA inactive ingredients for approved drug products	Yes	50,91,92

Table S2. Formulation parameters and loadings for laboratory-scale MPs. All values are mean ± SD. MN = Micronutrient.

Lab-Scale BMC Microparticles					
MN	MN (mg)	BMC (mg)	Loading (µg/mg)	Loading %	Encapsulation Efficiency (EE) (%)
Vitamin A	10	100	73 ± 7	7.3 ± 0.7	80 ± 8
Vitamin B2	15	200	67 ± 2	6.7 ± 0.2	96 ± 3
Vitamin C	20	200	63 ± 2	6.3 ± 0.2	69 ± 2
Vitamin D	2	100	12 ± 1	1.2 ± 0.1	61 ± 5
Zinc	17	100	11 ± 1	1.1 ± 0.1	7.6 ± 0.7
Iodine	5	100	21 ± 3	2.1 ± 0.3	44 ± 6

Lab-Scale HA-BMC Microparticles										
	HA-particle					BMC-HA microsphere				
MN	MN (mg)	HA (mg)	Loading (µg/mg)	Loading %	EE (%)	HA-MN (mg)	BMC (mg)	Loading (µg/mg)	Loading %	EE (%)
Fe	30	20	185 ± 5	18.5 ± 0.5	31 ± 2	11.3	200	6.0 ± 0.1	0.6 ± 0.01	61 ± 1
Vitamin B9	5	20	117 ± 21	11.7 ± 2.1	59 ± 11	5.2	100	1.7 ± 0.1	0.17 ± 0.01	29 ± 2
Vitamin B12	5	20	141 ± 17	14.1 ± 1.7	71 ± 9	2.5	100	2.3 ± 0.1	0.23 ± 0.01	67 ± 3

Lab-Scale Ge-BMC Microparticles										
	Ge-particle					BMC-Ge microsphere				
MN	MN (mg)	Ge (mg)	Loading (µg/mg)	Loading %	EE (%)	Ge-MN (mg)	BMC (mg)	Loading (µg/mg)	Loading %	EE (%)
Vitamin B3	15	20	58 ± 5	5.8 ± 0.5	14 ± 1	5	200	0.4 ± 0.1	0.04 ± 0.01	28 ± 7
Vitamin B7	17	20	320 ± 5	32 ± 0.5	70 ± 1	3	200	3.5 ± 0.6	0.35 ± 0.06	74 ± 13

Table S3. Subject characteristics of human study 1 and 2. All female, no significant difference in baseline characteristics between the study populations[a].

Baseline subject characteristics of study participants for human study 1 and 2		
Characteristics	Study 1	Study 2
n (number of subjects)	20	24
Age[b] [year]	22.8 ± 3.5	22.4 ± 1.9
Height [meter]	1.66 ± 0.06	1.64 ± 0.06
Weight [kg]	57.3 ± 4.1	57.8 ± 6.2
Body Mass Index [kg/m^2]	20.8 ± 1.5	21.3 ± 1.4
C-Reactive Protein[c] [mg/L]	0.62 (0.36, 1.07)	1.07 (0.72, 1.59)
Plasma Ferritin [µg/L]	11.6 (9.4, 14.5)	13.2 (10.5, 16.5)
% Iron Deficient[d] (PF < 15 µg/L)	65	58
Hemoglobin [g/L]	13.4 ± 0.85	13.2 ± 0.95
% Anemic[e] (Hemoglobin < 120 g/L)	5	8
% IDA[f]	5	4

[a]independent t-test, P < 0.05
[b]all such values are mean ± SD
[c]all such values are geometric mean (95% CI)
[d]ID, Iron deficiency, defined as PF < 15 µg/L
[e]Anemia, defined as Hb < 120 g/L
[f]IDA, iron deficiency anemia, defined as PF < 15 µg/L and Hb < 120 g/L

Table S4. Process design formulation parameters and loadings for MPs used in the second human study. Loading values are mean ± SD.

Human Study 2 MPs				
	Fe-HA MPs (Spray Dry)		Fe-HA-BMC MPs (Spinning Disc)	
Fe Isotope (mg of Fe/g of MP)	$FeSO_4$ Feed (g)	HA Feed (g)	HA-Fe Feed (g)	BMC Feed (g)
^{54}Fe (31.9 ± 0.7 mg/g)	5.57 (1.98g ^{54}Fe)	9.84	9.23	19.44
^{57}Fe (182.9 ± 3.8 mg/g)	3.78 (1.41g ^{57}Fe)	1.89	3.57	0.32
^{57}Fe (87.5 ± 1.0 mg/g)	0.80 (0.30g ^{57}Fe)	2.35	N/A	N/A
	VitA-BMC MPs (Spinning Disc Collected in Starch Bed)			
Vitamin A Isotope (mg of Fe/g of MP	VitA Feed (g)		BMC Feed (g)	
Vitamin A (34 ± 2.4 mg/g)	54		1026	

Table S5. Quality control tests for MPs used in both human studies. Loading values are mean ± SD. CFU = Colony forming units; LAL = Limulus Amebocyte Lysate; ppm = parts per millions; USP = United States Pharmacopeia.

Human study 1 MPs								
Formulation (Loading)	**Quality control tests**							
Fe isotope (mg of Fe/g of MP)	Kinetic-Chromogenic LAL Testing	Bioburden (Aerobic)	Bioburden (Soybean-Casein Digest Agar/Tryptic Soy Agar)	Bioburden (Sabouraud – Dextrose Agar)	ppm of DCM USP <467>	ppm of Residual Acetone USP <467>	ppm of Residual Ethanol USP <467>	ppm of Residual Hexane USP <467>
^{54}Fe (6.81 ± 0.13 mg/g)	< 0.047 EU/mg	< 1 CFU/ml	< 1 CFU/ml	< 1 CFU/ml	< 600	< 5000	< 5000	< 41
^{57}Fe (6.09 ± 0.20 mg/g)	< 0.004 EU/mg	< 1 CFU/ml	< 1 CFU/ml	< 1 CFU/ml	< 600	< 5000	< 5000	< 41

Human Study 2 MPs				
Formulation (Loading)	**Quality Control Tests**			
Fe Isotope (mg of Fe/g of MP)	LAL Bacterial Endotoxin (USP 40 <85>)	Bioburden (Aerobic) (USP 40 <61>)	Bioburden (Yeast-Mold) (USP 40 <61>)	ppm of DCM
^{54}Fe (31.9 ± 0.7 mg/g)	< 0.0500 EU/mg	100 CFU/g	< 100 CFU/g	131
^{57}Fe (182.9 ± 3.8 mg/g)	< 5.00 EU/mg	< 100 CFU/g	100 CFU/g	< 138

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MANUSCRIPT 4

THE EFFECT OF A NATURAL POLYPHENOL SUPPLEMENT ON IRON ABSORPTION IN ADULTS WITH HEREDITARY HEMOCHROMATOSIS

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Manuscript draft

Abstract

Background: Patients with hereditary hemochromatosis (HH) require frequent phlebotomies to prevent iron overload due to increased dietary iron absorption. Plant polyphenols are potent inhibitors of non-heme iron absorption.

Objective: To develop a natural polyphenol supplement that strongly chelates iron *in vitro* and assess its effect on non-heme iron absorption in patients with HH.

Design: We performed *in vitro* iron digestion experiments to measure iron solubility of 12 polyphenol rich food powders and then formulated a supplement (PPS) containing black tea powder, cocoa powder and grape juice extract. The effect of this PPS on iron absorption was assessed in otherwise healthy patients (n = 14) with HH homozygous for the p.C282Y variant in *HFE* gene. We measured fractional iron absorption (FIA) as stable iron isotope incorporation into erythrocytes from labelled test meals and test drinks in a multi-center, single-blind, controlled cross over-study.

Results: We found that black tea powder, cocoa powder and grape juice extract most effectively precipitated iron during *in vitro* digestion. A PPS mixture of these three extracts precipitated ~80% of iron when 2 g was added to a 500 g iron solution. In the iron absorption study, the PPS reduced FIA by ~40%: FIA from the meal consumed with the PPS was lower (3.01% (1.60, 5.64)) than with placebo (5.21% (3.92, 6.92)) (P = 0.026)), and FIA from the test drink with the PPS was lower (10.3% (7.29 14.6)) than with placebo (16.9% (12.8 22.2)) (P = 0.002).

Conclusion: Because this PPS sharply reduces dietary iron absorption in patients with HH, it could provide a new adjunct approach to the management of these patients; taken with meals, it could reduce iron absorption and body iron accumulation, and might thereby decrease the frequency of phlebotomy.

