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NOVEL APPROACHES IN MINERAL FORTIFICATION OF RICE AND CEREALS

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All this science I don't understand,

It's just my job five days a week

Elton John –
Rocket Man

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ABBREVIATIONS

AA	Ascorbic acid
CA	Citric acid
CHr	Reticulocyte hemoglobin content
CP	Crude protein
CRP	C-reactive protein
Dcytb	Duodenal cytochrome b
DIAAS	Digestible indispensable amino acid score
DMT-1	Divalent metal transporter
e.g.	Example given
EC	European Commission
EDTA	Ethylendiamin tetra-acetic acid
EFSA	European Food Safety Authority
EU	European Union
FAFe	Fractional iron absorption
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
Fe	Iron
FePP	Ferric pyrophosphate
FeSO$_4$	Ferrous sulfate
GM	Genetically modified
GRAS	Generally recognized as safe
HCP1	Heme carrier protein
HNO$_3$	Nitric acid
IAEA	International Atomic Energy Agency
ICN2	Rome Declaration on Nutrition
ID	Iron deficiency
LA:DGLA	Linoleic acid : dihomo-gamma-linoleic acid
MCH	Mean cell hemoglobin
MCV	Mean corpuscular volume

MT	Metric ton
NGO	Non-Governmantal Organization
NO	Nitric oxide
NS PO$_4$	Nano-sized ferric phosphate
NSeI	Nutrition-sensitive intervention
NSpI	Nutrition-specific intervention
NTBI	Non-transferrin bound iron
PA	Phytic acid
PDCAAS	Protein digestibility-corrected amino acid score
PF	Plasma ferritin
PP	Polyphenol
RBCs	Red blood cells
RBV	Relative bioavailability
SCOGS	Select Committee on GRAS Substances
SF	Serum ferritin
SFP	Soluble ferric pyrophosphate
SID	Standardized ileal digestibility
TfR	Transferrin receptor
TIBC	Total iron binding capacity
TSC	Trisodium citrate
UL	Tolerable upper intake level
WHO	World Health Organization
WFP	World Food Programme
YLD	Years lived with disability
Zn	Zinc
ZnO	Zinc oxide
ZnSO$_4$	Zinc sulphate

SUMMARY

Introduction

Iron deficiency (ID) and anemia remain major public health problems affecting individuals worldwide, but particularly in low- and middle-income countries. Symptoms of ID are manifold and include fatigue, weakness and reduced work productivity. Women of child-bearing age and children are particularly prone to suffer from ID due to increased iron losses and requirements, respectively. This is the case especially in low-income countries where iron amounts provided from the diets often do not meet individuals' needs.

Food fortification is one approach to combat ID and many foods have shown to be promising vehicles for delivering iron.

Rice is a major staple food for more than half the world's population and a main source of energy in many countries, however, its micronutrient content is considerably low in its consumed form. Fortification of rice is challenging due to its white color and consumption as intact grain. Iron phosphates, particularly ferric pyrophosphate (FePP), are the most widely used iron compounds for rice fortification due to their inert sensory profile, however, iron bioavailability from those compounds is relatively low. Different technologies for rice fortification exist, but extrusion and coating have been suggested as most viable approaches.

Objectives

The objectives of this thesis were to a) Investigate, how iron absorption enhancers and inhibtors influence human iron absorption from rice meals containing FePP-fortified extruded rice; b) assess, whether observed inhibiting/enhancing effects from single meal administrations are also present upon multiple meal administrations; c) investigate, if the technology used for rice fortification impacts iron or zinc absorption. The generated results aim to contribute to the scientific evidence required to establish a fortification formulation that provides optimal iron supply from FePP-fortified rice.

Design

We conducted five different absorption studies in Swiss women of child bearing age and in Ghanaian school-age children.

SUMMARY

In study 1, we assessed the effects of citric acid in combination with trisodium citrate (CA+TSC) on iron absorption from FePP-fortified extruded rice in healthy Swiss women. Study 2 investigated the effects of the zinc co-fortificant (zinc as sulphate or oxide) on iron absorption from FePP-fortified extruded rice in iron-depleted, but otherwise healthy, Swiss women. Study 3 contained of two parts, in the first part, we investigated iron absorption in healthy Swiss women from FePP-fortified rice produced via hot or cold extrusion; in the second part we investigated iron and zinc absorption in healthy Swiss women from coated and hot extruded rice. In study 4, we investigated iron absorption from FePP-fortified rice in combination with different zinc co-fortificants (ZnO or $ZnSO_4$) and absorption enhancers (CA+TSC, CA+EDTA) in Ghanaian school-age children, using a novel multiple-meal approach.

All studies had a cross-over design, where participants acted as their own controls. Unfortified rice, where a solution of the highly bioavailable $FeSO_4$ was added prior consumption served as a reference for fractional iron absorption (FAFe) in all, but one, studies.

Furthermore, we supported our *in vivo* bioavailability findings with *in vitro* iron solubility assessments and conducted micronutrient retention tests from FePP-fortified rice manufactured via coating, hot and cold extrusion.

Results

Study 1 showed a significantly increased FAFe, when FePP-fortifified rice was also extruded with CA+TSC (3.2 % FAFe) compared to addition of a CA+TSC solution to the rice prior consumption (1.7 %; *P < 0.05*). Furthermore, FAFe in the participants did not significantly differ between the rice extruded with FePP, CA+TSC and the $FeSO_4$-reference (1.7 %, *n.s.*).

In study 2, we showed, that the choice of the zinc co-fortificant affects iron absorption. FAFe from FePP-fortified rice in combination with ZnO (2.7 %) was significantly lower than from $ZnSO_4$ (4.5 %, *P < 0.03*). This was also reflected in iron bioavailability relative to $FeSO_4$ (RBV) of 62 and 38 % when co-fortified with $ZnSO_4$ and ZnO, respectively.

Study 3 showed, that the extrusion conditions affect iron absorption, thus, consumption of cold extruded FePP-fortified (1.8 %) rice caused a significantly higher FAFe than of hot extruded rice (1.1 %, *P < 0.05)* of the same composition. No significant differences were found in FAFe from coated rice (4.0 %) versus hot extruded rice of a similar composition

(5.1 %, *n.s.*). Similarly, no differences between fractional zinc absorption were found in coated (9.6 %) versus hot extruded rice (9.5 %, *n.s.*).

In study 4, we found significant differences in FAFe from rice fortified with FePP co-fortified with ZnO (2.3 %) compared to co-fortification with $ZnSO_4$ (3.5 %, *P < 0.05*). Addition of CA+TSC increased iron absorption in both conditions, however, absolute differences between ZnO (4.5 %) and $ZnSO_4$ (6.3 %, *P < 0.05*) remained. Furthermore, we found that addition of CA+EDTA to FePP and ZnO co-fortified rice also enhanced FAFe (6.4 %). Iron absorption from FePP-fortified rice fortified with ZnO, CA+EDTA or $ZnSO_4$, CA+TSC did not differ from the $FeSO_4$-reference (6.4 %, *n.s.*). Within this study, we successfully tested a novel multiple-meal approach, in which each type of labelled test meal was given twice daily for five consecutive days providing the high precision of using stable iron isotopes, while integrating the fortified rice as a regular part of a daily consumed diet.

Conclusions

The results presented in this thesis, provide a solid base for future rice-fortification recommendations. The almost doubling effect on iron absorption upon addition of the absorption enhancers CA+TSC or CA+EDTA allows to reduce the quantities of fortification iron and thus to minimize the amount of unabsorbed iron. Based on the presented work, the use of such absorption inhibitors for FePP-fortified rice in combination with $ZnSO_4$ as zinc co-fortificant is suggested. Further studies on storage stability, consumer acceptance as well as the performance of rice fortified with FePP, $ZnSO_4$, CA and EDTA are highly recommended. Furthermore, optimal extrusion conditions regarding maximized iron absorption should be evaluated.

ZUSAMMENFASSUNG

Einleitung

Eisenmangel und Anämie sind weltweit prävalent, am weitesten verbreitet sind diese Krankheiten jedoch in einkommensschwachen Ländern, wo die Menge an bioverfügbarem Eisen von der Nahrung oft nicht den Bedürfnissen der Population gerecht wird. Symptome des Eisenmangels sind vielfältig und beinhalten Müdigkeit, Schwäche sowie eine verminderte Produktivität. Frauen im reproduktionsfähigen Alter und Kinder sind besonders anfällig für Eisenmangel durch ihre erhöhten Eisen-Verluste beziehungsweise Bedürfnisse.

Fortifizierung von Nahrungsmitteln ist ein Ansatz, um Eisenmangel zu bekämpfen – viele Nahrungsmittel sind vielversprechende Vehikel zur Eisenfortifizierung.

Reis ist ein Grundnahrungsmittel für über die Hälfte der globalen Bevölkerung und eine Haupt-Kalorienquelle in vielen Ländern, jedoch ist sein Gehalt an Mikronährstoffen in der Form, in welcher er normalerweise konsumiert wird, vergleichsweise gering. Fortifizierung von Reis ist schwierig aufgrund seiner weissen Farbe und da er als intaktes Korn konsumiert wird. Eisenphosphate, insbesondere Eisenpyrophosphat (FePP), sind die am weitesten verbreiteten Eisenverbindungen zur Reis-Fortifizierung aufgrund ihrer vorteilhaften sensorischen Eigenschaften – die Eisenverfügbarkeit von diesen Verbindungen ist jedoch gering. Verschiedene Technologien zur Reis-Fortifizierung existieren, wobei Extrusion und Beschichtung die gängigsten sind.

Ziele

Die Ziele dieser Dissertation waren zu erforschen a) wie Verstärker beziehungsweise Inhibitoren der Eisenabsorption die humane Eisenbioverfügbarkeit von mit FePP fortifiziertem Reis beeinflussen; b) ob verstärkende/inhibierende Effekte nach Einzelgaben auch nach mehreren Gaben beobachtet werden können; und c) ob die Technologie, welche zur Reis-Fortifizierung verwendet wurde die Eisen- und Zink-Bioverfügbarkeit beeinflusst.

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Die generierten Resultate sollen zur wissenschaftlichen Evidenz beitragen, welche zur Etablierung einer Fortifizierungs-Formulierung mit optimaler Eisenbioverfügbarkeit nötig ist.

Aufbau

Fünf Absorptionsstudien wurden in Schweizer Frauen im gebärfähigen Alter und in ghanaischen Schulkindern durchgeführt.

In Studie 1 wurde der Effekt von mit FePP-fortifiziertem extrudierten Reis mit Zitronensäure in Kombination mit Tri-Natrium-Zitrat (CA+TSC) auf die Eisenabsorption in gesunden Schweizer Frauen getestet. Studie 2 erforschte den Effekt, welchen die Zink-Quelle [Zink als Sulfat ($ZnSO_4$) oder als Oxid (ZnO)] auf die Eisenabsorption in gesunden Schweizer Frauen mit geringem Eisenstatus von mit FePP-fortifiziertem extrudierten Reis, hat. Studie 3 bestand aus zwei Teilen, beide Teile untersuchten die Absorption von mit FePP-fortifiziertem Reis in gesunden Schweizer Frauen. Im ersten Teil wurde getestet, ob es Unterschiede in der Eisenabsorption gibt, nach Konsumierung von heiss oder kalt extrudiertem Reis. Im zweiten Teil wurde getestet, ob sich Eisen- beziehungsweise Zinkabsorption unterscheiden nach dem Konsum von extrudiertem oder beschichtetem Reis.

In Studie 4 wurde die Eisenabsorption von mit FePP extrudiertem Reis, welcher mit verschiedenen Zink-Quellen (ZnO oder $ZnSO_4$) und Absorptionsverstärkern [CA+TSC; CA+ Ethylendiamintetraessigsäure (EDTA)] fortifiziert wurde in ghanaischen Schulkindern mit erschöpften Eisenspeichern getestet.

Unfortifizierter Reis, welchem eine Lösung von höchst bioverfügbarem $FeSO_4$ bei der Verabreichung zugesetzt wurde, agierte als Referenz für die fraktionelle Eisenabsorption (FAFe) in allen Studien, mit der Ausnahme vom ersten Teil der Studie 3.

Weiters wurden unsere Ergebnisse der *in vivo* Bioverfügbarkeit durch *in vitro* Löslichkeitsversuche ergänzt und die Mikronährstoff-Retention von Reis, welcher mittels heisser beziehungsweise kalter Extrusion oder Beschichtung fortifiziert wurde.

Resultate

Studie 1 zeigte signifikante Unterschiede in FAFe, wenn der Reis mit CA+TSC extrudiert wurde, im Gegensatz zum Zusatz einer CA+TSC Lösung vor der Verabreichung (3.2 %

versus 1.7 % FAFe, *P < 0.05*). FAFe von mit CA+TSC extrudiertem Reis zeigte keine signifikanten Unterschiede in FAFe zur $FeSO_4$-Referenz (1.7 %, *n.s.*).

Studie 2 zeigte, dass die Zink-Quelle die Eisenabsorption beeinflusst. FAFe von FePP-fortifiziertem Reis in Kombination mit ZnO (2.7 %) war signifikant geringer im Vergleich zu $ZnSO_4$ (4.5 %, *P < 0.03*). Relative Bioverfügbarkeit (RBV) bezogen auf $FeSO_4$ von Reis fortifiziert mit ZnO oder $ZnSO_4$ war 38 beziehungsweise 62 %.

Studie 3 zeigte, dass die Extrusionsbedingungen die Eisenabsorption beeinflussen, Verabreichung von kalt extrudiertem Reis (1.8 %) hatte eine signifikant höhere FAFe zur Folge, als die von kalt extrudiertem Reis (1.1 %, *P < 0.05)* mit gleicher Zusammensetzung. Kein signifikanter Unterschied wurde gefunden in FAFe von beschichtetem Reis (4.0 %) verglichen mit heiss extrudiertem Reis von ähnlicher Zusammensetzung (5.1 %, *n.s.).* In gleicher Weise wurde kein signifikanter Unterschied zwischen fraktioneller Zink Absorption in beschichtetem (9.6 %) versus heiss extrudiertem Reis (9.5 %, *n.s.*) gefunden.

In Studie 4 fanden wir keine signifikanten Unterschiede in FAFe von FePP-fortifiziertem Reis, welcher mit ZnO (2.3%) oder $ZnSO_4$ (3.5 %, *P < 0.05*) co-fortifiziert wurde. Zugabe von CA+TSC erhöhte die Eisenabsorption in beiden Konditionen, jedoch blieben absolute Unterschiede zwischen ZnO (4.5 %) und $ZnSO_4$ (6.3 %, *P < 0.05*) bestehen. Weiters konnte gezeigt werden, dass die Zugabe von CA und EDTA zu FePP und ZnO co-fortifiziertem Reis auch die FAFe erhöhte (6.4 %). Eisenabsorption von Reis fortifiziert mit FePP und ZnO, CA+EDTA oder $ZnSO_4$, CA+TSC unterschied sich nicht signifikant von der $FeSO_4$-Referenz (6.4 %, *n.s.*). Mit dieser Studie konnten wir erfolgreich einen neuartigen Ansatz für eine ‚multiple meal' Studie testen, in welcher jede Art von Mahlzeit zweimal täglich über fünf zusammenhängende Tage verabreicht wurde, was einerseits die hohe Genauigkeit durch die Verwendung stabiler Isotope zum Vorteil hat, während zusätzlich der fortifizierte Reis in die täglich konsumierte Diät integriert ist.

Konklusion

Die Resultate die in dieser Dissertation präsentiert werden, stellen eine solide Basis für zukünftige Empfehlungen zur Reisfortifizierung bereit. Die annähernde Verdopplung der Eisenabsorption durch die Zugabe der Absorptionsverstärker CA+TSC oder CA+EDTA erlaubt, den Gehalt an Fortifizierungs-Eisen zu reduzieren und folglich die Menge von nicht absorbiertem Eisen, welches potenzielle Gefahren birgt, gering zu halten. Reis sollte mit

FePP und einem der beiden Absorptionsverstärker fortifiziert werden, wobei $ZnSO_4$ als Zink-Quelle dienen sollte. Weitere Studien sollten in Bezug auf die Lagerungsstabilität, Akzeptanz bei Konsumenten sowie den Effekt einer Fortifizierung mit FePP, $ZnSO_4$, CA+EDTA durchgeführt werden. Weiters sollten optimale Extrusionsbedingungen in Hinblick auf eine erhöhte Eisenabsorption erforscht werden.

1   RICE

2.1   History

Rice, whose name probably derived from the Greek word *oryza (Oxford Dictionary 2017)*, has fed more people over a longer time than has any other crop (Arendt and Dal Bello 2011, Maclean, Hardy et al. 2013). While originating in different parts of Asia and possibly Africa in the Neolithic era, the crop was likely introduced to Greece and neighboring areas of the Mediterranean in the 4th century B.C. and gradually spread from Greece and Sicily throughout Southern Europe and certain locations in North Africa (Maclean, Hardy et al. 2013). Rice was introduced to the New World by early European settlers and eventually brought to North America by African slaves in the late 17th century (Maclean, Hardy et al. 2013). Nowadays, rice is cultivated on every continent apart from Antarctica (Dexter 1998, Childs 2004). An estimated 120 000 varieties of rice exist worldwide (Khush 1997) and locations as well as climatic conditions for rice production vary greatly, however, the highest yields are suggested to be achieved in subtropical or warm temperate climates (Houston, Houston et al. 1970).

2.2   Morphology

In human nutrition, two rice species are predominant: *Oryza sativa* and *Oryza glaberrima*. The former is grown worldwide and the latter only in parts of West Africa (Khush 1997). *O. sativa* is indigenous to Asia and, like *O. glaberrima*, member of the *Oryzae* tribe in the monocotyledonous grass-family *Poaceae/Gramineae* (Schlechteri and Röhr 2002). The rice plant has been suggested as model plant in monocotyledons due to its small genome size and known genome sequence (Itoh, Nonomura et al. 2005). Along with cereals such as wheat and barley, rice belongs to the group of C_4 grasses, whose photosynthetic pathway, compared to C_3 grasses such as maize and sorghum, produces less biomass per unit of intercepted radiation and transpiration (Tuong, Bouman et al. 2005).

Embryogenesis, vegetative and reproductive phases illustrate the plant's developmental process and are delimited by seed dormancy, germination and onset of inflorescence

development, respectively (Itoh, Nonomura et al. 2005). During embryogenesis, the plant body plan is established while the zygote undergoes cell division to form a globular embryo, which then differentiates the shoot apical meristem (SAM) and radicle (Itoh, Nonomura et al. 2005). Subsequently, the vegetative development begins, where the plant repeatedly forms leaves and branches serving as lateral organs. This development can be divided into a juvenile and an adult phase, whereas the transition between those phases is not discrete, but continuous (Itoh, Nonomura et al. 2005). The following vegetative phase is evident through the flowering of the plant, thus, the SAM with its regularly formed foliage leaves and tillers is converted into an inflorescence meristem (Itoh, Nonomura et al. 2005). The meristem forms small degenerate leaves, called bracts and inflorescence branches, called spikelets (**Figure 1**). The rice plant is most sensitive to drought around flowering, due to a potential induction of spikelet sterility (Houston, Houston et al. 1970). Rice inflorescence is racemic, thus spikelets are formed on lateral branches rather than on the main axis (Itoh, Nonomura et al. 2005).
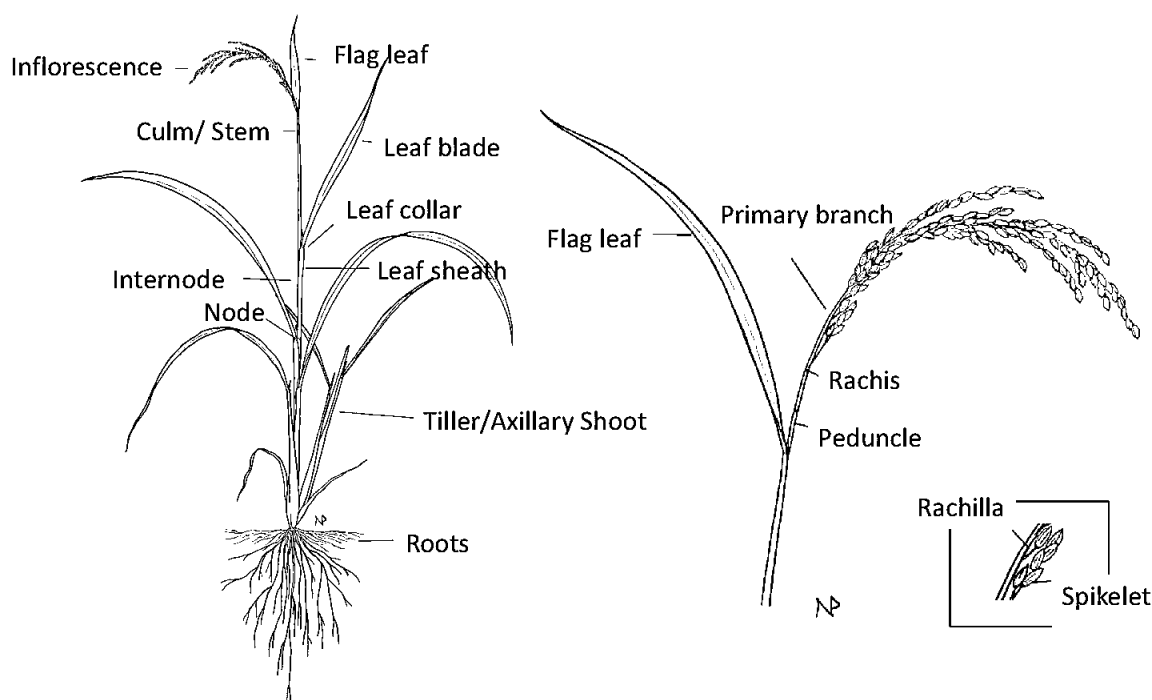
Figure 1. Stem morphology of *O. sativa* (Gramene).

A rice shoot consists of successive phytomers, whereas each phytomer consists of a node associated with a leaf and a subtending internode with a tillerbud at the base (Nemoto, Morita et al. 1995). Contrary to other *Poaceae* species, rice has numerous adventitious roots, whereas their development is coordinated with the development of a

corresponding leaf in each phytomer (Nemoto, Morita et al. 1995). Vegetative and reproductive development of rice depend primarily on the leaf emergence rate (Nemoto, Morita et al. 1995). While the synchrony between root initiation and leaf development differs among cultivars, the synchrony between root emergence and leaf development depends on environmental factors (Nemoto, Morita et al. 1995).

Spikelets consist of a caryopsis, four bracts and a rachilla; encased by the hull, which accounts for roughly 20% of the mass of a rice kernel (Fitzgerald and Starch 2004). The caryopsis without the hull is the so called brown rice, a single-seeded fruit with a pericarp (Fitzgerald and Starch 2004) containing 10% bran, 90% endosperm and 1% germ (**Figure 2**) (Atungulu and Pan 2014). Several tissues varying in structure, composition and function, are found in the seed, whereas the most important ones are testa, nucellus, embryo and endosperm (Fitzgerald and Starch 2004) (**Figure 3**).
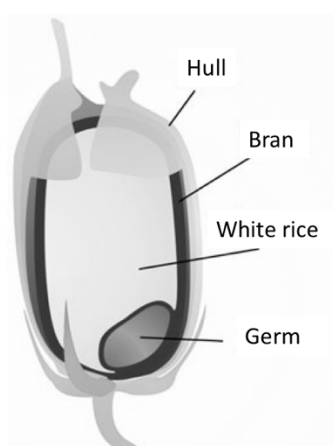
Figure 2. Schematic overview of a rice grain (Qureshi University).
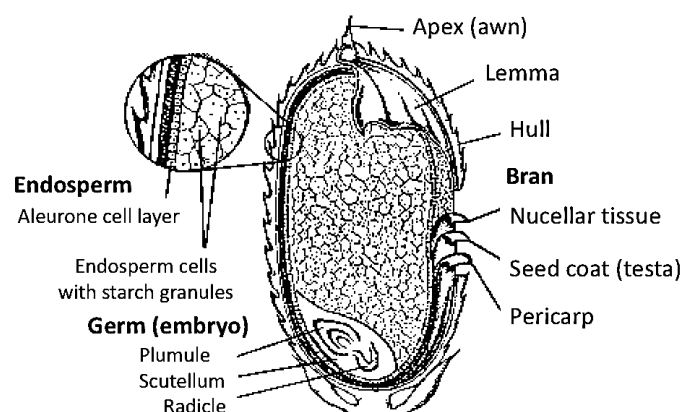
Figure 3. Detailed structure of a rice grain (Encyclopædia Britannica).

Approximately four months after seeding, paddy rice, consisting of an outer husk layer, germ and bran layers and an endosperm, is harvested (Fitzgerald and Starch 2004). Various milling steps are required to make the rice suitable for consumption (Atungulu and Pan 2014). Rough rice still contains an intact hull (Mitchell 2009), whereas removing the outermost husk layer results in brown and eventually white rice (**Figure 4**) (Muthayya, Sugimoto et al. 2014). Thus, the degree of milling determines the color of rice, which is an important quality parameter (Arendt and Dal Bello 2011). White rice mainly consists of the endosperm, filled with starch granules and protein bodies (Juliano and Bechtel 1985). While cell size, starch content and size of starch granules increase towards the center of the endosperm, protein content is highest in the periphery and decreases towards the center (Juliano and Bechtel 1985).
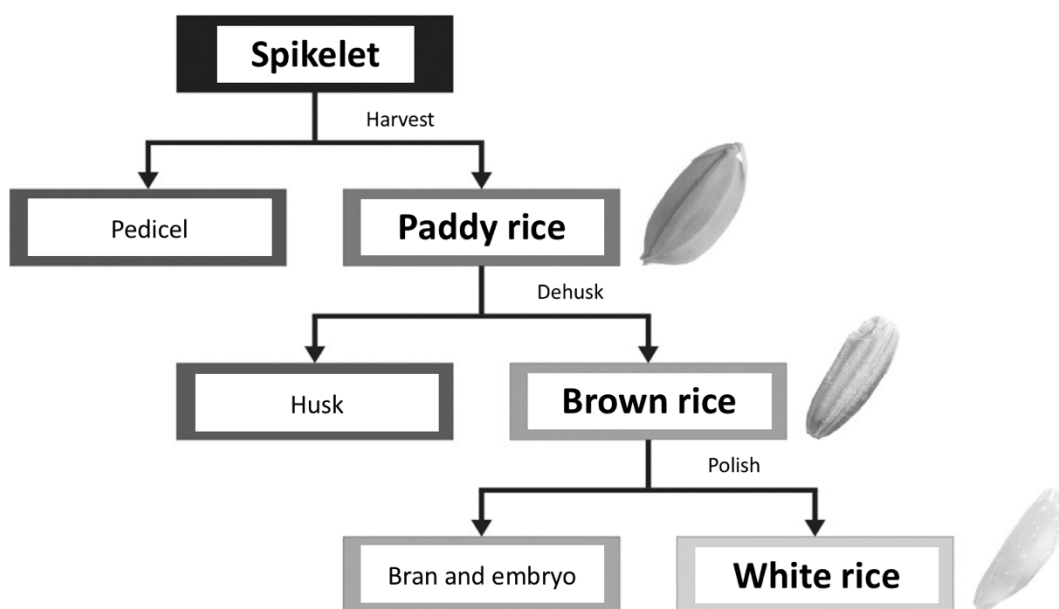
Figure 4. Rice grain processing (Sperotto, Ricachenevsky et al. 2012).

Depending on the size of the milling product, milled rice is divided into several fractions, such as head and broken rice (which can be further divided into second-head and brewer's rice) (Mitchell 2009). Milled rice has the shortest shelf-life as its surface cells easily hydrolyze due to lipases released from the bran layer and subsequent lipoxygenase oxidation of unsaturated fatty acids which can cause rancidity (Mitchell 2009, Atungulu and Pan 2014). Milling yield highly determines the economic value of rough rice and is usually indicated as milled rice yield or head rice yield (Atungulu and Pan 2014). Up to 40% of milled rice can be broken after milling, the broken rice can either be sold at a cheaper

price or used for rice flour production, in the brewing industry or as feed (Arendt and Dal Bello 2011).

2.3 Rice as staple food

2.3.1 Rice supply chain

Rice processing involves a sequence of (pre-)harvesting, cleaning, drying, storage, milling and distribution (Atungulu and Pan 2014). The rice supply chain entails stakeholders from both public and private sectors connecting rice producers (farmers, millers, collectors), traders, retailers, food processors, and customers (Muthayya, Sugimoto et al. 2014) (**Figure 5**).
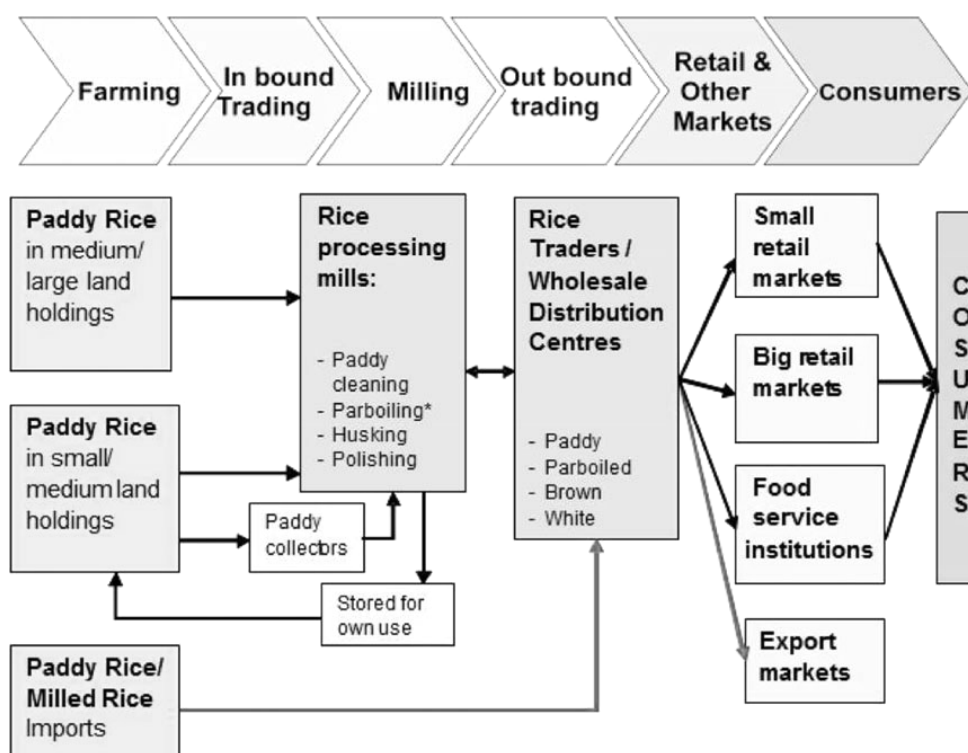
Figure 5. The rice supply chain in a rice growing country (Muthayya, Sugimoto et al. 2014).

2.3.2 Rice farming

The fields for rice production are prepared via soaking, plowing and puddling, the latter of which is required to control weeds, reduce soil permeability and ease seed-transplanting (Bouman, Lampayan et al. 2007). After puddling, the field is flooded for up to 4 weeks with subsequent seedling transplantation (Bouman, Lampayan et al. 2007). Globally, most of

the rice is grown under continuous (irrigated) or intermittent (rainfed) ponded water conditions (Bouman, Barker et al. 2007). In both irrigated and rainfed conditions, fields are mainly puddled and plants transplanted, whereas upland rice is grown under dryland conditions neither employing irrigation nor puddling (Bouman, Barker et al. 2007).

Rice production per weight unit requires two to three times more water than growing other cereals (Tuong, Bouman et al. 2005) with irrigated rice receiving 34 – 43% and 34 – 30% of the global irrigation and freshwater resources, respectively (Bouman, Barker et al. 2007). Most rice varieties can survive complete submersion for 3 – 4 days only, with the exception of some rainfed lowland rice, which can survive for up to 10 days (Bouman, Barker et al. 2007). In irrigated lowlands, which account for about 75% of the world's rice production, rice is grown in bunded fields, where irrigation with 'floodwater' (5 – 10 centimeters) is ensured for one or more crops per year, thus, exclusively relying on irrigation during the dry season (Bouman, Barker et al. 2007). About 19% and 4% of global rice is grown in rainfed low- and up-lands, respectively, where bunded rice fields are flooded with rainwater (100 centimeters or more for up to 10 days) for parts of the cropping season (Bouman, Barker et al. 2007). Rainfed rice areas are frequently affected by drought and suffer from multiple abiotic stresses leaving farmers in those areas in uncertainty about timing, duration and intensity of rainfall (Bouman, Barker et al. 2007). Soils in those growing areas may have poor physical and chemical properties and average yields are 2.3 and 1 metric tons (MT) per hectare in low- and up-lands, respectively (Bouman, Barker et al. 2007). Deepwater and floating rice are grown in flood-prone environments with average yields of ~1.5 MT per hectare (Bouman, Barker et al. 2007).

2.3.3 Rice production

Four types of rice account for almost the entire global rice production: *indica*, *japonica*, *aromatic*, and *glutinous*, whereas *indica* is the globally dominating type (Childs 2004). Glutinous, or waxy, rice has been categorized into a sub-group with distinct cooking characteristics from common rice (Houston, Houston et al. 1970). Rice can also be classified into long and short-grain types, whereas long-grain rice cooks to dry, fluffy products and short-grain rice to cohesive and moist ones (Houston, Houston et al. 1970).

The majority of rice is produced in Asia, with China, India, Indonesia and Bangladesh accounting for almost 70% of its global production (Childs 2004). According to the United Nations Food and Agriculture Organization (FAO), 723 million tons of paddy rice were produced in 2011 in over 100 countries (FAOSTAT). This resulted in 480 million tons of milled rice, whereas ~77% and 7% of the milled rice were used as food and feed, respectively and ~6% were wasted (FAOSTAT). From the late 1960s through 1990, global rice production exceeded the demand of a growing population, however, the picture changed due to a slower (and even decreased) production along with an annual consumption growth due to a growing population in rice-consuming countries (Childs 2004). An estimated 40% increase in annual rice production will be needed to meet the growing rice demand in the future (Atungulu and Pan 2014), which will have to be met by increased field yields as rice-growing areas as well as water supply are limited and pesticide use should be minimized in order to avoid serious environmental problems (Childs 2004).

2.3.4   Market

Main rice exporters are Thailand, Vietnam, the US, China, Pakistan, and India accounting for more than 80% of the world trade, whereas Indonesia, Nigeria, Iran, Iraq, the Philippines, and the EU are leading on the import side (Childs 2004). Staple foods, such as rice, are characterized by low value and high storability (Wopereis, Johnson et al. 2013), whereas their sales barely respond to price and income changes (Alavi, Bugusu et al. 2008). While 13 – 25% of commodities such as wheat, soybeans and corn are traded globally, only ~7% of rice is traded (Wopereis, Johnson et al. 2013, Muthayya, Sugimoto et al. 2014). Rice production is highly restricted to soil and climate conditions and substitution among buyers with rigid taste preferences is small, resulting in more volatile prices compared to other grains (Dawe 2002, Childs 2004). Due to its small trading volume, production-shortfalls in a major rice-importing country can highly influence international prices, rendering the international rice market thin, volatile and risky (Childs 2004, Wailes 2005). The Rome Declaration on Nutrition (ICN2) acknowledged trade as key element in achieving food security and nutrition and considered an excessive volatility of prices for food and

agricultural commodities as factors which negatively impact food security and nutrition (FAO 2014).

From 1950 to 1981, world rice prices averaged to USD ~934/ton and subsequently fell, averaging to USD 355/ton from 1985 to 1998 (Dawe 2002). In the 30 years between 1986 and 2016, however, the price per MT of rice increased almost 2.5-fold from 173 to 414 USD (Indexmundi). In May 2008, rice prices tripled in only a few months reaching an inflation-adjusted 30-year high (over 1000 USD/MT) due to a ban of rice exports from Vietnam, India and Egypt (Wopereis, Johnson et al. 2013) and plummeted back towards their pre-crisis level in September 2008 (~720 USD/MT) (Demont 2013). However, the peak prices then were well below those during the world food crisis in the early 1970s (after adjusting for inflation) (Dawe and Slayton 2010).

2.3.5 Biofuel

The number of hungry people has been estimated to increase by about 16 million for each percentage increase in food prices (Runge and Senauer 2007), which is striking given the 10% increase in rice prices from August 2015 to August 2016 (Indexmundi) (**Figure 6**). With biofuel as most rapidly growing renewable energy source in developed countries (Spiertz and Ewert 2009), maize, which is mainly used for ethanol production, becomes scarce as food or feed, whereas rising maize prices cause consumers to shift towards rice and wheat consumption, potentially leading to increasing prices for those commodities (Rosegrant 2008). In a joint letter to the European Commission (EC), 17 Non-Governmantal Organizations (NGOs) demanded the EC to suspend the European Union's mandatory biofuel target of 10% stating different reasons why the target is not sustainable, one being a potentially jeopardized food security for poor populations (2017). UN Human Rights Counselor *Jean Ziegler* demanded to '*stop this biofuels madness*' and claimed that a failure by the EU to cancel biofuel support '*would make them an accomplice to a crime against humanity*', Nestle Chairman and Chief executive *Peter Brabeck-Letmathe* stated, that '*to grant enormous subsidies for biofuel production is morally unacceptable and irresponsible*' (Tenenbaum 2008, Ziegler 2013). Another report estimated global calorie consumption to drop by 2% in most regions by 2020 if the trend towards biofuels is '*moderate*', whereas a

'*drastic*' biofuel expansion would reduce calorie consumption by more than 8% in Latin America and Sub-Saharan Africa (Tenenbaum 2008). Despite the opposing voices, the current EU target is to deliver 10% of energy in transport from renewable sources by 2020, however, biofuels produced from food-based feedstocks should not receive '*public support*' (Institute for European Environmental Policy 2014).
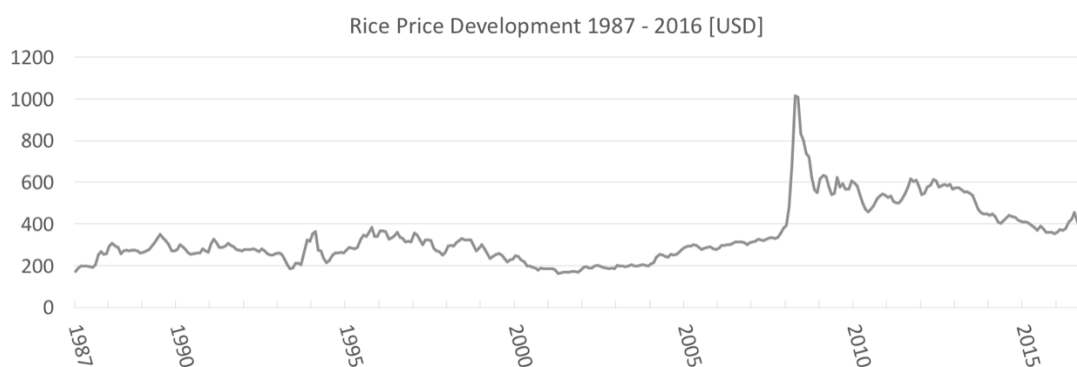
Figure 6. Rice price development from 1986 to 2016 in USD (Indexmundi).

2.3.6   Consumption

Rice is a staple food for more than half the world's population – its high popularity, even in the poorest sectors of urban populations, can be attributed to its ease of preparation, storage and cooking, low preparation costs, comparably low price and steady supply (Wopereis, Johnson et al. 2013). Among all staple crops, rice has had the fastest-growing consumption rate within the last decades (Wopereis, Johnson et al. 2013), especially In Africa (**Figure 7**).
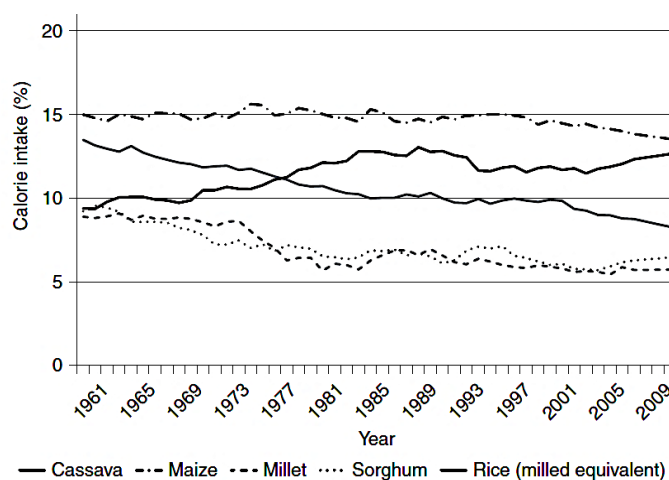
Figure 7. Contribution of staple food crops to Sub-Saharan African diets (Wopereis, Johnson et al. 2013)

Three models of rice consumption have been suggested: the *Asian*, the *Subtropic Developing Country*, and the *Occident model* with yearly per capita rice consumption of ~80 kg and more, 30 to 60 kg, and below 10 kg, respectively (Arendt and Dal Bello 2011). Global per capita rice consumption rose from 47 kg per year in 1970 to 54 kg in 2011; in Africa, per capita consumption more than doubled from 10.9 kg per year in 1970 to 23.5 kg in 2011, with roughly half of the rice being supplied locally (Muthayya, Sugimoto et al. 2014). In 2011, rice contributed to ~19% of the total global calorie intake, whereas it was on average ~30% in Asia, almost 10% in Africa and South America and 2.2 and 1.5% in Europe and North America, respectively (FAOSTAT). The highest contribution to calorie intake from rice was reported in Bangladesh (71%), Cambodia (63%) and Laos (61%) (FAOSTAT). With 29% calorie supply in low-income countries, policies affecting rice prices, production and trade have vastly impacted the poor (Wailes 2005).

2.3.7   Rice as commodity

Rice has been the single most important source of employment and income for rural people for centuries and fosters the strengthening of communities in rice growing areas (Tuong, Bouman et al. 2005). Over 400 new rice-containing products were placed on the market in the year 2000 (Arendt and Dal Bello 2011), owing to its flavor-carrying capability, hypo-allergenicity and bland flavor (Bryant, Kadan et al. 2001). In addition to being consumed directly, the major applications for rice are processed foods (breakfast cereals, puddings and bread) and beer production (Mitchell 2009). Rice bran can be used as livestock feed and medium for growing mushrooms and enzymes. Hulls are non-digestible, fibrous and abrasive and are used as animal feed, chicken litter or juice pressing aid (Mitchell 2009). Along with the husks, the rice straw hulls are also used as construction materials or for fuel production (Maclean, Hardy et al. 2013).

2.3.8   Rice preparation and quality assessment

Rice is considered a culturally sensitive commodity as its growing, selection and cooking are subject to regional, national and local preferences (Atungulu and Pan 2014). Many languages in rice-consuming countries use the words for rice and food synonymously (Steiger et al. 2014). Generally, two basic techniques for cooking rice are followed depending on and reflecting varietal differences and cultural preferences: cooking in large

(*Excess* or *American method*) or measured quantities (*Pilaf* or *Oriental method*) of water (Crowhurst and Creed 2001). Various modifications of those two techniques are used worldwide, such as 1) soaking and boiling in excess water; 2) boiling in excess water; 3) boiling without excess water; 4) rinsing and boiling without excess water and 5) frying and boiling without excess water (Atungulu and Pan 2014).

Three determinants for objective assessment of rice quality have been proposed: 1) instrumental measurement of cooking properties (investigating rice stickiness, hardness and firmness); 2) physicochemical analysis (determining the amylose content of rice and its effect on texture and eating quality as well as volumetric expansion); and 3) sensory evaluation (Rousset, Pons et al. 1995, Crowhurst and Creed 2001) and will not be further discussed within this dissertation.

2.4 Chemical Properties of rice

2.4.1 Composition of rice

Milled rice is predominantly a carbohydrate, mainly composed of starch (~78%) (Fitzgerald and Starch 2004), with small portions of pentosans, hemicelluloses and sugars (Houston, Houston et al. 1970). Protein is the second most abundant component of milled rice, constituting between 4 and 11% of the rice kernel (Fitzgerald and Starch 2004), depending on the rice type (Zhou, Robards et al. 2002). The protein content, which can affect the rice's texture, can be affected by climate and agronomic conditions (Hamaker 1993). Similar to fat, minerals and vitamins, the protein content of rice decreases by degree of milling as it is mainly found in the peripheral layers of the kernel (Zhou, Robards et al. 2002) **(Table 1)**.

In 2011, rice provided on average 14.7 g protein/capita/day in selected Asian countries, which is more than any other cereal (FAOSTAT). In contrast to other cereals, the primary storage protein for rice is glutelin instead of prolamin (Hamaker 1993). Due to a more evenly balanced amino acid profile of glutelin, rice has been suggested a richer protein source compared to other cereals despite its lower gross protein content (Hamaker 1993). Nevertheless, the essential amino acid lysine may be a limiting factor in rice (Young and Pellett 1994) **(Equation 1)**.

Evaluation of the dietary protein quality

Dietary protein quality evaluation should account for dietary amino acids as individual nutrients and data for digestible or bioavailable amino acids should be indicated where possible (FAO 2011). The measure for protein quality proposed by FAO – the digestible indispensable amino acid score (DIAAS; **Equation 1**) has replaced the earlier protein digestibility-corrected amino acid score (PDCAAS) (FAO 2011). While the PDCAAS used a single crude protein digestibility value, the DIAAS accounts for the ileal digestibility value of each amino acid (FAO 2011, Cervantes-Pahm, Liu et al. 2014). The apparent ideal digestibility of amino acids is defined as net appearance of ingested dietary amino acids from the digested tract proximal to the distal ileum; upon correction for the basal endogenous loss in pigs, it is termed the standardized ileal digestibility (SID).

Equation 1. Calculation of the Digestible Indispensable Amino Acid Score

$$\text{DIAAS [\%]:} \quad \frac{\text{Digestible dietary indispensable amino acid in 1 g dietary protein [mg]}}{\text{Dietary indispensable amino acid in 1 g reference protein [mg]}} \quad *100$$

Based on SID assessments in pigs, rice was ranked first among eight cereal grains, followed by dehulled oats and Nutridense maize and had the greatest SID of most amino acids compared to the other cereals (Cervantes-Pahm, Liu et al. 2014). Nevertheless, the DIAAS (%) of 64 for polished white rice is below the cut-off for a 'good' protein source (FAO 2011).

Table 1. Standardized ileal digestibility (SID) and digestible indispensable amino acid scores (DIAAS) for polished white rice (FAO 2011, Cervantes-Pahm, Liu et al. 2014).

Amino acid	SID	DIAA reference ratio
CP	88.07	
Arginine	92.45	
Histidine	91.39	1.33
Isoleucine	92.30	1.20
Leucine	93.84	1.13
Lysine	92.41	0.64
Methionine	94.66	
Phelynalanine	95.29	
Threonine	90.64	1.09
Tryptophan	94.70	1.12
Valine	94.34	1.28
Sulphur amino acids		1.83
Aromatic amino acids		2.36
Total amino acids [%]	94.05	
DIAAS [%]		64 (Lys)

CP, crude protein. SID was assessed in pigs.

2.4.2   Starch

Starch, the only quantitatively important digestible polysaccharide (Asp 1996), can contribute up to 80% of the daily calorific intake in some cultures, and has been suggested one of the most prominent agricultural products for the world's population (Fitzgerald and Starch 2004).

2.4.2.1   *Starch synthesis and structural properties*

Four main processes for starch synthesis have been suggested: (1) Acquisition of small sugars such as sucrose and glucose; (2) Synthesis of amylose and amylopectin; (3)

Organization of starch polymers into crystalline, semi-crystalline and amorphous regions; and, exclusive to rice and oats; (4) Organization of individual starch granules into compound starch granules (Fitzgerald and Starch 2004).

Biosynthetic pathways for starch in the cereal endosperm and the roles of several involved enzymes (ADP-Glc pyrophosphorylase, granule-bound and soluble starch synthase, starch debranching enzymes and starch phosphorylase) have been reviewed (James, Denyer et al. 2003, Jeon, Ryoo et al. 2010) and will not further be discussed within this dissertation. Rice starch granules are the smallest plant-produced starch granules and have an irregular polygonal shape with an average size of 2 – 7 μm (Wani, Singh et al. 2012). The granules evolved to store large amounts of glucose in a condensed, relatively dehydrated form to provide sufficient energy during germination and the early stages of plant growth and constitute ~80 – 90% of a milled grain's dry weight (Houston, Houston et al. 1970, Juliano and Bechtel 1985, Fitzgerald and Starch 2004, Arendt and Dal Bello 2011). Starch is arranged in endosperm cells with starch granules forming the disperse and proteins the continuous phase (Steiger et al. 2014). Its synthesis in rice becomes most active ~10 days after pollination (Fitzgerald and Starch 2004) and, similar to barley, begins at the apex of the grain and at the central region, whereas starch deposition occurs in the youngest cells near the aleurone layer, however, this process is not yet completely understood (Shannon, Garwood et al. 2009).

2.4.2.2   Amylose and amylopectin

Starch and the semi-crystalline granules are composed of amylose and amylopectin (Shannon, Garwood et al. 2009) and its properties depend on physical and chemical characteristics, such as mean granule size, granule size distribution, amylose/amylopectin ratio and mineral content (Wani, Singh et al. 2012). While amylose has a linear structure due to its 1-4 L-D-glucopyranosyl units, the polymers of amylopectin primarily contain (1-4) bonds with branches forming (1-6) linkages (Shannon, Garwood et al. 2009) (**Figure 8**). The average chain length for amylopectin is 20 – 30 glucosyl units, whereas for amylose it is 100 – 10 000 units (Shannon, Garwood et al. 2009), which translates to 25 – 50% and 30 – 60% of the branched fraction in rice compared to number and mass of amylose,

respectively (Zhou, Robards et al. 2002).

Amylopectin, unlike the less abundant amylose, is essential for starch-granule synthesis, whereas the contribution of amylose to the granule architecture is unknown (Buléon, Véronèse et al. 2007). Amylopectin conforms in crystalline double helices, which can be classified based on their three-dimensional packing structure (based on X-ray diffraction patterns) into A-, B-, and C-types, which can be natively found in cereals, tubers, and legumes, respectively (Wani, Singh et al. 2012, Doblado-Maldonado, Gomand et al. 2017). A- and B-type starches differ in their water content; B-type starch can accommodate more water molecules due to a large void in its structure (Wani, Singh et al. 2012). A-type crystallization is favored by higher temperatures, lower chain lengths and rather hydrophobic conditions, whereas the B-type is generally formed at lower temperatures with higher chain lengths (Buléon, Véronèse et al. 2007). In contrast, poorly water soluble V-type crystals are mostly formed by amylose in cereal starches treated with heat and moisture (Zobel 1988). Amylose has been studied intensively as it is considered a key indicator for cooking quality (Lee 1987, Fitzgerald and Starch 2004) and, furthermore, rice varieties of high and intermediate amylose content show low estimated glycemic scores *in vitro*, a benefit especially in individuals with impaired glucose tolerance (Hu, Zhao et al. 2004). Rice varieties are classified by their amylose content into common and waxy (or glutinous) varieties (Mitchell 2009), whereas waxy rice contains little or no amylose (Houston, Houston et al. 1970).
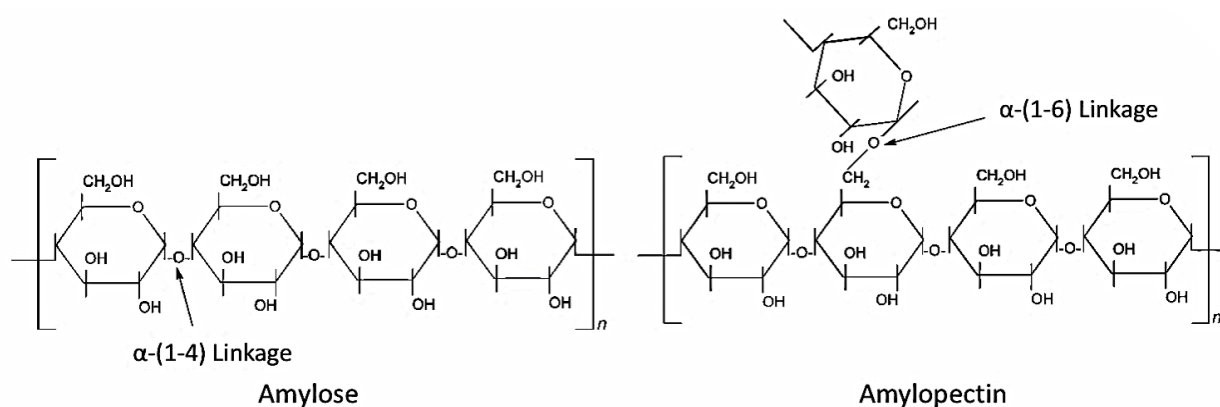
Figure 8. Chemical structure of starch – amylose and amylopectin units (Visakh et al. 2012).

Starch transformation

Raw rice grains undergo several transformation stages during cooking before they can be consumed. These processes include glass transition, gelatinization, swelling, pasting and leaching of amylose as well as retrogradation.

Glass transition

As described earlier, starch is semi-crystalline consisting of an amorphous and a crystalline phase. When applying thermal energy to an amorphous system, molecules move rendering the polymer viscous, rubbery and flexible (Zeleznak and Hoseney 1987). Glass transition of the amorphous phase occurs at temperatures at which the viscosity of the amorphous regions decreases while the mobility of the polymers increases, whereas glass transition of the crystallite phase occurs at temperatures, at which the solid becomes an amorphous liquid (Lund 1989).

Gelatinization

Starch gelatinization has been proposed one of the most important phase transitions in foods (Lund 1989) and has been described as a '*non-equilibrium melting process of partially-crystalline, kinetically metastable native starch in the presence of water*' or as '*disruption of molecular orders within the starch granule manifested by irreversible changes in properties such as granular, swelling, native crystallite melting, loss of birefringence and starch solubilization*' (Lund 1989). Intact starch granules are insoluble in cold water, but can reversibly absorb water and swell, however, with increasing temperature, starch molecules vibrate and intermolecular bonds break allowing to engage more water molecules, causing irreversible swelling and alteration of the granule structure, thus gelatinization (Lee 1987). In a nutshell: starch swells irreversibly upon heating in aqueous solutions and its crystalline structure collapses (Wani, Singh et al. 2012).

Several factors can influence starch gelatinization: Water can be considered as plasticizer, depressing the glass transition temperature of the continuous amorphous region, rendering the amorphous region sufficiently mobile for the metastable crystallites to melt upon heating to the melting temperature (Lund 1989). Salt and sugars also affect starch gelatinization temperature, sugars additionally delay pasting (Lund 1989).

Pasting

Pasting occurs concurrently with or after gelatinization as a result of continuous heating of starch in excess water and stirring, causing starch granules to swell and burst with subsequent leaching of amylose and granule disintegration, thus leading to the formation of a viscous material called paste (Wani, Singh et al. 2012). The most important pasting characteristic is viscosity, which determines the suitability of starch as thickening (in foods) or finishing agent (in the textile and paper industry) (Wani, Singh et al. 2012).

Starch Retrogradation

Starch retrogradation has been described as a non-equilibrium recrystallization of a completely amorphous but crystallizable polymer system (Slade and Levine 1988). Amylopectin recrystallizes from an amorphous state to a partially crystalline one, whereas moisture is redistributed and water molecules incorporated into the crystal lattice (Lund 1989, Englyst, Kingman et al. 1992). It has been suggested, that the amylose content affects starch retrogradation and textural properties of cooked rice, thus, cooked rice with low amylose content would retrograde slowly, whereas a high amylose content would facilitate retrogradation (Yu, Ma et al. 2009). Retrogradation causes formation of type 3 resistant starch, which is resistant to digestion, in contrast to retrograded amylopectin, which is digestible upon cooking (Boers, ten Hoorn et al. 2015).

An increased hardness of cooked rice after storage was reported, whereas rice high in amylose had the highest and waxy rice the lowest hardness upon storage, possibly due to leaching of amylose into cooking water, potentially generating a thick coating film on the rice grains (Yu, Ma et al. 2009). Hardness has been positively correlated with amylopectin retrogradation, indicating that both amylose and amylopectin retrogradation contribute to the hardness of cooked rice upon cooking and storage, respectively (Yu, Ma et al. 2009). This is in line with the suggestion, that amylose is responsible for short-term and amylopectin for longer-term rheological and structural changes of starch gels (Zhou, Robards et al. 2002).

3   IRON & ZINC

3.1   Iron

3.1.1   Iron homeostasis

Iron is the second most abundant metal on the planet and comprises 6.2 % of the Earth's crust (Bolm 2009, Erdman Jr, MacDonald et al. 2012). Despite its high abundance, iron scarcity is a prevalent condition for most organisms (Ganz 2013). Depending on its chemical environment, oxidation stages range from Fe^{2-} to Fe^{6+}, whereas ferric (Fe^{3+}) and ferrous (Fe^{2+}) are the only stable forms in an aqueous environment and naturally occur in biological systems (Lee, Clydesdale et al. 1979).

Along with other transition metals, iron shares two biologically important properties: (1) it has more than one relatively stable oxidation state, and (2) it can form complexes (Worwood and Hoffbrand 2005). It is able to participate in one-electron transfer reactions, such as the reduction of ribonucleotides to corresponding deoxy-ribonucleotides (Crichton 2016). Its readiness to accept or donate electrons, makes it highly reactive and toxic, however, its chemical reactivity is constrained by its association with prosthetic groups and proteins (Ganz 2013).

The human body contains ~3.5 – 4 g iron (Erdman Jr, MacDonald et al. 2012, Ganz 2013, Lane, Merlot et al. 2015), which roughly translates to 40 – 50 mg/kg in adults (Worwood and Hoffbrand 2005). Full term newborn infants contain ~80 mg/kg iron, which are utilized for growth (Erdman Jr, MacDonald et al. 2012) and are sufficient to cover the infants iron requirements for the first 4 – 6 months of life since breast-milk is a poor source of iron. The level of maternal iron stores is of little effect for fetal iron or breastmilk iron content and from approximately 6 months onwards iron stores in infants are depleted requiring the switch to an iron rich diet, traditionally corresponding with the weaning period, where solid foods are introduced. The marked decline in ID anemia prevalence in infants and young children in industrialized countries has been partly attributed to fortification of infant formulas and weaning foods (Lynch 2011). If adequate iron of sufficient bioavailability can be provided, stores gradually accumulate to ~5 mg/kg during childhood

(Worwood and Hoffbrand 2005). Due to a shift in the consumption of mainly animal source foods to a cereal-based diet of low micronutrient density, meeting micronutrient needs is challenging for infants, especially in the second 6 months of their life (Dewey 2013). Infants with a low birthweight do not have adequate iron stores and are at high risk for iron deficiency (ID) while being breastfed (Zimmermann and Hurrell 2007), and even healthy, exclusively breastfed infants (at 4 months of age) showed an ID- prevalence of 21 % (Dube, Schwartz et al. 2010).

The major cell types in postnatal iron homeostasis are duodenal enterocytes, macrophages and hepatocytes which absorb, recycle and store iron, respectively (Ganz 2013). The highest fraction of iron is found in hemoglobin (2000 – 3500 mg) (Zimmermann and Hurrell 2007, Ganz 2013), followed by myoglobin (400 – 500 mg), ferritin and hemosiderin (1000 mg), heme and non-heme enzymes (100 mg), an intracellular or transit pool (7 mg) and transferrin (3 mg) (Erdman Jr, MacDonald et al. 2012).

Four categories of metalloproteins are involved in the biological functions of iron:
1) globin-heme, non-enzymatic ferroproteins (e.g. hemo-, myo-, neuro- and cytoglobin);
2) heme-enzymes involved in electron-transfer and oxidase activities (e.g. cytochromes);
3) iron-sulfur clusters involved in electron transfer (e.g. NAPH dehydrogenases); and
4) activities that depend on iron as cofactor (e.g. phenylalanine, tyrosine and tryptophan hydroxylases) (Geuens, Brouns et al. 2003, Erdman Jr, MacDonald et al. 2012).

1) **Globin-hemes** can serve as a) transporters for oxygen, carbon mono- and dioxide, and nitric oxide, b) oxygen-stores, as well as c) scavengers of free radicals (Erdman Jr, MacDonald et al. 2012). In the mitochondria, Fe^{2+} is inserted into protoporphyrin IX, generating heme, the majority of which is subsequently incorporated into hemoglobin (Erdman Jr, MacDonald et al. 2012). One hemoglobin molecule can transport four oxygen molecules, therefore, blood carries 50 – 70 times more oxygen than plasma would alone (Erdman Jr, MacDonald et al. 2012). With roughly 1 mg/mL packed volume, erythrocytes show the highest iron concentrations (Ganz 2013). Erythrocytic hemoglobin, can form oxy- and desoxy-hemoglobin by binding, respectively releasing, oxygen depending on a molecule within the globin (Erdman Jr, MacDonald et al. 2012).

Fetal hemoglobin has a higher affinity for oxygen than adult hemoglobin, enabling the fetus to acquire and release oxygen from maternal hemoglobin at the placenta and *in utero*, respectively (Erdman Jr, MacDonald et al. 2012).

Myoglobin is the oxygen-store in the muscle and its functions equal those of hemoglobin (Erdman Jr, MacDonald et al. 2012). Neuroglobin is present in the human brain where it has been suggested to prevent neurons against hypoxic damage (Pesce, Dewilde et al. 2003). Cytoglobin, which is located in the nucleus, has been attributed a regulatory role involved in gene transcription upon stimulation (Geuens, Brouns et al. 2003).

2) **Heme** moieties are present in cytochromes, facilitating electron transfer reactions; in cytochrome oxidase and catalase, where they activate substrates; or as nitric oxide (NO) sensor in guanylate cyclase (Papanikolaou and Pantopoulos 2005).

3) **Iron sulfur clusters**, the most prevalent forms of non-heme iron in metalloproteins, are involved in electron transfer, transcriptional regulation, structural stabilization and catalysis (Papanikolaou and Pantopoulos 2005).

4) **Aromatic amino acid hydroxylases** are non-heme iron enzymes and are required by many organisms for a functioning central nervous system (Erlandsen, Kim et al. 2002).

3.1.2 Iron absorption

The human body has no known excretion mechanism for iron and body iron is efficiently conserved and recycled, whereas losses (such as losses during menstruation and cell desquamation) are compensated through gastrointestinal absorption, leading to a comparatively small proportion for required absorbed dietary iron (Hurrell and Egli 2010, Erdman Jr, MacDonald et al. 2012).

Heme and non-heme iron

Iron bioavailability depends on the iron source (Lee, Clydesdale et al. 1979), whereas two basic forms for dietary iron exist: heme iron, which is present in meat and fish products

and accounts for ~5 – 10% of dietary iron intake and non-heme iron, present in both animal source and plant-based foods, accounting for 90 – 95% (Hallberg 1981, Sharp and Srai 2007, EFSA 2015). Contamination iron in Western-type meals appears to be relatively small, nevertheless, in developing countries it can be present in large amounts (Hallberg, Björn-Rasmussen et al. 1983) and may thus be nutritionally relevant (WHO 2004). However, research on contamination iron is limited and will not be further discussed here.

The amounts of heme and non-heme iron consumed and the presence of factors influencing iron bioavailability and iron status have been suggested as main factors influencing dietary iron absorption (Hallberg 1981).

Heme iron has a bioavailability of 20 – 30% (EFSA 2015), ranging between ~10 – 40 % in iron repletion and ID, respectively (WHO 2004). Non-heme iron has a comparably lower bioavailability (1 – 10%), and is greatly influenced by other dietary components (Sharp and Srai 2007). Main factors that contribute to non-heme iron absorption include the subjects' iron status as well as iron content and composition of the administered meals (Rossander, Hallberg et al. 1979). Healthy people who received 50 mg iron supplements showed a decreased non-heme iron absorption, while heme iron absorption remained unaffected (Roughead and Hunt 2000).

Physiologic mechanisms of iron absorption

Iron status has been suggested as major determinant for the efficiency of iron absorption (Thankachan, Walczyk et al. 2008), however, dietary composition additionally and independently affects iron absorption from whole diets (Moretti, Zimmermann et al. 2006, Collings, Harvey et al. 2013).

The complex process of iron absorption involves at least three steps, namely: 1) digestion and release from the diet; 2) enterocyte uptake; and 3) transport from the enterocytes to the circulation (Salovaara, Sandberg et al. 2002). Acidic and proteolytic enzymes in the stomach and small intestine release iron from protein complexes (Worwood and Hoffbrand 2005). The uptake is decreased in the increasingly alkaline environment in the jejunum, likely due to the formation of insoluble ferric hydroxide complexes (Worwood and Hoffbrand 2005).

Iron absorption and excretion in men amounts to roughly 0.8 mg iron daily, whereas in women of childbearing age 1.4 mg are required to compensate for menstrual losses (Zimmermann and Hurrell 2007). Iron requirements are covered through ferroportin-mediated export from intestinal enterocytes, iron-recycling macrophages and iron storage tissues (Muckenthaler, Rivella et al. 2017).

Three major cell types are employed in postnatal iron homeostasis: 1) **duodenal enterocytes** that absorb dietary iron via receptor-mediated endocytosis of iron-bound transferrin (Ganz 2013, Muckenthaler, Rivella et al. 2017); 2) **macrophages** which recycle iron from erythrocytes and other cells; and 3) **hepatocytes** which store iron and release it, when needed (Ganz 2013). Iron absorption is highest from the duodenal enterocytes, and apical transport of heme and non-heme iron occurs through independent pathways (Sharp and Srai 2007). Iron absorbed by enterocytes, which is not taken up by transferrin, is mainly excreted in the feces and only little iron is excreted into the urine (Otten, Hellwig et al. 2006).

Non-heme iron absorption

Non-heme iron enters the gastrointestinal tract in its Fe^{3+} form and is converted to absorbable Fe^{2+} (Sharp and Srai 2007) via **Duodenal cytochrome b reductase** (Dcytb) and then transported across the brush border membrane via **Dimetal Transporter 1** (DMT-1) (Worwood and Hoffbrand 2005, Muckenthaler, Rivella et al. 2017), an active, cellular uptake mechanism for divalent cations (Gunshin, Mackenzie et al. 1997). Likely, most iron enters the labile pool, part of it may be incorporated into ferritin and subsequently lost during cell exfoliation (Worwood and Hoffbrand 2005). Iron is then transported across the serosal membrane by **ferroportin 1** and is subsequently taken up as Fe^{3+} by **transferrin** (Worwood and Hoffbrand 2005). Most recently, Caco-2 cell-lines have been suggested an alternative pathway for iron uptake from nanosized ferric phosphate (NS $FePO_4$) via clathrin-mediated endocytosis and micropinocytosis (Perfecto, Elgy et al. 2017).

Heme iron absorption

The uptake mechanism for heme iron is yet to be fully understood, earlier research suggested that **heme carrier protein** (HCP1) is involved (Sharp 2010, Pizarro, Olivares et al. 2016), which is upregulated by hypoxia and iron-deficiency and might also transport

folate (Zimmermann and Hurrell 2007). However, more recently, an essential contribution of HCP1 to heme iron transport has been questioned (Ganz 2013). Furthermore, **heme responsive gene-1** has been suggested to be involved in the transport of heme recovered from senescent erythrocytes (Ganz 2013).

Ferritin and Hemosiderin

Ferritin, a 24-subunit protein, is abundant in all cells and serves as cytosolic and mitochondrial iron depot which stores iron in a non-toxic form, whereas iron can be mobilized upon ferritin degradation (Wang, Knovich et al. 2010, Erdman Jr, MacDonald et al. 2012, Muckenthaler, Rivella et al. 2017). One ferritin molecule can reversibly bind up to 4500 iron atoms, however, only 20 – 50% of this capacity is usually occupied (Erdman Jr, MacDonald et al. 2012, Muckenthaler, Rivella et al. 2017). Serum ferritin is an acute phase reactant and serves as marker of acute and chronic inflammation, which is elevated nonspecifically in a wide range of inflammatory conditions (Wang, Knovich et al. 2010). Ferritin concentrations vary by age and sex, they are high at birth and during the first two months of life and subsequently fall throughout later infancy (WHO 2011). Ferritin is positively correlated with size of the total body iron stores in the absence of inflammation (WHO 2011). Low plasma (or serum) ferritin concentrations reflect depleted iron stores, but not necessarily the severity of the depletion (WHO 2011).

Hemosiderin is considered a degradation product of ferritin and can be found in tissues associated with iron storage, such as the liver, spleen and bone marrow (Anderson and McLaren 2012). It forms under conditions of iron excess, is insoluble and has a smaller particle size than cytosolic ferritin cores (Anderson and McLaren 2012).

Transferrin and Transferrin receptors

Transferrin is the main iron-carrier in the circulation as well as extracellularly and transports iron to cell-surface transferrin receptors (TfRs) on erythroblasts and other tissues (Erdman Jr, MacDonald et al. 2012), while maintaining Fe^{3+} in a redox-inert state (Abbaspour, Hurrell et al. 2014). Four molecular forms of transferrin exist: apotransferrin, mono-ferric A and B transferrin as well as di-ferric transferrin, however, all transferrin bound iron forms one physiologically homogenous pool (Brissot, Ropert et al. 2012). Total

iron content of transferrin corresponds to > 0.1% of body iron, however, it undergoes >10x daily turnovers to sustain erythropoiesis (Abbaspour, Hurrell et al. 2014). TfRs are the main means for iron transport into cells (Anderson and McLaren 2012), whereas iron- saturation of transferrin is approximately 30 % (Brissot, Ropert et al. 2012). To date, TfR1, which is expressed on all proliferating cells, and TfR2, mainly expressed on hepatocytes, have been identified (Anderson and McLaren 2012). Transferrin-bound iron distribution is mediated through blood plasma, which contains 2 – 4 mg iron (Ganz 2013) and has a half-life of roughly 75 minutes (Erdman Jr, MacDonald et al. 2012).

Non-transferrin bound iron

Non-transferrin bound iron (NTBI) is neither bound to transferrin nor does it correspond to heme or ferritin iron and its main form was suggested to be iron (III) citrate (Brissot, Ropert et al. 2012). NTBI may be involved in pathological conditions in relation to iron overload and elevated levels may cause cellular damage at both the cellular surface and intracellularly (Brissot, Ropert et al. 2012).

Ferroportin

Ferroportin is essential for dietary iron absorption as it recycles iron from senescent erythrocytes, mobilizes stored iron and transfers iron to the developing fetus (Drakesmith, Nemeth et al. 2015). Macrophages regulate ferroportin synthesis on transcriptional and translational levels (Drakesmith, Nemeth et al. 2015). Nevertheless, the structural basis and transport mechanisms of this multimembrane protein remain to be fully understood (Drakesmith, Nemeth et al. 2015).

Hepcidin

A more direct regulation of the iron homeostasis has been suggested via the predominantly hepatic peptide hepcidin. Hepcidin posttranslationally controls the membrane concentration of the iron exporter ferroportin; this interaction with ferroportin governs the iron-flux into plasma and the iron supply available to iron-consuming tissues (Ganz 2013). Ferroportin is the only known hepcidin-receptor (Drakesmith, Nemeth et al. 2015). Upon binding of hepcidin to ferroportin, the internalization, ubiquitination and

subsequent lysosomal degradation of ferroportin is triggered (Hentze, Muckenthaler et al. 2010, Muckenthaler, Rivella et al. 2017). Hepcidin expression is downregulated in case of ID, hypoxia and increased erythropoietic activity and upregulated when iron stores are replete or during inflammation (Worwood and Hoffbrand 2005, Erdman Jr, MacDonald et al. 2012).

The elimination of serum hepcidin is yet to be understood and it is still debated, whether its degradation is a consequence of or caused by kidney filtration (Silva and Faustino 2015). Hepcidin has recently been proposed as useful diagnostic test for ID in Gambian pregnant women (Bah, Pasricha et al. 2017).

3.1.3 Iron requirements

The calculation of iron requirements is based on factorial modeling, accounting for the following factors: basal iron losses, menstrual losses, fetal requirements in pregnancy, increased requirements during growth for the expansion of blood volume and increased tissue and storage iron (Otten, Hellwig et al. 2006).

A normal Western diet provides ~15 mg of iron daily, whereas half of it is released in its soluble form within the gut lumen, about 3 mg are taken up by mucosal cells and 1 mg is transferred to the portal blood (Worwood and Hoffbrand 2005). To maintain iron balance, adult men need to absorb ~1 mg iron daily, whereas needs for menstruating women range from 1.5 – 3.4 mg, and up to 4 – 5 mg for women in late stages of their pregnancy (Otten, Hellwig et al. 2006). Requirements are also increased during childhood, especially in periods of rapid growth in early childhood and adolescence (Otten, Hellwig et al. 2006) (**Figure 9**). Selected food sources for iron are given below (Table 2).
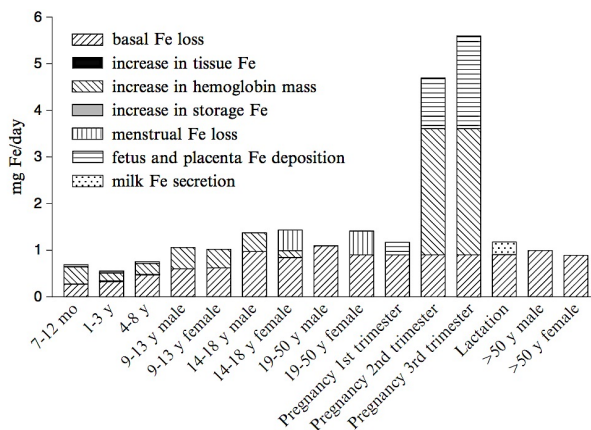
Figure 9. Estimated daily average iron requirements during different life stages (Anderson and McLaren 2012).