Introduction

Hereditary hemochromatosis (HH) is characterized by increased iron accumulation in tissue and organs, eventually leading to liver cirrhosis, hepatocellular carcinoma, diabetes, arthropathy, and heart disease.[1] It is one of the most common genetic diseases in Caucasian populations, mainly of Nordic or Celtic ancestry.[2-6] *HFE*-related HH accounts for 85–90% of all cases, associated to the homozygous p.C282Y mutation.[2] Iron overload in *HFE*-related HH is caused by relative hepcidin deficiency.[7] This is due to the conformation of HFE, which affects the signaling pathway that regulates hepcidin expression.[8] The lack of hepcidin leads to inappropriately high dietary iron absorption, and a lack of its effective regulation by body iron stores.[7-10]

The standard of care for HH patients is phlebotomy to reduce body iron accumulation.[11-13] The morbidity and mortality of HH patients is significantly reduced when the treatment is started before the development of cirrhosis and/or diabetes.[14,15] The frequency of the phlebotomies depends on iron status, measured by the transferrin saturation (TSat) and serum ferritin (SF) levels [5]. Frequent phlebotomies can be inconvenient and a burden for many patients.[16]

Previous studies reported equivocal effects of dietary iron intake (heme and non-heme) on iron status in HH.[17-20] A systematic review of studies assessing the effect of diet in HH concluded that despite limited evidence, dietary modification may be a beneficial adjunct strategy to limit iron accumulation.[21] Dietary modification would require limiting intake of heme and non-heme iron, as well as reducing the intake of iron absorption enhancers (e.g. vitamin C) and increasing intake of iron absorption inhibitors, such as phytate and polyphenols (PP). PP are widely distributed among plants and inhibit non-heme iron absorption by forming insoluble Fe-PP complexes in the intestinal lumen.[22] Polyphenol rich foods and beverages have been shown to be inhibitory in various iron absorption studies.[22-28] In patients with HH, the consumption of black tea with a meal led to a significant reduction in iron absorption from a single meal. Furthermore, HH patients in the tea drinking group, who consumed black tea with all main meals over one year, had a smaller increase in SF than the control group who consumed water with meals.[29] Therefore, patients with HH would most likely profit of the regular intake of a PP rich supplement to reduce their body iron accumulation and therefore frequency in required phlebotomies. However, the inhibitory effect of PP on iron absorption is strongly dependent on the PP structure.[22] A supplement containing silybin, a flavonoid

extracted from milk thistle (*Silybum marianum*), reduced serum iron response in HH patients,[30] whereas a supplement containing proanthocyanidins did not.[31]

The objectives of this study were: 1) to develop a natural PP supplement with high efficiency in chelating iron *in vitro*, and 2) to determine its inhibitory effect on iron absorption when provided with a non-heme iron-rich test drink and test meal in HH patients. Using *in vitro* digestion and measuring iron solubility, we screened various PP rich food powders to formulate a natural PP supplement (PPS) with maximum iron-chelating potential. The PPS was formulated in capsulates and administered to patients with HH. Fractional iron absorption was measured using stable iron isotopes. We hypothesized that iron absorption from an iron-fortified test drink and from a non-heme iron-rich test meal given with the PPS will be reduced significantly compared to when consumed with the placebo capsules.

Methods

Food analyses

Polyphenol rich food sources were selected based on a high content of gallic acid equivalents (GAE) according to data extracted from phenol explorer,[32-34] a low content of ascorbic acid (based on literature), and their availability in dried powder form. Total PP concentrations of all food powders and the PPS mixture were measured with a modified Folin-Ciocalteau method.[35] Concentrations were measured as GAE. Total iron (Fe) concentrations were measured by graphite furnace atomic absorption spectrophotometry (GFAAS, AA240Z; Varian), after complete mineralization of the sample by microwave digestion (MLS TurboWave; MLS GmbH). Total phytic acid (PA) concentrations were measured by a modified method by Makower.[36] Vitamin C content of the test meal was analyzed via HPLC (Waters Acquity H-Class) after stabilization and extraction in metaphosphoric acid and reduction via ditiothreitol.

Polyphenol rich foods in in-vitro digestion

The *in vitro* digestion method was used to determine the inhibitory effect of the PP rich food powders on solubility and bioaccessibility of iron. We mixed different doses of the PP rich food powders (60, 120, or 180 mg) into a 30 g iron solution containing 600 µg Fe (20 µg Fe/g). The tested doses of the PP rich food powders were equivalent to 1 g, 2 g and 3 g powder in relation to a 500 g solution containing 10 mg Fe (matching the test condition in stable iron isotope

study). *In vitro* digestion was performed in triplicate samples and each condition was tested twice. Using amylase (Takadiastase from *Aspergillus oryzae*, Sigma-Aldrich) samples underwent oral digestion for 10 minutes, followed by a pH reduction with 6M HCl to pH 2. A pepsin (from porcine gastric mucosa, Sigma-Aldrich) solution was added to simulate gastric digestion in a 37°C shaking water bath. After 2 h, the pH was increased with 0.5M KOH and a pancreatin (bile extract, from porcine and pancreatin from porcine pancreas, both from Sigma-Aldrich) solution was added. After 10 minutes of intestinal digestion, all samples were centrifuged at 3600 rpm for 15 minutes. The iron concentration of the supernatant was measured using GFAAS ($Fe_{soluble}$), whereas the total iron concentration (Fe_{total}) was calculated based on measured concentrations of the food powders. We estimated that PP-Fe are insoluble complexes and precipitate during the centrifugation, therefore we calculated $Fe_{insoluble} = Fe_{total} - Fe_{soluble}$. The PPS was formulated based on the three foods that exhibited the highest capacity to complex iron *in vitro* (**Table S1**). The dosage of 2 g PPS per 500 g meal/drink for the human absorption study was decided based the results of the one-way ANOVA comparing PP dosages (Table S1).

Stable iron isotope study participants and study sites

Study participants (n = 14) were patients with diagnosed hereditary hemochromatosis, all homozygous in the p.C282Y variant in the *HFE* gene. Other inclusion criteria were: Between 18 to 65 years of age, weight of below 80 kg, body mass index (BMI) within 18.5 – 25 kg/m^2, the last phlebotomy being at least 4 weeks prior to first test meal/drink administration, and expected to comply with the study procedures. Exclusion criteria were: Pregnancy and breastfeeding, acute illness or infection, metabolic or chronic diseases, use of long-term medication and consumption of mineral and vitamin supplements 2 weeks prior and during the study period, a scheduled phlebotomy during the study period, and participation in any other clinical study within the last 30 days.

The study was conducted at the Santo Antonio Hospital - Porto University Hospital Center (CHUP), in Porto, Portugal and at the Laboratory of Human Nutrition at ETH in Zurich, Switzerland. The study arm in Portugal was conducted between August 2019 and October 2019 and the study arm in Switzerland between December 2019 and August 2020. The study was performed according to the Declaration of Helsinki, and ethical approval for the study was provided by the ethical review committees of ETH Zürich and the Canton of Zürich (BASEC

2019-01776) in Switzerland, and of the Porto University Hospital Center (CHUP), Portugal (2019.127(107-DEFI/111-CE)). The study was registered at clinicaltrials.gov (NCT03990181). Informed signed consent was obtained from all the participants.

Study design and procedures

This was a multi-center, partially-randomized, single-blind, placebo-controlled cross-over study (**Figure 1**). The study included four experimental conditions: 1) isotopically labelled test meal (containing 8 mg native Fe and 2 mg ^{57}Fe as ferrous sulfate [$FeSO_4$]) consumed with the supplement containing 2 g of the PPS powder (Meal-PPS); 2) isotopically labelled test meal (containing 8 mg native Fe and 2 mg ^{58}Fe as $FeSO_4$) consumed with the supplement containing 2 g of maltodextrin (placebo) (Meal-Placebo); 3) isotopically labelled test drink (water fortified with 8 mg Fe with natural isotopic composition as $FeSO_4$ and 2 mg ^{57}Fe as $FeSO_4$) consumed with 2 g of the PPS powder supplement (Drink-PPS); and 4) isotopically labelled test drink (water fortified with 8 mg Fe with natural isotopic composition as $FeSO_4$ and 2 mg ^{57}Fe as $FeSO_4$) consumed with the placebo supplement (Drink-Placebo).

Study participants were recruited at their regular phlebotomy visit at the Santo Antonio Hospital- Porto University Hospital Center (CHUP) in Porto, Portugal, the Department of Internal Medicine of the University Hospital Zurich, and the Blood Transfusion Service Zurich in Switzerland. In Porto, if a patient was interested, informed consent was obtained and a questionnaire administered to assess in- and exclusion criteria approximately one to two months before study Day 1. In Zurich, if a patient was interested, informed consent was obtained and in- and exclusion criteria were checked in a screening on study Day 1. On the study days, all participants were fasting, meaning no food intake after 8 pm and no drinks after midnight on the evening before. In the morning of study Days 1, 3, 22 and 24, participants came to the study center for administration of the experimental conditions Meal-PPS, Meal-Placebo, Drink-PPS, or Drink-Placebo. The order of the experimental condition was partially randomized, meaning the same stable iron isotope was not consumed within the same study week.

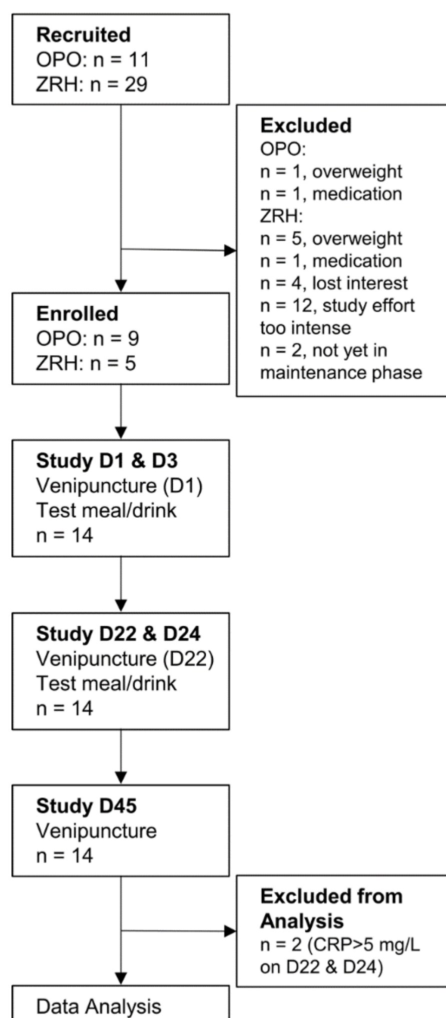

Figure 20: Study participant flow.

Venous blood samples were collected by venipuncture on study Days 1, 22, and 43 for analysis of hemoglobin (Hb) concentration, and indices of iron status and inflammation. On study Days 1 and 22, we further measured serum hepcidin, and on study Days 22 and 43 we determined stable iron isotope incorporation into erythrocytes.

The test meals (Meal-PPS and Meal-Placebo) consisted of four whole grain toast bread slices (Olivers Toast, Vollkorn, Migros) (115 g), 48 g of cashew paste (Cashewmus, Alnatura), two bean tarts (80 g), and 44 g apricot-pumpkinseed fruit leather. The toast, cashew paste, and all ingredients for the bean tarts and fruit leather were purchased in bulk and frozen until use. The bean tarts and the fruit leather were prepared in one batch and frozen until use. Each bean tart contained a prebaked wheat flour pastry case (Törtchenbödeli, Midor, Migros), blended canned white beans (Soisson Bohnen, MClasic, Migros), egg yolks (53 g+,

Freilandhaltung, Migros), grounded peelend almonds (Mandeln gemahlen geschält, MClassic Migros), and refined sugar (Feinkristallzucker, Migros) (at a ratio of 15:32:32:24:13). The fruit leather contained apricots and pumpkinseeds in a ratio of 10:3, which were homogenized with water and thin layers of the purée were dried for 6 hours at 80°C. To the test meals we administered 210 g of deionized water for drinking, and the labelled $FeSO_4$ solution was added to the toast bread. The test drinks (Drink-PPS and Drink-Placebo) were 470 g of deionized water mixed with 10 g of the $FeSO_4$ solution containing 8 mg Fe with natural isotopic composition and 2 mg labelled Fe, plus twice 10 g rinsing water. Total weight of the test meals (including drinking water), and of the test drinks provided, were both 500 g. This corresponds to the same ratio as in the *in vitro* experiments.

Preparation and administration of stable iron isotopes, test meals, drinks and PPS

We labelled $FeSO_4$ with ^{57}Fe-enriched and ^{58}Fe enriched elemental iron (with 95.56% and 99.89% isotopic enrichment, respectively, all Chemgas, Boulogne-Billancourt, France) as previously described [37]. The $FeSO_4$ solutions were pre-weighted in individual doses into Teflon vials and stored at 4°C until use. The PPS mixture was prepared in one batch by mixing milled black tea powder, cocoa powder and grape juice extract in equal parts. Black, 000 sized gelatin capsules were manually filled each with 1 g (± 5 mg) of the PPS mixture, or maltodextrin for the placebo supplements (Maltodextrin 6, Nutricia GmbH). The meals were thawed the night before administration, and the toast slices were heated before consumption and spread with the cashew paste. The isotopically labelled $FeSO_4$ solution (providing 2 mg iron) was poured directly onto the bread for the test meals, or provided diluted in water in a small glass with the test drinks. The vial was rinsed twice with 1 ml water, which was also poured on the bread, or in the glass. Participants were asked to start with taking two capsules of the PPS or placebo supplement, and then to consume the test meal or drink. The isotope solution was consumed at once in the middle of the drink, the glass was rinsed twice with 10 g of water and consumed by the participant.

Blood analyses

Venous blood samples were immediately processed after withdrawal. EDTA whole blood was used for the analysis of Hb on the sampling day using an automated hematology analyzer (Sysmex XE-5000 analyzer, Sysmex Corporation, both study sites). Heparinized whole blood

was aliquoted for analysis of isotopic composition. Blood collected in serum tubes was centrifuged, and serum samples aliquoted for SF analysis (Porto: Elecsys Ferritin assay; Roche Cobas analyzer; Zurich: Immulite; Siemens Healthcare Diagnostics), and for determination of C-reactive protein (CRP), alpha-1-acid glycoprotein (AGP) and soluble transferrin receptor (sTfR) using a multiplex ELISA method.[38] Serum iron (SFe) and total iron binding capacity (TIBC) were measured using colorimetry, and sHep using a commercial ELISA Kit (DRG Hepcidin 25, DRG Instruments GmbH). All aliquoted samples collected in Portugal were stored at -20°C until shipment on dry ice to ETH Zurich and further stored at -20°C until analysis. TSat was calculated using the formula SFe/TIBC*100. Acute inflammation was defined as CRP concentrations > 5 mg/L or alpha-1-acid glycoprotein (AGP) >1 g/L.

We determined fractional iron absorption (FIA) and calculated the amounts of ^{57}Fe and ^{58}Fe isotopic labels in blood on study Day 22 and 43, based on the shift in iron isotope ratios in the erythrocytes and on the estimated amount of iron circulating in the body.[39] We performed the analyses by multicollector-inductively coupled plasma mass spectrometry (MC-ICP-MS, Neptune; Thermo Finnigan) as previously described.[40] We calculated circulating iron in the body based on hemoglobin and blood volume, derived from the participant's height and weight,[41] and assuming an 80% incorporation of absorbed iron into erythrocytes.[39]

Sample size calculation and statistical analysis

A priori we calculated a sample size of 18 to be adequate to detect a 50% reduction in fractional iron absorption from the iron-rich meal/drink when consumed with the PPS, taking into account a standard deviation of 0.4, an effect size of 0.92, and a probability of an α error of 0.05 to reach a power β of 0.8. These assumptions were based on data from iron absorption studies performed in HH and healthy individuals [24,29]. To account for dropouts, we anticipated a sample size of 20. After 14 participants had completed the study, we ran an interim analysis. The data of these participants reached a power of 0.799 and 0.862 to explain our hypothesized differences in FIA from the iron-enriched meals and drinks, respectively, when consumed with the PPS compared to placebo.

We used IBM SPSS statistics (Version 24) for statistical analysis. To test for differences in the percentage of precipitated Fe after *in vitro* digestion between the PP doses (1, 2 and 3 g), a one-way ANOVA with post hoc Bonferroni correction was run for each PP food, and the PPS. A repeated-measures ANOVA was performed with percentage of precipitated Fe as the

dependent variable, the dose as the repeated measure and the PP food as between-subject factor. Post hoc Bonferroni corrected comparisons were made to test for significant differences between the PP foods. We assessed the correlation of precipitated iron with total PP concentration using Spearman's rho test. We tested data of the human absorption study for normality by Shapiro-Wilk tests. Normally distributed data are presented as means ± standard deviation (SD), log transformed normal data as geometric mean with 95% confidence interval (95%CI), and non-normal data as median and interquartile range (IQR). We tested between-group differences in FIA using log-transformed data with dependent samples T-tests. Predictors of iron absorption were estimated using linear regression with log FIA as dependent variable, and sex, meal matrix (drink vs. meal), intake of PPS, log SF, log sHep, and log CRP as independent variables, reported are standardized β. The α-level of significance was set at 0.05.

Results

Precipitation of iron with different PP foods and doses in vitro

Grape juice extract, black tea powder and cocoa powder showed the strongest ability to complex iron, with no significant differences between them. When 2 g of their powders was digested in a 500 g solution, containing 20 µg/g Fe, 77-86% of the total iron was precipitated (Table S1, and **Figure 2**). We found differences in percentage of precipitated iron *in vitro* between different PP foods ($p < 0.001$) and between PP doses ($p < 0.001$), as well as a significant PP food x dose interaction ($p < 0.001$) indicating that the dose-effect was dependent on the PP food. Results of the post hoc comparisons are shown in Table S1. All other PP food sources had significantly lower ability to complex iron (all at 2 g): cloves and cinnamon precipitated around 62–64%, chestnut flour, anise and marjoram around 44–48%, oregano, common sage, and coffee powder around 25–32%, and turmeric around 15% (Table S1, and Figure 2). For black tea powder, grape juice extract, and cocoa there was a significant increase in precipitated Fe from 1 g to 2 g of the food powder, but not when the doses were increased from 2 g to 3 g (Table S1). The PPS containing grape juice extract, black tea powder and cocoa powder led to a 78.7 ± 2.9% reduction in soluble iron when 2 g of the powder underwent *in vitro* digestion in 500 g solution containing 20 µg/g Fe (Figure 2, and Table S1). The PPS mixture showed a significant increase in precipitated Fe from dose 1 g to 2 g, and from 2 g to 3g (Table S1, and Figure 2). There was a moderately strong relationship of total PP

concentrations (as GAE) of the food powders (**Table S2** with their ability to precipitate iron (r_s = 0.629, $P < 0.001$) (**Figure 3**).