The tolerable upper intake level (UL) is the maximum amount of daily nutrient intake, which likely poses no risk of adverse effects for healthy people (Otten, Hellwig et al. 2006). For iron the UL is 45 mg for both adolescents and adults whereas for infants and children it is 40 mg (Trumbo, Yates et al. 2001, Otten, Hellwig et al. 2006). The UL for iron is based on gastrointestinal distress as critical adverse event and should not be exceeded routinely, however, people with conditions such as hereditary hemochromatosis or certain liver diseases may not be protected by the UL (Otten, Hellwig et al. 2006).

Table 2. Selected food sources for heme and non-heme iron (Anderson and McLaren 2012).[1]

Selected heme iron sources	Iron supply per serving [mg]
Pork liver sausage[2]	9.4
Breaded, fried oysters[2]	6.0
Braised lean beef chunk [2]	3.2
Breaded, fried clams[2]	2.2
Roasted dark meat turkey[2]	2.3
Roasted duck[2]	2.3
Roasted chicken breast[2]	1.1
Canned white tuna[2]	0.8
Selected non-heme iron sources	
Iron-fortified ready-to-eat cereal[3]	18
Boiled soybean[3]	8.8
Boiled lentils[3]	6.6
Cooked spinach[3]	6.4
Boiled kidney beans[3]	5.2
Boiled black beans[3]	3.6
Whole egg (1 piece)	1
Whole wheat bread (1 slice)	0.9

[1] Data compiled from the USDA Food Composition Database (USDA)
[2] Values are indicated per 85 g (3 ounces).
[3] Values are indicated per 237 g (1 cup).

2.1.4 Factors influencing non-heme iron absorption

A recent review identified ascorbic acid (AA) and meat (including poultry and fish) among the key dietary enhancers of iron absorption, whereas tannins, calcium and dairy products, polyphenol (PP), phytatic acid (PA), certain animal proteins (such as milk and egg) and micronutrients (such as zinc and copper) were proposed as inhibitors (Collings, Harvey et al. 2013). However, earlier multiple regression analyses suggested that 16.4% of the variance in iron absorption was accounted for by animal tissue, PA and AA, whereas calcium and PP were no significant predictors (Reddy, Hurrell et al. 2000).

No conclusions can be drawn on the effects of vitamin A, carotenoids and non-digestible carbohydrates on iron absorption as the existing scientific evidence is limited and/or contradictory (Hurrell and Egli 2010). A compilation of the major suggested enhancers and inhibitors of iron absorption is given below. However, for certain substances findings are conflicting and an extensive review would exceed the scope of this dissertation.

2.1.5 Enhancers of non-heme iron absorption

Common strategies to improve iron absorption are: 1) the use of organic acids (such as AA) or EDTA in combination with the iron compound; (2) using a fortification iron form that is protected from dietary inhibitors; or (3) degradation/removal of PA (Hurrell 2002).

Organic acids

Organic acids can enhance non-heme iron absorption through reducing Fe^{3+} to Fe^{2+}, the most potent enhancer being AA (Hallberg 1981, Sharp and Srai 2007) even in whole diets (Collings, Harvey et al. 2013). Saloovara et al. organized organic acids based on their effect on iron uptake from Caco2 cells into four groups, whereas representatives of each group were mainly structurally related and showed similar properties and iron uptake (Salovaara, Sandberg et al. 2002).

- *Ascorbic acid*

Ascorbic acid can increase the absorption of native and fortification iron several-fold through its reducing and chelating properties (Hurrell 1997), given its stability in a food vehicle is ensured (Teucher, Olivares et al. 2004). It mainly acts in the stomach and

duodenum (Hurrell 2002) and can form soluble complexes with iron at low pH and decreases the formation of insoluble complexes in the gut (Hurrell 1997).

In adults, iron absorption from a breakfast meal containing ~3 mg iron more than doubled (0.40 mg vs. 0.16 mg absorbed iron), when administered with orange juice containing 70 mg AA (Rossander, Hallberg et al. 1979). Intracellularly, AA distributes electrons to DCYTB to facilitate enzymatic reduction of Fe^{3+} in the luminal membrane of human enterocytes and it has been hypothesized, that it causes an iron-dependent activation of IRP2, which blocks ferroportin mRNA-translation (Scheers and Sandberg 2014).

Meals containing low to medium levels of absorption inhibitors require AA addition at molar ratios AA/Fe of 2 : 1, whereas in the presence of high levels of inhibitors, the addition should amount to at least 4 : 1 (Teucher, Olivares et al. 2004). Due to its relatively high cost, instability and potential to cause sensory changes it is generally not used as an enhancer in fortified staple foods (Teucher, Olivares et al. 2004).

Iron chelators

Chelating ligands prevent iron from binding with water and subsequent formation of ferric hydroxides; instead, they increase the solubility and thus bioavailability of iron (Zhu, Glahn et al. 2009). Certain chelating agents, however, may form strong iron-complexes and are poor iron-donors, hence inhibiting iron absorption (Salovaara, Sandberg et al. 2002). Whether chelated iron is transported across the intestinal mucosal barrier via DMT-1 and when it is released from its chelating ligand, remains unclear (Zhu, Yeung et al. 2006).

- *Citric acid (CA)*

Iron absorption upon addition of CA was increased in adults consuming rice meals (1 g CA/ 3 mg iron as $FeSO_4$) (Gillooly, Bothwell et al. 1983) or oat-based beverages (60 mg CA/ 1.25 mg iron as FePP) (Zhang, Önning et al. 2007). In contrast, CA addition to a Latin American meal containing maize, rice and black beans (1 g CA/ 4.3 mg native iron) even decreased iron absorption (Hallberg and Rossander 1984). CA addition to a $FeSO_4$-fortified fish sauce (molar ratio to iron ~2.5) did not increase iron absorption in Thai women, however, the iron reference dose in this study was not administered within the sauce, which may have impacted the results (Walczyk, Tuntipopipat et al. 2005).

Soluble ferric pyrophosphate (SFP), where ferric iron is chelated to citrate and pyrophosphate ligands, has been suggested a promising source for fortification-iron in foods due to its solubility over wide pH-ranges (Zhu, Glahn et al. 2009). Iron uptake from SFP in Caco2-cells was equal to or higher than from simple iron salts such as $FeCl_3$ or $FeSO_4$ and significantly increased Caco2-ferritin formation (Zhu, Glahn et al. 2009). The underlying mechanism is likely a reduction from ferric to ferrous iron, which, upon reduction, is taken up in the ionic form at the brush border site in the small intestine after dissociation from pyrophosphate and citrate ligands (Zhu, Glahn et al. 2009).

Furthermore, *in vitro* iron dialyzability from whole-wheat bread increased with CA addition and even showed enhanced phytate degradation (Porres, Etcheverry et al. 2001). *In vitro* iron diffusibility from plain wheat flour increased in the presence of CA (Hazell and Johnson 1987) and CA in low-molecular weight human milk fractions equimolar to iron increased iron uptake in Caco2 cells (Palika, Mashurabad et al. 2013). However, a maximal iron absorption at relative concentrations of CA/iron of 2 : 1 has been suggested, followed by a decrease when exceeding 20 : 1 (Glahn, Lai et al. 1998).

- *EDTA and NaFeEDTA*

EDTA chelates through its four negatively charged carboxylic acid and two amine groups, whereas its effectiveness depends on the stability constant between EDTA and the metal, which is influenced by pH and molar ratio (Hurrell 1997). Of the nutritionally important metals, Fe^{3+} has the highest stability constant (Hurrell 1997). The proposed mechanism for NaFeEDTA absorption is a firm bonding of Fe^{3+} to EDTA in the stomach, followed by iron release and subsequent absorption in the duodenum and EDTA excretion in the stool (Hurrell 1997). A major advantage of NaFeEDTA over many other iron fortificants is that it prevents iron from binding with PA, reflecting in a 2 – 3 times higher iron absorption compared to $FeSO_4$ in meals containing a considerable amount of PA (Hurrell 1997, R 2002). Furthermore, NaFeEDTA was suggested to prevent fat-oxidation reactions during storage (Hurrell 1997, Hurrell, Reddy et al. 2000), and unlike other soluble iron compounds, it causes no peptide-precipitation when added to fish or soy sauce (Hurrell 2002).

While NaFeEDTA and Na_2EDTA increased iron absorption from $FeSO_4$-fortified wheat-based infant cereal 2 to 4-fold, Na_2EDTA did not influence iron absorption from water

insoluble FePP (Hurrell, Reddy et al. 2000). However, the authors suggested, that this effect may be iron compound or even batch-specific. According to the European Food Safety Authority (EFSA), NaFeEDTA raises no safety concerns regarding genotoxicity or carcinogenicity as long as daily EDTA exposure remains below 1.9 mg EDTA/ kg bodyweight (Additives and Nutrient Sources added to 2010). However, NaFeEDTA is not considered as GRAS, given an insufficient amount of scientific data (SCOGS).

A study in non-anemic weanling rats showed similar iron absorption from $FeSO_4$ or NaFeEDTA containing meals administered over 7 days, whereas non-heme iron content was lower in the liver and spleen in the NaFeEDTA compared to the $FeSO_4$ group and the authors hypothesized that free EDTA may travel to major iron storage sites (such as liver and spleen) upon absorption and subsequently mobilizes or redistributes iron (Zhu, Yeung et al. 2006).

Other enhancers

The nature of the *'meat factor'*, enhanced non-heme iron absorption when ingested with meat, remains unclear, but some attribute the effect to the protein fraction or other parts of muscle tissue in meat (Hurrell and Egli 2010) and it has been suggested that it origins from peptide digestion products rather than free amino acids (Hurrell, Reddy et al. 2006). However, other muscle tissue components may be involved and an enhanced iron absorption may not only be due to a single peptide fraction but rather a multitude of small peptides (Hurrell and Egli 2010).

Enhancers of heme iron absorption

The digestive enzymes trypsin and mucin showed enhanced heme iron uptake in Caco2-cells, and trypsin increased heme iron absorption in humans when combined with hemoglobin (Pizarro, Olivares et al. 2016). Furthermore, a recent study in women showed reduced heme iron bioavailability when heme (derived from rabbit and calf blood) was ingested with fish and chicken, whereas heme ingested with beef showed no such reduction (Pizarro, Olivares et al. 2016).

Inhibitors of non-heme iron absorption

Common dietary inhibitors of native food and fortification iron absorption include PA, phenolic compounds, calcium and certain milk or soy proteins (Hurrell, Juillerat et al. 1992, Hurrell 2002). Eggs, oxalate, succinate and cystein have been proposed as potential absorption inhibitors as well (Hallberg 1981).

- *Phytic acid*

Phytate is the principal storage form of phosphorous in cereals, legumes and oleaginous seeds (Sharp and Srai 2007, Gibson, Bailey et al. 2010, Gibson, Bailey et al. 2010). Phytic acid complexes metal ions, such as zinc, iron and calcium, but not copper, thus rendering them unavailable for intestinal absorption (Gibson, Bailey et al. 2010). It is the main inhibitor of iron absorption, whereas its negative effect is dose-dependent and already starts at concentrations as low as 2 – 10 mg/meal (Hurrell and Egli 2010). Phytate degradation is desirable – unless otherwise possible and the iron/PA molar ratio should be <0.7 : 1 to prevent from an inhibition of iron absorption (Hurrell 2002). Several approaches to remove or degrade phytate exist, such as aqueous extraction, heat treatments, extrusion cooking or roller-drying, soaking, germination fermentation or the addition of exogenous phytases (Hurrell 2004).

 In healthy volunteers, PA decrease in wheat flour from 1g/100g to 0.1g/100g caused a 2-fold increase in iron absorption, whereas complete dephytinization lead to a 5-fold increase (Hurrell 2002). Phosphorous alone has shown an inhibitory effect on iron absorption potentially reflecting dietary phytate in its inhibitory properties (Cook and Reddy 2001).

- *Polyphenolic compounds*

Polyphenolic compounds are abundant in all plant products and can hinder iron absorption dose-dependently via binding of iron to tannic acids in the intestine (Otten, Hellwig et al. 2006, Jaramillo, Briones et al. 2015). Besides chelating iron in the intestinal lumen, PP may also play a more complex role in the regulation of iron absorption at cellular and molecular levels (Sharp 2010).

- *Calcium*

Contrary to other iron absorption inhibitors, calcium negatively affects both non-heme and heme iron absorption, possibly during initial uptake into the enterocytes (Hurrell and Egli 2010). It has been suggested, however, that this effect is limited when investigated in multiple-meal studies including more diverse diets (Reddy and Cook 1997). Furthermore, milk has shown a decreased iron absorption from meals with low iron bioavailability (Hallberg 1981). In contrast, a recent review indicated no significant inhibitory effects on iron absorption for calcium, milk or phytate, however, the amount of reviewed data was limited (Collings, Harvey et al. 2013).

- *Zinc*

Zinc was suggested an iron absorption inhibitor and will be discussed in **Chapter 2.4**.

- *Hemoglobin*

Hemoglobin as food additive is added as dried red blood cells (Hurrell 1997). Iron absorption from this source is relatively high as iron contained within the porphyrin-ring of the heme-molecule is protected from major iron absorption inhibitors (Hurrell 1997). In women, heme iron bioavailability from hemoglobin was similar to that of heme only (Pizarro, Olivares et al. 2016). Despite its high absorption and protein provision, hemoglobin as food additive has two major disadvantages, which make it less useful for food fortification, due to its relatively low iron content and rather intense color (Hurrell 1997). Additionally, technical difficulties regarding collection and processing of animal blood as well as a potential cultural aversion limit its usability for mass fortification (Hurrell 1997).

2.1.6 Single vs. multiple meal administrations

Absorption from isolated meals may exaggerate the importance of dietary non-heme iron bioavailability compared to multiple meal administrations (Cook, Dassenko et al. 1991, Singh, Sanderson et al. 2006, Collings, Harvey et al. 2013). Thus, a varied diet allows a more realistic evaluation of the nutritional effect of non-heme iron bioavailability (Reddy, Hurrell et al. 2006). When comparing iron absorption from bean meals with high respectively low PP contents administered twice daily, iron absorption from the high PP

meals was 27% lower than from the low PP meals, however, when similar meals (different bean varieties) were fed along with rice or potatoes twice daily for five consecutive days, no difference in iron absorption could be shown (Petry, Egli et al. 2012). Another study suggested a far less pronounced facilitating effect of vitamin C on iron absorption, when consuming fortified wheat rolls over five days compared to a single consumption (Cook and Reddy 2001). Tidehag et al. found an almost 80% higher iron absorption in ileostomy subjects from a low-fibre diet administered as single meal compared to the average iron absorption from the same meal given over five consecutive days (Tidehag, Hallmans et al. 1996). A high-fibre diet following the same procedures showed a 48% higher iron absorption from the single meal, however, this difference was not statistically significant (Tidehag, Hallmans et al. 1996). Furthermore, the inhibiting effect of calcium on iron absorption was less pronounced in males and females consuming a varied diet over 5 days or a complete diet compared to a single consumption of a similar diet (Reddy and Cook 1997). Thus, the extent of enhancement or inhibition is less pronounced when the absorption is assessed over several days in contrast to single meal administrations, however, single meal studies are useful to identify relative effects of enhancers or inhibitors on iron absorption (Reddy, Hurrell et al. 2000).

2.1.7 Iron deficiency and anemia

2.1.7.1 Iron deficiency

Iron deficiency (ID) ID is the most common micronutrient deficiency worldwide, affecting almost 1/3 of the world's population (de Benoist, McLean et al. 2013). Red blood cells become hypochromic and microcytic, when produced under iron-restricted conditions, however, it may take months until clinical symptoms become apparent (Anderson and McLaren 2012). Causes for ID are manifold and include physiologic, environmental, pathologic, drug-related and genetic reasons (Camaschella 2015). The risk for ID is highest, when iron requirements exceed the energy needs, which is likely the case in infants and children, adolescents, menstruating or pregnant women (Zimmermann and Hurrell 2007). Decreased hemoglobin values are often a sign of advanced ID (Anderson and McLaren 2012), nevertheless, serum ferritin has been suggested as the most sensitive and specific marker to assess ID, however, iron status should be determined as a whole rather than

relying on single test results (Camaschella 2015), as measurements may be affected by confounding factors, rendering no single measurement ideal for all clinical circumstances (Worwood and Hoffbrand 2005). Most widely used parameters for ID assessment and special considerations for their application are indicated below **(Table 4)**.

2.1.7.2 Anemia

Anemia is characterized by a decreased quantity of red blood cells often in combination with reduced hemoglobin levels or altered blood cell morphology (Kassebaum, Jasrasaria et al. 2014). Thus, any condition causing a shortage of functional hemoglobin or decreased red blood cell mass may cause anemia (Kassebaum 2016).

For a systemic analysis of global anemia burden, Kassebaum identified 17 causes of anemia, whereas the main causes were attributed to ID, followed by hookworm, sickle cell disorders, thalassemia, schistosomiasis and malaria (Kassebaum, Jasrasaria et al. 2014, Pasricha 2014). More than half of the anemia cases were attributed to ID (Kassebaum 2016), which is indicated by serum ferritin levels < 30 µg/L, whereas in combination with inflammation or other conditions, the cutoffs are < 100 or 300 µg/L, respectively (Camaschella 2015).

Anemia accounted for 65.5 million years lived with disability (YLD) in 1990 and 68.4 million YLD's in 2010, corresponding to 11.2% and 8.8% of all YLD in the given period, respectively (Kassebaum, Jasrasaria et al. 2014). Despite the absolute increase in YLD's from 1990 to 2010, anemia prevalence decreased from 40.2% to 32.9%, whereas the decrease occurred in both sexes and for all severities of anemia (Kassebaum, Jasrasaria et al. 2014). Nevertheless, total anemia prevalence had a more pronounced reduction in males than in females, and females had higher anemia prevalences in virtually all regions (Kassebaum, Jasrasaria et al. 2014). In contrast, male children showed a higher anemia prevalence than female ones, possibly due to the prevalence of mild anemia resulting from hookworm infestation (Kassebaum, Jasrasaria et al. 2014).

The prevalence of ID anemia can be an indicator for zinc deficiency, although they are not causally related, but the distribution of iron and zinc in foods and dietary components that impact their absorption, are similar (Brown, Rivera et al. 2004). While anemia is not necessarily an indicator for zinc deficiency, it is indicated to assess zinc status in regions with high ID anemia prevalence (Brown, Rivera et al. 2004).

Table 4. Indicators for ID compiled from (Worwood and Hoffbrand 2005, Zimmermann and Hurrell 2007, Camaschella 2015).

Parameter	Properties	Cutoff values for ID[1] or anemia[2]		Confounders & Considerations
Hemoglobin (g/L)		6 months – 5 years	<110	Low specificity and sensitivity as isolated parameter.
		6 years – 11 years	<115	
		Non-pregnant women	<120	
		Pregnant women	<110	
Serum ferritin (µg/L)	Non-invasive measure of iron stores in healthy subjects.	5 years or younger	<12	Most useful parameter for measuring iron status. Diagnosis of ID anemia in anemic patients. Increased as acute phase protein and by damage to iron-rich organs (tissue ferritin); decreased by ascorbate deficiency. Limited usefulness in pregnancy (WHO 2011).
		Children > 5 years/ adults	<15	
		Presence of infection (all ages)	<30	
		ID anemia	<10	
Serum iron	Reflects iron supply to tissues.	Serum transferrin saturation	<15%	Short-term fluctuations render single value difficult to interpret.
		→ insufficient for erythropoiesis	normal	
Total iron binding capacity (TIBC)				Elevated TIBC indicates ID; decreased serum iron with normal/reduced TIBC indicates infection and inflammation.
Serum transferrin receptors	Reflect numbers of erythroid precursors and iron supply to the bone marrow.			Valuable indicator for deficient body iron stores in anemia of chronic disease. Increased by enhanced erythropoiesis and ID, not greatly affected by acute-phase response, but potentially by malaria, age and ethnicity. Limited application due to high costs and lack of international standards.

Continued

Parameter		Cutoff	Comment
Red cell protoporphyrin	Accumulation of protoporphyrin due to restricted iron supply to the eryhtron.		Useful screening test in the field. Reflects reduced iron supply over previous weeks, but may not reflect current iron supply. Increased levels may be due to sidero-blastic anemias and lead poisoning.
	5 years or younger	>70	
	Children > 5 years/adults	>80	
	Children > 5 years/ adults on washed red blood cells	>40	
Reticulocyte hemoglobin content (CHr) (pg)			Sensitive parameter – decreases within days of onset of ID erythropoiesis; can be confounded by increased MCV and thalassemia.
	Infants and young children	<27.5	
	Adults	≤28.0	
Red cell MCV/MCH (cu µm)	Reflects iron supply to erythroid marrow.		May be reduced in other disorders of hemoglobin synthesis in addition to ID.
	MCV: Children older than 11 years and Adults	<82	
	MCH	<27	
Red cell ferritin			Little practical application due to elaborate preparation.
% of hypochromic red cells	Reduced iron supply to erythron results in increasingly hypochromic new red cells.		
Transferrin saturation (%)		<16	Inexpensive, but limited use due to diurnal variation in serum iron and is affected by many clinical disorders
s-TfR-to-SF ratio			Assay specific, quantitative estimate of total body iron. May not be used in cases of high inflammation (elevated SF independent of iron stores).

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2.2 Zinc

2.2.1 Zinc homeostasis

Zinc is the second most abundant trace element in the human body (Crichton 2016). Five stable zinc-isotopes exist: ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn and ^{70}Zn with natural abundances of 48.9%, 27.9%, 4.1%, 18.6% and 0.6%, respectively (Erdman Jr, MacDonald et al. 2012). Zinc is the only metal which is represented in each of the six enzyme-classes: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases (Andreini, Banci et al. 2006, Erdman Jr, MacDonald et al. 2012, Crichton 2016). Unlike transition metals, such as iron or copper, zinc does not undergo redox reactions and is considered a pro-antioxidant, serving catalytic or structural purposes (Andreini, Banci et al. 2006, Maret 2013). Outside of physio- or pharmacological ranges, zinc ions can become pro-oxidants with pro-inflammatory and pro-apoptotic properties (Maret 2013). Lethal zinc doses have been established in rats, however, in humans, doses for acute toxicity may be achieved only under rarest circumstances. Estimated doses of 235 – 650 mg zinc have been associated with poisoning of individual humans, with symptoms such as e.g. nausea, abdominal cramping and vomiting (Maret and Sandstead 2006), however, no natural or anthropogenic sources of environmental zinc in foods have been identified as human health hazards.

The large majority of protein sequences in the human proteome is annotated as putative **zinc-fingers** or contains protein domains associated to other zinc-binding domains (Andreini, Banci et al. 2006). Zinc fingers can be defined as any small, functional, independently folded domain coordinated by one or more zinc ions that stabilize their structure (Laity, Lee et al. 2001). Structure as well as function of zinc fingers are very diverse and range from DNA or RNA binding to protein-protein interactions and membrane association (Laity, Lee et al. 2001). Zinc further facilitates the stabilization of the tertiary structure of several proteins, is involved in DNA replication and reverse transcription as well as in DNA damage recognition, DNA repair, apoptosis and cell cycle control (Hartwig, Asmuss et al. 2002, Stefanidou, Maravelias et al. 2006). Some suggested 2800 human proteins are zinc-binding, corresponding to 10% of all proteins in the human proteome (Andreini, Banci et al. 2006). The most prevalent zinc-coordinating protein-residues are cysteine and histidine (Blindauer 2008).

2.2.2 Zinc absorption

Dietary zinc absorption has been estimated at 20 – 40% (Tapiero and Tew 2003, Otten, Hellwig et al. 2006). Zinc homeostasis is regulated by several dozen proteins, that store, release, transport, bind or sense zinc, however, a regulatory hormone analogue to hepcidin in iron metabolism has not been identified (Maret 2013). Most zinc is absorbed in the distal duodenum or proximal jejunum, where specific zinc transporters (Zip4, ZnT1) are increasingly expressed (Otten, Hellwig et al. 2006, Erdman Jr, MacDonald et al. 2012). In humans, zinc transport is facilitated by two transporters: SLC30 and SLC 39, which belong to the ZIP and ZnT families, respectively (Eide 2006). SLC 39 transports zinc and/or other metal ion substrates from the extracellular space or organellar lumen into the cytoplasm, whereas SLC30 works in opposition, transporting zinc and/or other metal ions from the cytoplasm into the lumen of intracellular organelles or the outside of the cells (Eide 2006).

An energy-independent mechanism, both saturable and non-saturable, transports zinc across the brush border (Erdman Jr, MacDonald et al. 2012). Several transporters control cellular zinc and its traffic through the plasma membrane and between cytosol and various cellular compartments on transcriptional, translational or protein levels (Maret 2013). The active transport carrier mechanism predominates at low to normal luminal zinc concentrations (0.1 – 1.8 mM), whereas at higher concentrations, the non-saturable mechanism for zinc absorption becomes prominent (Erdman Jr, MacDonald et al. 2012).

Zinc-histidine complexes are better absorbed than zinc sulfate (Schölmerich, Freudemann et al. 1987), however, dietary zinc absorption has been described as a saturable process, primarily determined by the amount of ingested zinc and the absence or presence of absorption enhancers or inhibitors, such as dietary phytate, whereas the zinc status of the individual does not seem to be a major determinant of absorption efficiency (Erdman Jr, MacDonald et al. 2012, Krebs 2013). WHO additionally names protein as a dietary factor to influence zinc absorption and utilization (WHO 1996).

Zinc is present in most cells and the total zinc content in adults averages from 1.5 g to 2.5 g (Erdman Jr, MacDonald et al. 2012). Zinc is present in all organs, tissues, fluids and secretions – the highest amounts can be found in the skeletal muscle (57%) and bone

(29%), whereas smaller amounts are present in tissues and blood (14%) as well as in plasma (0.1%) (Tapiero and Tew 2003, Erdman Jr, MacDonald et al. 2012). Cellular zinc concentration is rather high, which is why it can hardly be considered as a trace element at the cellular level (Maret 2013). Zinc uptake and turnover in the central nervous system and bones can be comparably slow, whereas they are relatively fast in pancreas, liver, kidney and spleen (Erdman Jr, MacDonald et al. 2012).

Zinc is excreted from the body mainly through the feces and losses may range from less than 1 mg/day with zinc-poor diets to over 5 mg/day with zinc-rich diets (Otten, Hellwig et al. 2006). Less than 10% of zinc are lost through the urine, however, the losses may increase as a consequence of starvation or trauma (Otten, Hellwig et al. 2006). Skin cell turnover, sweat, semen, hair and menstruation losses cause additional zinc losses (Otten, Hellwig et al. 2006). Upon insufficient dietary zinc intake, the body tries to reduce endogenous losses and to maintain zinc concentrations in hair, skin, heart and skeletal muscle, whereas zinc concentrations in plasma, liver, bone and testicles decrease (Erdman Jr, MacDonald et al. 2012).

No zinc stores have been identified in the human body (Gibson 2012), however, metallothionin-bound zinc has been suggested as a potential short-term zinc pool (Zlotkin and Cherian 1988, Erdman Jr, MacDonald et al. 2012). Nevertheless, metallothionins, do not only serve as a zinc storage, but also operate as zinc acceptors and donors (Maret 2013).

2.2.3   Zinc requirements

The estimated average requirement for zinc ranges between 2.5 and 4 mg in infants and children, 9.4 mg in adult men and 6.8 mg in women of child-bearing age, with increases during pregnancy (9.5 – 10.5 mg) and lactation (10.4 – 10.9 mg) (King, Brown et al. 2016). The values are based on factorial analysis of metabolic studies of zinc absorption and defined to match at least the total daily zinc losses (Otten, Hellwig et al. 2006). Special considerations have to be made for children, people who consume vegetarian diets and people suffering from alcoholism (Otten, Hellwig et al. 2006). The UL for zinc is based on

adverse effects of excess zinc on copper metabolism as critical effect and ranges between 4 mg in infants and 40 mg in women during pregnancy or lactation (Otten, Hellwig et al. 2006, King, Brown et al. 2016).

2.2.4 Zinc sources

Dietary sources for zinc include red meat, seafood, whole grains and some breakfast cereals (**Table** 3). Cereals are the primary plant source of zinc which is mainly found in the germ and bran portions of grains. Thus, whole grains are richer in zinc than unfortified refined ones, hence as much as 80% of zinc is lost during milling (Otten, Hellwig et al. 2006, Erdman Jr, MacDonald et al. 2012). Nuts, seeds and legumes contain relatively high amounts of zinc, whereas tubers, refined cereals, fruits and vegetables contain lower amounts (Brown, Rivera et al. 2004).

Table 3. Zinc content, and phytate/zinc molar ratios of selected foods (Brown, Rivera et al. 2004).

Food groups	Zinc content [mg/100 g]	Phytate/zinc [molar]
Liver, kidney, Beef, pork	2.9–6.1	0
Poultry (chicken, duck, etc.)	1.8–3.0	0
Seafood (fish, etc.)	0.5–5.2	0
Eggs (chicken, duck)	1.1–1.4	0
Dairy (milk, cheese)	0.4–3.1	0
Seeds, nuts (sesame, pumpkin, almond, etc.)	2.9–7.8	22–88
Beans, lentils (soy, kidney bean, chickpea, etc.)	1.0–2.0	19–56
Whole-grain cereal (wheat, maize, brown rice, etc.)	0.5–3.2	22–53
Refined cereal grains (white flour, white rice, etc.)	0.4–0.8	16–54
Bread (white flour, yeast)	0.9	3
Vegetables	0.1–0.8	0–42
Fruits	0–0.2	0–31

2.2.5   Factors influencing zinc absorption

WHO classifies zinc bioavailability (%) into 1) high (~ 50%), 2) moderate (~ 30%) and 3) low (~ 15%), when consuming: 1) refined diets low in cereal fibre with PA/zinc molar ratios <5 and an adequate non-vegetable protein content; 2) mixed diets containing animal or fish protein, with PA/zinc molar ratios between 5 and 15; and 3) unrefined, unfermented and ungerminated cereal grains, a negligible animal protein intake and possibly fortified with inorganic calcium salts; respectively (WHO 1996).

2.2.5.1 Absorption enhancers

Dietary factors to enhance zinc absorption are: meats, liver, eggs and seafood, possibly due to certain amino acids that improve zinc solubility and the lack of chemical constituents inhibiting zinc absorption (Erdman Jr, MacDonald et al. 2012).

Early studies indicated, that an increased protein content in a meal enhances zinc absorption, especially when the protein source was animal, however, this effect was not shown in humans consuming cow's milk, possibly due to a high casein or calcium content (Gibson 2012). In contrast, zinc absorption from ZnO from a plant-based meal increased in subjects consuming milk and yogurt (Rosado, Díaz et al. 2005). Studies investigating human zinc bioavailability from different milks have indicated a greater absorption from human compared to cow's milk as well as a higher zinc absorption from whey-predominant compared to casein-predominant formula (Hotz 2005). Zinc absorption in healthy Dutch women was enhanced by 62 % when high-phytate rice was served with milk compared to water (Talsma, Moretti et al. 2017). In the same study decreased absorption-enhancing properties were shown, when milk was diluted with water, whereas ultra-high temperature treatment of the milk showed no such effect (Talsma, Moretti et al. 2017).

Rat and *in vitro* dialyzability models have suggested a positive effect of total protein content on zinc bioavailability; more specifically, amino acids, such as cysteine, methionine and tyrosine, showed associations with higher zinc absorption in rats (Hotz 2005). It was further shown that zinc uptake from sweetened, condensed milk was greater than from cow's or human milk (Hotz 2005).

2.2.5.2 Absorption inhibitors

Zinc from plant-based foods is suggested to be of lower availability for intestinal absorption compared to that of animal sources (Hunt 2002, Gibson 2012) as it can be inhibited by food components that form insoluble zinc complexes or compete at absorption sites and therefore inhibit zinc uptake (Erdman Jr, MacDonald et al. 2012).

Phytic acid

Several studies reported an inhibitory effect of phytate on zinc uptake in rat models, as well as *in vitro* (dialyzability, Caco-2 cells) (Hotz 2005). Similar to iron, PA negatively affects human zinc absorption in a dose-dependent manner, whereas only higher inositol phosphates influence zinc absorption, while lower inositol phosphates have no effect (Erdman Jr, MacDonald et al. 2012). PA/zinc ratios >10 increase the risk of poor zinc utilization, hence, removing phytate from the food can increase zinc availability (Erdman Jr, MacDonald et al. 2012). Recently, the inhibitory effect of high dietary phytate has been shown to be greatly reduced during late pregnancy and early lactation (Hambidge, Miller et al. 2017). Approaches to remove or degrade phytases are indicated above (**Chapter 2.1.5**).

Other inhibitors

Earlier studies suggested a negative impact of dietary fiber on zinc absorption, nevertheless, this has later been attributed to co-existing phytate in those foods (Gibson 2012). Maillard reaction products, which are formed when reducing sugars and amino acids undergo heat treatment, can complex zinc and make it less available for absorption (Gibson 2012).

Dietary calcium has been suggested to decrease zinc absorption, but this effect likely depends on the calcium source and is not relevant for people at adequate levels of zinc intake, regardless of the phytate content (Gibson 2012). The impact of calcium on zinc bioavailability likely depends on the physicochemical environment and mineral content of a meal (Hotz 2005).

2.2.6   Zinc deficiency

Unlike iron, zinc is a type 2 nutrient, which is required for multiple general metabolic functions and responds to inadequate intakes with a reduced excretion or tissue catabolism (King 2011). Zinc deficiency can impair a multitude of systems, including the central nervous, reproductive, integumentary, skeletal, gastrointestinal and immune systems (Erdman Jr, MacDonald et al. 2012). Low zinc levels not only harm individuals directly, but can also lead to susceptibility and progression into other diseases, especially infectious diseases in childhood (Gómez-Galera, Rojas et al. 2010). Naturally occurring zinc deficiency has long been considered unlikely and it was only in 1961, when zinc deficiency was reported to be an issue in parts of the Middle East. In 2002, WHO estimated almost half of the global population to suffer from a suboptimal zinc status (Erdman Jr, MacDonald et al. 2012). A missing reliable indicator for zinc status is one reason why it took relatively long for zinc to be recognized as important mineral for public health concerns and the actual extent of global zinc deficiency is yet unknown (Wieringa, Dijkhuizen et al. 2015).

Dietary zinc contents are estimated to be inadequate for 15 – 20% of the global population, whereas the estimated prevalence for zinc deficiency varies by food composition, total energy and zinc availability and the PA/zinc molar ratio (Wessells and Brown 2012). Regions at highest risk for inadequate zinc intake are South Asia, where zinc intake is suggested inadequate for ~30% of the population, followed by Sub-Saharan Africa (~ 26%), while Latin America is at lowest risk (6%) followed by high-income countries and China (~ 8% for both) (Wessells and Brown 2012).

2.2.7   Parameters for zinc status assessment

2.2.7.1 Individual versus population-based assessment

Nutritional status can be assessed in a population, thus, in individuals sharing a common trait, or on an individual level (Brown, Rivera et al. 2004). Individual assessment can lead to specific treatment, whereas assessment on a population level is used for planning and evaluating population-based interventions, thus, techniques may be used that misclassify some individuals but can reveal populations at risk provided a sampling technique

representative of the whole population is used (Brown, Rivera et al. 2004). More research is needed linking indicators of functional outcomes and total body zinc content in order to understand both the sensitivity and specificity of zinc status biomarkers more clearly (Wieringa, Dijkhuizen et al. 2015).

An expert working group classified the population indicators for zinc deficiency into biochemical, dietary and functional (De Benoist, Darnton-Hill et al. 2007). Biochemical indicators have been suggested as objective and quantitative means for assessing a population's zinc status, whereas functional indicators may be associated with zinc status albeit being unspecific but rather associated with other nutritional deficiencies or infections (De Benoist, Darnton-Hill et al. 2007). An overview of parameters that may indicate zinc deficiency is given below **(Table 6)**.