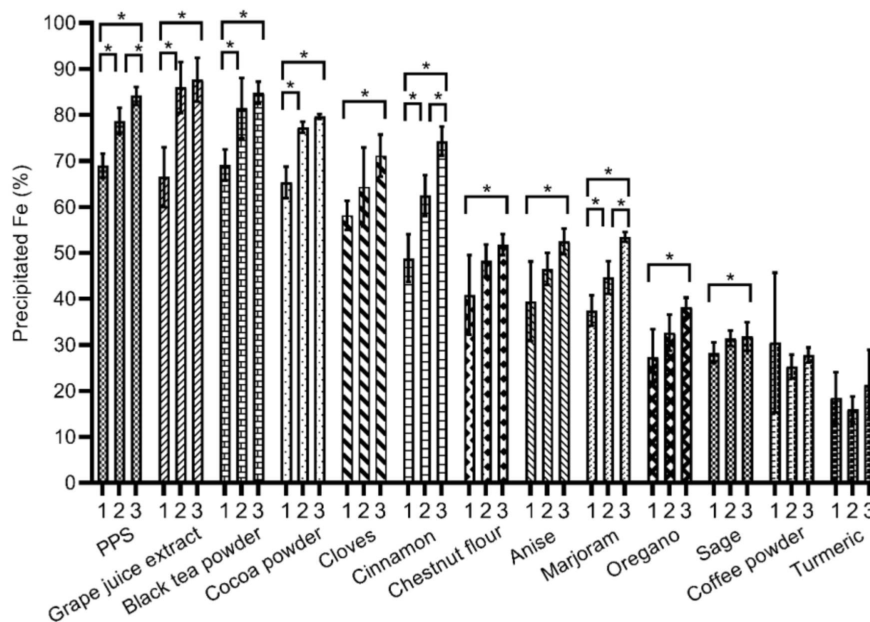

Figure 21: Percentage of precipitated iron after *in vitro* digestion in a 20 µg Fe/g solution with the PPS and other food powders at different doses of 1 g, 2 g and 3 g. Significant differences between doses within a PP food are indicated with * (one-way ANOVA with Bonferroni corrections, $p < 0.05$).

Characteristics of the polyphenol food mixture

The PP concentration of the PPS was 129 ± 3.41 mg GAE/g, and in cocoa powder, grape juice extract, and black tea powder the measured concentrations were 41.4 ± 1.20 mg GAE/g, 178.7 ± 8.97 mg GAE/g, and 135.9 ± 3.82 mg GAE/g, respectively. The measured iron concentration of the PPS was 183.6 ± 15.3 µg/g Fe, and PA concentration was 1.13 ± 0.091 mg/g. Therefore, 2 g of the PPS contained 259.15 ± 6.82 mg total PP (as GAE), 367.22 ± 30.64 µg Fe, and 2.26 ± 0.18 mg PA.

Characteristics of the test meal

Each test meal portion contained 8.02 ± 0.005 mg of native Fe, 1.06 ± 0.06 g phytic acid, 26.9 ± 0.1 mg Vitamin C, and 300.8 ± 17.4 mg total polyphenols (gallic acid equivalents).

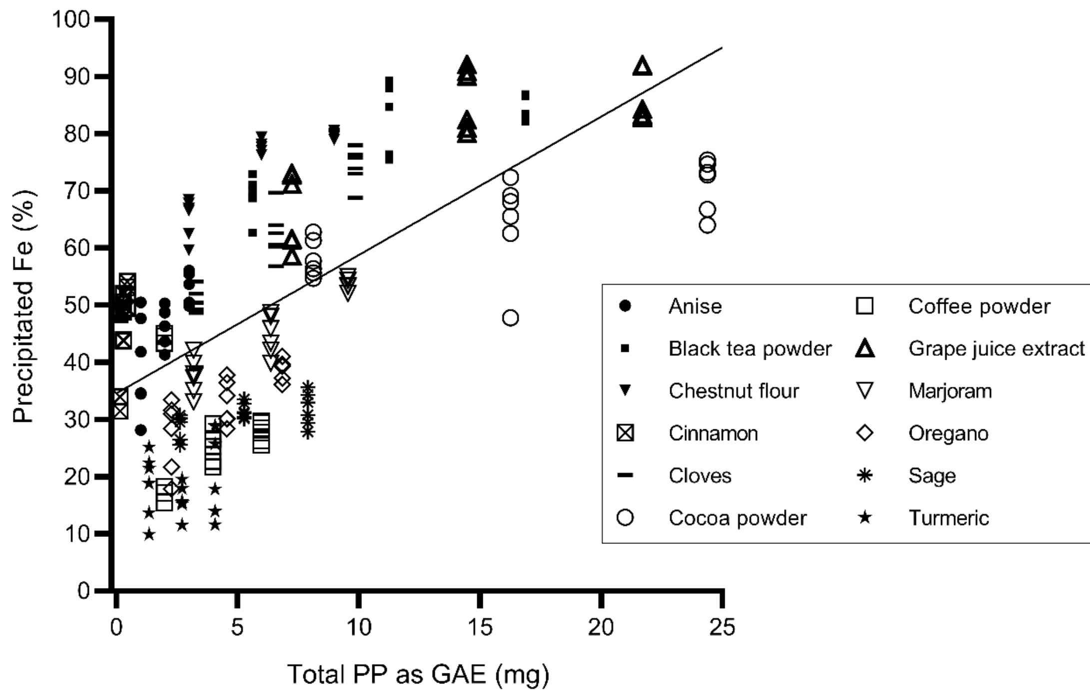

Figure 22: The correlation of percentage precipitated iron with measured PP content (mg) as gallic acid equivalent (GAE). Spearman's rho = 0.629, p < 0.001 (1-tailed).

Human study participants

Among the approached HH patients in Porto, 11 interested participants were recruited, and nine of these fulfilled all inclusion criteria. Among the HH patients in Zurich, 29 participants were recruited, and five of these fulfilled all inclusion criteria. We were able to enroll and complete the study with 14 participants. On study Day 22, an increased CRP (CRP > 5 mg/L) was measured in two participants, therefore their FIA data of Day 22 and 24 were not included in the analysis (Figure 1). Of these 14 study subjects, seven were female and seven male. Participant characteristics, baseline anthropometric measurements, indices of inflammation, iron status and hepcidin concentrations are shown in **Table 1**.

Table 11: Study participant characteristics, baseline anthropometric measurements and iron status based on samples from study Day 1 and 22.

	All Study participants	Female participants	Male participants
n	14	7	7
Age (y)[a]	44.8 ± 9.86	43.0 ± 9.9	46.6 ± 10.2
Weight (kg)	68.1 ± 10.6	60.7 ± 8.1	75.5 ± 7.0
Height (cm)	172.6 ± 12.7	161.7 ± 4.9	183.6 ± 6.7
BMI (kg/m2)	22.8 ± 2.4	23.2 ± 2.8	22.4 ± 2.2
CRP (mg/L)[b]	0.75 (0.32, 1.74)	0.88 (0.17, 4.51)	0.63 (0.21, 1.93)
AGP (g/L)	0.57 ± 0.19	0.57 ± 0.15	0.56 ± 0.25
Hb (g/dL)	15.4 ± 1.57	14.2 ± 0.80	16.6 ± 1.18
SF (ng/mL)[c]	69.8 (54.4 - 114.6)	59.0 (43.5 - 74.6)	86.0 (65.0 - 223.5)
sTfR (mg/L)	3.99 (3.61 - 4.37)	3.96 (3.70 - 4.19)	4.00 (3.33 - 5.37)
SFe (µg/dl)	164.5 ± 36.7	164.4 ± 32.5	164.5 ± 43.2
TIBC (µg/dl)	308.0 (282.7 - 335.6)	307.9 (281.7 - 400.2)	308.0 (283.0 - 314.6)
TSat (%)	50.41 (44.11, 57.61)	49.2 (40.0, 60.6)	51.6 (40.9, 65.2)
sHep (ng/mL)	3.49 (2.26, 5.37)	2.95 (1.53, 5.69)	4.13 (1.97, 8.67)

BMI, body mass index; Hb, hemoglobin; SF, serum ferritin; CRP, C-reactive protein; AGP, α1-acid glycoprotein; sHep, serum hepcidin; sTfR, soluble transferrin receptor; SFe, serum iron; TIBC, total iron binding capacity; TSat, transferrin saturation. Data is based on samples collected on study Days 1 and 22.
[a] values are mean ± standard deviation, all such values.
[b] values are geometric means and 95% confidence interval, all such values.
[c] values are median and interquartile range, all such values.

Iron absorption

The geometric mean and 95% CI of the total iron absorption from the meal with and without the PPS (Meal-PPS vs. Meal-Placebo) was 0.313 (0.167, 0.586) mg Fe and 0.522 (0.393, 0.693) mg Fe, respectively (*P* = 0.035). FIA from the meal with and without the PPS (Meal-PPS vs. Meal-Placebo) was 3.01% (1.60, 5.64) and 5.21% (3.92, 6.92), respectively (*P* = 0.026) (**Figure 4A**). Without the meal matrix, the total iron absorption from the drink with and without the PPS (Drink-PPS vs. Drink-Placebo) was 1.07 (0.756, 1.52) mg Fe and 1.69 (1.28, 2.22) mg Fe, respectively (*P* = 0.003). FIA from the drink with and without the PPS (Drink-PPS vs. Drink-Placebo) was 10.3% (7.29 14.6) and 16.9% (12.8 22.2), respectively (*P* = 0.002) (**Figure 4B**). The intake of the PPS led to a 42 and 39% reduction in FIA in both, meals (*P* = 0.026) and drinks (*P* = 0.002), respectively. We identified a meal matrix effect (Drink-Placebo vs. Meal-Placebo) which was independent from the effect of the polyphenol supplement, and led to a 69% reduction in FIA (*P* < 0.001). The pooled FIA geometric mean of both meals (3.96% (2.82, 5.56)) and both drinks (13.2% (10.5, 16.6)) also differed (*P* < 0.001) (70% reduction).

FIA in this study was predicted by: meal matrix (β = 0.711, $P < 0.001$); hepcidin concentration (β = -0.294, $P = 0.008$); sex (β = 0.278, $P = 0.007$); and PPS (β = 0.271, $P = 0.002$). These parameters explained 65% of the variability in FIA ($R^2 = 0.694$, $R^2_{adjusted} = 0.653$). In contrast, FIA was not associated with CRP (β = 0.081, $P = 0.347$) or ferritin (β = -0.077, $P = 0.530$) (data not shown).

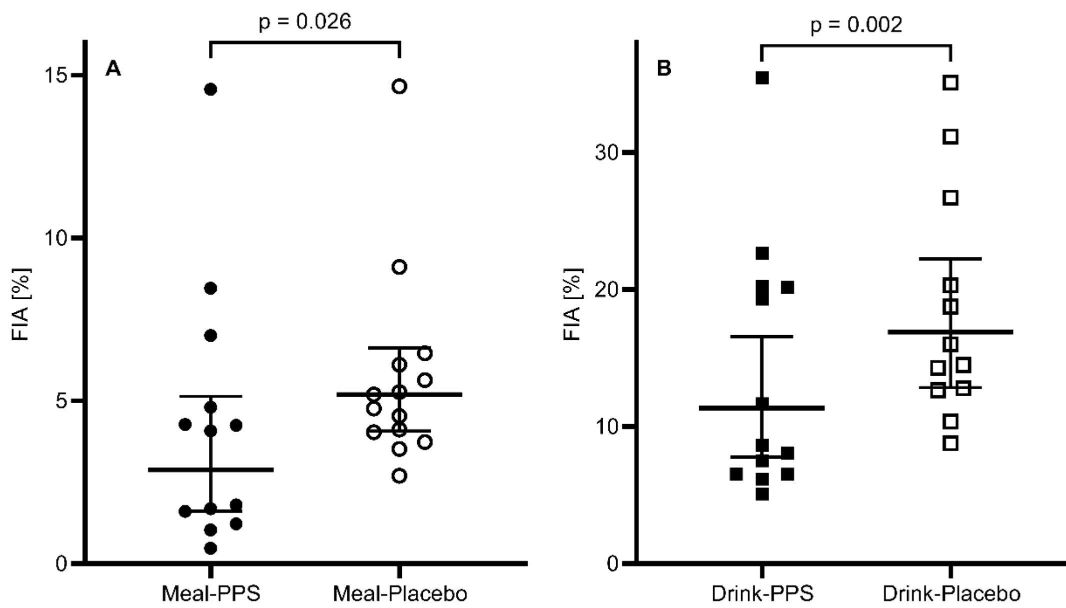

Figure 23: A: FIA from test meals and **B**: test drinks consumed either with the PPS (Meal-PPS & Drink-PPS) or with the placebo supplement (Meal-Placebo & Drink-Placebo). Shown are individual datapoints and the geometric mean with the 95% CI, p = 0.026, and 0.002, respectively, paired samples T-test.

Discussion

The main findings of this study are: 1) the natural polyphenol rich foods used in the PPS strongly complexed iron *in vitro* and reduced its solubility by ~80%; and 2) in the iron absorption study, the PPS reduced FIA from an iron rich meal and drink by ~40% in adults with HH.

We tested the capability of various PP rich food powders to precipitate iron *in vitro*. The selection of these food powders was based on their measured total PP content as GAE. Coffee has a high concentration of total PP[42] and has shown to reduce iron absorption form a meal by 61%.[22] Nevertheless, in our *in vitro* experiments, coffee powder precipitated iron by 25–30%, this low precipitation is most likely due to the decreased solubility of the PPs in cold (room temperature and body temperature) water compared to coffee brewed in hot water. Turmeric had the lowest capability to precipitate iron, which is in line with a human absorption study showing that iron absorption was not impaired by the addition of turmeric to a test meal.[25] Overall, the capability to precipitate iron *in vitro* correlated well with the total PP content of the food powders. This is in agreement with the results from an iron absorption study in which the reduction in FIA correlates with the total PP content.[24]

The effect size of the PPS in our study (40%) is smaller compared to a previously reported 70% reduction in FIA from a meal consumed with black tea in HH patients[29] and compared to the 60-90% reduction in iron absorption from a meal consumed with either black tea, coffee, cocoa, or peppermint tea in heathy subjects.[24] This difference might be attributed to the following mechanisms. The administration method of the PPS in gelatin capsules might have led to a delay in their release and therefore an incomplete mixture of the PPS with the test meal (compared to administration of PP rich beverages). To solve this issue, other galenic forms, such as a pressed tablet, might overcome the delay in release due to encapsulation. Otherwise, the instruction to take the supplement 15 minutes before each meal (rather than at the start of the meal) might improve food/PPS mixing in the gastrointestinal tract. Another difference is that in prepared tea and beverages, the polyphenols are already dissolved from their matrix and are available to form PP-Fe complexes. The polyphenols from our PPS required to be first dissolved in the stomach before being able to form PP-Fe complexes. And lastly, we administered 2 g of a mixture of powdered Ceylon tea, cocoa and a grape juice extract, which also contains a broad range of different polyphenolic structures.

The iron absorption measured in our study in homozygous p.C282Y patients with hemochromatosis was overall lower than reported by Kaltwasser *et al.*[29] who measured iron absorption in a similar patient group from a meal which was consumed with either tea or water. The meal consisted of beef, rice, spinach and potatoes, and was consumed with 1.5 g Ceylon tea, extracted for 5 minutes in 250 ml water. The FIA from this meal with tea was 6.9% compared to 22.1% when consumed with water, which would result in a ~70% reduction in FIA.[29] These measured FIA values are higher compared to the FIA values from our test meal with PPS (3.0%) and with placebo (5.2%). The overall lower FIA from the test meal in our study can most likely be attributed to the high content in PA and PP. The meal matrix per se had a highly inhibitory effect on FIA, it induced a significant reduction of ~60%, and was a main determinant of FIA in the logistic regression. The molar ratio of PA:Fe in the meal was 9.3:1, which is classified as highly inhibitory.[43] The major contributor of PA to the meal was the cashew paste, followed by toast bread > fruit leather > bean tarts. Besides the study by Kaltwasser and colleagues, only few studies have measured iron absorption in hemochromatosis patients using stable or radio iron isotopes and have reported higher iron absorption values. Iron absorption from ferric citrate in a chicken soup meal was reported to be 30.0%,[44] iron absorption from a composite meal containing wheat bread, hamburger, gravy, lettuce and tomato juice was reported to be 36.4%,[45] and non-heme iron absorption from a meal containing beef, wheat bread, french fries and a vanilla milkshake was 41.3%.[46] An iron absorption study without a meal matrix reported 74% iron absorption from a ferric ascorbate reference dose.[47] These studies included patients diagnosed with hemochromatosis before *HFE* was discovered, which may differ from our specified patient group being homozygous for the p.C282Y variant in *HFE*. Further, our methodology may have caused a lower FIA, our meal was highly inhibitory due to the high PA, high native PP content, and during the preparation of the iron solution drink, iron hydroxide formation may have occurred.

According to Phenol-Explorer, flavanols make up for 72% of the total PP in black tea and 16% are accounted to hydroxybenzoic acids. In cocoa powder, flavanols are also the largest represented class and make up for 93% of the total PP in cocoa powder. The widest represented PP in grapes (*Vitis vinivera*) belong to the classes of anthocyanins (82%) and flavanols (16%).[32-34] Therefore, the main represented components in our PPS can be accounted to flavanols. Flavanols contain catechol or galloyl groups, which are good proton donors and binding sites for ferric iron creating octahedral insoluble PP-Fe complexes.[22,24,48]

The iron accumulation rate in hemochromatosis patients has a large variation (1.2–241 µg SF/L), on average serum ferritin rises by 99 µg/L in a year.[49] Considering the following assumptions: our study test meal is consumed with the PPS three times per day; a mean Hb concentration of 15.4 g/dL; an average concentration of 3.47 mg Fe/g Hb; and 1 µg/L SF represents 8 mg of stored body iron,[50,51] the PPS could potentially lead to a yearly decrease of ~230 mg in absorbed iron, which would correspond to one unit of phlebotomized blood (450 mL). Or in other words, this is equivalent to ~29 µg SF and is 1/3rd of the average yearly increase in SF in hemochromatosis patients.[49] However, the estimated mitigation in yearly SF increase is lower compared to the longitudinal study performed by Kaltwasser and colleagues,[29] showing that regular tea drinking with all three main meals for one year led to a larger decrease in SF in the tea drinking group compared to the control group (77 µg SF difference).[29] Nevertheless, the regular intake of our PPS with all main meals may be less cumbersome than brewing and regular tea drinking for some individuals.

A strength of our study is that, with the two experimental conditions of test drink and test meal, we have demonstrated that the PPS is able to reduce iron absorption by ~40% in both conditions, independently of the food matrix and other iron absorption inhibitors. Although the FIA from our test meal is generally low, likely due to the high PA content, this may have weakened our overall study result. A test meal with a lower PA content (but therefore also a lower non-hem iron concentration) would require measuring iron absorption from multiple meals. As stable iron isotopes need amounts of milligrams to be detectable and should not contribute more than 20% to the total iron content of the meal.[52]

To summarize, our PPS has shown to reduce iron solubility in vitro by ~80%, and iron absorption in HH patients by ~40%, which could possibly correspond to the reduction of one phlebotomy session per year. However, to assess its efficacy a placebo controlled longitudinal study would be required. There is increasing evidence suggesting that dietary PP may have a protective role against chronic diseases.[53-55] Thus, the additional daily intake of 6 g of the PPS (containing black tea powder, cocoa powder and grape juice extract) may provide additional health benefits for patients with HH. In conclusion, the intake of our PPS shows promising results in the reduction of dietary iron intake in patients with HH and may also be of advantage in other iron overload diseases, which are caused by excess dietary iron absorption.