Socioeconomic indicators associated with zinc deficiency include maternal education, income, employment and access to health, water and sanitation services, however, they should not be used for monitoring and evaluating interventions that address nutrient deficiencies (Brown, Rivera et al. 2004). As mentioned earlier, a suite of markers may reflect zinc status more accurately than a single marker (Lowe, Fekete et al. 2009). Major obstacles for identifying the extent and severity of suboptimal zinc status are the lack of sensitive, specific and agreed-upon biomarkers (King 2011). For a complete understanding of zinc status, the cellular zinc metabolism has to be assessed along with whole-body zinc homeostasis (King 2011).

Recent studies suggested the ratio of the fatty acids linoleic acid : dihomo-gamma-linoleic acid (LA:DGLA) as a biomarker for *in vivo* zinc status, hence, decreased DGLA and increased LA:DGLA concentrations were associated with lower plasma zinc in birds and humans (Reed, Qin et al. 2014, Knez, Stangoulis et al. 2016). However, additional dietary intervention trials to support those finding are required (Knez, Stangoulis et al. 2016).

Table 6. Parameters indicating zinc deficiency in individuals or populations. [1]

Parameter	Properties	Indicator for zinc deficiency	I/P[2]	Confounders & Considerations
Biochemical parameters				
Serum/plasma zinc [μmol/L][3]	Currently most accurate and widely used available biomarker; reference values accounting for age, sex, fasting status, time of sample collection.	Low values in > 20% of population indicate high risk of zinc deficiency. Children (PM/AM): <8.7/9.9 Women (PM/AM): <9.0/10.1 Severe zinc deficiency: <7.65	P	Confounders: low serum albumin, elevated white blood cell counts, pregnancy/lactation, hormones, recent meals, time of day, age, sex, physiologic factors, systemic infections or inflammation, minor muscle catabolism, contamination of collection tubes, variations in tissue zinc. Not indicated for diagnosis or treatment of individuals; should be combined with other assessment methods.
Hair zinc conc.	Proposed as longer-term index of zinc status, not affected by diurnal variation, prolonged fasting, meal composition or acute infection.		P	Concentrations differ according to sex, age, season. Limited reference data. Less invasive than venipuncture, easier handling/storage of samples.
Urinary zinc conc.				Data are limited; only useful in subjects with moderate zinc status at baseline.
Enzymes	Plasma or serum alkaline phosphatase, 5'nucleotidase, ribonuclease, lactic dehydrogenase, delta-aminolevulinic acid dehydrogenase, extra-cellular superoxide dismutase.			Currently not recommended for assessment: high between-study variability, many enzymes affected by other nutrients as well.

Continued

Metallothionin	Circulating metallothionin may be correlated with zinc intake.			May be influenced by infection and stress.
Cells	Reflect long-term zinc status.			No established reference values, difficulty of separating cells in the field; large blood volumes required.
Molecular techniques	Measurement of mRNA for proteins, whose expression is regulated by metal ions.			Specialized equipment/techniques required.
Kinetic markers	Exchangeable zinc pools, serum zinc turnover rates.	—		Costly and not useful for population assessment.
Functional parameters				
Height-for-age[4], Length-for-age, (Weight-for-age)[5]	Can only be assessed in children.	P	% of stunting children (< -2 SD below age-specific median of reference population) <5 years of age	Prevalence of zinc deficiency may be higher in children due to higher required nutrient density and higher infection rates. Major disadvantage: long delay between intervention and detectable outcomes.
Dietary parameters				
Dietary zinc intake	Assessment of risk for zinc-deficiency rather than zinc status.	P	Elevated risk, when prevalence or probability of inadequate intakes (based on EAR) exceeds 25%.	24-hour recall most appropriate. Assessments should be repeated periodically and should include environmental or economic indicators.

[1] Information compiled from (Brown, Rivera et al. 2004, De Benoist, Darnton-Hill et al. 2007, Gibson, Hess et al. 2008, Lowe, Fekete et al. 2009, Erdman Jr, MacDonald et al. 2012, Wessells and Brown 2012, Wieringa, Dijkhuizen et al. 2015). [2] I = Individual assessment; P= Population assessment. [3] Serum or plasma zinc concentrations are not completely comparable, but will be referred to interchangeably, as custom in the common literature (Brown, Rivera et al. 2004). [4] Stunting = Height for age < -2SD of WHO Child Growth Standards median (WHO 2010); Countries showing ~ 20%, ~ 29% and ~ 43% stunting prevalence being at low, moderate and high risk for zinc inadequacy, respectively (Wessells and Brown 2012). [5] The experts suggest height- or length-for-age as best-known functional outcome associated with inadequate zinc status in populations, as they are widely responsive to supplemental zinc and standardized methods are existent and widely used (De Benoist, Darnton-Hill et al. 2007). Height- or length-for-age is preferred over weight-for-age as linear growth is likely to be the primary response to increased zinc intake rather than weight gain, which occurs as a result of increased linear growth (Brown, Rivera et al. 2004).

2.2.8   Zinc intoxication

Although zinc deficiency is common in certain populations, rare cases of acute zinc toxicity in humans upon exposure to excessive zinc amounts have been reported (Erdman Jr, MacDonald et al. 2012). Indicators for excess zinc intake are copper status (serum or plasma concentration) and superoxide dismutase activity in erythrocytes, whereas the use of iron status as a proxy for excess zinc intake or declines in the serum concentration of high density lipoproteins may be problematic or not well defined (Brown, Rivera et al. 2004). Typical symptoms of acute zinc toxicosis include abdominal pain, diarrhea, nausea and vomiting (Erdman Jr, MacDonald et al. 2012). An overview of clinical symptoms for zinc deficiency and overload is given below (Figure 10).
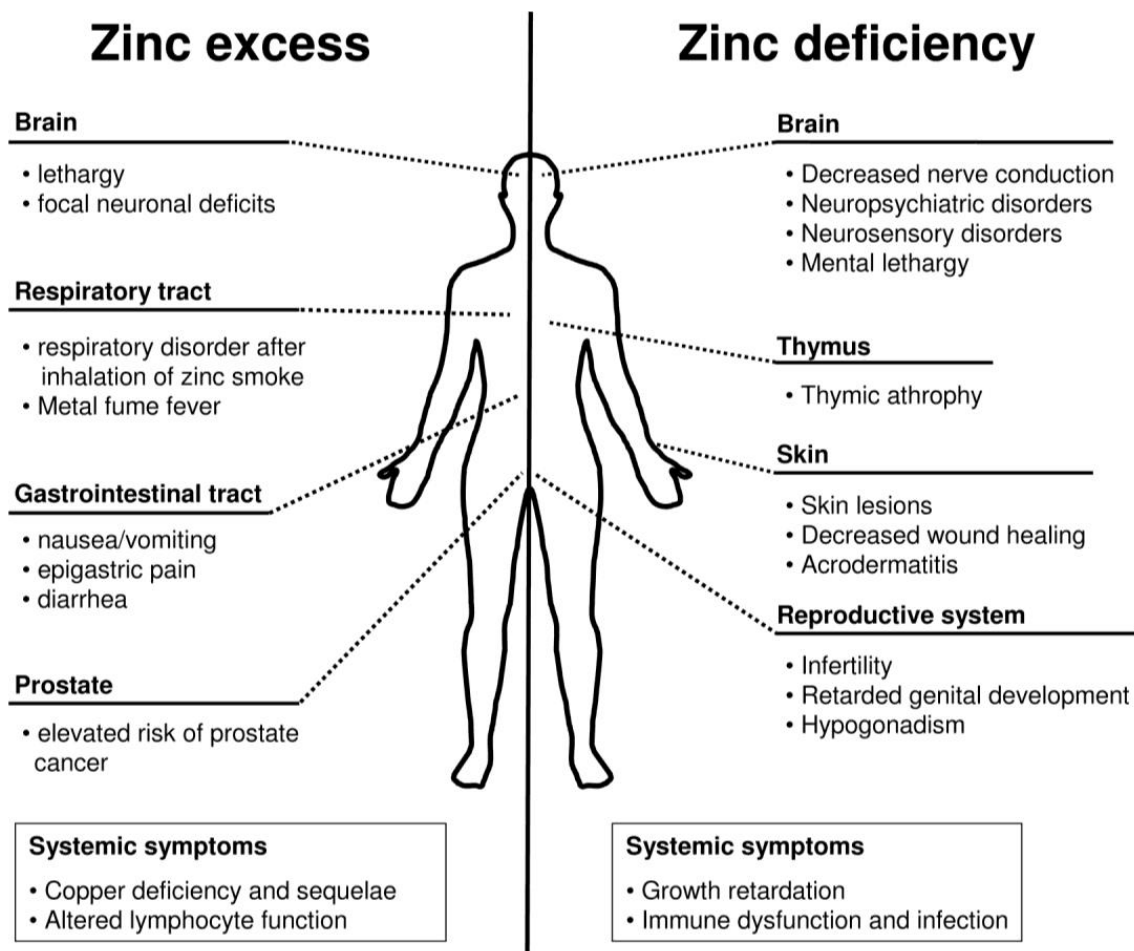
Figure 10. Comparison of the effects of zinc intoxication versus deficiency (Plum et al., 2010). Effects that could not be attributed to a certain organ system or affect several organs are classified as systemic symptoms.

2.3 Evaluating iron and zinc bioavailability from fortified foods

Successful fortification approaches require an adequate nutrient bioavailability for the consumer (Darnton-Hill 1998). Hence, bioavailability should be assessed before evaluating efficacy or effectiveness of a fortified food. The Food and Agriculture Organization (FAO) defines bioavailability as '*the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action*' (USDA 2015), in other words it is described as '*the concentration of a given compound or its metabolite at the organ level*' (Holst and Williamson 2008). Bioavailability cannot be determined by *in vitro* methods in its entirety, as many factors may be disregarded by this assessment (Sandberg 2005), thus, the terms uptake or absorption are suggested when referring to those methods (Etcheverry, Grusak et al. 2012). Further research is required to establish reliable *in vitro* methods paired with validation studies to test the agreement between their outcomes (Fernández-García, Carvajal-Lérida et al. 2009).

2.3.1 Assessment of iron and zinc absorption *in vivo*

Human absorption studies

The chemical balance technique, a rather insensitive, imprecise and time-consuming method, has been used in early years to directly measure iron absorption from the whole diet through comparison of iron content in food and feces, but gives no information on iron absorption from different meals (Hallberg 1981, Consaul and Lee 1983) and cannot distinguish between unabsorbed dietary and endogenous losses (King, Raynolds et al. 1978).

The use of isotopic tracers allows to determine the metabolic fate of minerals derived from a specific diet (Turnlund 1991). Isotopes of a trace element are often used for measuring iron bioavailability and are considered as gold standard given that 1) the isotopic label is metabolized in the same way as the mineral in the food or supplement it is being used; and 2) the amount of given label does not interfere with the examination process (IAEA 2012). Isotopes, differing in their atomic masses but which are otherwise similar, can be administered as radioisotopes, which emit ionizing radiation, or non-radioactive naturally

occurring stable isotopes (IAEA 2012). With the introduction of radioisotopes, single food items could be labeled and their absorption measured. Thus, studies using intrinsic radio-labels in food contributed to the knowledge on iron bioavailability from specific foods (Turnlund 1991). Radioisotopes can be easily detected; however, they expose the participant to radiation and may decay prior to analysis, which makes the use of stable isotopes favorable (Turnlund 1991). Another major advantage of using stable isotope is the possibility to simultaneously investigate the absorption of multiple isotopes of the same mineral or multiple minerals (IAEA 2012).

Methodologically, iron bioavailability can be assessed after manufacturing the isotopically labeled iron compound(s), quantitative oral administration and subsequent recovery from feces, plasma or red blood cells (IAEA 2012). The latter was done in the below described human absorption studies **(Manuscripts 1 – 4)** and will be explained in more detail.

The majority of absorbed iron (~ 80%) is incorporated into red blood cells (RBCs) within 10 – 12 days and this enrichment remains stable over the roughly 120-day-lifespan of RBCs (IAEA 2012). This method requires 1) a fixed fraction of absorbed iron incorporated into RBCs; 2) hemoglobin in RBCs containing a constant fraction of iron; 3) no exchange of labeled iron with iron in plasma once incorporated in RBC; and 4) a reliable estimate of blood volume for the studied population. The calculations for the amount of absorbed iron include hemoglobin and blood volume as parameters (IAEA 2012).

Early studies to determine zinc absorption in humans using stable isotopes employed the method of fecal monitoring, however, urinary enrichment with stable isotopes of zinc has been suggested an easier, less invasive, but yet accurate, method to determine fractional zinc absorption (Friel, Naake et al. 1992) with the required compliance of subjects being only minimal (King, Lowe et al. 1997). The double isotope method, which was adapted from a method determining calcium absorption, entails oral and intravenous administrations of different isotopes with subsequent measurement of both isotopes in the urine (Rauscher and Fairweather-Tait 1997). While the need for a more detailed method validation has been suggested earlier (Rauscher and Fairweather-Tait 1997), the

double isotopic tracer ratio technique has been used extensively (Brnić, Wegmüller et al. 2014, Wegmüller, Tay et al. 2014, Brnić, Wegmüller et al. 2016).

2.3.2   Animal studies

Rodents have been considered inappropriate to reflect bioavailability of dietary iron in humans (Cook, Dassenko et al. 1991, Fairweather-Tait, Lynch et al. 2005), partly because they can endogenously synthesize AA, which promotes iron absorption (Atanasova, Mudway et al. 2004). Juvenile and adult rats have a relatively high intestinal phytase activity, thus increasing the availability of minerals for absorption, whereas in rat pups this activity is low (Hotz 2005). To estimate zinc bioavailability, *in vitro* dialyzability and Caco-2 cell models as well as animal models (rat/rat pups) have been suggested (Hotz 2005). However, those methods entail certain disadvantages: *in vitro* methods may not be able to reflect biological feedback mechanisms present *in vivo* (Hotz 2005).

2.3.3   Assessment of iron and zinc uptake *in vitro*

In vitro methods can assess bio-accessibility – thus, the amount of ingested nutrient potentially available for absorption (solubility, dialyzability and a gastrointestinal model) – and bioavailability (Caco-2 model) (Etcheverry, Grusak et al. 2012). Human bioavailability of iron and zinc has been categorized into availability, uptake, absorption, retention utilization and body stores, whereas no single *in vitro* method can be used to completely mirror *in vivo* iron or zinc bioavailability (Fairweather-Tait, Lynch et al. 2005). Although many efforts to validate comparability between *in vitro* and *in vivo* results have been made, there is still need for further endorsement (Etcheverry, Grusak et al. 2012) and precise standardization to increase inter-laboratory comparability of results (Sandberg 2005).

2.3.4   Solubility and dialyzability

In vitro digestion systems have been suggested as rapid and useful alternatives to animal and human models, despite the fact that differences between *in vitro* and *in vivo* findings may occur (Miller, Schricker et al. 1981, Sandberg 2005, Hur, Lim et al. 2011). Solubility and dialyzability are considered less useful predictors of iron absorption, however, they

can serve as tools to understand factors that may affect iron absorption (Sandberg 2005) and to determine effects of isolated variables rather than evaluating overall iron bioavailability from a complex meal (Reddy, Hurrell et al. 2000). The effects of milk, proteins, tea and organic acids cannot be predicted by solubility or dialyzability measurements, nor the dialyzability of iron bound to large molecules (Sandberg 2005).

A fast and inexpensive method to determine soluble iron in fortified rice via colorimetry has been suggested as tool for identifying formulation and storage issues, however, obtained results may over-estimate the *in vivo* effects (Johns, Parker et al. 2014).

Dialyzability involves a digestion process and a selective dialysis membrane and its use has been suggested to screen foods or mineral compounds regarding the direction, but not the magnitude, of absorption (Fairweather-Tait, Lynch et al. 2005). An *in vitro* method for estimating iron availability from complex meals via a simulated two-step (gastric and intestinal) enzymatic digestion, even allowing for discrimination between high and low molecular weight soluble iron complexes when using dialysis tubings has been described by Miller et al. (Miller, Schricker et al. 1981) and has been adapted for fortified rice (Manuscript 1). The Miller method estimated human iron absorption in terms of magnitude more closely than did a comparison of human and rat iron absorption, however, meals with widely different non-heme iron concentrations cannot be compared (Schricker, Miller et al. 1981).

In vitro dialyzability has also been recommended to assess zinc bioaccessibility as it is the only *in vitro* method that has been validated against human studies (Etcheverry, Grusak et al. 2012).

Caco-2 cells

Caco-2 cells, which behave like intestinal cells upon culture, belong to a human epithelial cell line derived from a human colonic adenocarcinoma and might represent physiologic *in vivo* conditions more accurately than other *in vitro* methods (Etcheverry, Grusak et al. 2012). When combined with a simulated digestion procedure, Caco-2 cells can predict mineral availability and uptake, however, they do not provide measures for retention,

utilization or storage as opposed to algorithms, which may additionally predict all those factors (Fairweather-Tait, Lynch et al. 2005). Caco-2 models with subsequent digestion have been recommended for the assessment of calcium and iron uptake (Etcheverry, Grusak et al. 2012).

Additionally, the use of Caco-2 cells can help to identify undiscovered factors in food influencing absorption or interactions between food components and they can predict the response direction for major modifiers in iron absorption, however, it is unclear whether the effects have the same magnitude (Fairweather-Tait, Lynch et al. 2005). In the case of iron, uptake by the cells can be estimated via ferritin formation or radio-isotope uptake (Etcheverry, Grusak et al. 2012).

2.3.5   Computer-controlled gastrointestinal models

Those models consist of four sections (representing stomach, duodenum, jejunum, ileum), which aim to stimulate digestive processes in the stomach and small intestine, however, they have only been used in few studies as they are rather expensive and time-consuming, (Sandberg 2005).

2.3.6   Mathematical approaches

Early models for estimating the amount of absorbable iron from a meal considered 1) total iron, 2) heme iron, 3) non-heme iron, 4) AA and 5) meat, poultry and fish based on a reference level for body iron stores of 500 mg (Monsen, Hallberg et al. 1978). A more recent approach used the iron absorption from a wheat roll free from known inhibitors, adjusted to a reference dose absorption of 40% and multiplied by the expected effect of different influencing factors, which included PP, PA, seafood, calcium, egg, soy protein and alcohol (Hallberg and Hulthén 2000). This approach can be applied to predict iron absorption from various diets, to translate physiological into dietary iron requirements and to estimate the effects expected by dietary modification, however, limiting factors include the potential influence of non-detectable phytate as well as the quantification of AA at the time of consumption (Hallberg and Hulthén 2000).

Regression models to predict iron absorption have identified animal tissue, PA and AA as useful predictors for non-heme iron absorption, however, those efforts do not take into account interactions that occur when two or more active compounds are present in the same meal and they are based on meals that are consumed mainly in the Western hemisphere (Reddy, Hurrell et al. 2000, Armah 2016).

2.4   Iron – zinc interactions

2.4.1   Simple vs. complex food matrices

Several studies have investigated the effect of zinc on iron absorption from aqueous solutions (Crofton, Gvozdanovic et al. 1989, Rossanderhulten, Brune et al. 1991). A recent review concluded that the inhibiting ratio of Zn/Fe depends on the concentration of both administered cations and the administration vehicle (Olivares, Pizarro et al. 2012). A threshold for inhibiting low-dose iron (0.5 mg) bioavailability from aqueous solutions at Zn/Fe (wt/wt) ratios of $\geq$ 5.9 : 1, has been suggested, whereas with higher iron doses (10 mg) the inhibition occurred at wt/wt ratios of 1 : 1 (Olivares, Pizarro et al. 2012). This has been attributed to the higher quantity of ionic species available for enterocytic uptake, where zinc and iron minerals may compete for the same molecular transporters, possibly DMT-1 (Olivares, Pizarro et al. 2007, Espinoza, Le Blanc et al. 2012), or a currently unidentified transporter (Tandy, Williams et al. 2000, Olivares, Pizarro et al. 2012). Zip-14, which can transport iron and zinc, could be the site of interaction between those minerals (Iyengar, Pullakhandam et al. 2012). A recent review suggested no competition for DMT-1 uptake by iron and zinc, however, zinc may affect DMT-1 expression on a transcriptional level (Knez, Graham et al. 2015). The transcription of ferroportin, the only known cellular iron exporter protein, is induced by zinc (Knez, Graham et al. 2015). Ferroportin has also shown affinity for zinc, however, its low reactivity may not strongly influence zinc homeostasis (Mitchell, Shawki et al. 2014).

Given within a food matrix, iron and zinc interact with dietary ligands, which decrease the number of cations available for enterocytic transport (Olivares, Pizarro et al. 2012). No effect was reported when adult subjects were given milk with added $ZnSO_4$, at Zn/Fe molar ratios between 0.6:1 and 2:1 (Olivares, Wiedeman et al. 2012). This agrees with studies

showing no inhibitory effect on iron absorption when preterm infants received premature milk formula with $ZnSO_4$ or human milk at Zn/Fe ratios of 1:1 and 4:1 (Friel, Serfass et al. 1998). Non-heme iron absorption from a hamburger meal after addition of $ZnSO_4$ at Zn/Fe molar ratios of 4.3:1 did not significantly affect iron absorption (Rossanderhulten, Brune et al. 1991). In contrast, four studies investigated the effect of $ZnSO_4$ on iron absorption when added to cereals, but only one study reported a detrimental effect on iron bioavailability when $ZnSO_4$ was added to wheat based dumplings, while in the same study, ZnO addition at the identical 0.9:1 Zn/Fe ratio did not affect iron absorption (Herman, Griffin et al. 2002).

An *in vitro* study on iron solubility from rice analogues fortified with micronized FePP showed a greater inhibition of iron solubility by $ZnSO_4$ compared to ZnO (Johns, Parker et al. 2014), which was in agreement with *in vitro* findings from our laboratory (Manuscript 2).

3 FOOD FORTIFICATION

An expert panel from the *2012 Copenhagen Consensus* considered hunger and education as the world's most important challenges in order to advance global welfare, particularly in developing countries. Hence, they suggested interventions to reduce chronic undernutrition in pre-schoolers to tackle those challenges (Lomborg 2013). Furthermore, ICN2 considered the elimination of malnutrition as imperative for health, ethical, political social, and economic reasons (FAO 2014). Worldwide, roughly 800 million people suffer from starvation, whereas an approximate 2 – 3 billion suffer from micronutrient deficiencies, particularly vitamin A, iodine, iron and zinc deficiencies (Atungulu and Pan 2014, FAO 2014). Estimates suggest, that undernutrition in general (including fetal growth restriction, stunting, wasting, suboptimal breastfeeding, and vitamin A and zinc deficiencies) causes over 3 million child deaths per year, corresponding to 45% of all child deaths in 2011, hence macro- and micronutrient undernutrition remain the dominant nutritional problems in most developing countries (Lomborg 2013).

3.1 What is food fortification?

According to the WHO, fortification *'refers to the addition of micronutrients to processed foods'*, thus, it is *'the practice of deliberately increasing the content of an essential micronutrient, i.e. vitamins and minerals (including trace elements) in a food, so as to improve the nutritional quality of the food supply and provide a public health benefit with minimal risk to health'* (Uauy 2005, WHO 2006). Food fortification is one among several nutrition interventions to combat micronutrient deficiencies and is considered a nutrition-specific intervention (NSpI), addressing the immediate causes of undernutrition, in contrast to nutrition-sensitive interventions (NSeI), which can be multisectorial (e.g. agriculture, education, social support) (Lawrence, Wingrove et al. 2016). NSpIs are well suited to treat acute micronutrient deficiencies, whereas NSeIs are suggested in chronic situations; however, a strategically implemented combination of multiple interventions has been suggested as most viable approach (Lawrence, Wingrove et al. 2016). Thus, food fortification is a complement to other efforts in disease prevention and management along

with efforts to improve water, sanitation and hygiene (Hoogendoorn 2017). The aim of a fortification program *'… is for all people to be able to obtain from their diet all the energy, macro- and micronutrients they need to enjoy a healthy and productive life'* (WHO 2006). Nevertheless, fortification approaches should be complementary to food-based strategies and should not be a replacement for dietary diversification (Uauy 2005). Fortification must not be confused with supplementation, where relatively large doses of micronutrients are administered via different application forms (pills, capsules, syrups), requiring a high degree of consumer compliance (WHO 2006). Enrichment is synonymous to fortification, irrespective of whether or not the nutrients were originally in the food prior processing (WHO 2006).

Fortification of a staple food affects an entire population and can be a cost-effective and quick means to reach those at-risk, however, concerns may be raised regarding possible over-dosage and increased production and consequently retail costs (WHO 2006). It is key to select appropriate foods to be fortified with the essential micronutrients lacking in a diet (Dexter 1998). One cannot conclude, that iron fortification of foods is without risk for a consumer, however, potential risks are likely much lower than those associated with ID (Prentice, Mendoza et al. 2017).

3.1.1   Types of fortification

Food fortification can be classified into: 1) Mass fortification, where foods are fortified that are widely consumed by a general population; 2) Targeted fortification, where foods are fortified that are designed for specific population subgroups; or 3) Market-driven fortification, which is more widespread in industrialized countries and allows food manufacturers to voluntarily fortify foods available in the market place (WHO 2006), with a major constraint being that certain populations are hard to reach (Horton S 2008); 4) Household and community fortification; or 5) Biofortification (**Chapter 3.6.4**). Mass fortification has been suggested as preferred option when the majority of a population is facing an unacceptable risk of being or becoming deficient in specific micronutrients (WHO 2006). Targeted fortification is relatively simple for infants or children; however, women of child-bearing age are harder to reach and a widely consumed product can reach broader populations easily (Hurrell 1997). Household and community fortification – a combination

of supplementation and fortification via micronutrient based powders or tablets – is costlier than mass fortification approaches, but may be useful for improving local foods for infants or in cases where universal fortification is not possible (WHO 2006).

3.1.2 Mandatory vs. voluntary fortification

Fortification approaches can also be classified into mandatory or voluntary (WHO 2006). Mandatory food fortification is legally emposed to food producers, in situations where a proportion of the general population is at risk of being or becoming deficient in one or many specific micronutrients (WHO 2006). Food vehicles fortified on a mandatory basis can be basic commodities (e.g. flour, salt) as well as processed foods or ingredients thereof (WHO 2006). While basic commodities are more suitable for mass fortification, certain processed formulated foods are advantageous for targeted fortification approaches (WHO 2006). The government is responsible for ensuring that the combination of food vehicle and fortificants will be efficacious and effective for the target group, while still being safe for both target and non-target groups (WHO 2006).

Voluntary fortification describes, when a food manufacturer freely chooses to fortify certain foods provided the permission by the food law or after being encouraged by the government to do so (WHO 2006). The extent of federal control over voluntary fortification should be commensurate with the inherent level of risk (WHO 2006). The public health impact from voluntary food fortification can range substantially, but is likely less than that of mandatory fortification (WHO 2006).

While mass fortification is usually mandatory, market-driven fortification is always voluntary within regulatory limitations enforced by the government, whereas targeted fortification can be mandatory or voluntary (WHO 2006).

Key determining factors for choosing whether mandatory or voluntary fortification approaches are more appropriate have been suggested as: 1) Significance of the public health need; 2) Size and scale of food industry sector; 3) Level of awareness among the population about nutritional needs; 4) Political environment; and 5) Food consumption patterns.

Programmatic considerations for food fortification have been identified as 1) Need; 2) Coverage and utilization; 3) Appropriate fortificants at the right level; 4) Quality assurance

and adherence to fortification standards; and 5) Long-term industry and government commitment and capacity strengthening (Hoogendoorn 2017).

3.2 Reasons for food fortification

In its nutrition policy, the World Food Programme (WFP) emphasizes, that cereals dispensed via general food distribution should be fortified in order to achieve nutritional objectives (eg. micronutrient needs) of targeted populations (Roks 2014). Sixteen minerals are essential in human nutrition, whereas eleven of them are sufficiently abundant in food and drinking water, thus, deficiencies from those nutrients arise only rarely (Gómez-Galera, Rojas et al. 2010). In populations consuming monotonous, plant-based diets as is the case in resource poor settings in developing countries iron, zinc, iodine, calcium and selenium are present in only limited amounts, causing potential deficiencies (Gómez-Galera, Rojas et al. 2010). Iron is considered one of the most limiting micronutrients and it has been suggested that more than half of the ID anemia cases could be addressed with enhanced iron amounts in the diet (Gómez-Galera, Rojas et al. 2010). Important factors for food fortification programs are that compliance and coverage of the program are documented and that that information is used to determine when the potential health impact of a program should be evaluated (Pachón, Spohrer et al. 2015). Constraining factors for successfully developing food fortification programs can be technical, socio-economic, infrastructural and political ones (Darnton-Hill 1998).

3.3 Food vehicles for fortification

The lack of an obvious food vehicle that can reach targeted recipients is a major constraint (Darnton-Hill 1998). Nevertheless, examples for fortified foods are manifold: cereal fortification with thiamine, riboflavin and niacine; margarine-fortification with vitamin A, milk fortification with vitamin D, infant-formula fortification with iron, or wheat-fortification with folic acid (WHO 2006). Iodized salt was introduced in Switzerland and the USA in the early 1920s and is now used in most countries worldwide (WHO 2006). Cereal flours are the most frequently used vehicles for iron fortification that reach the entire population, however, their fortification levels are rather low (Hurrell 1997). The main disadvantages of using certain cereal products as iron-fortification vehicles are the

comparatively high levels of PA and their sensitivity to fat oxidation during storage (Hurrell 1997). Widely used condiments (eg. fish sauce, bouillon cubes) in developing countries also have the potential to act as fortification vehicles (Walczyk, Tuntipopipat et al. 2005, Cercamondi, Duchateau et al. 2016). Iron fortification of salt and sugar is technically challenging especially with respect to storage and sensory properties. Furthermore, refined sugar in certain developing countries is only consumed by people in the higher socioeconomic strata (Hurrell 1997).

3.4   Iron and zinc compounds for fortification

A search in the US Food and Drug Administration (FDA) database, for substances that are generally recognized as safe (GRAS), reveals 23 iron compounds, whereas 12 listings relate to packaging. For zinc, the database only lists seven compounds: zinc gluconate, acetate, carbonate, chloride, oxide, sulfate and hydrosulfite, whereas the latter is used for packaging (SCOGS). Zinc oxide (ZnO) and zinc sulphate (ZnSO$_4$) are suggested for food fortification due to their low-cost profile (de Romaña, Lönnerdal et al. 2003).

Iron compounds can be divided into four groups: 1) Freely water-soluble; 2) Poorly water soluble but soluble in dilute acids; 3) Water soluble but poorly soluble in dilute acid and 4) Protected iron compounds (Hurrell 1997). The more water-soluble a compound, the higher its absorption (Hurrell 1997). For wheat flour, fortification compounds of choice include FeSO$_4$, Fe-fumarate, and NaFeEDTA, whereas electrolytic iron is a second choice fortificant (Hurrell, Ranum et al. 2010). A major challenge in iron fortification is that iron compounds which exhibit the highest bioavailability impair product quality (Lee, Clydesdale et al. 1979), thus bioavailable forms of iron are usually chemically reactive and often produce undesirable effects in the food (Dexter 1998). FeSO$_4$, which is completely water-soluble, has been attributed a relative bioavailability of 100% (Hurrell 1997), however, in rice fortification it should only be used sparingly with certain technologies (dusting or coating) due to an interaction with the rice matrix and sensory changes over time (Steiger et al. 2014). Its solubility in water, can also cause losses after washing/rinsing of rice if the water is discarded (Steiger et al. 2014). The compound of choice for rice fortification is ferric pyrophosphate (FePP), which is advantageous in terms of color and low reactivity with the

rice matrix and other nutrients, however, it has a relative bioavailability of 21 – 74 at more than double the cost compared to $FeSO_4$ (Hurrell 1997, Steiger et al. 2014). Ferric orthophosphate is used for rice fortification in the US, however, given its low bioavailability its value as fortificant in rice has been questioned (Alavi, Bugusu et al. 2008, Steiger et al. 2014). Efforts to improve its bioavailability targeted the particle size (bioavailability inversely related to particle size), use of solubility enhancers or identified potential absorption inhibitors (Hilty, Arnold et al. 2010, Parker, Mosites et al. 2015). Safety concerns about Nano-sized ferric phosphate (NS PO_4) have recently been addressed in rats and human cell lines and the compound has been proposed to be safe for ingestion (Perfecto, Elgy et al. 2017, Von Moos, Schneider et al. 2017). Most recent approaches employed combinations of organic and inorganic materials as fortificants – a hybrid of protein fibrils and nanosized iron particles showed a comparable bioavailability to $FeSO_4$ in rats, while maintaining organoleptic acceptability in solid and liquid foods and, furthermore, no tissue accumulation was reported (Shen, Posavec et al. 2017). However, data on human bioavailability are lacking as well as assesments regarding toxicity of those formulations.

3.5 Economic value of food fortification

Fortification is a high-priority investment and may be well applied for widespread deficiencies, especially those associated with high-costs and that are prevalent in hard-to-reach target-populations, and/or if the fortification cost is not too high and processing of the product more centralized (Horton 2006). Experts estimated, that each dollar spent on reducing chronic undernutrition has a pay-off of at least USD 30, and that a bundle of interventions at the cost of USD 100 per capita (including e.g. micronutrient provision) could reduce chronic undernutrition in developing countries by 36% (Lomborg 2013). It has been estimated, that home fortification with zinc and other micronutrients would cost USD 4.5 per child annually, whereas for large-scale wheat flour fortification the cost would amount to USD 0.24 or USD 0.06 per capita when fortifying with $ZnSO_4$ or ZnO, respectively; the costs for iron fortification were estimated at approximately USD 0.12 (Horton 2006). The costs for rice fortification are estimated between USD 10 and 20 per

milled ton, which converts into an additional cost of USD 0.36 – 2.18 per year for consumers with a daily per capita intake of 100 – 300 g of rice (Alavi, Bugusu et al. 2008).

3.6   Micronutrient fortification of rice

Paddy rice is a good source of thiamine, riboflavin and niacin, however, nutrient contents decrease by increasing the degree of milling and due to other processing steps (Houston, Houston et al. 1970, Piccoli, Grede et al. 2012) (Table 1), leaving rice-eating populations at high risk for vitamin and mineral deficiencies (Houston, Houston et al. 1970, Muthayya, Sugimoto et al. 2014). Washing the rice prior consumption can remove most of the protein, ash, water-soluble vitamins, minerals and fats (Atungulu and Pan 2014). The major nutritional problems in rice-consuming countries have been identified as inadequate and unbalanced dietary intake and, in further consequence, protein-energy malnutrition, nutritional anemia as well as deficiencies of vitamin A, iodine, and certain vitamins and minerals (Juliano 1993, Piccoli, Grede et al. 2012).

It has been suggested, that rice fortification has the potential to substantially increase micronutrient intake among populations where rice is a staple food (Piccoli, Grede et al. 2012, De Pee 2014), thus to eliminate micronutrient deficiencies given the sensory characteristics of the end-product are not discernibly changed and that consumers incorporate fortified rice into their regular diet (Steiger et al. 2014). In its recommendation for rice fortification, WFP states, that *'. . . premix kernels must conform to shape, size and color of the rice it is blended with, so that sensory differentiation is difficult'* (Holden 2015). Fortification levels need to substantially contribute to the micronutrient intake while still being safe at higher consumption-levels in the population, furthermore, the fortified rice has to be palatable, have a long shelf life and needs to be distinguishable from unfortified rice (De Pee 2014).

First attempts for rice fortification focused on thiamin addition and enrichment became economic when technological advances made it possible to commercially synthesize large quantities of thiamin and riboflavin (Dexter 1998). Rice fortification programs can be implemented through social safety nets, voluntarily, or through legislation by the government (Tsang, Moreno et al. 2016). US-regulations adopted in 1958 established a

food standard for enriched rice – while enrichment was not mandatory, specified quantities of certain nutrients (riboflavin, thiamin, niacin, iron) in case manufacturers chose to enrich, were given in order to restore the milled rice to the nutritional level of brown rice (Hunnell, Yasumatsu et al. 1985, FFI 2016). To date, rice fortification is mandatory in six countries: Costa Rica, Nicaragua, Panama, Papua New Guinea, the Philippines and the US (FFI 2016) (**Figure 11**). However, in the Philippines, although rice enrichment is mandatory, reports have suggested challenges in law enforcement (Dexter 1998).
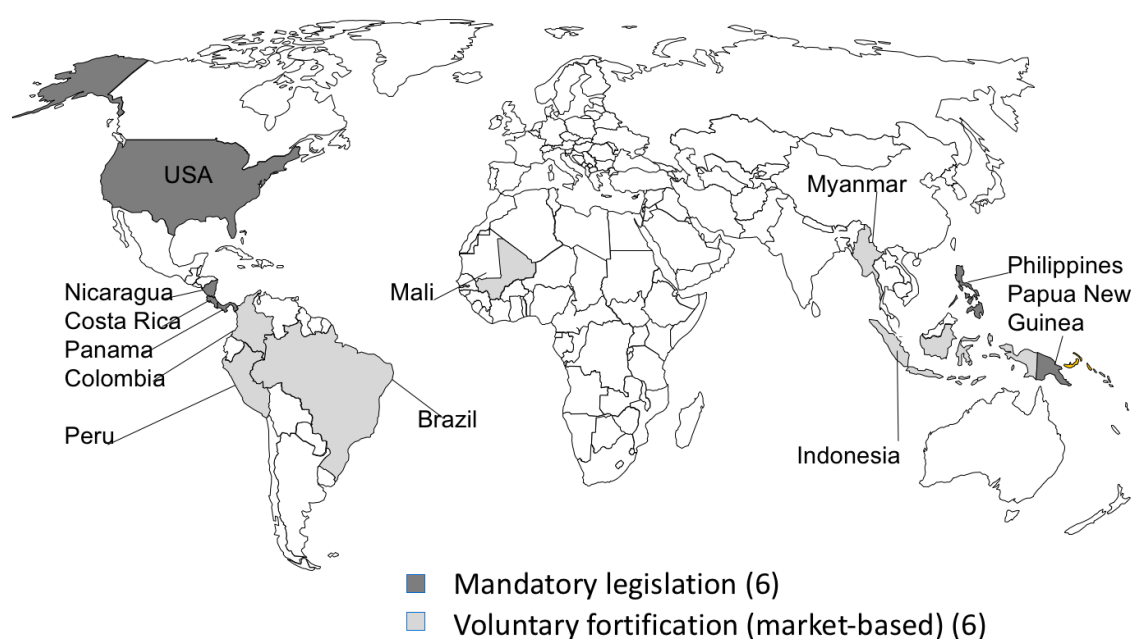
Figure 11. Rice fortification global status (FFI 2016).

High initial investment and increased production cost, lack of government leadership and consumer hesitation towards the product as well as a potential lack of understanding of the benefits provided from fortified rice have been identified as the main obstacles for scaling up rice fortification (Piccoli, Grede et al. 2012). In addition to the production costs for fortified rice, there may be costs for blending fortified with regular rice and for scrutinizing the value chain regarding production, blending and distribution (Roks 2014).

Key targets for successfully scaling up rice fortification have been identified as follows (Hurrell 1997, De-Regil et al. 2014): 1) Stability of micronutrients and compounds in

different cooking processes; 2) Relative bioavailability of micronutrients proposed for fortification and their interactions; 3) Acceptability of organoleptic changes; 4) Effects of different phytate contents on absorption; 5) Most appropriate delivery platforms for reaching target populations; and 6) Effectiveness of different rice fortification methods. The supply chain reaching from the farmer to the mill and eventually to the distributor may encompass additional caveats for implementing rice fortification (Dexter 1998, Muthayya, Sugimoto et al. 2014).

3.6.1   Technologies

Rice fortification remains technically challenging, especially due to the size difference between rice kernel and micronutrients, leading to heterogeneity and subsequent nutrient losses if both were simply mixed (Steiger et al. 2014). Hot and cold extrusion, coating, and dusting have been identified as the four major methods for rice fortification (Alavi, Bugusu et al. 2008).

3.6.2   Premix approaches

A premix is *, . . . a mixture of a micronutrient(s) and another ingredient, often the same food that is to be fortified, that is added to the food vehicle to improve the distribution of the micronutrient mix within the food matrix and to reduce the separation (segregation) between the food and micronutrient'* (WHO 2006) and '*contains all the vitamin and/or mineral addition added to a small portion of the grain product to be enriche*d' *(Max 1949)*. Dexter proposed two types of rice enrichment for commercial use: powder and whole grain enrichment (Dexter 1998). Powder enrichment contains a pre-blended mixture of vitamins and minerals added to the rice, whereas grain type enrichment refers to the addition of nutrients to the kernel surface or matrix in high concentrations and subsequent blending with regular grains to obtain desired enrichment levels in the final product (Dexter 1998).

3.6.2.1 Extrusion

In 1966, *Roche Products Ltd.* described a process to produce ceral grains from a dough similar to pasta production and subsequent cutting to grain-like shapes (Schnyder 1966).

During extrusion, compressive and shear forces developed in the stock, allow the material to be heavily deformed without fracturing and create a wide variety of cross-sectional configurations (Oberg 2012). The extrusion of starchy foods results in a gelatinized product, with partially or completely destroyed crystalline structures and molecular fragmentation of starch polymers, protein denaturation and complexes of lipids with starch or protein (Hagenimana, Ding et al. 2006). The dough, mainly consisting of water, rice flour, nutrients and potential additives, is moved through the extruder with screws – generating increasing pressure shear and heat – and is subsequently cut into grain-like structures (Alavi, Bugusu et al. 2008). Moisture contents between 30 – 40% have been suggested to obtain optimal processing and end-product properties (Steiger et al. 2014). First attempts to produce this 'a*rtificial rice*' have been conducted in the early 1950s, with a mix of wheat flour, waxy and non-waxy rice flour and resulted in a '*poor appearance*' (Hunnell, Yasumatsu et al. 1985).

Common extrusion equipment requires a hammer mill for rice flour production, mixers, single or twin screw extruders and dryers (Alavi, Bugusu et al. 2008). Extruded rice has the advantage of incorporating vitamins and minerals into the food matrix (Atungulu and Pan 2014). Cold, warm and hot extrusion differ in the processing temperature, consequently leading to differences in color and consistency of the products (De-Regil et al. 2014), thus, extrusion conditions highly influence properties of extruded rice (Hagenimana, Ding et al. 2006).

During hot extrusion, the starch melting temperature is exceeded, whereas cold extrusion is conducted at temperatures above glass transition but below starch melting temperatures (Steiger et al. 2014) with increasing processing costs by increased extrusion temperatures (Oberg 2012). Hot extrusion results in products with a sheen, consistency and transparency similar to natural rice, unlike cold extruded rice which is slightly laced and has an opaque appearance (Atungulu and Pan 2014), and results in a high degree of gelatinization (Steiger et al. 2014).

Warm and hot extrusion

The product temperatures for hot extruded rice range between 80 – 100 °C, the heat is partly derived from heated barrels and partly from shear (Steiger et al. 2014). Warm

extrusion is conducted at intermediate temperature ranges, with product temperatures between 60 – 90 °C, allowing partial but not full melting of amylopectin, which largely affects structural properties of rice kernels (Steiger et al. 2014). After warm or hot extrusion, rice protein no longer forms networks, but rather appears as protein assemblies throughout the kernels, starch is then the new continuous phase and overtakes the role of a structuring agent (Steiger et al. 2014).

Cold extrusion

Is a process similar to hot extrusion, but employing simpler equipment (a so called pasta press), which requires no additional thermal energy input (Alavi, Bugusu et al. 2008). Product temperatures range between 30 – 40 °C, causing no starch gelatinization (Steiger et al. 2014).

3.6.2.2 Coating

In 1950, a '*process for increasing the vitamin and mineral content of cereal grains, which consists in impregnating the cereal grains with a solution containing vitamins and mineral substances and coating the impregnated grains with an edible coating comprising a film/forming agent, an adhesive agent and a plasticizer, which is insoluble in cold water but removable in hot water . . . The coating . . . does not flake off from te grain surfaces during mixing and shaking. At cooking temperature, the protective coating is removed*' has first been described (Roche 1950). The proposed coating is insoluble in water (below 55 ^{o}C), soluble in water above 80 ^{o}C and unaffected by enzymatic action (Roche 1950). Rice kernels fortified via coating use waxes and gums combined with the fortification mix to create a liquid which is sprayed on the grain-surface of intact premix-kernels in several layers (Steiger et al. 2014). Waxes and gums allow micronutrients to stick to the rice kernel (Alavi, Bugusu et al. 2008), forming a protective coat, that does not rinse off the surface when washed, thus preventing the rice from high micronutrient losses (De-Regil et al. 2014) and releasing the nutrients through the polymers via diffusion (Peil, Barrett et al. 1982). The product may not be appealing to potential customers due to odors from waxes and solvents in the final product (Alavi, Bugusu et al. 2008) and differences in color (Steiger et al. 2014).

3.6.3 Non-premix approaches

3.6.3.1 Dusting

Rice grains are dusted with a powder from the micronutrient mix and are supposed to stick to the grain surface due to electrostatic forces (Alavi, Bugusu et al. 2008). Dusting is most effective when conducted soon after milling white or parboiled rice as both temperature and moisture at the grain surface facilitate powder adherence (Atungulu and Pan 2014). This comparatively cheap method has been suggested as inappropriate for developing countries, where rice is washed and rinsed before cooking (Alavi, Bugusu et al. 2008, Atungulu and Pan 2014, De-Regil et al. 2014, Steiger et al. 2014). Furthermore, if the difference in market price between broken and intact kernels offsets the production costs of extruded kernels, extrusion can potentially be cheaper than dusting as dusting requires the use of intact grains, whereas in extrusion can be broken rice kernels processed (Steiger et al. 2014).

The stability of the micronutrients used for rice fortification have to resist heat, humidity and various drying steps which can lead to losses between 0 – 20% in coating or extrusion depending on different parameters, while dusting is considered to cause the smallest of all losses provided that no serious stress is applied to the kernels (Steiger et al. 2014).

3.6.3.2 Parboiling

This process aims to transfer the nutrients from the bran to the inner endosperm layer prior to milling and usually includes soaking, steaming and drying of the rice (Atungulu and Pan 2014). Traditional rice parboiling entails soaking rough rice in water at ambient temperatures overnight with subsequent boiling or steaming leading to gelatinization of the starch and expansion of the grain (Atungulu and Pan 2014). Recent investigations have explored the addition of fortificants to the soaking water of dehusked rice (De-Regil et al. 2014). Parboiling may improve textural properties, however, challenges when using parboiling technology include the destruction of natural antioxidants in the rice and a more rapid rancidity and development of mold due to the moist processing conditions; the required drying step adds an additional cost factor (Atungulu and Pan 2014). Vitamin levels may be met with this method, however, nutrients such as iron and zinc are not elevated

in white rice after parboiling, rendering other fortification approaches more advisable (Steiger et al. 2014).

3.6.3.3 Spraying

Spraying is a fortification method used in Columbia which employs certain aspects of coating and dusting. A liquid micronutrient premix, which is applied to the rice, is sprayed at high pressure, allowing the grains' pores to absorb the premix (Tsang, Moreno et al. 2016). Problems with this method are heterogeneity of the fortified rice, micronutrient losses during food preparation as well as mold formation due to the high humidity during the fortification process (Tsang, Moreno et al. 2016).

3.6.4   Biofortification

Biofortification aims to increase the bioavailable concentrations of essential elements in edible portions of crop plants through agronomic intervention or genetic selection (White and Broadley 2005), hence, it employs fertilization, conventional breeding and/or genetic (metabolic) engineering approaches (Sperotto, Ricachenevsky et al. 2012). It takes advatange of the consistent daily consumption of large amounts of food staples, which are highly prevalent in diets of the poor, thus targeting low-income households (Bouis, Hotz et al. 2011).

Fertilization can increase leaf mineral concentrations and improve yields, however, this increase is not necessarily transmitted to the plant's fruit, seed or grain (White and Broadley 2005). Main drawbacks of this method include inefficiency, cost and environmental factors and with regards to iron, fertilization is even more challenging owing to its insolubility (Sperotto, Ricachenevsky et al. 2012). Foliar application, intended to increase final concentrations in crops, is hardly feasible for large-scale implementation given the need for repeated applications (Sperotto, Ricachenevsky et al. 2012).

Conventional breeding, which is rather time consuming and only possible between closely related individuals (De Steur, Blancquaert et al. 2015), has been disqualified as option for micronutrient rice-biofortification due to its rather limited success in the past (Bhullar and

Gruissem 2013) given a narrow genetic variability of iron content in the rice germplasm (Boonyaves, Wu et al. 2017).

Genetic engineering approaches rely on the information related to the genetic network controlling iron uptake, transport, and storage (Boonyaves, Wu et al. 2017) with key targets for their application and adoption being an increase in yields, improved disease resistance, and lower cost of rice production (Demont and Stein 2013). Research on genetically modified rice has intensified since the rice genome has been decoded. Main areas of interest for genetic modification on the rice crop were targeted towards 1) Improving nutritional qualities; 2) Developing more resistant pest-, fungal-resistant and herbicide-tolerant strains; 3) Engineering seeds capable to survive submergence, drought or salinity (Calpe 2006). Unlike for other cereals, traits have been developed for rice that focus on consumer benefits, so called second-generation genetically modified (GM) crops (Demont and Stein 2013).

Biotechnological approaches can be multi-dimensional and efforts have been targeted mainly toward enhancing provitamin A and folate levels in the endosperm as well as on iron biofortification and enhancement of essential amino acids (Bhullar and Gruissem 2013). While plants are able to synthesize vitamins, in terms of mineral accumulation they rely on their availability in the soil (De Steur, Blancquaert et al. 2015).

It has been suggested, that commercialization of GM rice would substantially contribute in alleviating poverty, hunger and malnutrition, also for other GM crops and their global acceptance (Demont and Stein 2013). Several strategies to enhance iron content in rice grains targeted iron uptake and translocation within the plant as well as the storage of iron in the endosperm, however, more promising results have been achieved with approaches combining the facilitation of iron uptake and its storage (Bhullar and Gruissem 2013). A target content for iron of 15 µg/g biofortified rice (dry weight) has been suggested to ensure adequate iron supply (Bouis, Hotz et al. 2011). Most recent efforts achieved rice endosperm concentrations of 10.46 µg iron/g (Boonyaves, Wu et al. 2017).

3.6.4.1 Golden Rice

In the early 21st century, research samples of genetically fortified rice have been developed by a team of public and private research organizations. '*Golden Rice*', which contains the vitamin A precursor b-Carotene not only in the leaves but also in the grains, aims to reduce vitamin A deficiency (Childs 2004, Wesseler and Zilberman 2014) and has been suggestedthe potential to significantly reduce the disease burden of vitamin A deficiency provided its support by the government and public (Bhullar and Gruissem 2013). India, Indonesia, the Philippines and Viet Nam incorporated *Golden Rice* in their breeding programs, however, no country has approved its use by farmers (Bhullar and Gruissem 2013) and an overregulation of requirements for approval of GM crops, particularly for *Golden Rice*, has been claimed. Those requirements are based on the notion that the technology leads to an '*unpredictable and uncontrolled modification of the genome*' (Wesseler and Zilberman 2014). Expected to be introduced in 2002, *Golden Rice* still awaits approval and a publication from 2014 estimated annual losses for India of at least 1 424 680 life years or USD ~707 million, disregarding indirect health costs of vitamin A deficiency, have been suggested for each year that its introduction gets postponed (Wesseler and Zilberman 2014). Being subject of a controversial debate, *Golden Rice* is in the third phase of agricultural development and is supported by the US National Academy of Sciences and other key scientists worldwide (Moghissi, Pei et al. 2016).

Nevertheless, opposing opinions towards *Golden Rice* exist and main criticisms include fears, that this rice would promote monoculture, that real needs of farmers might be ignored, and that it could impose adverse impacts on the cultivation of conventional and organic rice, which could harm rural livelihoods; critics further argue that vitamin A capsule distribution is more effective as it can be monitored and evaluated for coverage and impact more easily (Lee and Krimsky 2016).

3.7   Costs for rice fortification

The core costs for rice fortification programs have been identified as: production, transportation to the point of blending, blending, sales/distribution costs, quality control/assurance and additional planning (Roks 2014). The micronutrients in fortified rice account for only ~5% of the fortified kernel and thus contribute a relatively small cost

element (Roks 2014). A micronutrient premix can be reasonably priced when produced at a scale of 3 – 5 MT, resulting in 100 MT of fortified kernels and consequently in 10 000 MT fortified rice (at a 1:100 blending ratio) (Roks 2014).

Investment costs for rice-premix factories have been suggested to account for USD 4, 0.75 and 0.30 million for hot, cold extrusion, and coating technology, respectively (Alavi, Bugusu et al. 2008). Coating technologies require a lower initial financial investment, however, the cost per MT of fortified rice is comparable (Steiger et al. 2014). Therefore, introducing premix-approaches for rice fortification should not be considered, if rice consumption is less than 100 g per day, hot extrusion facilities should be considered when estimated demands of rice-premix are at least 1500 MT per year (Alavi, Bugusu et al. 2008).

Rice blending can be done manually or automatically and the chosen method dictates the cost. Labor costs depend on the mode of operation and on local salaries. Simple blenders can be acquired for USD 200, whereas the price for sophisticated dosing equipment can amount up to USD 20 000 (Dexter 1998), however, no additional costs occur after the initial investment (Roks 2014).

Another cost factor is attributed to project management staff and it has been claimed, that at least one full-time project manager is needed per country for at least 2 years for overseeing the project and informing stakeholders (Roks 2014). However, actual total costs for rice fortification cannot be pinned down to one number that applies globally (Roks 2014). Validation of beneficiary or consumer acceptability are key and need to be ensured before program implementation. Effectiveness and efficacy studies may be redundant in the future, as scientific evidence in this field is constantly growing (Roks 2014).

An important consideration regarding the shelf life of fortified rice is, its expiration date, which is specified as one year upon blending with regular rice, hence decreasing the expiration date of unfortified rice in most cases (Roks 2014). Recommendations by WFP demand that vitamin and mineral premix kernels remain viable for at least 24 months upon storage (up to 30 °C at 75 % relative humidity) (Holden 2015).

3.8   Fortified rice – country and research examples

Sustainable commercialization of iron-fortified rice requires political support, cooperation of partners and a variety of social marketing activities as well as a consistent quality of the product; nevertheless, affordable prices for the customer and ensuring consumer accessibility to the rice are key determinants (Angeles-Agdeppa, Saises et al. 2011).

In Colombia, rice is the main energy and protein source for low-income groups, however, its role in the diet is affected by its price, but also by the price of wheat and other carbohydrate sources (Tsang, Moreno et al. 2016). First efforts to introduce fortified rice into the food supply started at the turn of the millennium and were driven by rice millers, leading to 35% of the marketed rice being fortified in 2015 (Tsang, Moreno et al. 2016). Due to increased rice smuggling from neighboring countries, Colombian rice millers desired a regulatory and mandatory scheme for fortification, however, given the lack of control by the federal government and possible increases in the price of fortified rice upon introduction of an official label, their position changed (Tsang, Moreno et al. 2016). Recently, an improvement of nutritional outcomes in Columbia due to fortified rice has been questioned (Tsang, Moreno et al. 2016).

In Brazil, fortified rice represents only $1-3\%$ of the rice market, which has been considered too small a fraction to create substantial public health impact, future growth of the fortified-rice category in Brazil has been predicted to be constrained in scope and speed (Milani, Carnahan et al. 2017).

Costa Rica's rice fortification guidelines include B and E vitamins, selenium, and zinc, however, the rice is not fortified with iron (Martorell, Ascencio et al. 2015).

3.8.1   Efficacy and effectivenes of fortified rice

In Mexican women of child-bearing age, fortified rice meals providing an average of 13 mg iron per day (as micronized FePP) administered over 5 days per week for 6 months lead to a reduction in anemia and ID of 10.3 and 15.1% (compared to the control group), respectively (Hotz, Porcayo et al. 2008).

In contrast, a study in Indian school-age children of normal iron status, who received rice meals either low or high in iron (6.25 mg or 12.5 mg iron as FePP) or unfortified for 6 days

per week over 6 months, revealed no between-group differences in the prevalence of anemia, ID and IDA (Thankachan, Walczyk et al. 2008).

In Brazilian infants (10 – 23 months) weekly administrations of iron-fortified rice (containing 56.4 mg elemental iron per serving) over 18 weeks increased hemoglobin levels and reduced anemia (Arcanjo, Santos et al. 2012).

In ID Indian children fortified rice meals (containing 20 mg iron as micronized ground FePP) administered daily over 7 months significantly increased iron stores and reduced ID prevalence (Moretti, Zimmermann et al. 2006).

A study in the Philippines in anemic school-age children who were fed rice meals fortified with 12 mg iron either as $FeSO_4$ or FePP decreased the anemia prevalence by almost 50%, whereas in the control group, anemia reduction was ~35%, however, part of this reduction in all groups was attributed to deworming procedures during the study (Angeles-Agdeppa, Capanzana et al. 2008).

In general, iron fortified rice has shown the potential to substantially improve iron statuses of vulnerable populations, however, further efforts are needed to translate the scientific findings into 'real life' situations, thus, to ensure that the fortified rice reaches the customer.

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MANUSCRIPT 1

Co-fortification of ferric pyrophosphate and citric acid/trisodium citrate into extruded rice grains doubles iron bioavailability through *in situ* generation of soluble ferric pyrophosphate citrate complexes.

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Abstract

Background: Iron fortification of rice is a promising strategy to improve iron nutrition. However, it is technically challenging as rice is consumed as intact grains, and ferric pyrophosphate (FePP), which is usually used for rice fortification, has low bioavailability.

Objective: To investigate whether the addition of a citric-acid/trisodium-citrate mixture (CA/TSC) prior to extrusion increases iron absorption in humans from FePP-fortified extruded rice grains.

Design: We conducted an iron absorption study in iron-sufficient young women (n=20), each consuming four different meals (4 mg iron/meal): 1) extruded FePP-fortified rice (No Ca/TSC); 2) extruded FePP-fortified rice with CA/TSC added prior to extrusion (CA/TSC extruded); 3) extruded FePP-fortified rice with CA/TSC-solution added after cooking prior to consumption (CA/TSC solution); and 4) non-extruded rice fortified with a ferrous sulfate ($FeSO_4$) solution added after cooking prior to consumption (Reference). Iron absorption was calculated from erythrocyte-incorporation of stable iron isotopes 14 days after administration. In *in vitro* studies, we assessed the soluble and dialyzable iron from rice meals where CA/TSC was added at different preparation stages and from meals with different Fe:CA:TSC ratios.

Results: Fractional iron absorption was significantly higher from CA/TSC extruded meals (3.2%) compared to No CA/TSC (1.7%) and CA/TSC solution (1.7%; all P<0.05) and was not different from the $FeSO_4$ reference meal (3.4%; n.s.). *In vitro* solubility and dialyzability were higher in CA/TSC extruded rice compared to rice with No CA/TSC and CA/TSC solution and solubility increased with higher amounts of added CA and TSC in extruded rice.

Conclusions: Iron bioavailability is nearly doubled when CA/TSC is extruded with FePP into fortified rice, resulting in iron bioavailability comparable to that of $FeSO_4$. We attribute this effect to an in situ generation of soluble FePP-citrate moieties during extrusion and/or cooking due to close physical proximity of FePP and CA/TSC in the extruded rice matrix.

This clinical trial was registered at clinicaltrials.gov as NCT02176759.

Introduction

Iron deficiency (ID) is common in children and young women in both developing and industrialized countries (Kassebaum, Jasrasaria et al. 2014). ID is considered the most common cause for anemia (Kassebaum, Jasrasaria et al. 2014) which adversely affects cognition (McCann and Ames 2007, Szajewska, Ruszczynski et al. 2010, Perignon, Fiorentino et al. 2014) and physical development of infants and children (Lozoff, Castillo et al. 2014), immune status and morbidity from infections (WHO 2001) and economic performance (Horton and Ross 2003, Plessow, Arora et al. 2015).

Iron fortification of foods can be an effective approach to control anemia and ID (Gera, Sachdev et al. 2012). Rice is a staple food for half the world's population (Muthayya, Sugimoto et al. 2014) and is therefore a promising vehicle for iron fortification. It is commonly consumed as intact grains, which can be fortified with iron via extrusion following a grain-premix approach (Brooke 1968). Nevertheless, achieving good organoleptic properties and high iron bioavailability is challenging. Fortification of extruded rice grains with ferric pyrophosphate (FePP) results in acceptable organoleptic characteristics (Moretti, Lee et al. 2005, Steiger et al. 2014), and has been shown to improve iron status in feeding trials (Moretti, Zimmermann et al. 2006, Hotz, Porcayo et al. 2008, Beinner, Velasquez-Meléndez et al. 2010).

Abbreviations: AA, Ascorbic acid; AAS, Atomic absorption spectrophotometry; CA, Citric acid; CA/TSC extruded, Extruded Rice containing [57]FePP, micronutrients and CA/TSC; CA/TSC solution, Extruded Rice containing [57]FePP and micronutrients, CA/TSC solution added prior consumption; CRP, C-reactive protein; ID, Iron deficiency; Fe, Iron; FePP, Ferric pyrophosphate; [57]FePP, Isotopically labelled ferric pyrophosphate; $FeSO_4$, Ferrous sulfate; [58]$FeSO_4$, Isotopically labelled ferrous sulfate; Hb, Hemoglobin; No CA/TSC, Extruded Rice containing [57]FePP and micronutrients; RBV, Relative Bioavailability; Reference, Regular Basmati Rice, [58]$FeSO_4$ solution added prior consumption; PF, Plasma ferritin; SFP, Soluble ferric pyrophosphate; TSC, Trisodium citrate

However, the bioavailability of FePP in rice is typically only 20-50% compared to $FeSO_4$ (Moretti, Zimmermann et al. 2006), which has high iron bioavailability in humans (Hurrell 1997). $FeSO_4$ cannot be added to extruded rice because it causes adverse discoloration (Moretti, Lee et al. 2005). If iron bioavailability from FePP-fortified rice could be increased, FePP amounts could be reduced (De Pee 2014), thus reducing costs and providing better sensory performance, hence augmenting the acceptability in rice eating populations (Khanh Van, Burja et al. 2014). Moreover, it could reduce the amount of iron passing unabsorbed into the colon, thus reducing adverse effects on the gut microbiome and inflammation (Zimmermann, Chassard et al. 2010, Jaeggi, Kortman et al. 2015).

In vitro experiments in Caco-2 cell models have indicated that soluble ferric pyrophosphate (SFP), where the ferric iron is chelated to citrate and pyrophosphate ligands, has a higher bioavailability than FePP alone (Zhu, Glahn et al. 2009). In the current study, we hypothesized that adding a mixture of citric acid (CA) and trisodiumcitrate (TSC) to rice flour prior to extrusion would result in the formation of SFP-moieties from the pressure and heat treatment during extrusion and subsequent boiling, and that this would result in higher iron bioavailability. To test this, we assessed the effect of CA/TSC either extruded with FePP or added as a solution before or after cooking on *in vitro* solubility and dialyzability of iron from FePP-fortified rice. Then, in a stable iron isotope study in generally healthy women, we compared iron absorption from rice meals fortified with a FePP:CA:TSC mixture added prior to extrusion, and compared it to meals where rice was fortified with FePP only and where CA/TSC was added as a solution after cooking prior to consumption. All meals were compared to a similar reference meal fortified with $FeSO_4$. Iron absorption was assessed by measuring stable iron isotopic-label incorporation in red blood cells 14 days after administration.

Methods

Human absorption study

Subjects

We enrolled twenty women from the student and staff population of ETH Zurich and University Zurich, Switzerland. Inclusion criteria were: 1) age between 18 and 40 years; 2) body weight < 65 kg; 3) apparently healthy with no chronic diseases or medications (except for oral contraceptives); 4) nonsmokers; 5) no blood donation or substantial blood loss within 4 months prior to the beginning of the study; and 6) no pregnancy or lactation. The baseline characteristics of the subjects are shown in **Table 1.** Informed written consent was obtained from all participants. Based on previous data from iron absorption studies in our laboratory, we calculated that a sample size of 20 would be adequate to detect an intra-subject difference of 30% in fractional iron absorption with a beta of 0.8 and an α of 0.05. The ethical committee of the canton Zurich reviewed and approved the study (KEK-ZH-Nr. 2014-147).

Design of the human absorption study

A randomized crossover design with four different rice meals was performed with each woman serving as her own control. Each woman consumed four different isotopically labeled test meals based on: (1) extruded ^{57}FePP-fortified rice (No CA/TSC), (2) extruded ^{57}FePP-fortified rice with CA/TSC added prior to extrusion (CA/TSC extruded), (3) extruded ^{57}FePP-fortified rice with a CA/TSC solution added prior to consumption (CA/TSC solution), and (4) one reference meal consisting of rice fortified with a ^{58}FeSO$_4$-solution added prior to consumption. Meals were served over four weeks with a two weeks period between test meals **(Figure 1)**. The reference meal was given one day prior to one of the test meals. The order of the test meal and the reference meal administration was randomized. The total duration of the study was 44 days.

Study procedures

The study was conducted between September 2014 and November 2014 at the Laboratory for Human Nutrition in Zurich, Switzerland. During screening (1 to 2 wk before

the first meal administration), body weight and height were measured and a urine sample was collected to conduct a pregnancy test. Eligible participants were invited to participate. Participants were instructed not to eat after 8.00 pm until the next morning or to drink fluids after 12 am on the day prior to each visit, except for the screening visit. The study was single-blind. In our study design, 18 meal administration schemes were possible. To harmonize it with the 20 participants, each scheme was listed twice in a column of Microsoft Excel (2013; Microsoft Corporation, Redmond, WA). The 36 schemes and the 20 participants, listed in separate columns, were then allocated by sorting random numbers generated by Microsoft Excel. Either on day 1 or 2 before consuming the first meal (Figure 1) all participants underwent a baseline venipuncture for the measurement of hemoglobin (Hb), plasma ferritin (PF) and C-reactive protein (CRP). Depending on the order of the reference meal administration, the study started with a blood sampling and the reference meal on day 1, or on day 2 with a blood sampling and the first test meal.

On days 16 and 30, participants received their second and third test meals after undergoing venipuncture for measurements of Hb, PF, CRP and isotopic iron composition following the same procedure as on day 2. Depending on the randomization schedule, participants received the reference meal either on day 1, 15 or 29. On day 44, 14 days after the last test meal administration, whole blood samples were collected to measure Hb, CRP and isotopic iron composition (Figure 1).

All meals were administered in the morning after an overnight fast. The participants completetly consumed the meals under direct supervision of the investigators. The empty bowls were then rinsed twice with 10 and 20 ml 18 MΩ·cm water, which was consumed by the participants. Participants were not allowed to eat or drink for at least 3 h after the test meals.
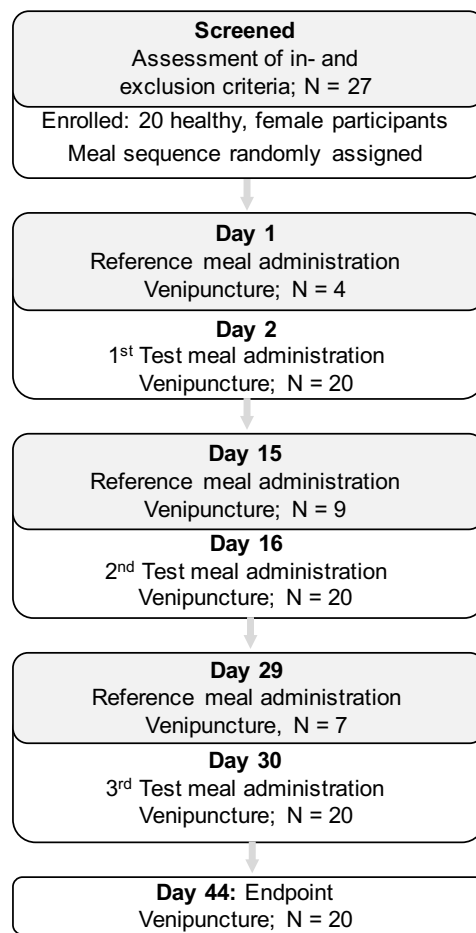
Figure 1. Schematic diagram of the study design.

Preparation of isotopically labeled extruded rice grains

The labeled ferric pyrophosphate (^{57}FePP) was prepared by Dr. Paul Lohmann GmbH KG, Emmerthal, Germany, from elemental enriched iron (95.8% ^{57}Fe enrichment, Chemgas, Boulogne, France), using a scaled down process as used for the synthesis of the commercial compound (Batch Nr. 1076133). Three hundred-twenty g long-grain rice flour (Tipo 150'000, 14#00065, Riseria Taverne SA, Taverne, Switzerland) were first manually mixed with 2.69 g isotopically labelled ^{57}FePP, 0.84 g zinc oxide (ZnO, D99013; Jungbunzlauer Suisse AG, Basel, Switzerland), 0.90 g vitamin A (Vitamin A palmitate 250 S/N, UE 01303005; DSM Nutritional Products Ltd., Basel, Switzerland), 0.63 g thiamine (Thiamine-mononitrate, UQ30903267; DSM Nutritional Products Ltd., Basel, Switzerland), 0.06 g folic acid (UT13110140; DSM Nutritional Products Ltd., Basel, Switzerland), and 0.34 g vitamin B$_{12}$ (0.1% Cyanocobalamin, UT13110014; DSM Nutritional Products Ltd., Basel, Switzerland). Afterwards, the whole mixture was mixed in a mixer (KM 410,

Kenwood Ltd.; Havant Hants, Great Britain). Then, the flour mixture was divided into two batches and 0.11 g CA and 3.04 g TSC (Citric Acid anhydrous, SZBE0090V; Trisodium Citrate Dihydrate, BCBL6643V; both Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) were added to one batch resulting in an Fe:CA:TSC molar ratio of 1:0.1:2.1. The water content of the two batches was subsequently adjusted to 25% by slowly adding 18.2 MΩ·cm water using a mixer (KM 410). The two batches were separately extruded using a Brabender single-screw extruder (DSE 20/24 DO-Corder DN20; Brabender GmbH & Co KG, Duisburg, Germany) with a custom-made brass die and cutter under hot extrusion conditions. The parameters for extrusion were as follows: barrel-temperature 90 °C, drive 120 rpm, pressure 90 bar, feeding rate 280-300 g/h. The extruded grains were air-dried overnight to reach a moisture content of approximately 10%. There was a minimal color difference comparing the No CA/TSC to the CA/TSC extruded rice grains (the CA/TSC grains were slightly darker); this difference was no longer apparent in the blended rice both prior to and after cooking.

Test meal preparation

The test meals and the reference meal consisted of 48 g and 50 g Basmati rice, respectively, and 30 g vegetable sauce (21). To reach a fortification level of 80 ppm, 2.236 g (± 0.004) isotopically labelled extruded rice were added to the No CA/TSC and CA/TSC solution test meals and 2.084 g (± 0.003) isotopically labeled extruded rice containing also CA/TSC were added to the CA/TSC extruded test meal. No extruded rice was added to the reference meal. All meals were administered along with 300 ml 18.2 MΩ·cm water. The vegetable sauce was prepared in bulk as previously described (21) and stored frozen until administration. Basmati rice and extruded rice were cooked in an oven (20 minutes; 220 °C; BOSCH, Switzerland) using a separate glass bowl for each participant one day prior to administration and refrigerated at 4 °C overnight. On the administration day, the defrosted vegetable sauce was added to the precooked rice and the composite meal was heated in a microwave oven for 1 min at 600 W.

Prior to consumption, 1 ml of CA/TSC solution (equal to 1.26 mg CA and 36.16 mg TSC) was added to the CA/TSC solution meals, and 3.962 mg (± 0.026) iron in form of a labeled ^{58}FeSO$_4$ solution, which was produced as previously described (Cercamondi, Egli et al.

2013) from enriched elemental iron (99.90% ^{58}Fe enrichment, Chemgas, Boulogne, France), were added to the reference meal.

Test meal analysis

Iron content in the Basmati rice and vegetable sauce was analyzed via atomic absorption spectrophotometry (AAS, GTA 120 or AA240FS; both Agilent Technologies AG, Basel, Switzerland) after mineralization by microwave digestion (MLS TurboWave, MLS GmbH; Leutkirch, Germany) using HNO_3. Iron content in extruded rice was analyzed as previously described for blood samples (Zimmermann, Troesch et al. 2009). Phytic acid (PA) content was determined as previously described (Cercamondi, Egli et al. 2010). The ascorbic acid (AA) content in the vegetable sauce was analyzed via HPLC (Acquity H-class UPLC system, 4824949; Waters AG, Dättwil, Switzerland) after stabilization and extraction in meta-phosphoric acid and reduction via dithiothreitol (Parviainen and Nyssönen 1992).