Acknowledgments

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Authors' contributions

SB, LS, CZ, DM, and MBZ designed the in vitro experiments; LS performed and analyzed the *in vitro* experiments; SB, JB, and MBZ designed the human study; MJT, GP, JHH, ILD, FV, NA, ES and BMF recruited the study subjects; SB, NK, MJT, GP, and JB performed the study, SB, NK and CZ analyzed the samples and performed statistical analyzes; SB, GP, FV, BMF, JB and MBZ participated in the data interpretation; SB wrote the first draft of the manuscript; all authors edited and approved the manuscript and final version.

Conflict of interest

None of the authors declare a conflict of interest.

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Supplementary

S Table 1: Mean and standard deviation (SD) of the percentage of precipitated iron in a 20 µg Fe/g solution at different doses of the polyphenol rich foods (1, 2 and 3 g).

PP Food	% precipitated Fe per food dose			Differences between PP foods[3]
	1 g	2 g	3 g	
PPS	68.9 ± 2.7 [A]	78.7 ± 2.9 [B]	84.2 ± 2.0 [C]	-
Grape juice extract[1, 2]	66.4 ± 6.6 [A]	86.0 ± 5.5 [B]	87.7 ± 4.7 [B]	a
Black tea powder	69.0 ± 3.5 [A]	81.7 ± 6.4 [B]	84.8 ± 2.2 [B]	a
Cocoa powder	65.3 ± 3.5 [A]	77.5 ± 1.2 [B]	79.7 ± 0.5 [B]	a
Cloves	58.1 ± 3.3 [A]	64.2 ± 8.7 [AB]	71.1 ± 4.6 [B]	b
Cinnamon	48.9 ± 5.2 [A]	62.3 ± 4.3 [B]	74.3 ± 3.2 [C]	b
Chestnut flour	40.9 ± 8.6 [A]	48.2 ± 3.5 [AB]	52.0 ± 2.1 [B]	c
Anise	39.5 ± 8.6 [A]	46.5 ± 3.5 [AB]	52.6 ± 2.8 [B]	c
Marjoram	37.6 ± 2.3 [A]	44.7 ± 3.5 [B]	53.8 ± 1.1 [C]	c
Oregano	27.3 ± 6.2 [A]	32.8 ± 3.9 [AB]	38.3 ± 2.1 [B]	d
Common sage	28.0 ± 2.4 [A]	31.6 ± 1.3 [AB]	31.8 ± 3.0 [B]	d
Coffee powder	30.6 ± 15.0 [A]	25.3 ± 2.8 [A]	27.9 ± 1.7 [A]	d
Turmeric	18.6 ± 5.8 [A]	15.9 ± 2.7 [A]	21.1 ± 7.7 [A]	e

PP: Polyphenol; PPS: Polyphenol supplement.
[1]Shown are means ± standard deviation (SD)
[2]Different capital letters indicate significant differences between PP doses within each PP food (one-way ANOVA for each PP food, pairwise comparisons with Bonferroni correction), with $p<0.05$.
[3]Different small form letters indicate significant differences between PP sources, (repeated measures ANOVA, pairwise comparisons with Bonferroni correction), with $p<0.05$.

S Table 2: Measured total polyphenol concentration (mg GAE/g) of all polyphenol rich food powders (mean ± standard deviation).

PPF	Total PP (mg GAE/g)
Grape juice extract	120.6 ± 5.6
Cloves	135.5 ± 7.5
Black tea powder	93.7 ± 8.5
Ceylon cinnamon	54.8 ± 4.7
Marjoram	53.2 ± 2.9
Cocoa powder	49.9 ± 2.7
Common sage	43.9 ± 3.7
Oregano	38.0 ± 1.9
Coffee	33.2 ± 2.6
Turmeric	22.6 ± 4.1
Star anise	16.7 ± 0.9
Chestnut flour	2.5 ± 0.2

GAE: Gallic acid equivalents; PP: Polyphenol: PPF: Polyphenol rich foods.

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GENERAL DISCUSSION AND CONCLUSION

The aim of this doctoral thesis was to investigate and unravel extrinsic and intrinsic factors that affect iron absorption in iron deficiency (ID) and iron overload. To do so, our objectives were fourfold: 1) to assess if asymptomatic *H. pylori* infection blunts the iron absorption from iron fortification compounds (**Manuscript 1**); 2) to assess and compare iron absorption, hepcidin and iron status in women carrying homozygous variants of the rs855791 polymorphism in the TMPRSS6 gene (**Manuscript 2**); 3) to determine iron absorption from microparticles encapsulating ferrous sulphate in a pH sensitive polymer (BMC) (**Manuscript 3**); and 4) to develop and test a natural polyphenol rich supplement to reduce iron absorption in patients with hereditary hemochromatosis (**Manuscript 4**).

To accomplish these aims, we: 1) performed a retrospective, pooled analysis of stable iron isotope absorption studies in preschool children and women of reproductive age and assessed their status of *H. pylori* infection by serology (**Manuscript 1**); 2) conducted a stable iron isotope absorption study in iron replete Taiwanese women of reproductive age carrying the homozygous variants of the rs855791 polymorphism (**Manuscript 2**); 3) performed two iron absorption studies in women to assess the effect of encapsulation of ferrous sulphate in BMC, with respect to different iron loaded microparticles, and two different food vehicles for fortification (maize and wheat flour) (**Manuscript 3**); and to achieve our final aim, we 4) screened polyphenol rich food powders for their ability to precipitate iron in *in vitro* digestion, and assessed the capability of a mixture of the thee most powerful complexing food powders in reducing iron absorption from a meal and a drink in hereditary hemochromatosis patients using stable iron isotopes (**Manuscript 4**).

Hereafter, the four manuscripts are discussed separately, covering the summary of their main findings, their limitations, contextualization and suggestions for future research. This is then followed by overall study limitations, and a conclusion of this thesis.

Main findings, limitations, contextualization and future directions

How does asymptomatic Helicobacter. pylori infection affect iron absorption in women and children, and do these groups also benefit from iron fortification programs?

Manuscript 1 addresses the effect of asymptomatic *H. pylori* infection on iron absorption of various iron compounds ($FeSO_4$, NaFeEDTA, and FeFum) from a broad range of different meals (pearl millet porridge and paste, wheat bread, and fermented sorghum porridge). The two major risk groups for iron ID, women of reproductive age and preschool children, were investigated in this retrospective pooled analysis with iron absorption studies from Benin, Senegal and Haiti. We have reported a prevalence of *H. pylori* seropositivity of 51.3% in women and 54.4% in children, which are comparable to values reported in literature.[1] The geometric mean of the fractional iron absorption (FIA) values in the *H pylori* infected versus non-infected groups did not differ. FIA of *H. pylori* infected and non-infected women was: 9.73% and 8.52%, respectively. In *H. pylori* infected and non-infected children FIA was 9.77%, and 8.94%, respectively. The model that assessed determinants of iron absorption in women (n = 80) included data of 213 FIA measures, and included status of *H. pylori* infection, test meal matrix, Fe compound, the addition of an iron absorption enhancer or the addition of an inhibitor, age, hemoglobin (Hb), and serum ferritin (SF) adjusted for infection (CRP) as potential predictors variables. In women, being positive for a serological *H. pylori* test was not a determinant of iron absorption. The same model in children (n = 90) included data of 232 FIA measures, and in addition to the variables above, sex was also included to the statistical model. Nonetheless, *H. pylori* was also not a determinant of iron absorption in children. Therefore, our findings show that being serologically positive for *H. pylori* does not affect iron absorption and suggests the asymptomatic *H. pylori* infection does not impair FIA from an iron fortified meal.

Our main finding suggests that the increased risk for ID and iron deficiency anemia (IDA) in *H. pylori* infection[2] is most likely through increased bleeding from *H. pylori* induced gastritis.[3,4] However this finding is also disrupted, as in endoscopy of *H. pylori* infected patients with IDA, no lesions and negative fecal occult blood have been reported.[5-11] Not all *H. pylori* infections end up causing clinical diseases, but around 20% of infected individuals develop gastritis, peptic ulcer disease and/or gastric cancer.[12-14] Whether and what form of disease develops, depends on the virulence of the strain.[14,15] The immune response of the host may also predict

whether the *H. pylori* infection causes a corpus predominant or antral predominant gastritis, however this mechanism is also not fully clear. A corpus predominant infection induces reduced gastric acid production[16,17] and the body predominant induces higher gastric acid output.[16,18] The excretion of hydrochloric acid is needed for the solubility and absorption of non-heme iron.[19] Therefore it could be hypothesized that only the corpus predominant infection results in an increased risk for ID and/or IDA. We did not assess this, which is a limiting factor in our study.