Preparation of fortified extruded rice grains for in vitro trials

The preparation of the rice grains produced for the *in vitro* experiments was similar to the one for isotopically labeled rice; the only difference was that regular FePP (Batch Nr. 124466; Dr. Paul Lohmann GmbH KG, Emmerthal, Germany) was used and no vitamins or ZnO were added. Thus, the fortified extruded rice grains contained rice flour, FePP and either CA/TSC (in varying molar ratios relative to FePP) or no CA/TSC. Additionally, one type of rice grains contained SFP with sodium citrate (SFP + SC, 30109381; Dr. Paul Lohmann GmbH KG, Emmerthal, Germany) and one type contained $FeSO_4$ (Ferrous sulfate-7-hydrate, 288096; Dr. Paul Lohmann GmbH KG, Emmerthal, Germany).

In vitro experiments

Determination of iron solubility

Extruded rice kernels with CA/TSC added at extrusion contained 4.92 (± 0.14) mg/g iron. All other rice kernels (No CA/TSC, CA/TSC prior cooking and CA/TSC post cooking) contained 4.38 (± 0.14) mg/g iron and the Fe:CA:TSC ratios were the same as those for the present *in vivo* study. For comparing the solubility from different Fe:CA:TSC molar ratios, the following CA:TSC molar ratios respective to 5 mg iron were used – the Fe amounts (± SD) in mg/g iron are given in brackets: 0.1:1.7 [4.78 (± 0.11)]; 0.1:2.1 [4.60 (± 0.04)]; 0.1:2.4

[4.79 (± 0.06)]; 0.3:4.7 [4.25 (± 0.05)]; 0.9:17.2 [3.39 (± 0.02)]; SFP [4.98 (± 0.06)]; $FeSO_4$ 0:0 [6.23 (± 0.15)]. To assess iron solubility from extruded rice kernels, a modified method of Miller et al (Miller, Schricker et al. 1981) was used. Briefly, all extruded samples were prepared in triplicate, and unfortified long-grain rice (Jasmine rice Bio, Migros, Switzerland) with a $FeSO_4$ solution served as a reference. Thirty ml boiling water were added to 1 g extruded or unfortified rice, respectively, and cooked in an oven (BOSCH, Switzerland) for 20 minutes at 150 °C. After cooling down to 35-40 °C and homogenization with a Polytron homogenizer (PT 1200 E; Kinematica AG, Lucerne, Switzerland), aliquots of 250 mg were taken in triplicate from each sample for mineralization and subsequent determination of total iron concentration. 1 ml of a $FeSO_4$ solution (5 mg Fe/ml) was added to the unfortified reference sample before aliquoting.

To the remainder of each sample, 0.1 g amylase (Takadiastase from *Aspergillus oryzae*, BCBM 5345; Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) were added and samples were incubated for 10 minutes at room temperature with subsequent adjustment to pH 2 with 6 M HCl. From each sample, three aliquots of 3 ml were mixed with 26 µl pepsin solution [1.6 g pepsin (Porcine, P70000; Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) in 10 g 0.1 M HCl] and were incubated on a shaking water bath for 2 h at 37 °C and 150 rpm. Thereafter, all samples were centrifuged for 15 minutes at 3600 rpm and the iron concentration in the respective supernatants was measured. The retrieved iron concentration values were summed up from each triplicate and this sum was extrapolated to the weight of the sample after initial cooking. The solubility [%] was expressed as the quotient of the prior extrapolated iron content measured in the supernatant divided by the total iron content in the respective aliquots.

Determination of iron dialyzability

Iron dialyzability of the following meals was tested: 1) CA/TSC extruded, 2) CA/TSC prior cooking, 3) CA/TSC post cooking and 4) No CA/TSC. For determining the iron dialyzability of extruded rice kernels, we adapted the methods described by Miller et al. (Miller, Schricker et al. 1981) and by Hurrell et al. (Hurrell, Lynch et al. 1988). Briefly, extruded rice samples were analyzed in triplicate, the reference sample containing long grain rice was only prepared once. 1 g extruded rice and 49 g long grain rice, respectively 50 g long grain rice (reference), were mixed in a glass beaker and 150 g boiling 18.2 MΩ·cm water were

added. The beakers were covered, put in an oven at 100 °C for 20 minutes and homogenized (PT 1200 E) after cooling down to 35 – 40 °C, then 0.5 g amylase were mixed into each sample, followed by a 10 min incubation period at room temperature and adjustment to pH 2 with 6 M HCl. For each sample, four Erlenmeyer flasks were prepared and 40 g rice and 1.3 ml pepsin solution were added in each flask. Additionally, 200 µl $FeSO_4$ solution (250 mg $FeSO_4$ in 10 g 0.1 M HCl) were added to the reference sample only. Then, all samples were covered with parafilm and incubated on a shaking water bath (2h hours at 37 °C and 150 rpm).

Prior to titration, dialysis membranes (SpectraPor 1, 2.4 mm O; Spectrum Laboratories, Compton CA, USA) were boiled in water for about 30 min; afterwards one end was sealed with dialysis standard closures (35 mm, Spectrum Laboratories, Compton CA, USA). Five ml of pancreatin solution [0.4 g pancreatin (Porcine, P-1750; Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) in 25 ml 0.1 M $NaHCO_3$ and 2.5 g bile extract in 25 ml 0.1 M $NaHCO_3$ were mixed and filled up to 100 ml with 0.1 M $NaHCO_3$] were added to each sample. Determination of the amount of $NaHCO_3$ and incubation of sample after pancreatin addition were done according to Hurrell et al. (22), except that samples were not frozen overnight.

For the extraction of dialysates, the dialysis membranes were removed from the samples and the dialysates transferred into acid-washed pre-weighed PE-bottles, which contained 700 µl HNO_3 65%. The samples were stored at 4 °C over night, diluted and measured by graphite-furnace AAS (GF-AAS, GTA 120; Agilent Technologies AG, Basel, Switzerland) on the subsequent day. The dialyzability [%] was expressed as the quotient of dialyzable iron from the supernatant divided by the dialyzable iron from the reference ($FeSO_4$).

Blood analysis

PF and CRP were measured by using the IMMULITE 2000 system (Siemens Healthcare Diagnostics, Zürich), following manufacturer's instructions. Hb was measured in whole blood on the day of collection by using either a Sysmex XE_5000 (Sysmex Corporation, Japan) or an Advia 2120 haematology analyzer (Siemens Healthcare, USA); anemia was defined as Hb < 12 g/dl (WHO 2001). ID was defined as PF < 15 mg/l and ID anemia as Hb < 12 g/dl and PF < 15 mg/l (WHO 2001). Reference CRP concentrations for healthy individuals were < 5 mg/l (Dati, Schumann et al. 1996). The isotopic composition in the

blood samples and the calculation of iron absorption were conducted as previously described by our laboratory (Zimmermann, Troesch et al. 2009).

Statistical analysis

Data were analyzed using SPSS (version 22.0, 2013; SPSS Inc, Chicago, IL) and Microsoft Excel (2013; Microsoft Corporation, Redmond, WA). The fractional iron absorption from the different meals within the same participant and the *in vitro* results were compared by repeated-measures ANOVA followed by Bonferroni corrected pairwise comparisons. Results for age, and anthropometric features, Hb, PF and CRP were presented as means ±SDs if normally distributed, otherwise the results were presented as geometric means with 95% CI. Calculations for iron and PA concentration of the composite meals were based on the means from the analysis of single components (regular Basmati rice, extruded rice, vegetable sauce; N=3), SDs were adapted by calculating the square root of the squared and summed SDs from each single component. Differences were considered as significant at $P<0.05$. Non-normally distributed data were logarithmically converted for statistical analysis and reconverted for reporting. The study was powered to detect an intra-subject difference of 30% in fractional iron absorption with an α level of 0.05.

Results

Participant characteristics

All 20 subjects completed the study and all data were included in the analysis. At baseline (day 1 or day 2), four participants were iron deficient without anemia, two participants were iron deficient anemic and three participants had elevated CRP concentrations (5.8 – 18.3 mg/l). Five participants had a CRP concentration above 5.0 mg/l during at least one study visit. One participant had elevated CRP concentrations throughout the study, one participant had an increased CRP during three visits and two participants had an increased CRP during one visit (Table 1). No adverse events were reported.

Table 1. Anthropometric features, iron and inflammatory status of study participants (N=20), assessed prior to first meal administration

Age[1], y	26 ± 3
Weight[1], kg	56.9 ± 4.8
Height[1], m	1.66 ± 0.05
BMI[1], kg/m^2	20.6 ± 1.6
Hemoglobin[2], g/l	130 (126, 135)
C-reactive protein[2], mg/l	1.0 (0.6, 1.8)
Plasma ferritin[1], ng/ml	40.3 ± 26.1

[1]Values are means $\pm$ SDs.
[2]Values are geometric means (95% CI).

Test meal composition

Iron concentration in the isotopically labelled extruded rice grains with CA/TSC and without CA/TSC was 191.7 (± 4.8) mg/100 g and 177.5 (± 5.2) mg/100 g, respectively. The native iron content was 0.29 (± 0.02) mg Fe in each composite test meal, which also contained 0.08 g (± 0.00) phytic acid. The PA and iron concentrations and the PA / Fe molar ratios of the different test meals and the reference meal are summarized in **Table 2.** The composite reference meal contained 0.29 (± 0.02) mg Fe and 0.07 (± 0.00) g PA. The sauce given with each meal contained 0.4 (± 0.0) mg AA. The AA content was determined only in the vegetable sauce as its content in rice is negligible.

Table 2. Test meal composition and values for iron absorption (N=20) [1]

Meal	Total iron content per meal[2] [mg]	Fe:CA:TSC [Molar Ratio]	Phytic acid : Iron[Molar Ratio]	Fractional iron absorption[3] [%]	Total absorbed iron per meal[3] [mg]	RBV[4] compared to Reference [%]
No CA/TSC addition	3.98 (± 0.01)	n.a.	1.56 : 1	1.7[a] (0.6, 4.3)	0.06[a] (0.02, 0.16)	45[a]
CA/TSC extruded	3.86 (± 0.01)	1:0.1:2.1	1.59 : 1	3.2[b] (1.1, 9.0)	0.11[b] (0.04, 0.32)	83[b]
CA/TSC solution	3.98 (± 0.01)	1:0.1:2.1	1.56 : 1	1.7[a] (0.6, 4.9)	0.06[a] (0.02, 0.18)	46[a]
Reference	4.27 (± 0.01)	n.a.	1.45 : 1	3.4[b] (1.2, 9.9)	0.14[b] (0.05, 0.39)	100[b]

[1]Micronutrient content in meals No CA/TSC addition; CA/TSC extruded; CA/TSC solution: zinc 4 mg, vitamin A 200 µg, thiamin 0.5 mg, folic acid 150 µg, vitamin B$_{12}$ 1µg. CA/TSC extruded: Extruded Rice containing [57]FePP, micronutrients, Trisodium Citrate and Citric Acid (CA/TSC); CA/TSC solution: Extruded Rice containing [57]FePP and micronutrients, CA/TSC solution added prior consumption; No CA/TSC: Extruded Rice containing micronutrients and [57]FePP; Reference: regular Basmati Rice, [58]FeSO$_4$ solution added prior consumption. RBV: Relative bioavailability.

Different superscript letters within a column indicate significant differences between values at P<0.05; Bonferroni corrected repeated measures ANOVA.

[2]Values are means (±SD), based on the means from the analysis of single components (regular Basmati rice, extruded rice, vegetable sauce; N=3 for all components), SDs were adapted by calculating the square root of the squared and summed SDs from each single component].

[3]Values are geometric means (95% CI), N=20.

[4]Relative bioavailability (RBV) was calculated for each meal from the fractional iron absorption [%] respective to the fractional iron absorption from the reference meal, N=20.

Iron absorption measurements

Type of meal significantly affected iron absorption (P<0.02). Fractional and total iron absorption from the CA/TSC extruded meal was sharply higher than from the No CA/TSC meal (P<0.01, **Table 2**). There was no significant difference in iron absorption between the CA/TSC extruded meal and the $FeSO_4$ reference meal; the relative bioavailability (RBV) of the CA/TSC extruded meal was 83%. Fractional iron absorption from the different meals followed a similar pattern in most subjects **(Figure 2).**
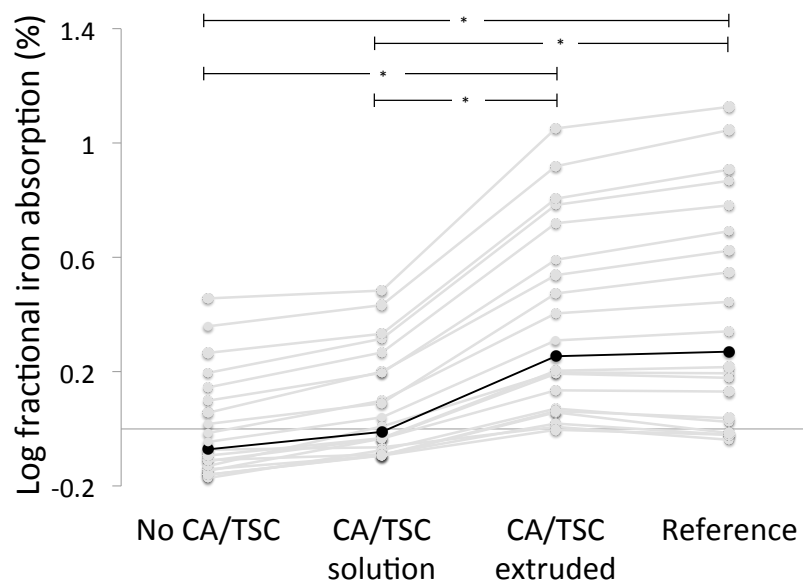
Figure 2. Log-transformed fractional iron absorption [%] in healthy women (N=20), who consumed a composite rice meal either containing 1) extruded rice fortified only with [57]FePP and micronutrients (No CA/TSC); 2) extruded rice fortified with [57]FePP, micronutrients, Trisodium Citrate (TSC) and Citric Acid (CA) added prior to consumption (CA/TSC solution); 3) extruded rice fortified with [57]FePP, micronutrients, Trisodium Citrate (TSC) and Citric Acid (CA) added prior to extrusion (CA/TSC extruded); 4) rice meal without extruded rice fortified with [58]$FeSO_4$ solution added prior consumption (Reference).
Each dot represents the log transformed fractional iron aborption [%] for one subject, connected dots represent are from one subject, the black line represents the mean absorption from all subjects (N=20). Horizontal bars with asterisks indicate signifcant differences in a repeated measures ANOVA (Bonferroni correction; P<0.05).

In vitro measurements

Extrusion of CA/TSC with FePP led to a significant increase in iron solubility and dialyzability compared with the other fortification approaches tested **(**9.6% and 10.7%; P<0.01; **Figures 3 & 4).** No addition of CA/TSC showed significantly lower solubility and dialyzability than the other conditions (2.7%; P<0.01). CA/TSC addition as a solution prior

(7.1%) or post (6.2%) cooking to meals containing FePP-fortified extruded rice grains was not a determining factor for iron solubility, whereas it was for iron dialyzability (P<0.03), where the iron dialyzability was higher when adding CA/TSC after cooking (5.6%) rather than before cooking (4.6%).

We found higher iron solubility with increasing molar ratios of CA/TSC in the extruded rice: CA/TSC addition at molar ratios of Fe:CA:TSC of 1:0.9:17.2 increased iron solubility by more than 7 fold compared to no CA/TSC addition (19.9 vs. 2.7%, P<0.05) and showed a trend toward higher solubility than rice extruded with $FeSO_4$ (17.1%) (P=0.07, **Figure 5**). Iron solubility at molar ratios of Fe:CA:TSC of 1:0.1:2.1, the same ratio as used in the current *in vivo* study, led to a 3.5-fold increase of iron solubility compared to no CA/TSC addition (9.6 vs. 2.7%, P<0.05).
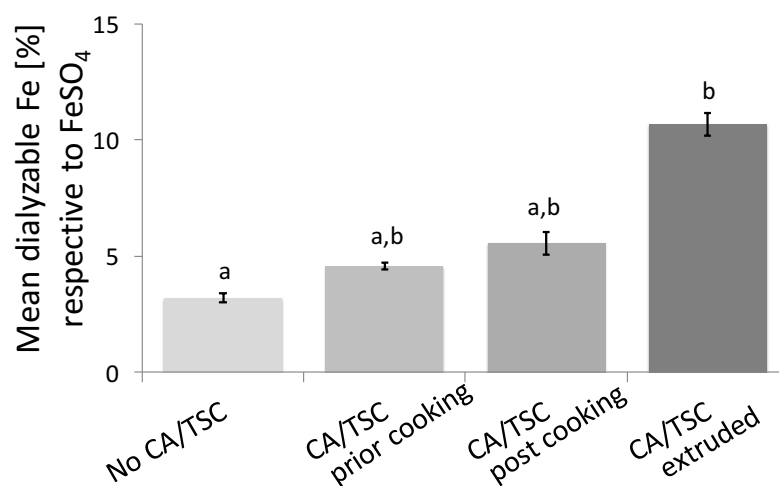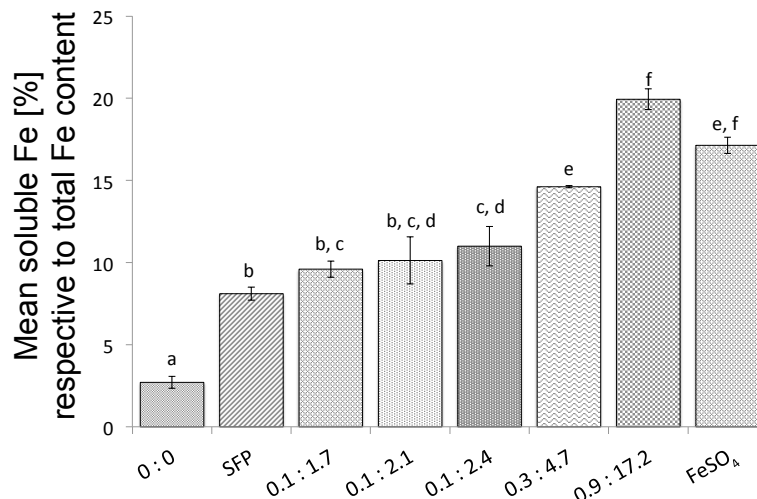

Figure 3. Bars represent the mean iron solubility [%] from extruded rice fortified with 5 mg iron per g extruded rice. CA/TSC was either added before, after cooking or at extrusion (all N=3). Molar ratios for Fe:CA:TSC for all samples are 1:0.1:2.1. Error bars with whiskers indicate standard deviations. Different letters on the bars indicate significant differences in a univariate ANOVA (Bonferroni correction; P<0.01).
CA/TSCextruded: Extruded Rice containing Ferric Pyrophosphate (FePP), Trisodium Citrate and Citric Acid (CA/TSC); CA/TSC prior cooking: Extruded Rice containing FePP and CA/TSC solution added prior cooking;
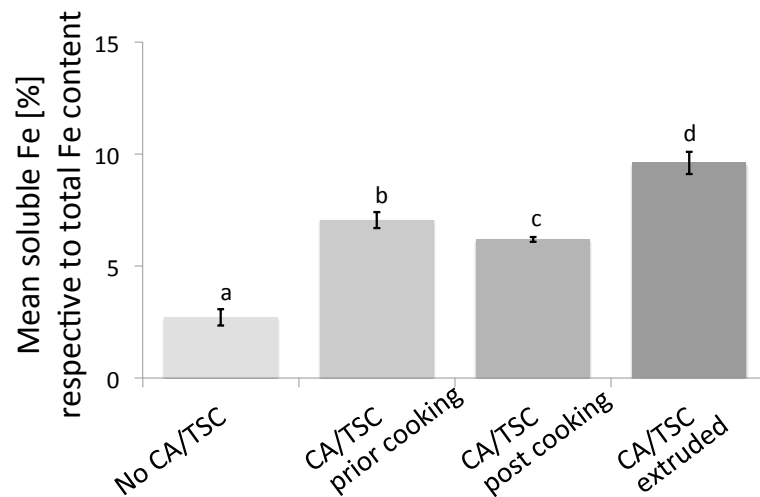
Figure 4. Bars represent the mean relative iron dialyzability [%] (FeSO$_4$ added as solution = 100% dialyzability) from extruded rice (fortified with 5 mg iron) assessed after digestion *in vitro* and subsequent mineralization. Citric acid and trisodium citrate (CA/TSC) were either added before or after cooking or at extrusion (all N=3). Molar ratios for Fe:CA:TSC for all samples are 1:0.1:2.1. Error bars with whiskers indicate standard deviations. Different letters on the bars indicate significant differences in a univariate ANOVA (Bonferroni correction; P<0.03).
CA/TSCextruded: Extruded Rice containing Ferric Pyrophosphate (FePP) and CA/TSC; CA/TSC prior cooking: Extruded Rice containing FePP and a CA/TSC solution was added prior cooking; CA/TSC post cooking: Extruded Rice containing FePP and a CA/TSC solution was added after cooking; No CA/TSC: Extruded Rice containing FePP.

Figure 5. Bars represent the mean iron solubility [%] after *in vitro* digestion from extruded rice with varying molar ratios of Citric acid : trisodium citrate (CA:TSC) relative to 5 mg iron as ferric pyrophosphate (N=3). Solubility data for ferrous sulfate (FeSO$_4$; N=2) are used for comparison. The values are means; error bars with whiskers indicate standard deviations. Different letters on the bars indicate significant differences in a univariate ANOVA (Bonferroni correction; P<0.05).

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Discussion

The main finding of this study is that addition of CA/TSC prior to extrusion nearly doubles iron absorption from FePP-fortified rice. Our findings suggest the addition of CA/TSC may be a breakthrough in efforts to increase the typically low iron absorption from FePP in extruded rice (Moretti, Zimmermann et al. 2006). This approach may also prove effective in other FePP-fortified foods, such as bouillon cubes, infant cereals and salt (WHO 2006). Evidence of the effect of other iron absorption enhancers on iron absorption from FePP is limited. AA enhances the bioavailability of iron from FePP of different particle sizes (Fidler, Davidsson et al. 2004). In contrast, the addition of EDTA to FePP-fortified wheat-based infant cereals did not significantly increase iron absorption (Hurrell, Reddy et al. 2000). Earlier studies that examined the effects of CA on iron absorption produced mixed results. The addition of 60 mg CA to an oat-based beverage fortified with 1.3 mg ferric ammonium citrate increased iron bioavailability by 54%, which was borderline significant (Zhang, Önning et al. 2007). In a rice meal fortified with 3 mg $FeSO_4$, addition of 1 g of CA increased iron absorption threefold (Gillooly, Bothwell et al. 1983). In contrast, CA added (at a molar ratio ~2.5 to iron) to fish sauce fortified with 4 mg $FeSO_4$ did not increase iron absorption (Walczyk, Tuntipopipat et al. 2005), and the addition of CA to a composite meal of maize, rice and black beans decreased iron absorption (Hallberg and Rossander 1984).

In our study, the addition of a CA/TSC solution after cooking prior to consumption did not have an enhancing effect on iron absorption. This indicates that co-extrusion and/or boiling is essential for *in situ* formation of SFP in the extruded rice kernel. Our *in vitro* solubility and dialyzability data suggest that extrusion is necessary to achieve a substantial increase in iron solubility and dialyzability via CA/TSC addition. It is likely that *in situ* formation of SFP is facilitated by extrusion followed by cooking, possibly due to mixing and the resulting close physical proximity of FePP and CA/TSC in the extruded rice matrix. Further studies are required to determine if these effects will occur when applying other rice fortification techniques, such as coating, similarly characterized by physical proximity of fortificants, but not by the heat and pressure treatment of extrusion.

In contrast to our *in vivo* data, we found higher iron solubility and dialyzability in meals where CA/TSC was added as a solution compared to no addition. Furthermore, the dialyzability of the CA/TSC extruded meal was only 12% of the $FeSO_4$-reference,

contrasting with the 84% RBV observed in vivo. Discrepancies between in vitro and in vivo methods assessing iron bioavailability are well documented (Fairweather-Tait, Lynch et al. 2005) and the usefulness of dialyzability and solubility is likely limited to comparative, but not quantitative assessment (Fairweather-Tait, Lynch et al. 2005).

High *in vitro* bioavailability of SFP was also recently reported in Caco-2-cells (Zhu, Glahn et al. 2009). The authors suggested it to be due to a shielding effect by the surrounding pyrophosphate and citrate ligands, preventing the interaction of iron with potential inhibitors (Zhu, Glahn et al. 2009). SFP is commercially available but it has not been used as iron fortificant in rice, likely because it leads to strong discolorations of the kernels (data not shown). It is likely that the formation of SFP in our extruded FePP:CA:TSC kernels occurs at least partly during cooking, which explains the low tendency for kernel discoloration when CA/TSC is added with FePP prior to extrusion.

Our solubility data do not indicate a solubility plateau with increasing amounts of CA/TSC in relation to FePP; there was a continuous increase of solubility with increasing ratios of CA:TSC to iron (Figure 5). This suggests that higher absorption enhancing effects might be obtained by increasing the amounts of CA/TSC relative to FePP to a higher ratio than we used in our in vivo study. However, the comparable iron absorption *in vivo* of the CA/TSC addition prior to extrusion and $FeSO_4$ (which is water soluble and the reference standard for iron absorption) suggests that the potential for a further increase in human iron absorption may be limited. Clearly, further research is needed on the optimal ratio of CA/TSC addition to enhance iron absorption from FePP in extruded rice. However, these studies should also include careful sensory evaluation because higher CA/TSC concentrations may alter the integrity, color and taste of the extruded rice kernels.

In our study, the subjects were generally iron-sufficient, with a mean PF of 40.3 µg/l. Therefore, performing our study in iron-deficient subjects would have very likely resulted in higher fractional and total iron absorption. We have previously reported the RBV of FePP in extruded rice is dependent on iron status (Moretti, Zimmermann et al. 2006) because absorption from $FeSO_4$ is up-regulated in subjects with low iron stores to a greater extent than for FePP (Zimmermann, Biebinger et al. 2011). In the present study, we did not find a significant difference in absorption from rice extruded with FePP and CA/TSC versus $FeSO_4$, however, it is possible that iron absorption could be higher from $FeSO_4$ in iron-deficient subjects.

A strength of this study is the combined *in vitro* and *in vivo* approach. We measured iron absorption *in vivo* from iron fortified rice intrinsically labeled with stable isotopes at high precision and accuracy; in parallel, we assessed *in vitro* solubility and dialyzability which both support the hypothesis that heat treatment and co-localization are required for the *in situ* generation of soluble FePP. A limitation of our study is that the standardized meals employed contained negligible amounts of PA, a potent inhibitor of iron absorption (Hallberg, Brune et al. 1989, Hurrell 2004). While rice meals tend to have lower PA contents than other cereal-based meals, further studies are required to investigate whether the enhancing effect of CA/TSC is as pronounced in meals with considerable amounts of PA such as e.g. rice meals containing beans or legumes. We did not rigorously evaluate and compare the sensory properties of the different extruded rice grains. However, on careful inspection, we could detect only minimal color differences in the blended rice prior to and after cooking. Due to the high costs of isotopic labels, the blending ratio of fortified to native rice in our study was 1:25, which is higher than the blending ratios used in current fortified rice programs, which vary between 1:100 to 1:200. It will therefore be important to test sensory properties as well as storage stability of extruded rice fortified at a 1:100 blending ratio, as these conditions are closer to commercially available fortified rice blends.

In conclusion, our findings show that iron absorption from FePP-fortified rice can be nearly doubled by adding CA and TSC prior to extrusion. CA and TSC are low cost food ingredients with various applications in the food industry (Muthayya, Sugimoto et al. 2014) and are both considered GRAS (SCOGS). With the use of CA/TSC, iron absorption from FePP can be significantly enhanced, which would allow the amount of FePP used in rice fortification to be decreased, lowering production costs, while maintaining biological efficacy and avoiding the adverse health effects associated with high iron doses. Further studies are needed to investigate the impact on sensory properties and the storage stability of extruded rice containing CA/TSC, particularly at higher CA/TSC to iron ratios. Also, the effect of CA/TSC in fortified rice needs to be investigated in meals containing phytate-rich ingredients such as beans or legumes. Finally, addition of CA/TSC should be considered in other foods where FePP is the iron fortification compound of choice such as for bouillon cubes and salt, as the enhancing effect may not be restricted to fortified rice.

Authorship Contributions

DM, LH, CIC and MZ designed the studies. LH, CIC, HA, CZ and DW conducted the experiments. LH and CZ analyzed data. LH, CIC, MZ and DM wrote the paper. LH, CIC, MZ and DM had primary responsibility for final content. All authors read and approved the final version of the paper.

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MANUSCRIPT 2

Iron bioavailability from ferric pyrophosphate in extruded rice co-fortified with zinc sulfate is greater than when co-fortified with zinc oxide in a human stable isotope study.

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Abstract

Background: Extruded rice grains are often co-fortified with iron and zinc. However, it is uncertain if the addition of zinc to iron-fortified rice affects iron absorption, and whether this is zinc-compound specific.

Objectives: We investigated whether zinc, added as oxide (ZnO) or sulfate ($ZnSO_4$), affects human iron absorption from extruded rice fortified with ferric pyrophosphate (FePP).

Methods: In 19 iron-depleted Swiss women (plasma ferritin ≤16.5 μ//L) between 20 and 39 y of age with normal BMI (18.7 – 24.8 kg/m^2), we compared iron absorption from 4 meals containing fortified extruded rice with 4 mg iron and 3 mg zinc. Three contained extruded rice labeled with FePP (^{57}FePP): 1) one without added Zn (^{57}FePP-Zn); 2) one co-fortified with ZnO (^{57}FePP+ZnO); 3) one co-fortified with $ZnSO_4$ (^{57}FePP+$ZnSO_4$). The fourth meal contained extruded rice without iron or zinc, extrinsically labeled with ferrous-sulfate (^{58}FeSO$_4$) added as a solution after cooking. All four meals contained citric acid (CA). Iron bioavailability was measured by isotopic iron ratios in red blood cells. We also measured relative *in vitro* iron solubility from ^{57}FePP-Zn, ^{57}FePP+ZnO and ^{57}FePP+$ZnSO_4$ expressed as fraction of $FeSO_4$-solubility.

Results: Geometric mean fractional iron absorption (95% CI) from ^{57}FePP+$ZnSO_4$ was 4.5% (3.4, 5.8%) and differed from ^{57}FePP+ZnO: 2.7% (1.8, 4.1%) (P<0.03); both did not differ from ^{57}FePP-Zn: 4.0% (2.8, 5.6%). Relative iron bioavailability (RBV) compared to ^{58}FeSO$_4$ was 62%, 57% and 38% from ^{57}FePP+$ZnSO_4$, ^{57}FePP-Zn and ^{57}FePP+ZnO, respectively. In vitro solubility from ^{57}FePP+$ZnSO_4$ differed from ^{57}FePP-Zn (14.3%) (P<0.02) but not from ^{57}FePP+ZnO (10.2% vs. 13.1%) (P=0.08).

Abbreviations: AA, Ascorbic acid; AAS, Atomic absorption spectrophotometry; CA, Citric acid; CRP, C-reactive protein; ID, Iron deficiency; Fe, Iron; FePP, Ferric pyrophosphate; ^{57}FePP, Isotopically labelled ferric pyrophosphate; ^{57}FePP-Zn, Rice extruded with ^{57}FePP and CA; ^{57}FePP+ZnO, Rice extruded with ^{57}FePP, ZnO and CA; ^{57}FePP+$ZnSO_4$, Rice extruded with ^{57}FePP, $ZnSO_4$ and CA; $FeSO_4$, Ferrous sulfate; ^{58}FeSO$_4$, Isotopically labelled ferrous sulfate; Hb, Hemoglobin; No CA/TSC, Extruded Rice containing ^{57}FePP and micronutrients; Hb, Hemoglobin; ICP-MS, Inductively-coupled plasma mass spectrometry; PA, Phytic acid; PF, Plasma ferritin; RBV, Relative Bioavailability; Reference, Regular Basmati Rice, ^{58}FeSO$_4$ solution added prior consumption; PF, Plasma ferritin; SFP, Soluble ferric pyrophosphate; TSC, Trisodium citrate; ZnO, Zinc Oxide; $ZnSO_4$, Zinc Sulfate

Conclusions: In iron-depleted women, iron absorption from FePP-fortified extruded rice co-fortified with $ZnSO_4$ was 1.6 (1.0, 2.3) fold that of rice co-fortified with ZnO. These findings suggest $ZnSO_4$ may be the preferable zinc co-fortificant for optimal iron bioavailability of iron-fortified extruded rice. This clinical trial was registered at clinicaltrials.gov (NCT02255942).

Introduction

Iron and zinc deficiency are major public health concerns, and fortification of staple foods with both minerals is a useful approach to combat these deficiencies. Rice, the staple food for almost half the world's population (Muthayya, Hall et al. 2012), may be an important fortification vehicle. Ferric pyrophosphate (FePP) is the iron compound of choice for rice fortification, due to its white color and low reactivity with the rice matrix. However, iron from FePP generally has a relative bioavailability (RBV) of only ≈50% compared to $FeSO_4$ (Allen, De Benoist et al. 2006), and the RBV of FePP in rice meals is reported to be even lower, only 15-24% (Moretti, Zimmermann et al. 2006). To increase the low iron bioavailability of FePP in rice, ligands acting as solubilizing agents have been suggested, such as citric acid (CA) (Johns, Patel et al. 2015) or EDTA, which has been used in large scale rice fortification efforts (Parker, Mosites et al. 2015, Perignon, Fiorentino et al. 2016).

The zinc compounds commonly used for food fortification are zinc oxide (ZnO) and zinc sulfate ($ZnSO_4$) (Herman, Griffin et al. 2002, Olivares, Pizarro et al. 2007, Olivares, Wiedeman et al. 2012). Several human studies have reported on zinc – iron interactions in fortified water (Crofton, Gvozdanovic et al. 1989), wheat flour (Herman, Griffin et al. 2002, Olivares, Pizarro et al. 2013) and other cereals (Hettiarachchi, Liyanage et al. 2010). The effect of zinc on iron bioavailability from fortified foods, liquids and supplements was recently reviewed (Olivares, Pizarro et al. 2012). The review reported generally no effect on iron absorption with Zn:Fe molar ratios below 2:1 (in aqueous solutions or wheat flour) (Herman, Griffin et al. 2002, Olivares, Pizarro et al. 2007) whereas higher ratios resulted in a decreased iron absorption (in aqueous solutions, wheat flour or hamburger meals) (Rossanderhulten, Brune et al. 1991, Olivares, Pizarro et al. 2007, Olivares, Pizarro et al. 2013). However, one study reported that iron absorption in children was decreased with $ZnSO_4$ as a fortificant, but not with ZnO at Zn:Fe molar ratios of 1:1 (Herman, Griffin et al. 2002). There are no studies investigating the effect of zinc addition on iron absorption from fortified rice in humans.

Typically, rice is fortified with a kernel-premix approach, where one part of fortified premix kernels is mixed with 100 or 200 parts of natural rice (Moretti, Lee et al. 2005). The premix kernel is fortified at high nutrient levels, which results in local nutrient concentrations 100 times higher than the fortification concentration applied to other cereals. It is possible that the high iron and zinc concentrations in the fortified kernel may influence their solubility which could result in an interaction during digestion and absorption (Johns, Parker et al. 2014, Johns, Patel et al. 2015). Potential interactions between iron and zinc are more likely in the extruded rice kernel – premix approach than in other fortified foods (e.g. wheat, maize).

Therefore, the aim of this study was to investigate whether the choice of zinc compound would influence human iron absorption from FePP-fortified rice. Our hypotheses were: 1) co-fortification of zinc into extruded rice grains containing FePP would reduce iron absorption; and 2) this effect would not be specific to the zinc compound used. We produced iron fortified rice grains co-fortified with isotopically labeled FePP and either ZnO or $ZnSO_4$. Iron absorption was assessed by measuring erythrocyte incorporation of stable iron isotopic labels in young women with a low iron status 14 days after test meal administration.