We assessed asymptomatic *H. pylori* infection by serology and assumed that the presence of IgG titers in the serum of the study participants corresponds to a positive asymptomatic *H. pylori* infection. This has most likely produced false positive results. Hence, the prevalence of asymptomatic infection is most likely overestimated in our study, which may have caused a bias to our FIA data. False positive results are caused by the slow decline of IgG titers in serum over several months after an eradication therapy.[20] Other major limiting factors were its cross-sectional design precluding definitions of causality, and the potential of unrecognized bias from unmeasured covariates.

Future studies which determine the factors that contribute to disease development, and specifically to the development of a corpus or antral predominant gastritis are needed. These results would potentially contribute to better understanding of the increased risk of ID and IDA in *H. pylori* infected individuals.

Nevertheless, most of the *H. pylori* infected individuals remain asymptomatic lifelong (80%). For these, our study provides evidence that their iron absorption from iron fortified foods is not affected by their infection. Iron fortification programs are likely to be beneficial for the overall population, irrespective of the presence of (asymptomatic) *H. pylori* infection. Moreover, our findings suggest that, asymptomatic *H. pylori* infection is unlikely to be a major contributor to the etiology of ID and IDA in the population and settings we have studied.

Is the SNP rs855791 in the TMPRSS6 gene associated with iron absorption, hepcidin and iron status, and is the TT homozygous genotype more susceptible to develop iron deficiency?

In **Manuscript 2** we performed an iron absorption study in Taiwanese women of reproductive age comparing the homozygous variants carrying the TT (n = 45) and the CC (n = 35) alleles of the single nucleotide polymorphism (SNP) rs855791. Recent publications have shown increased evidence that this SNP is associated with iron status indices and erythrocyte traits.[21-27] In our study we reported no differences in SF, Hb and hepcidin (Hep) concentrations, but serum iron concentrations and transferrin saturation (TSat) were lower in the group carrying the TT variant. We further showed that the normalized Hep values for TSat differ between variants, which has also been reported in other studies[26,27] and was suggested as a biochemical indicator to predict *TMPRSS6* mutations in patients with chronic ID.[28] Nonetheless, the geometric mean of FIA in these two variants were comparable (TT: 7.99%, and CC 6.50%). The model that assessed differences in FIA between variants and controlled for Hb, SF, TSat, soluble transferrin receptor (sTfR), Hep, CRP and menstrual blood losses estimated a significantly lower FIA in the TT variants than in the CC variant.

To reduce inter-individual variability,[29] we normalized our FIA data using an adapted version of the formula suggested by Cook *et al*.[30] and used a SF concentration of 15 µg/l. With this approach we were able to report a significantly reduced FIA in the TT variants (18.5%), compared to CC variants (26.6%). The use and limitations of normalizing FIA data is further discussed in the upcoming section (Overall Limitations) of this General Discussion. Nevertheless, the correlation of FIA to SF was much weaker in the TT variants (r = -0.45) than in the CC (r = -0.79), suggesting a poorer upregulation of FIA at low SF in the TT variants. An atypical FIA to SF correlation has also been reported in a study assessing iron absorption in variants of a polymorphism in the transferrin protein (G277S).[31]

The administration of two identical test meals in this study had two advantages: It increased statistical power and allowed us to make intra-individual comparisons. The intra-individual correlation of FIA from the two meals, which was administered on two alternate days, was significantly lower in the TT variants (r = 0.67) compared to the CC variants (r = 0.86). These results suggest a higher intra-individual variability of iron absorption in variants carrying the TT alleles. The higher variability was also reflected in the models that assessed predictors of

iron absorption. The model was controlled for SF, Hb, TSat, sTfR, Hep, CRP and menstrual blood losses. This model explained 38% of the variability in FIA in TT variants, and significantly contributors were: Hep, sTfR, Hb and TSat. The same model for the CC variants explained much more of the variability in FIA (67%), in which SF was the solely predicting variable. Taken together, our findings suggest TT variants are less able to upregulate FIA when body iron stores are low and are therefore may be more prone to develop ID when dietary iron intakes are low.

We aimed to perform this study in iron sufficient participants based on the hypothesis that a basal BMP/SMAD activity would be required for a measurable Hep concentration,[32] which would allow to quantify the effect of the TMPRSS6 polymorphism. Whether this is a limitation in the study is arguable. On one side, we have only detected a difference in FIA after normalizing FIA to a low SF of 15 µg/l, and potentially a study in iron deplete women may have given clearer results. On the other hand, in iron replete women carrying the TT variant, we reported a higher intra-individual variability and lower explicable value of the model predicting FIA. Based on these study results, we were able to reveal differences in the regulation of systemic iron homeostasis between these two studied variants of the *TMPRSS6* gene.

During the recruitment process for this study, we experienced that many women had to be excluded due to low SF (< 30 µg/l). In order to include these interested participants, we supplemented them with iron (n_{TT} = 34, n_{CC} = 15) (27 mg Fe as FeFum, daily for 2-3 months), and reinvited them for screening. Out of these, we enrolled 20 TT and 12 CC participants into the study one week after discontinuing supplementation. Wheby *et al*. have reported a drop within 6 days in SF after discontinuation of iron supplementation.[32] However, the authors administered higher amounts of iron (120 mg Fe), compared to in our study. Moretti *et al*. have administered two doses of 40,80,160, and 240 mg Fe supplements to iron deficient women, and reported a drop in SF to normal levels after 3-4 days after supplementation discontinuation (study data of the study with 80 and 160 mg Fe, personal communication).[33] The transient elevation in serum ferritin due to supplementation often lasts for 4-5 days with higher dosing of 60-120 mg Fe (personal communication from PD Dr. med. Jeroen Goede). The iron supplementation group did not show a majOr decrease in SF from study day one to endpoint (17 days): the median (and interquartile range) drop in SF was 3.30 (-4.95 - 11.58) µg/l in TT variants and 1.00 (-2.13 - 7.35) µg/l in CC variants, and the change in

SF concentrations over the study period were comparable in both genetic variants, suggesting the iron supplementation is likely not to have caused a bias to our main study outcome. However, our study results could have been affected if we had: a) changed our inclusion criteria and include participants with lower SF concentrations; or b) extended with recruitment, but with the probability of not reaching our needed sample size. After completing the study and data analysis, option a) might have been preferable and may have allowed us to study a broader range of SF concentrations.

We can only speculate about the relative importance of the studied SNP to the etiology of ID. There is a complex interplay of many genes involved in iron metabolism, with a long list of SNPs which may contribute to the final phenotype. Furthermore, other factors such as diet and inflammation are among others, important modulators of iron status. However, our data do contribute to the overall understanding of iron metabolism and homeostasis in humans.

How is the performance of an iron microparticle encapsulated in a pH-sensitive polymer in terms of iron absorption and protection from adverse sensory changes? What future developments are required before implementation into a food fortification program?

Manuscript 3 reports the development of a microparticle to encapsulate micronutrients in a pH-sensitive polymer. Besides iron, also other micronutrients have been encapsulated individually using the same approach: iodine, zinc, vitamin A, B_2, niacin, biotin, folic acid, B_{12}, C and D. The iron microparticle contains the highly bioavailable ferrous sulfate, embedded in HA, and encapsulated in BMP, which is the pH-sensitive polymer. For this manuscript, we performed two stable iron isotope absorption studies in healthy women of reproductive age and assessed the iron absorption from three different microparticles.

The first human study investigated the iron absorption and heat-stability of the initially developed Fe-HA-BMC microparticle, which had a very low iron loading of 0.6% (not reported in the manuscript). In this study the 0.6%-Fe-HA-BMC microparticles were added to a white maize porridge either before or after cooking. Despite the particles itself were red, these microparticles had good sensory performance in banana milk, inducing minimal color changes. In the first study, iron absorption from these were low. Their relative bioavailability (RBV) to free $FeSO_4$ were for both conditions (added before or after cooking) ~0.43%. Therefore, these microparticles remained stable during cooking, but likely had a slow dissolution in the duodenum where iron absorption takes place. A review on the effect of iron encapsulation on absorption from $FeSO_4$, concluded that a capsule to Fe compound ratio of ≥60:40 decreases the RBV by ~20%.[34] The capsule to Fe compound ratio for the microparticles in study one was 98:2.

Further development of these microparticles and changing their production method from a two-step modified inverse emulsion process to spray drying and spinning disk atomization, resulted in an increased iron loading of 3.19 and 18.29%, resulting in a capsule to Fe compound ratio of 88:12 and 44:56, respectively. The second human iron absorption study assessed the FIA of these from wheat bread. The main outcome from this study was that the RBV from the higher loaded microparticles (18.29%) was 89%, whilst the RBV of the 3.2% loaded particles was 71%. The same review as cited above summarized that at capsule:Fe compound ratios of ≤50:50, the RBV is similar to $FeSO_4$.[34]

To successfully implement these microparticles in food fortification, they also need to protect the food from sensory changes due to oxidation induced by iron.[35] Banana milk is a commonly used food vehicle to test, whether an iron compound is susceptible to induce off-color changes.[36,37] Both microparticles, with the higher loading, have induced unacceptable color changes, resulting in a grey-blueish banana milk. Next to the poor performance in a polyphenol rich matrix, the red color of the microparticles itself would change the appearance of a fortified wheat flour. The red color of the particles also indicates the oxidation of ferrous sulfate to ferric oxyhydroxide – which has a red color and has many disadvantages in terms of bioavailability. Ferric iron has reduced solubility in water,[38] and needs to be reduced to ferrous iron in the duodenum before uptake into the enterocytes is possible by DMT-1.[39]

Therefore, future development of this iron microparticle should emphasize: 1) to reduce the sensory changes induced by the iron microparticle; and/or 2) investigate their performance in food vehicles of a dark color with low polyphenol content and low fat (to prevent lipid oxidation). This could be, for example a mixed flour with a higher content of bran/germ particles.