Methods

Human Study

Subjects

The female participants were recruited from the student and staff population of ETH Zurich and University Zurich, Switzerland. Exclusion criteria were: 1) known metabolic, chronic or gastrointestinal diseases (based on self-report), 2) long-term medication (except for oral contraceptives) and smoking, 3) pregnancy and lactation, 4) blood donation or comparable blood loss in the 4 months preceding the study, 5) plasma ferritin (PF) concentrations > 15 µg/L, 6) prior participation in an iron stable isotope study, 7) age < 18 or > 40 years, 8) BMI > 25 kg/m^2, 9) weight > 65 kg, 10) male gender. We amended the inclusion criteria in the protocol and included one subject with a PF of 16.5 µg/L to reach the predefined sample size of twenty subjects. Furthermore, we included 3 subjects

with a normal BMI whose weight was between 65 and 66.5 kg. We enrolled twenty women **(Figures 1** and **2)** with a low iron status (PF ≤16.5 µg/L) aged between 20 and 39 years (25; CI: 23,27), with a normal BMI (18.7 – 24.8 kg/m^2) and a body weight <66.5 kg. Informed written consent was obtained from all participants. The ethical committee of the canton Zurich reviewed and approved the study protocol and its amendment (KEK-ZH-Nr. 2014-0508).
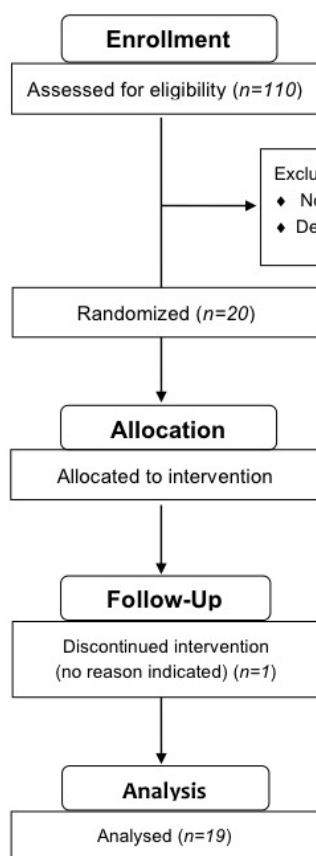
Figure 1. CONSORT flowchart for the human absorption study.
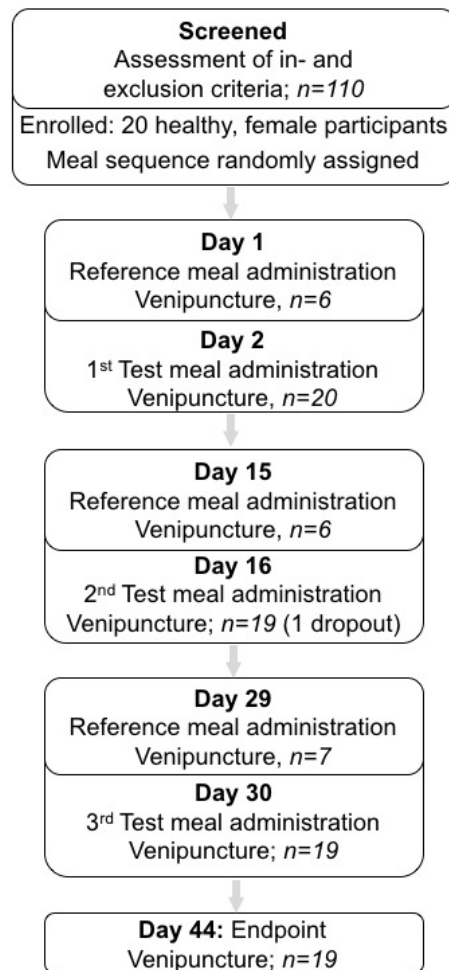
Figure 2. Schematic diagram of the study design. Three different study meals were administered to Swiss women (20 – 39 y; *n=19*) with a low iron status (plasma ferritin ≤16.5 µ/L). Each meal was given with a two wk delay. One reference meal was administered prior to one of the test meals.

Design of the human study

A randomized crossover design with four different rice meals was used with each woman serving as her own control. The allocation to the different groups was done after enrollment; subjects were randomly assigned to a predefined schedule of all possible test meal combinations to assure a balanced design (Hackl, Cercamondi et al. 2016). Each woman consumed four different isotopically labeled test meals based on 1) rice extruded with ^{57}FePP only (^{57}FePP-Zn); 2) rice extruded with ^{57}FePP and ZnO (^{57}FePP+ZnO); 3) rice extruded with ^{57}FePP and $ZnSO_4$ (^{57}FePP+$ZnSO_4$); and 4) extruded rice containing no iron nor zinc fortified with ^{58}FeSO$_4$ added as solution prior to consumption (Reference). Meals were served over six wk with a two wk break in between the test meals. The reference meal was given one day prior to one of the test meals. The order of the test meal and the reference meal administrations was randomized between participants.

Study procedures

The study was conducted between May and July, 2015. During screening (1 to 2 wk before the first meal administration), body weight and height were measured and blood and urine samples collected to assess iron status and to conduct a pregnancy test, respectively. Thus, all participants underwent baseline venipuncture for measurement of hemoglobin (Hb), plasma ferritin (PF) and plasma C-reactive protein (CRP) as part of the screening procedure. Depending on the order of the reference meal administration, the study started with the reference meal on day 1 or on day 2 with the first test meal (^{57}FePP-Zn; ^{57}FePP+ZnO or ^{57}FePP+$ZnSO_4$).

On days 16 and 30, participants received their second and third test meals (^{57}FePP-Zn; ^{57}FePP+ZnO or ^{57}FePP+$ZnSO_4$), respectively, after undergoing venipuncture for measurements of Hb, PF, CRP and isotopic iron composition, following the same procedure as on day 2. Depending on the randomization schedule, some participants received the reference meal either on day 1, 15 or 29. On day 44, 14 days after the last test meal administration, whole blood samples were collected to measure Hb and isotopic iron composition.

All meals were administered in the morning after an overnight fast which consisted in abstaining from eating after 2000 h and drinking fluids after midnight until the next morning on the day prior to each test meal administration. The participants consumed

the meals in the presence of the investigators. After complete meal consumption, the empty bowls were rinsed twice with a total of 20 ml 18.2 MΩ cm water, which was consumed by the participants. Participants were not allowed to eat or drink for at least 3 h after finishing their meals.

Preparation of isotopically labeled extruded rice grains

Four different batches were prepared for extrusion. Batch 1 contained 160 g long-grain rice flour (Tipo 150'000, Riseria Taverne SA, Taverne, Switzerland) which was manually mixed with 1.35 g isotopically labelled ^{57}FePP (Dr. Paul Lohmann GmbH KG, Emmerthal, Germany), 0.36 g ZnO (Jungbunzlauer Suisse AG, Basel, Switzerland), 0.18 g citric acid (CA; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 0.11 g monoglycerides (Dimodan; Danisco, Copenhagen, Denmark), 0.002 g sucralose (Tate & Lyle, London, United Kingdom), 0.01 g flavour (Cream Flavour, Givaudan, Dübendorf, Switzerland) and 0.88 g of a vitamin premix [containing a desired amount of 366.3 IU vitamin A (Vitamin A palmitate 250 S/N), 0.4 mg thiamine (Thiamine-mononitrate), 0.15 mg folate, 1.1 µg vitamin B-12 (Cyanocobalamin; all DSM Nutritional Products Ltd., Basel, Switzerland), 4.0 mg niacin (Nicotinamidum; Hänseler, Herisau, Switzerland), and 0.4 mg vitamin B-6 (Pyridoxin hydrochloride; Hänseler, Herisau, Switzerland) per serving of rice]. Afterwards, all ingredients were mixed in a mixer (Kenwood Ltd.; Havant Hants, Great Britain). Batches 2 and 3 were similar to Batch 1 with the difference, that 0.78 g ZnSO$_4$ (Zinc-sulfate-1-hydrate, Dr. Paul Lohmann GmbH KG, Emmerthal, Germany) were used as the zinc compound instead of ZnO (Batch 2) or no Zn was used at all (Batch 3). Batch 4 contained neither Fe nor Zn compounds, but was otherwise similar to batches 1, 2 and 3.

 The labeled ^{57}FePP (94.8% ^{57}Fe, Chemgas, Boulogne, France) was prepared as previously described (Hackl, Cercamondi et al. 2016). The water content of the different flour mixtures was adjusted to 25% prior to extrusion by slowly adding 18.2 MΩ.cm water using a mixer (KM 410). The extruded rice grains were produced with a Brabender single-screw extruder (DSE 20/24 DO-Corder DN20; Brabender GmbH & Co KG, Duisburg, Germany) with a custom-made brass die and cutter under hot extrusion conditions. The parameters for extrusion were as follows: barrel-temperature 85 – 95 °C, drive 120 rpm, pressure 90 – 110 bar, feeding rate 280 – 300 g/h. The extruded grains were air-dried overnight to reach a moisture content of approximately 10%.

Test meal preparation

The test meals and the reference meal consisted of 48 g Basmati rice and 30 g vegetable sauce. To reach a fortification level of 80 ppm, 2 g ($\pm$ 0.005) isotopically labeled extruded rice (Batch 3) were added to the ^{57}FePP-Zn meals, 2 g ($\pm$ 0.022) to meals ^{57}FePP+ZnO (Batch 1) and 2 g ($\pm$ 0.004) to meals ^{57}FePP+ZnSO$_4$ (Batch 2). The reference meals contained 2 g ($\pm$ 0.022) extruded rice (Batch 4). All meals were administered with 300 ml 18.2 MΩ.cm water. The vegetable sauce was prepared in bulk as previously described (Moretti, Zimmermann et al. 2006) and stored frozen until one day prior to administration, when it was refrigerated at 4 °C overnight. It contained 44% Chinese cabbage, 21% carrots, 21% zucchini, 12% onions, 2% oil and salt and had a low ascorbic acid (AA) content. Basmati rice and extruded rice were mixed and cooked in an oven (20 minutes; 220 °C; BOSCH, Switzerland) using a separate glass bowl for each participant one day prior to administration and were also refrigerated at 4 °C over night. On the administration day, the vegetable sauce was added to the precooked rice and the composite meal was heated in a microwave for 1 min at 600 W.

Prior to consumption, 4 mg ($\pm$ 0.022) iron in form of a labeled ^{58}FeSO$_4$ solution (Fe-metal: 99.90% enriched; Chemgas, Boulogne France), which was produced as previously described (Cercamondi, Egli et al. 2013), were added to the reference meal only.

Test meal analysis

Iron and zinc contents in the Basmati rice, vegetable sauce and extruded rice for in vitro experiments were analyzed by atomic absorption spectrophotometry (AAS; GTA 120 or AA240FS; both Agilent Technologies AG, Basel, Switzerland) after microwave-assisted mineralization with HNO$_3$ (MLS TurboWave, MLS GmbH; Leutkirch, Germany). The iron content in extruded rice for was measured by isotopic dilution ICP-MS. The phytic acid (PA) concentration was determined as described earlier (Cercamondi, Egli et al. 2010).

In vitro iron solubility

For the solubility experiments, the same isotopically labeled fortified rice was used as in the human absorption study. Iron solubility was determined as recently described (Hackl, Cercamondi et al. 2016). However, the relative solubility [%] was calculated instead of the

fractional solubility [%] to facilitate comparability between the tested rice-types. Relative solubility was expressed as the solubility of the compound divided by the solubility of the reference sample (FeSO$_4$).

Blood analysis

PF and CRP were measured from plasma samples collected on the administration days and frozen until analysis by the University Hospital Zurich using a Cobas 8000 analyzer (Roche Diagnostics, USA). Hb was measured in whole blood on the day of collection by using either a Sysmex XE_5000 (Sysmex Corporation, Japan) or an Advia 2120 haematology analyzer (Siemens Healthcare, USA). Anemia was defined as Hb <12 g/dL; ID as PF <15 µg/L and ID anemia as Hb <12 g/dL plus PF <15 µg/L (WHO 2001). The expected high sensitivity CRP concentrations for healthy individuals were <5 mg/L. Iron bioavailability was measured via multi-collector ICP-MS (Neptune, Thermo Finnigan, Germany) by assessing the shift of the isotopic iron ratio in red blood cells after mineralization and subsequent anion-exchange chromatography (Hotz, Krayenbuehl et al. 2012). This ratio was used to calculate total absorbed isotopic label (fractional absorption) using the participant's blood volume (Turnbull, Cleton et al. 1962) and assuming 80% of the absorbed iron to be incorporated into red blood cells (IAEA 2012). The calculation was conducted using the principles of isotopic dilution and taking into account that isotopic labels are not mono-isotopic (Walczyk, Davidsson et al. 1997, Agency 2012, Cercamondi, Duchateau et al. 2016). We corrected for previously incorporated ^{57}Fe by using the isotopic ratio value 14 days after first test meal administration as a new baseline value for the following test meal administration (Cercamondi, Duchateau et al. 2016). RBV from each meal was calculated based on fractional iron absorption [%] respective to fractional iron absorption from the reference meal for each subject (*n=19*).

Statistical analysis

Data were analyzed using SPSS (version 22.0, 2013; SPSS Inc, Chicago, IL) and Microsoft Excel (2013; Microsoft Corporation, Redmond, WA). Fractional iron absorption from the

different meals within the same participant was compared by linear mixed models followed by Bonferroni correction for multiple comparisons. RBV from each meal was calculated based on fractional iron absorption [%] respective to fractional iron absorption from the reference meal for each subject and compared by linear mixed models *(n=19).* P-values were Bonferroni corrected by multiplying the raw P-value obtained in the multiple comparison by the number of comparisons made and assigning a maximum P value of 1.0. Meals administered were fixed effect factors in the model while subjects were defined as random factors (intercept). A sample size of 16 participants was considered sufficient to detect a nutritionally relevant intra-subject difference in fractional iron absorption of 30% with a β of 0.8 and α of 0.05. For the calculation, we assumed a SD for the change in iron absorption between the different meals of 0.2 units in the log transformed fractional absorption. To compensate for possible drop-outs, 20 women were recruited in our study. Results for age, and anthropometric features, Hb, PF and CRP were presented as means $\pm$SDs if normally distributed, otherwise the results were presented as geometric means (95% CI). Calculated iron and zinc contents in composite meals were based on the means from the analysis of single components (regular Basmati rice, extruded rice, vegetable sauce; *n=3*), SDs were adapted by calculating the square root of the squared and summed SDs from each single component. *In vitro* solubility was analyzed with univariate analysis of variance (ANOVA) and multiple comparisons were conducted using Bonferroni correction. Differences were considered as significant at $P<.05$. Non-normally distributed data were logarithmically converted for statistical analysis and reconverted for reporting.

Results

Participant characteristics

One participant dropped out after the first meal administration without giving a reason, and 19 participants completed the study. At baseline (day 1 or day 2), 13 participants were iron deficient without anemia, one participant was iron deficient anemic and 2

participants had elevated CRP concentrations (**Table 1**). Three participants had CRP concentrations above 5.0 mg/L during one study visit apart from the baseline.

Table 1. Anthropometric features, iron and inflammatory status of study participants, assessed prior to first meal administration[1].

Age, y	25 (23, 27)
Weight, kg	59 ± 5.0
Height, m	2 ± 0.1
BMI, kg/m^2	21 ± 1.6
Hb, g/dL	13 ± 0.8
Plasma ferritin μg/L	12 ± 5.5
Plasma CRP, mg/L	1 (0.4,1.6)

[1] Values are means ± SDs or geometric means (95% CIs), *n=19*. CRP: C-reactive protein; Hb: Hemoglobin.

Test meal composition

Values in the text are means ± SDs or geometric means (95% CIs) unless otherwise indicated. The iron and zinc concentrations and the Zn:Fe molar ratios in the different test meals and the reference meal are summarized in **Table 2.** The iron concentrations in the isotopically labelled extruded rice grains from meals ^{57}FePP-Zn, ^{57}FePP+ZnO, and ^{57}FePP+ZnSO$_4$ were 236 ± 5.9, 239 ± 2.7 and 237 ± 1.8 mg/100 g, respectively. The zinc concentrations in the same grains were 3 ± 0.0, 185 ± 2.5, and 193 ± 8.0 mg/100 g in meals ^{57}FePP-Zn, ^{57}FePP+ZnO, and ^{57}FePP+ZnSO$_4$, respectively. The extruded rice grains in the reference meal contained 4 ± 0.2 mg/100 g iron and 3 ± 0.2 mg/100 g zinc. The native iron content was 0.5 ± 0.01 mg iron in each composite test meal, which also contained 1 ± 0.03 mg zinc and 0.1 g ± 0.01 PA. The sauce given with each meal contained 1 ± 0.02 mg AA/100 g. The AA content was determined only in the vegetable sauce as its content in rice was assumed to be negligible.

**Table 2.** Composition of administered study meals, human iron absorption and *in vitro* iron solubility[1]

Meal	Total iron content per meal [mg][2,5]	Total zinc content per meal [mg][2,5]	Zn : Fe [MR]	Fractional iron absorption [%][3]	RBV compared to Reference [%][3,4]	Relative iron solubility [%][5]
[57]FePP-Zn	4.4 ± 0.10	1.2 ± 0.03	1 : 5	4.0[b] (2.83, 5.62)	57[a,b]	14[a] ± 1.9
[57]FePP+ZnO	4.4 ± 0.05	4.3 ± 0.05	1 : 1.3	2.7[c] (1.80, 4.10)	38[a]	13[a,b] ± 0.9
[57]FePP+ZnSO$_4$	4.4 ± 0.03	4.4 ± 0.14	1 : 1.3	4.5[b] (3.42, 5.83)	62[b]	10[b] ± 0.4
[58]Fe-Reference	4.5 ± 0.01	1.2 ± 0.03	1 : 5	9.3[a] (7.10, 12.15)	n.a.	n.a.

[1]Values are means ± SDs or geometric means (95% CIs). Labeled means in a column without a common letter differ, P<0.05; Bonferroni corrected linear mixed model. Designated micronutrient content in all meals: vitamin A 366.3 IU, folate 0.15 mg, niacin 4 mg, thiamin 04 mg, vitamin B-6 0.4 mg, vitamin B-12 1.1 µg per serving. MR: Molar Ratio; RBV: Relative bioavailability. [2] Calculations are based on the means from the analysis of single components (regular Basmati rice, extruded rice, vegetable sauce; *n=3*), SDs were adapted by calculating the square root of the squared and summed SDs from each single component. [3] *n=19.* [4] RBV from each meal was calculated based on fractional iron absorption [%] respective to fractional iron absorption from the reference meal for each subject, *n=19.* [5] *n=3.*

[57]FePP-Zn: Extruded Rice containing [57]FePP, Citric acid (CA) and micronutrients (MN); [57]FePP+ZnO: Extruded Rice extruded with [57]FePP, ZnO, CA and MN; [57]FePP+ZnSO$_4$: Extruded Rice extruded with [57]FePP, ZnSO$_4$, CA and MN; [58]FeSO$_4$ solution added prior consumption. RBV: Relative bioavailability.

Iron absorption

Type of meal significantly affected iron absorption (P<0.001). Iron absorption from ^{57}FePP+ZnSO$_4$ was 1.6 (1.0, 2.3) fold higher than from ^{57}FePP+ZnO (4.5 vs. 2.7%, P for difference: P<0.03, Table 2, **Figure 3**). There was no difference in iron absorption between ^{57}FePP-Zn (4.0%) and ^{57}FePP+ZnO (P=0.12) or ^{57}Fe+ZnSO$_4$ (P=0.48). Fractional iron absorption was significantly different in the reference meal (9.3%) compared to meals ^{57}FePP-Zn, ^{57}FePP+ZnSO$_4$ and ^{57}FePP+ZnO (all, P<0.010). RBV from ^{57}FePP-Zn (57%) did not differ from ^{57}FePP+ZnO (32%; P=0.08) or ^{57}Fe+ZnSO$_4$ (62%; P=1.00). However, the RBV of ^{57}FePP+ZnO differed from ^{57}Fe+ZnSO$_4$ (P=0.015).
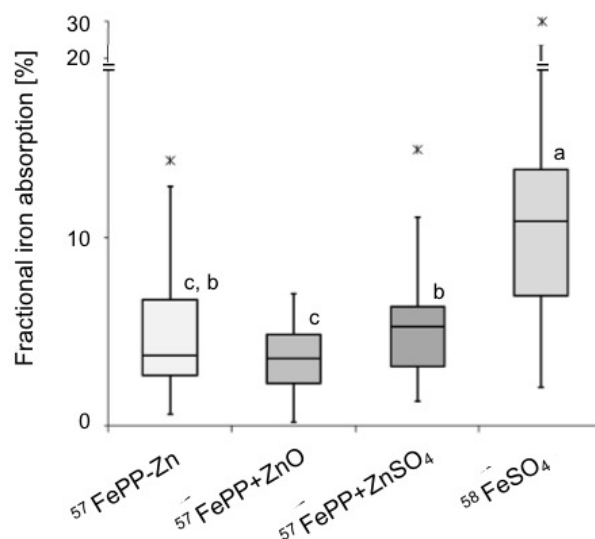
Figure 3. Boxplots for the fractional iron absorption [%] from four different rice meals in Swiss iron-depleted women.
Horizontal bars show the median fractional iron absorption for each meal; each box represents the first to third quartile, the whiskers represent the lowest and highest data points regardless of outliers. Asterisks indicate outliers 1.5 interquartile ranges above the 3rd quartile. Different letters indicate significant differences, Bonferroni corrected linear mixed model (P<.05); *n=19*.
^{57}FePP-Zn: rice extruded with ^{57}FePP, a vitamin premix, citric acid (CA); ^{57}FePP+ZnO: rice extruded with ^{57}FePP, ZnO, a vitamin premix and CA; ^{57}FePP+ZnSO$_4$: rice extruded with ^{57}FePP, ZnSO$_4$, a vitamin premix and CA; FeSO$_4$: extruded rice with a vitamin premix and CA; ^{58}FeSO$_4$ added prior consumption. Extruded rice containing ^{57}FePP, ^{67}ZnO, a vitamin premix and citric acid/trisodium citrate (CA/TSC).

In vitro iron solubility

In the *in vitro* solubility experiments, there was no significant difference in iron solubility comparing ^{57}FePP+ZnO to ^{57}FePP+ZnSO$_4$ (13.1 vs 10.1%; P=0.08). Iron solubility from ^{57}FePP+ZnSO$_4$ differed significantly from ^{57}FePP-Zn (14.3%, P<0.02; Table 2).

Discussion

This study is the first to investigate iron absorption from iron-fortified rice when co-fortified with two commonly used zinc fortificants, ZnO and ZnSO$_4$. Since fortified rice is typically produced using a kernel-premix approach, where only a small fraction of the kernels (1:100 or 1:200) is fortified with high nutrient concentrations (Johns, Patel et al. 2015), interactions between nutrients, such as iron and zinc, may be more pronounced compared to other fortification approaches where no kernel-premix is used. Our study, conducted at a premix kernel level of 1:28, suggests that co-fortification of ZnO in FePP-fortified rice results in lower iron absorption from FePP than co-fortification with ZnSO$_4$. One possible explanation for these findings is that the presence of ZnO, unlike ZnSO$_4$, counteracts the acid-driven dissolution of FePP in the stomach due to its high acid-binding capacity (Lawlor, Lynch et al. 2005) and that this process results in a lower overall solubility of FePP, and thus lower bioavailability. Our data do not suggest that the presence of Zn *per se* reduces iron absorption from FePP, as the co-fortification with ZnSO$_4$ did not reduce iron absorption. Our observation that ZnSO$_4$ does not influence iron absorption, is consistent with the majority (Olivares, Pizarro et al. 2012) but not all (Herman, Griffin et al. 2002) studies investigating the effect of ZnSO$_4$ on iron bioavailability from cereals.

Several studies have investigated the effect of zinc on iron absorption in humans from aqueous solutions (Crofton, Gvozdanovic et al. 1989, Rossanderhulten, Brune et al. 1991) in the absence of food. Olivares et al. report a threshold for the inhibition of iron bioavailability at Zn:Fe molar ratios of ≥5:1, for low-dose iron (0.5 mg), whereas with higher iron doses (10 mg Fe) the inhibition occurred at molar ratios of 0.8:1 (Olivares, Pizarro et al. 2012). In more complex food matrixes such as milk, no effect was reported when adding ZnSO$_4$ at Zn:Fe molar ratios between 0.6:1 and 2:1 (Olivares, Wiedeman et

al. 2012). Similarly, in preterm infants that were given a formula with $ZnSO_4$ or human milk at Zn:Fe ratios of 1:1 and 4:1, no inhibitory effect was observed (Friel, Serfass et al. 1998). Likewise, non heme iron absorption from a hamburger meal after addition of $ZnSO_4$ at molar ratios Zn:Fe of 4.3:1 was unaffected (Rossanderhulten, Brune et al. 1991). In contrast, in one of four studies investigating the effect of added $ZnSO_4$ on iron absorption from cereals, there was a negative effect of $ZnSO_4$ on iron bioavailability from wheat-based dumplings, while in the same study, ZnO addition at the identical 0.9:1 Zn:Fe ratios did not affect iron absorption (Herman, Griffin et al. 2002). The mechanism(s) responsible for the interaction of zinc on iron absorption is uncertain. It is possible that zinc and iron may compete for the same transporters on the apical surface of the enterocyte, one of which may be the dimetal transporter 1 protein (DMT1) (Dati, Schumann et al. 1996, Espinoza, Le Blanc et al. 2012), or a yet unidentified transporter (Olivares, Pizarro et al. 2012). However, it has been reported that a direct competition for a single transporter at the enterocyte is unlikely and that zinc inhibits iron uptake through mixed inhibition (Iyengar, Pullakhandam et al. 2009). Recently, ferroportin, the iron exporter protein on the enterocyte basolateral membrane, has been shown to have affinity for zinc, but this may not have a strong impact on zinc homeostasis (Mitchell, Shawki et al. 2014). A negative interaction between iron and zinc after absorption, at the extracellular or intracellular level, is also possible but unlikely at the amounts consumed in this study (Olivares, Pizarro et al. 2012).

In contrast to our *in vivo* results, our data from the experiments comparing *in vitro* iron solubility of $^{57}FePP+ZnO$ and $^{57}FePP+ZnSO_4$ do not support our hypothesis that ZnO would decrease iron solubility from FePP to a greater extent than $ZnSO_4$. Iron solubility from $^{57}FePP+ZnSO_4$ was lower than from $^{57}FePP-Zn$, whereas iron solubility from $^{57}FePP+ZnO$ was not significantly different from $^{57}FePP-Zn$. A recent *in vitro* study in rice analogues fortified with micronized FePP reported greater inhibition of iron solubility by $ZnSO_4$ compared to ZnO (30% vs. 24% inhibition) (Johns, Parker et al. 2014). A limitation of *in vitro* iron solubility experiments is that they greatly oversimplify the highly complex physicochemical and physiological events underlying human digestion (Hur, Lim et al. 2011). Thus, in vitro solubility may provide qualitative, but not quantitative information on potential bioavailability (Fairweather-Tait, Phillips et al. 2007) and measurements *in vivo* are more likely to mimic the true interactive effects of Zn on iron bioavailability from

fortified rice (Etcheverry, Grusak et al. 2012).

Recent findings suggest that the presence of ligands (such as CA/TSC) in fortified rice can substantially increase iron absorption in humans and enhance *in vitro* solubility from FePP-fortified rice with ZnO added at Zn:Fe molar ratios of 0.8:1 (Hackl, Cercamondi et al. 2016). This observation is supported by *in vitro* findings (data not shown), where iron solubility was more than doubled from rice co-fortified with either ZnO or $ZnSO_4$ in combination with CA/TSC compared to no CA/TSC addition. Future human absorption studies should be performed to determine if additional CA/TSC added to the fortified rice can counteract the adverse effect of ZnO on iron absorption from FePP in extruded rice. Also, the effect of other ligands/chelating agents on iron bioavailability from FePP fortified rice in the presence of various zinc fortification compounds requires further investigation.

Our data suggest that in iron deficient populations with a per capita rice consumption of 200 g/day, and the suggested FePP fortification level of 7-8 mg Fe/100g rice (De Pee 2014), our measured absorption of 2.71% would result in 0.44 mg/day of absorbed iron, providing approximately ≈30% of daily iron requirements in young women (Deutsche Gesellschaft für Ernährung 2013). Although this would be a meaningful contribution to iron requirements, it should be remembered that these absorption values are from iron deficient but otherwise healthy Swiss women free from common infections and consuming meals virtually free of iron absorption inhibitors. Therefore, additional approaches to further enhance iron bioavailability from FePP-fortified extruded rice should be the focus of future research.

Our data suggest that replacing ZnO with $ZnSO_4$ may increase iron absorption from FePP-fortified extruded rice grains. However, other factors also need to be considered when selecting a potential zinc fortificant for extruded rice, such as potential interactions with other co-fortificants besides iron, shelf life stability, and sensory properties. For example, increased losses (30% higher) of retinyl palmitate during storage have been reported when extruded rice was fortified with $ZnSO_4$ rather than ZnO (Pinkaew, Wegmuller et al. 2012). Compared to $ZnSO_4$, ZnO may be preferable due to its superior color masking properties and attractive cost profile; the commercial price for $ZnSO_4$ is 3-4 higher than for ZnO (personal communication Dr. Paul Lohmann GmbH KG, Emmerthal, Germany). On the other hand, the increased cost of $ZnSO_4$ may be justified when compared to the cost of additional FePP that would need to be added to ZnO co-fortified rice to obtain

comparable iron delivery.

Our study has several strengths: 1) we tested the effect of zinc on iron absorption using intrinsically-labeled FePP extruded into rice grains; 2) we included young women with low iron status, a target population for a rice fortification program; and 3) each subject acted as her own control, allowing for paired comparisons. Our study also has limitations: due to the high cost of the stable isotope labels, we tested a 1:28 (fortified kernel / natural rice) blending ratio in extruded rice. Also, single meal absorption studies with iron stable isotopes tend to overestimate the effects of enhancers and inhibitors of iron absorption (Hunt and Roughead 2000). We did not assess zinc absorption from the fortified rice, and the choice of zinc fortificant may affect fractional zinc absorption from rice. $^{58}FeSO_4$ in the Reference was not extruded in the rice as it would alter the color and potentially taste of the rice. This different form of application may have an impact on iron absorption, however, $FeSO_4$ may not be used as iron-fortificant in rice and in our study it only served as a highly bioavailable comparator. Finally, the test meals contained a sauce with low PA content; other sauces and complete diets may contain higher amounts of PA and additional absorption inhibitors. Investigations assessing iron absorption from fortified rice as an integral part of a diverse diet administered over a longer time span will be required to confirm our findings; such a study is currently underway (clinicaltrials.gov: NCT02714075). Nevertheless, our study suggests that $ZnSO_4$ may be preferable to ZnO for co-fortification into FePP fortified extruded rice to optimize iron absorption.

Authorship Contributions

DM, LH, RH, MP, PWJ and MZ designed the studies. LH, DM and CZ conducted the experiments. LH and CZ analyzed data. LH, DM and MZ wrote the paper. LH, DM and MZ had primary responsibility for final content. All authors read and approved the final version of the paper.

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MANUSCRIPT 3

Cold Extrusion but Not Coating Affects Iron Bioavailability from Fortified Rice in Young Women and Is Associated with Modifications in Starch Microstructure and Mineral Retention during Cooking

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Abstract

Background: Rice can be fortified using hot or cold extrusion, or coating, but the nutritional qualities of the resulting rice grains have never been directly compared.

Objective: In fortified rice produced by coating or hot or cold extrusion, we compared 1)iron and zinc absorption using stable isotopes; 2)iron and zinc retention during cooking; and 3)starch-microstructure.

Methods: We conducted two studies in young women: in study 1 (*n=19*; Age:26.2$\pm$3.4 y; BMI:21.3$\pm$1.6 kg/m^2), we compared fractional iron absorption (FAFe) from rice fortified through hot (HER1) versus cold (CER) extrusion; in study 2 (*n=22*; Age:24$\pm$4 y; BMI:21.2$\pm$1.3 kg/m^2), we compared FAFe and fractional zinc absorption (FAZn) from rice produced by hot extrusion (HER2) versus coating (COR) relative to a rice extrinsically fortified with FeSO$_4$ (reference). All rices were fortified with ferric pyrophosphate and zinc oxide. We assessed retention during standardized cooking experiments and characterized the rice-starch microstructure.

Results: FAFe (95% CI) was greater from CER: 1.8%(1.4, 2.4%) than from HER1: 1.1%(0.7,1.7%) (P=0.036). There was no difference in FAFe between HER2: 5.1%(3.7,7.1%) and COR 4.0%(2.9,5.4%) (P=0.14) but FAFe from COR was lower than from the reference meal 6.6%(4.9,9.0%) (P=0.003) and geometric mean FAZn (95% CI) did not differ between HER2 9.5%(8.0,11.7%) and COR 9.6%(8.7,10.7%) (P=0.92). Cooking in a rice to water ratio of 1:2 resulted in iron and zinc retentions > 80%; cooking in excess water did not affect iron retention from HER but caused iron losses of 25% from CER and COR. Distinct variations in starch microstructure were found in CER and HER1.

AA, Ascorbic acid; AAS, Atomic absorption spectrophotometry; CA, Citric acid; CA/TSC , Citric acid and trisodium citrate mixture; CER, Cold extruded rice containing [57]FePP, ZnO, TSC and micronutrients; COAT, Coated rice containing nutrient mix (including FePP) ; COR, Coated rice containing [57]FePP, [67]ZnO, CA/TSC and microutrients; CRP, C-reactive protein; CTC, Clinical Trials Center; ID, Iron deficiency; FAFe, Fractional iron absorption; FAFe$_{corr}$, Corrected fractional iron absorption; FAZn, Fractional zinc absorption; Fe, Iron; FePP, Ferric pyrophosphate; [57]FePP, Isotopically labelled ferric pyrophosphate; FeSO$_4$, Ferrous sulfate; [58]FeSO$_4$, Isotopically labelled ferrous sulfate; Hb, Hemoglobin; HER1, Hot extruded rice containing [57]FePP, ZnO, TSC and micronutrients; HER2, Hot extruded rice containing [57]FePP, [67]ZnO, CA/TSC and micronutrients; IFNH, Institute of Food, Nutrition and Health; PA, Phytic acid; PF, Plasma ferritin; PZn, Plasma zinc; RBV, Relative bioavailability; Reference, Regular Basmati Rice, [58]FeSO$_4$ solution added prior consumption; SAXS, Small-angle X-ray scattering; SFP, Soluble ferric pyrophosphate; TSC, Trisodium citrate; WAXS, Wide-angle X-ray scattering; Zn, Zinc; [67]Zn, [70]Zn, Isotopically labelled zinc

Conclusions: Iron absorption was 64% higher from cold than from hot extruded rice, with no difference between coated versus hot extruded rice. Lower extrusion temperatures may generate a more readily digestible starch structure, allowing for greater iron release *in vivo,* but lower mineral retention during cooking.

Registered at clinicaltrials.gov as NCT02176759.**Abstract**

Keywords: iron deficiency, extrusion, coating, rice, fortification

Introduction

Iron and zinc deficiency remain major public health problems (1) affecting many individuals in both developing and industrialized countries (2-4). Rice is a staple food for more than 3 billion people (5), and its fortification could be an important strategy to combat micronutrient deficiencies (6). However, iron fortification of rice is challenging because rice is mainly consumed as intact grains and sensory properties, particularly its white color, are important for its marketability and consumer acceptance (7, 8).

Extrusion and coating techniques are currently the two main technologies available for large scale fortification; they are typically used to fortify a small fraction (1 – 2 %) of the rice kernels (9, 10). In coating, a suspension of nutrients with waxes and polymers is applied to the surface of intact natural rice kernels (9, 10) maintaining the grain's inner starch structure. In contrast, during extrusion, artificial rice kernels are generated by forcing moistened fortified rice flour through a restricted opening exposing it to high shear and pressure (9). Cold extrusion is conducted at 30 – 40 °C and results in marginal or no starch gelatinization (9, 10) in contrast to hot extrusion, conducted at 80 – 110 °C, where starch is gelatinized (9, 11, 12). Starch consists of amylopectin and amylose in a three-dimensional network, characterized by semi-crystalline domains embedded into an amorphous glassy matrix, which is composed of alternating crystalline and amorphous lamellae at a fixed repeating distance. The microstructure of starch may predict the release of minerals and bioavailability in hot and cold extruded rice.

While both hot and cold extrusion have been shown to be feasible (13) and efficacious (14-16); iron absorption from rice produced with these two extrusion techniques has not been directly compared. Moreover, there are few data on human mineral bioavailability or efficacy from coated rice (17, 18) and no published data on zinc absorption from rice fortified through extrusion or coating (19).

To provide data for rice fortification programs on the choice of fortification technique and mineral fortification levels, our study objectives were: 1) to measure and compare iron absorption from hot extruded versus cold extruded rice; 2) to measure and compare iron

and zinc absorption from hot extruded versus coated rice; and 3) to assess iron and zinc retention from rice produced with these different technologies after varying pre-treatments and cooking procedures. Our hypotheses were: there would be 1) no significant difference in iron absorption from hot extruded versus cold extruded fortified rice; and 2) no significant difference in iron or zinc absorption from hot extruded versus coated fortified rice.