These mixed flours also have a higher phytic acid content, and the microparticle might be a favorable compound to use, they may minimize the formation of insoluble Fe-phytic acid complexes. However, this is an assumption, as in study one, the test meal had a high phytic acid content, but the FIA were significantly lower from the meals fortified with the lowest Fe loaded microparticles than from free $FeSO_4$. We speculate that the FIA values lowering effect in this study was more due to the high content of BMC and HA of the initially developed microsphere. The highest loaded Fe microparticles would hypothetically perform better in a meal high in phytic acid. Whether the microparticle protects iron from binding in a phytic acid rich environment would require further investigation and needs to be assessed by *in vitro* digestion and iron dialyzability, but the magnitude of the protective effect needs to be confirmed in a human study.

For now, the only advantage of these microparticles is, that the highest loaded microparticle had similar FIA than from free $FeSO_4$. Their disadvantages are the poor protection from sensory changes, their red color, and their high production costs. Whether the burden of ID justifies the disadvantages of the microparticles is debatable and requires careful evaluation on regional levels. If further development of these microparticles are able to improve their

sensory performance and mask the red color, the 18.29% loaded microparticle may be a suitable vector for food fortification.

Does a natural polyphenol rich food supplement reduce iron absorption in patients with hereditary hemochromatosis, and what hypothetical effect would it have in reducing the frequency in required phlebotomies?

In **Manuscript 4** we reported on the development of a natural polyphenol supplement that contains polyphenol rich food powders (black tea powder, cocoa powder and grape juice extract), from *in vitro* experiment to a human iron absorption study in patients with hereditary hemochromatosis. The main outcomes from this study are: 1) the iron solubility is significantly reduced by ~80% *in vitro* through the addition of 2 g of a mixture of black tea powder, cocoa powder and grape juice extract (named as the polyphenol supplement: PPS); and 2) the iron absorption from an iron rich meal, and a drink containing the equivalent amount of iron as the meal, is significantly reduced by ~40% when consumed with the PPS in adults with diagnosed hemochromatosis, carrying the homozygous C282Y variant.

Regular phlebotomy treatment is required two to four times per year for patients with hemochromatosis that are treated in their maintenance phase.[40] This treatment is considered simple, safe- and cost-effective.[41] But, for the majority of patients, this treatment is inconvenient and many patients report side effects such as tiredness, fainting and loss of appetite.[42] The reduction in required phlebotomies would not just address these side effects but also reduce health care costs as well as the amount of time invested for the patients.

Using the following assumptions: a person consumes the study test meal three times per day; has a mean Hb of 15.4 g/dl; an average concentration of 3.47 mg Fe/g Hb; and the removal of one unit of blood is 450 ml, a 40% reduction in absorbed iron would translate to a reduction in iron accumulation of ~230 mg per year, which corresponds to a removal of one unit of blood.

Looked at in another way, the average daily iron intake in UK men was assessed to be 13.5 mg/d and 9.8 mg/d in women.[43] Using the FIA reported in our test meal (3.0% and 5.2% with the PPS and without), this would correspond to a reduction in accumulated iron of ~110 mg in men and ~80 mg in women. Translated to units of blood these would be: 0.42 units in men and 0.35 units in women.

The FIA of 5.2% from the meal with the placebo supplement was relatively low, likely due to the high phytic acid content of the test meal. Compared to literature, iron absorption studies

in patients with hereditary hemochromatosis have reported higher FIA ranging from 20-40%.[44-47] Considering the fact that the PPS has led to a 40% reduction in FIA in either test condition, with a food matrix and without, the 40% reduction could most likely be translated to any other meal with varying amounts of phytic acid. This last calculation uses the average daily iron intake from the same study in UK men and women as mentioned previously,[43] and the FIA from our test drinks (10.3 and 16.9% with and without the PPS). Based on these variables, the PPS could result in a yearly reduction of ~325 mg Fe in men and ~240 mg Fe in women, or 1.3 and 1.1 units of blood, respectively.

Either way, reducing required phlebotomies by 0.5 units of blood or 1.3, the regular use of this PPS could potentially be an effective adjunct treatment to reduce required phlebotomies. Nevertheless, a prospective, placebo-controlled study is required to precisely define the efficacy of the PPS when consumed long-term.

A change in the galenic form of the supplement might also be relevant for future development. The gelatin capsules used in this study were size 000, for some participants these were difficult to swallow. To reduce the size of the supplement, dried extracts from the black tea and cocoa powder could be used. An extract of black tea contains polyphenols that are capable to reduce iron absorption.[48] Whether the iron chelating polyphenols from the cocoa could also be extracted would need further study and experimental analysis. Using extracts would certainly decrease supplement size, and therefore improve acceptability for consumers.

The second change in the galenic form of the supplement, which could be considered in future developments, is the compression of the powder into a pill. This would also address the issue of the delayed dissolution of the gelatin capsule, which may have reduced the mixing of the PPS with the meal in the digestive tract in our absorption study. For now, this delay in capsule dissolution might be compensated by taking the supplement for example 15 minutes before meal intake.

Besides patients with hemochromatosis, other iron overload diseases characterized by increased iron absorption from the diet include β-thalassemia. Due to ineffective erythropoiesis, patients with β-thalassemia suffer from anemia concurrent with tissue iron overload which can lead to organ damage (heart, liver, endocrine).[49] Without intervention, these patients have increased morbidity.[50] The conventional management is transfusion therapy to supply normal erythrocytes and iron chelation therapy with Deferoxamine,

Deferiprone or Deferasirox.[49] Therefore, the reduction in dietary iron intake and absorption could be beneficial in these patients to reduce their iron accumulation and therefore co-morbidities. However, the reducing effect of our PPS in these patients needs to be assessed and confirmed in an iron absorption study – which is currently planned in Thai adults with iron-loading thalassemia.

To summarize, the PPS is a promising approach as an adjunct treatment in the maintenance phase for patients with hereditary hemochromatosis. It may also be used to reduce iron accumulation in patients with β-thalassemia. However, this effect, as well as its efficacy in a long-term study assessing changes in the frequency of phlebotomy, requires further research.

Other Limitations

Iron bioavailability has large variations in humans, and FIA from a test meal ranges from 1% to 58%.[32] It is estimated that ~50% of the variation in iron bioavailability can be attributed to two factors: iron status and food matrix.[51] A striking negative correlation between SF and iron absorption has been reported,[52] based on these data, the equation $\log(FIA_C) = \log(FIA_O) + \log(SF_O) - \log(SF_C)$ has been developed.[53] FIA_O and SF_O are the observed iron absorption and SF concentration, whereas FIA_C and SF_C the corrected iron absorption, and SF concentration. This equation assumes a slope of -1 in the correlation of SF to FIA. This may be the case in the majority of all iron absorption studies, which do not assess and compare iron absorption between two genetic polymorphisms that are involved in iron homeostasis. This equation was used in the Manuscript 1 to standardize the FIA data to a SF concentration of 40 µg/l. Whereas in Manuscript 2, we studied a polymorphism in the *TMPRSS6* gene, in which we reported a significant difference between the two variants in the correlation of SF to FIA. One of the variants of this polymorphisms (carrying homozygous T alleles) did not show this typical inverse correlation. In this variant we reported a slope of -0.74, compared to a slope of -1.28 in the other variant (homozygous C alleles). An atypical inverse correlation of SF to FIA has also been reported in an iron absorption study of homozygous variants of the G277S polymorphism in the transferrin protein.[31] Hence, using the formula proposed by Cook *et al*.[53] in the study reported in Manuscript 2 would have overestimated the normalized iron absorption in the variants carrying both T alleles and underestimated the FIA in the CC variants. Whether the adapted formula, which includes the slope of the regression line, is the “true” approach remains to be confirmed by other confirmatory studies. Nevertheless, the main analysis based on linear mixed models, which was controlled for: variant, Hb, SF, TSat, sTfR, Hep, CRP, confirmed our hypothesis that the TT variant is associated with lower iron absorption.

Concluding remarks

With this thesis, covering extrinsic and intrinsic aspects of iron absorption in ID and iron overload, we have shown that:

1) Asymptomatic *H. pylori* infection appears not to significantly reduce non-heme iron absorption from fortified foods; therefore, reduced absorption may not be a major contributor to the etiology of ID and anemia in these individuals.
2) Individuals carrying two T alleles of the single nucleotide polymorphism rs855791 in the *TMPRSS6* gene may be less able to upregulate iron absorption when their iron stores are depleted. Hence, these individuals may be more susceptible to ID when dietary iron is low.
3) Microencapsulation of ferrous sulfate in a pH-sensitive polymer is a novel approach to deliver a highly bioavailable iron compound in fortified foods. However, despite the high relative bioavailability of the highest loaded microparticle (~90%), sensory performance in food models was poor and requires further development.
4) A natural polyphenol supplement reduced iron absorption from a meal in patients with hereditary hemochromatosis by ~40%. This patient group might benefit from a polyphenol supplement as concurrent treatment to phlebotomy, to reduce the frequency of phlebotomy. However, this needs confirmation in a long-term study. Another target group for this product could be patients with iron-loading β-thalassemia, and this will be studied in a planned iron absorption study in Thai patients with this disorder.

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