Methods

Subjects

We enrolled women from the student and staff population of ETH Zurich and University Zurich, Switzerland. Inclusion criteria were: 1) female and aged between 18 and 40 years; 2) BMI between 19 and 26 kg/m^2 and body weight <69 kg; 3) apparently healthy with no chronic diseases or intake of medication (except for oral contraceptives); 4) non-smoker; 5) no blood donation or substantial blood loss within 4 months prior to the study's start; 6) not pregnant or lactating; 7) no intake of mineral or vitamin supplements from at least 2 weeks before or during the study, and 8) no prior participation in a study where iron or zinc stable isotopes were administered. Informed written consent was obtained from all participants. The ethics committee of the canton of Zurich reviewed and approved the studies (KEK-ZH-Nr. 2015-021); the trial was registered at clinicaltrials.gov as NCT02176759.

Human absorption studies

We performed two studies using a single-blind, randomized crossover design where different rice meals were administered, with each woman serving as her own control. Different participants were included in each study. They were allocated to the different groups after enrollment and participants were assigned to a predefined schedule of all possible test meal combinations (20). In study 1 (**Figure 1**), each woman consumed two different isotopically labelled test meals containing [57]FePP-fortified rice, produced through (1) hot extrusion (HER1) and (2) cold extrusion (CER). Meals were served with a two wk period between test meals. The total study duration was 28 days.

In study 2 (**Figure 2**), each woman consumed three different isotopically labelled meals containing ^{57}FePP and ^{67}ZnO co-fortified rice, produced through (1) hot extrusion (HER2), (2) coating (COR), and (3) one reference meal consisting of hot extruded rice fortified with a ^{58}FeSO$_4$-solution added prior to consumption (reference). Meals were served with a four wk period between test meals, to ensure wash-out of the stable zinc isotopes administered on day 2 (21). The reference meal was given one day prior to one of the test meals (day 1 or 29). The total study duration was 44 days (Figure 2).

In both studies, body weight and height were measured during screening (1 to 2 wk before the first meal administration), and a pregnancy test was conducted. All meals were administered in the morning after an overnight fast. The participants completely consumed the meals under direct supervision of the investigators. The empty bowls were then rinsed twice with a total of 20 mL 18 MΩ·cm water, which was also consumed by the participants. Participants were not allowed to eat or drink for at least 3 h after complete test meal consumption.

Study 1

The study was conducted between August and September 2015 at the Institute of Food, Nutrition and Health. All participants underwent baseline venipuncture for the measurement of hemoglobin (Hb), plasma ferritin and plasma C-reactive protein (CRP) on day 1 before receiving their first test meal (CER or HER1). On day 15, participants received their second test meal (CER or HER1) after undergoing venipuncture for measurements of Hb, plasma ferritin, plasma CRP and isotopic iron composition following the same procedure as on day 1. On day 29, 14 days after the last test meal administration, whole blood samples were collected to measure Hb, plasma ferritin, plasma CRP and isotopic iron composition **(Figure 1).**
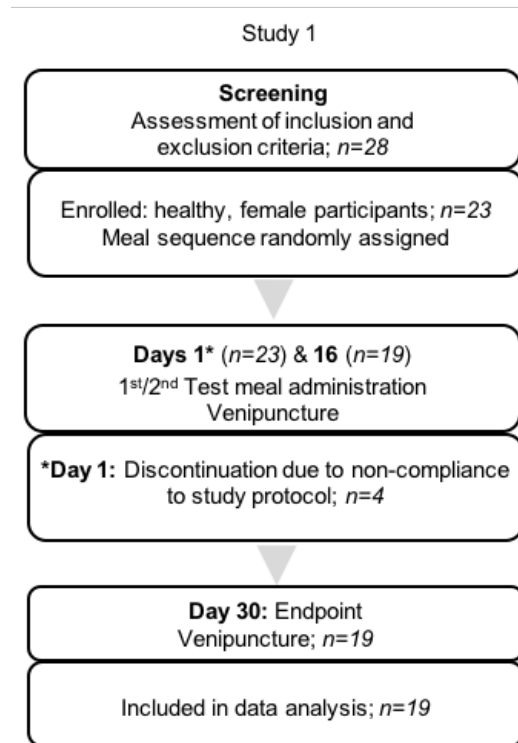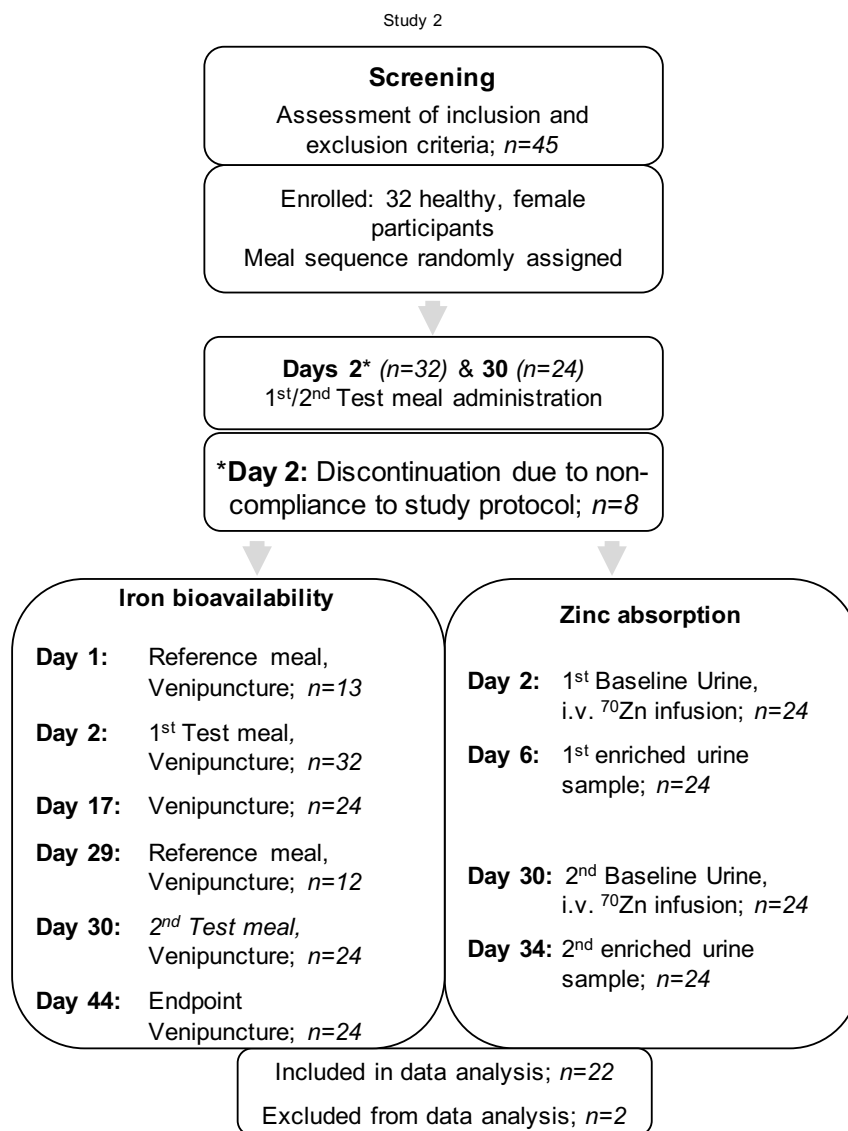
Figure 1. Schematic diagram of study 1. Two different study meals were administered to healthy Swiss women (*n = 19*). Meals were given with a 2-wk delay.

Study 2

The study was conducted between September and December 2015 at the Institute of Food, Nutrition and Health and the Clinical Trials Center. Participants were divided into three cohorts, which only differed in the study dates and the sequence for the reference meal administrations. Depending on their assignment to a cohort, participants received their reference meals on day 1 or 29 at IFNH. On days 2 and 30 either meals COR or HER2 were administered at Clinical Trials Center, the administration-sequence of the meals was randomized. Baseline spot urine samples were collected in the morning of days 2 and 30 before each test meal administration, enriched spot urine samples were collected in the morning of days 6 and 34 after an overnight fast to determine fractional zinc absorption (FAZn) via double isotopic tracer ratio technique (22). Additionally, all participants underwent baseline venipuncture for the measurement of Hb, plasma ferritin, plasma CRP and plasma zinc (PZn) before receiving the first test meal. After complete consumption of their test meals on days 2 and 30, participants received iv doses of ^{70}ZnO as previously described (21). On day 17, 15 days after the first test meal administration, a blood sample

was collected to measure Hb, plasma ferritin, plasma CRP and isotopic iron composition in the blood. On day 30, after a 4 wk wash-out period for zinc, participants received their second test meal (COR or HER2) after undergoing venipuncture for measurements of Hb, plasma ferritin, plasma CRP and isotopic iron composition following a similar procedure as on days 1 or 2. On day 44, 14 days after the last test meal, whole blood samples were collected to measure Hb, plasma ferritin, plasma CRP and isotopic iron composition **(Figure 2).**

Figure 2. Schematic diagram of study 2. Two different study meals were administered to healthy Swiss women (*n = 22*). Meals were given with a 4-wk delay. One reference meal was administered one day before either one of the test meals.

Preparation of isotopically labelled extruded rice grains and iv doses

Isotopically labelled extruded rice grains for all meals were extruded on a Brabender single-screw extruder (DSE 20/24 Do-Corder). Grains for meals HER1, HER2 and the reference were produced under hot extrusion conditions as previously described (20). The only difference was that regular ZnO was used in Study 1, whereas in Study 2 isotopically labelled ^{67}ZnO was used. Cold extruded rice grains (CER) were of the same composition as those for meal HER1, however, barrel temperatures at extrusion differed (**Table 1**).

Coated rice was produced by Wright Enrichment Inc. (Crowley, USA), following a down-scaled coating process comparable to their large-scale production technique using the same premix ingredients and labelled compounds of ^{57}FePP and ^{67}ZnO as for HER2. Labelled ^{57}FePP was produced by Dr. Paul Lohmann GmbH KG (Emmerthal, Germany), mimicking FePP-powder (Study 1: Fe-metal: 94.8% enriched; Study 2: Fe-metal: 96.0% enriched). Labelled ^{67}ZnO was obtained from Chemgas (Boulogne, France; Zn-metal: 90.6% enriched).

Mixtures HER1 and CER consisted of rice flour, ^{57}FePP, ZnO (Jungbunzlauer Suisse AG, Basel, Switzerland), citric acid (CA; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and a vitamin premix, which was similar to a recently described one (23). Mixtures HER2 and COR contained rice flour, ^{57}FePP, ^{67}ZnO, a vitamin premix, CA and trisodium citrate (CA/TSC; Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The reference mix contained rice flour, CA/TSC and the same vitamin premix as HER2 and COR. Doses for iv administration were prepared from ^{70}ZnO (^{70}ZnO-metal: 95.5% enriched, Chemgas, France) at the Cantonal Pharmacy of the University Hospital Zurich according to Good Manufacturing Practice as recently described (21).

Table 1. Test meal mineral composition and mineral absorption by healthy young women from various rice meals in Studies 1 and 2.[1]

Study	Meal	Barrel T. [°C]	Total Fe, mg/meal	Total Zn, mg/meal	FAFe, %	RBV iron, %	FAFe$_{corr}$, %	Total absorbed Fe, mg/meal	FAZn, %	Total absorbed Zn, mg/meal
1	CER	30 – 40	4.4 ± 0.12	4.0 ± 0.70	2.17[a] (1.39, 3.40)	NA	1.81 (1.36, 2.41)	0.08 (0.03, 0.12)	NA	NA
	HER1	80 – 92	4.4 ± 0.73	4.0 ± 0.37	1.19[b] (0.71, 2.00)	NA	1.07 (0.68, 1.69)	0.05 (0.02, 0.08)	NA	NA
2	Reference	NA	4.5 ± 0.01	1.2 ± 0.03	6.62[a] (4.86, 9.01)	100[a]	4.58 (3.47, 6.05)	0.26 (0.15, 0.37)	NA	NA
	HER2	80 – 92	4.4 ± 0.21	6.9 ± 0.27	5.14[a,b] (3.70, 7.13)	79[a,b] (57.7, 108.0)	3.64 (2.50, 5.31)	0.17 (0.11, 0.24)	9.54 (7.87, 11.57)	0.49 (0.38, 0.60)
	COR	NA	4.4 ± 0.11	7.1 ± 0.18	3.95[b] (2.89, 5.42)	61[b] (46.9, 78.9)	2.82 (2.08, 3.81)	0.12 (0.06, 0.18)	9.63 (8.70, 10.66)	0.59 (0.53, 0.66)

[1] Values are means ± SDs, $n = 3$ or geometric means (95% CI), $n = 19$ (Study 1) or $n = 22$ (Study 2). Within a study, labeled values in a column without a common letter differ, P < 0.05, paired samples t-test (Study 1) or repeated measures ANOVA with Bonferroni corrected multiple comparisons (Study 2). CA, Citric acid; CER, Cold extruded rice containing [57]ferric pyrophosphate ([57]FePP), zinc oxide (ZnO), citric acid (CA) and a vitamin premix; COR, Coated rice containing [57]ferric pyrophosphate ([57]FePP), [67]zinc oxide ([67]ZnO), citric acid and trisodium citrate (CA/TSC) and a vitamin premix, MR Fe/Zn/PA/CA/TSC = 1.0:1.9:0.9:0.05:0.8; FAFe, Fractional iron absorption; FAFe$_{corr}$, FAFe corrected for 40 µg/L; FaZn, Fractional zinc absorption; HER1, Hot extruded rice containing [57]FePP, ZnO, TSC and a vitamin premix, MR Fe/Zn/PA = 1.0:1.1:0.9; HER2: Hot extruded rice containing [57]FePP, [67]ZnO, CA/TSC and a vitamin premix, MR: 1.0:1.9:0.9:0.04:0.8; PA, Phytic acid; RBV: Relative bioavailability; Reference: Hot extruded rice containing CA/TSC and a vitamin premix, MR = 1.0:0.2:0.9:0.06:1.3; T., Temperature; TSC, Trisodium citrate. [58]FeSO$_4$ solution added prior consumption,

Test meal preparation

All test meals and the reference meal consisted of 48 g basmati rice, and 30 g vegetable sauce [31] and were administered with 300 mL 18 MΩ·cm water. To reach an iron fortification level of 80 ppm, 1.7 g (± 0.004) isotopically labelled extruded rice were added to CER and 1.7 g (± 0.006) to HER1 test meals; 1.7 g (± 0.003) isotopically labelled extruded and 2.1 g (± 0.003) isotopically labelled coated rice were added to meals HER2 and COR, respectively. Reference meals contained 1.9 g (± 0.005) extruded rice. Rice was served with a standardized vegetable sauce as previously reported (23).

Prior to consumption, 4.0 mg (± 0.038) iron in form of a labelled $^{58}FeSO_4$ solution, which was produced from enriched elemental iron (99.90% ^{58}Fe enrichment, Chemgas, Boulogne, France) as previously described (24), was added to the reference meal (Study 2) only.

Test meal analysis

Iron and zinc contents in basmati rice and vegetable sauce were analyzed by AAS after mineralization by microwave digestion (MLS TurboWave, MLS GmbH; Leutkirch, Germany) using nitric acid. Iron and zinc contents and isotopic composition were determined by isotopic dilution ICPMS. Phytic acid (PA) content was determined as described (25, 26). The ascorbic acid content in the vegetable sauce was analyzed by HPLC (Acquity H-class UPLC system, 4824949; Waters AG, Dättwil, Switzerland) after stabilization and extraction with meta-phosphoric acid and reduction by dithiothreitol (27). Iron solubility from HER1 and CER rice was determined after a modification of the method of Miller et al.(28) with digestive enzymes (amylase, pepsin) as recently described (20). Relative solubility was expressed as the solubility of the compound divided by the solubility of the reference sample ($FeSO_4$) as recently described (23).

Rice structure analysis

We investigated the starch microstructure of hot and cold extruded rice and determined the overall degree of starch crystallinity, the type of starch polymorphism, and measured the lamellar distance in the semi-crystalline starch domain as well as the lattice parameter

of the unit cell of the starch helices. Small- and Wide-angle X-ray scattering experiments were performed, data analysis and modeling are described in Supplemental Methods.

Blood analysis

Plasma ferritin, plasma CRP and Hb were measured on the day of collection as recently described (23); anemia was defined as Hb <12 g/dL (29). ID was defined as plasma ferritin <15 µg/L and ID anemia as Hb <12 g/dL and plasma ferritin <15 µg/L (29). Reference CRP concentrations for healthy individuals were <5 mg/L (30). Plasma separation and subsequent PZn determination, as well as spot urine collection and analysis, were performed as recently described (26). Determination of iron and zinc isotopic composition in blood, respectively urine samples, and subsequent calculation of iron and zinc fractional absorption were performed as previously described (19, 31, 32). Absorption values from two participants in study 1 were below the detection limit of 0.4% fractional absorption, therefore, random positive numbers below this threshold were generated in Microsoft Excel (2013; Microsoft Corporation, Redmond, WA) and used for subsequent analysis. FAFe$_{corr}$ was calculated from FAFe and corrected to a plasma ferritin level of 40 µg/L as described (33).

Micronutrient retention

Fortified rice samples, which differed in their nutrient concentrations compared to those samples used in the iron absorption studies, were tested regarding their mineral (iron and zinc) contents after six different preparation procedures. The rice samples were 1) medium whole grain rice kernels coated with a mixture of nutrients and waxes produced by Wright Enrichment Inc., 2) hot extruded or cold extruded rice both produced at ETH Zurich and consisting of rice flour, water and a mixture of nutrients and additives. All samples had a nutrient profile and production parameters similar to those described for the human absorption studies. Fortified rice and unfortified basmati rice were weighed and mixed at a 1:50 (w/w) ratio (hereafter referred to as rice meals).

Rice meals were cooked in separate glass bowls (20 min, 220 °C) with a 1:2 (w/w) or a 1:6 (w/w; excess water method) rice/water ratio and were either rinsed or soaked before cooking, or not. Each preparation condition was applied to each rice meal and all

experiments were conducted in triplicate. For rinsing, the rice meals were rinsed twice with a total of 300 mL 18 MΩ·cm water, and the water was decanted between the rinsing steps. For soaking, rice meals were soaked in their bowls for 30 min in 300 mL 18 MΩ·cm water, then the water was decanted and water for cooking was added (see above). Rice meals prepared with the 1 : 2 or 1 : 6 rice/water ratio alone received no prior treatment. After cooking, all meals were cooled (30 min, 8 °C) and excess water, if present, was decanted.

For determination of mineral retention, cooled rice meals were dried in an oven (110 °C). Then, meals were weighed again and homogenized in a rotor mill (Retsch ZM1, GmbH & Co.KG, Germany) with a Titanium sieve (sieve openings: 250 µm). Milled samples were stored in zip-loc bags under ambient temperature until further analysis by atomic absorption spectrophotometry (AAS, GTA 120 or AA240FS; both Agilent Technologies AG, Basel, Switzerland). Mineral contents retrieved in the dried rice meals were expressed as fraction of the amounts present in the uncooked meals. Results obtained from meals prepared with the 1 : 2 rice/water ratio were defined as 100% retention and results from other methods expressed as fraction thereof (= relative retention).

Sample size calculation and statistical analysis

Data were analyzed using SPSS (version 22.0, 2013; SPSS Inc, Chicago, IL) and Microsoft Excel (2013; Microsoft Corporation, Redmond, WA). The power calculation was performed with the software GraphPad StatMate for Windows (Version 2, GraphPad Software, LA Jolla, CA) anticipating that analyses would be performed on log transformed data, as iron absorption data is typically highly skewed. Based on previous data from iron absorption studies from FePP fortified rice conducted in our laboratory[34], we expected an SD of $\log_{10}$ fractional iron absorption of 0.23, and a correlation coefficient of fractional absorption from different fortified rice test meals within the same subjects of r=0.73. We expected a fractional iron absorption (not log transformed) from FePP fortified rice to be 3% of the administered dose. A 30% change in iron absorption would result in an increase in fractional absorption to 3.9%, which in a $\log_{10}$ scale would translate to an effect size of 0.11. To achieve this, a sample size of 16 participants was estimated to be necessary using paired *t*-tests with a power of 0.8 and α of 0.05. We anticipated dropout rates of 20% and

35% in studies 1 and 2, respectively and aimed at recruiting 20 subjects for study 1 and 24 subjects for study 2. Subjects who discontinued participation on the first study day were replaced.

FAFe from the different meals within the same participant was compared by paired samples *t*-test (study 1) or repeated-measures ANOVA followed by Bonferroni corrected pairwise comparisons (study 2). FAZn was compared by paired samples *t*-test. Results for iron solubility were compared by unpaired *t*-test. Results for age and anthropometric features, Hb, plasma ferritin, plasma CRP and PZn were presented as means ± SDs, if normally distributed, otherwise they were presented as geometric means with a 95% CI. Calculations for iron, zinc and phytic acid concentrations of composite meals were based on the means from the analysis of single components (regular basmati rice, extruded rice, vegetable sauce; *n = 5* or *3*), SDs were adapted by calculating the square root of the squared and summed SDs from each single component. Results for iron and zinc retention were compared with univariate general linear model with "preparation method" and "rice fortification technique" and their interaction as fixed factors and the "relative mineral retention" as dependent variable. We used multiple comparisons to distinguish specific effects between individual preparation methods and P values were Bonferroni corrected. Differences were considered as significant at P <0.05. Non-normally distributed data were logarithmically converted for statistical analysis and reconverted for reporting. $FAFe_{corr}$ (corrected to a plasma ferritin level of 40 µg/L) was calculated from FAFe as described earlier (33).

Results

Study 1

Twenty-three women were recruited and started the study, four discontinued participation because they could not comply with the study protocol, thus, 19 participants completed the study and were included in the analysis **(Table 2).** At baseline (day 1), two participants were iron deficient (ID), one participant was ID anemic, and one had an elevated plasma CRP concentration (6.4 mg/L).

Iron and zinc concentrations in the isotopically labelled extruded rice grains in meals CER and HER1 were 229 ±6.8 and 234 ±4.2 mg/100 g, and 166 ±3.5 mg/100 g and 163 ±0.9 mg/100 g, respectively. The native iron and phytic acid concentrations in the composite meals were 0.5 ±0.13 mg and 0.1 ±0.01 g, respectively, the sauce given with each meal contained 0.8 ±0.0 mg/100 g ascorbic acid.

FAFe from CER was greater than from HER1 (P = 0.036) (Table 1). In the *in vitro* simulated digestion experiments, CER had a greater relative iron solubility compared to HER1 (19.4% vs 13.6%) (P = 0.026). Starch structure differed between CER and HER1: both CER and basmati rice were characterized by A-type starch polymorphism (parallel packing of double helices). In contrast, HER1 showed a V-type polymorphism (antiparallel packing of single helices, Supplemental Figure 1, Supplemental Table 1). For further details on starch and structural measurements, please refer to the online supporting material.

Study 2

Thirty-two women were recruited for the study, eight women were excluded on study day 1 as they could not comply with the study protocol and 24 women started the study (Table 2). Two women were excluded during the study because they received heparin treatment which may influence hepcidin and iron metabolism (35). Therefore, 22 participants completed the study. At baseline (Day 1 or 2), four participants were ID, none suffered from ID anemia or had elevated plasma CRP concentrations. Two participants had elevated CRP concentrations (5 – 10 mg/L) during one study visit apart from baseline.

Iron and zinc concentrations in HER2 were 237 ±12.3 mg/100g and 344 ±16.1 mg/100g, COR contained 194 ±5.4 mg iron/ 100 g and 291 ±8.6 mg zinc/ 100 g and the extruded reference rice contained 7 ±0.4 mg iron/ 100 g and 3 ±0.2 mg zinc/ 100 g, respectively. The native iron, phytic and ascorbic acid concentrations were comparable to study 1 (above).

FAFe from COR did not differ significantly from HER2 (4.0% vs 5.1%; P = 0.14), but FAFe from COR was lower than from the reference meal (6.6%; P = 0.03, Table 2). FAZn was 9.7% from both HER2 and COR and did not differ (P = 0.91). Relative bioavailability from

HER2 (79%) did not differ from COR (61%) (P = 0.45) or the reference (P = 0.41). However, relative bioavailability from COR differed from the reference (P = 0.003).

Table 2. Baseline characteristics of the female participants in study 1, assessing differences between iron fortified rice produced with hot and cold extrusion and study 2, comparing iron and zinc absorption from rice produced with coating or hot extrusion[1]

	Study 1 (*n = 19*)	Study 2 (*n = 22*)
Age, y	26 ± 3	24 ± 4
Weight, kg	59.4 ± 4.8	59.0 ± 4.55
Height, m	1.65 ± 0.06	1.66 ± 0.05
BMI, kg/m^2	21.3 ± 1.6	21.2 ± 1.30
Hemoglobin, g/L	134.7 ± 9.7	134.2 ± 9.36
Plasma C-reactive protein, mg/L	0.7 (0.4, 1.2)	1.1 ± 1.0
Plasma ferritin, µg/L	46.2 ± 30.5	41.2 ± 31.29
Plasma zinc, µg/dL	n.a.	85.5 (79.06, 92.42)

[1] Values are means ± SDs or geometric means (95% CIs); n.a.: not assessed.

Mineral retention

Iron and zinc concentrations (per 100 g fortified kernels) were 511 ±43.8 mg and 730 ±24.7 mg, 392 ±7.1 mg iron and 619 ±6.0 mg zinc and 389 ±5.4 mg iron and 591 ±3.0 mg zinc in the coated rice, the hot extruded rice and the cold extruded rice, respectively. Both the preparation method as well as the rice fortification technique affected iron and zinc retention in cooked rice (**Table 3**). We found a significant interaction between rice fortification technique and preparation method for iron retention (P<0.001) but not for zinc retention (P=0.052). Retention upon using the 1 : 2 rice/water ratio was 90%, 88% and 87% for iron and 82%, 108% and 109% for zinc in the coated rice, the hot extruded rice and the cold extruded rice, respectively (Table 3). Cooking the rice at a 1 : 2 rice/water ratio, regardless of pre-treatment, showed overall relative iron and zinc retentions above 80% for all different types, except for coated rice with prior soaking (~60% relative zinc retention). Cooking in excess water without pre-treatment did not affect relative iron retention from the hot extruded rice; however, the coated rice and the cold extruded rice retained ~3/4 of iron; all types retained ~4/5 of zinc. Excess water cooking with prior

rinsing resulted in 87%, 72% and 48% iron retention in hot extruded rice, cold extruded rice and coated rice, respectively, whereas for zinc, retention from all three types of rice ranged from 60% to 75%. Cooking in excess water with prior soaking showed a similar pattern.

Table 3. Relative in vitro retention (percent) with different cooking methods for iron and zinc from fortified rice produced with 3 distinct rice fortification techniques undergoing 6 different cooking and preparation methods.[1]

Mineral and rice fortification technique / Rice fortification	Cooked in 1 : 2 rice/water [w/w]			Cooked in 1 : 6 rice/water [w/w]			P values[2]		
	No pretreatment	Rinsing	Soaking	No pretreatment	Rinsing	Soaking	Preparation method[3]	Rice fortification	Interaction
Iron retention									
Hot extrusion	100.0^b±3.1	102.0$^{b,+}$±4.0	109.5$^{a,+}$±1.5	99.8^{b,*}±2.3	87.5$^{c,+}$± 3.5	81.3^{c,*}±1.8	<0.001	<0.001	<0.001
Cold extrusion	100.0^a±2.3	97.8$^{a,+}$±2.2	95.6$^{a,*,+}$±1.3	76.4$^{b,+}$±3.0	72.3$^{b,+}$± 9.0	68.7^{b,*} ±3.7			
Coating	100.0^a±2.4	101.7^{a,*}±6.2	80.6^{b,*}±1.0	75.4$^{b,+}$±13.1	47.5^{c,*}±9.7	44.3$^{c,+}$±5.3			
Zinc retention									
Hot extrusion	100.0^a±2.4	95.3^b±1.9	81.6$^{b,+}$±1.9	81.3^b±6.8	63.7$^{c,+}$±10.3	58.7^d±2.1	<0.001	0.001	0.052
Cold extrusion	100.0^a±2.1	97.1^a±2.8	82.2$^{c,+}$±1.7	78.8^c±2.7	64.6^d±6.2	61.1^d±4.0			
Coating	100.0^a±1.8	82.9^a±2.0	59.6^{b,*}±2.1	81.3^a±19.9	59.1^b±4.8	53.6^b±4.4			

[1]Values are means ± SD, n = 3. [2]Models were calculated for each micronutrient separately. [3]Effects of cooking and pretreatment. Relative retention was calculated as fraction of mineral retention after preparation with the 1:2 rice/water ratio (second column). Retention upon cooking with a 1 : 2 rice/water ratio was 88%, 87% and 90% for iron and 108%, 109% and 82% for zinc from the hot extruded, the cold extruded and the coated rice, respectively. Univariate GLM and multiple comparisons (Bonferroni corrected, P<0.05) for each micronutrient. Different letters within a row and symbols within a column, indicate significant differences.

Discussion

Our results from study 1 show a higher iron absorption from cold (CER) compared to hot extruded rice (HER1). The *in vitro* digestion experiments suggest this may be due to higher iron solubility from CER meals. The higher solubility from CER may be explained by differences in starch microstructure and amorphous content: the V-type polymorphism found in HER1 restricts swelling and stabilizes the rice kernel structure, similarly as in parboiled rice (36-38). These findings are in agreement with observed differences in differential scanning calorimetry (Supplemental Figure 3) and mechanical properties (Supplemental Figure 4). Thus, compared to hot extrusion, lower extrusion temperatures appear to generate a more readily digestible starch structure, allowing for greater iron release in the proximal gut for absorption (39). Further investigations should focus on identifying ideal extrusion conditions for duodenal mineral delivery from extruded rice, regarding kernel starch and non-starch structure, porosity and integrity after cooking as well as during *in vitro* and *in vivo* digestion.

In study 2, we found no significant differences in iron and zinc absorption from fortified rice produced with coating or hot extrusion. While iron bioavailability from extruded fortified rice has been extensively investigated (14, 20, 23), no evidence on mineral absorption from coated rice in humans existed so far. Our findings suggest coating as a viable rice fortification technique, allowing to widen the technological portfolio for implementation of rice fortification, as coating technology may provide advantages in settings where extrusion cannot be readily implemented. However, while we did not detect a statistically significant difference in iron and zinc absorption between the techniques, we cannot exclude a smaller impact on iron absorption from cold extruded rice, which may not be captured with the power of this study. The study further suggests that 200 g of raw fortified rice containing either HER2 or COR could meet the daily requirements of ~2 – 3 mg absorbed zinc in adults (40). With regard to iron, 200 g of raw rice would cover half or two thirds of their daily iron requirements (41) in women of reproductive age with sufficient iron stores (serum ferritin 40µg/L). However, our study

did not account for higher phytic acid amounts in more diverse diets, whose inhibitory effect on FAZn may be higher (42).

Iron absorption from hot extruded fortified rice depends on the presence of other ingredients in the fortified kernel. The difference in corrected fractional iron absorption (FAFe$_{corr}$) from HER2 (3.6%) compared to HER1 (1.1%) may be attributed to two factors: 1) the presence of the CA/TSC buffering system, as relative bioavailability of HER2 was ~80% (n.s. compared to FeSO$_4$), which is consistent with our previous findings (20); and 2) the slightly higher ZnO amount present in HER1 compared to HER2. We have recently shown that ZnO per se can reduce FePP absorption from rice (23), but whether an incremental increase of ZnO would affect human iron absorption is unclear. Thus, while the difference in iron absorption from HER1 and HER2 is very likely due to the presence of CA/TSC, we cannot exclude an effect of ZnO. Whether CA/TSC addition improves iron absorption from cold extruded rice should be investigated.

Relative iron and zinc retention in coated and both extruded rice meals cooked with the 1 : 2 rice/water ratio regardless of pre-treatment was >80%, with the exception of coated rice that was soaked prior cooking. After cooking in excess water, regardless of pre-treatment, iron retention was highest in hot extruded rice meals (>80%), followed by cold extruded (69 – 76% retention) and coated rice meals (44 – 75% retention). Prior rinsing or soaking had the most detrimental effect on mineral retention in all meals. After excess water cooking, zinc retention in all three types of meals was lowest, when meals were rinsed or soaked before cooking with retention values ranging from 54% (coated rice) to 65% (cold extruded rice). In extrusion, minerals are embedded in the rice kernel matrix protecting the kernel from micronutrient losses caused by pre-cooking treatments, whereas in coated rice micronutrients are located on the grain surface (9). This likely explains the comparatively low retention from coated rice after excess water cooking, regardless of pre-treatment. Because of the higher kernel solubility upon cooking with the A-type starch polymorphism in CER, we expected higher nutrient losses in cold than hot extruded rice. Thus, hot extruded rice seems advantageous over cold extruded and coated rice regarding iron and zinc retention. Nevertheless, the chosen fortification technology should account for the prevailing local preparation method.

A strength of these studies is the precise measurement of human iron absorption using isotopically labelled rice that closely matches commercially fortified rice; both in the manufacture of the labelled fortification compounds and the rice kernels, produced by down-scaled versions of the large-scale process. However, our studies also have limitations. The standardized meals consisting mostly of white rice contained negligible amounts of PA, which can inhibit iron (43) and zinc (42) absorption and may not be fully generalizable to settings where legumes and whole grains are consumed along with rice. Further, we tested the rice meals in generally iron and zinc replete subjects and cannot exclude a different absorption in a depleted population, which would be targeted for a mass fortification program. We considered visual differences between fortified and unfortified rice after cooking as being minimal, however, we did not scrutinize the sensory properties of the different rice types. Due to expected unavoidable losses of iron and zinc stable isotopes during the down-scaled production, the kernels were manufactured for a 1:25 fortified/unfortified rice blending ratio, while typical fortification programs employ ratios of 1:100-200.

In summary, both coating and extrusion, employing the processes that were used for the production of the fortified kernels used in this study, appear to be viable rice fortification techniques. In both processes, we recommend the use of FePP combined with CA/TSC. Our data suggest the structure of the rice-kernel matrix affects nutrient release and bioavailability, therefore, further experiments to optimize the kernel microstructure are needed. While coating is generally less expensive than extrusion (10), large-scale fortification renders the cost per metric ton of fortified rice for both techniques comparable (9), and regardless of the technology used, fortified grains contribute to <1% of the wholesale price for fortified rice (44).

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Supplemental Material

Characterization Techniques

SAXS and WAXS

Small and wide-angle X-ray scattering (SAXS and WAXS) experiments were performed using a Bruker AXS Micro equipped with a microfocused beam (50 W, 50 kV, 1 mA) with the $\lambda_{CuK\alpha}$ = 0.15418 nm radiation in order to obtain direct information on the scattering patterns. The scattering intensities were collected by a Dectris 2D Pilatus 100K X-ray detector (83.8 cm × 33.5 cm, 172 µm resolution resolution). An effective scattering vector range of 0.1 nm^{-1} < q < 25 nm^{-1} was obtained, where q is the scattering wave vector defined as $q = 4\pi \sin \theta / \lambda_{CuK\alpha}$ with a scattering angle of 2θ. Entire rice kernels were measured at room temperature. Deconvolution of the WAXS scattering profile allows for the evaluation of the degree of crystallinity which is defined as the ratio between the total area corresponding to the crystalline diffraction peaks and the total area under the scattering curve, $\chi = A_{crystalline}/A_{total}$ (1). Moreover, the presence of diffraction peaks at specific q-values allows for the unambiguous assignment of the starch polymorphism and the determination of the unit cell (2). The evaluation of the SAXS scattering peak and the adjustment of this peak by the para-crystalline model (1) allows for the evaluation of the semi-crystalline lamellar distance and size domain, as well as for the determination of the crystalline and amorphous layer thickness within the domain.

Differential scanning calorimetry

Differential scanning calorimetry experiments were conducted on a Mettler Toledo DSC1 STAR^e System apparatus in a temperature range from 25 to 90 °C at heating and cooling rates of 5 K min^{-1} under nitrogen atmosphere. Milled rice kernel flour was mixed with water (1:10) and 40 µL aluminum pans were used.

Texture analysis

The mechanical properties of cooked rice were measured in compression mode with the help of a universal tensile testing Zwick Z010 machine equipped with a 10 N load cell at the strain rate of 50 mm min^{-1}. The measurements were performed per triplicate at room temperature after cooling at 8°C for 15 min the mixtures of rice kernels in water (1:4) which were cooked at 120 °C for 17.5 min.
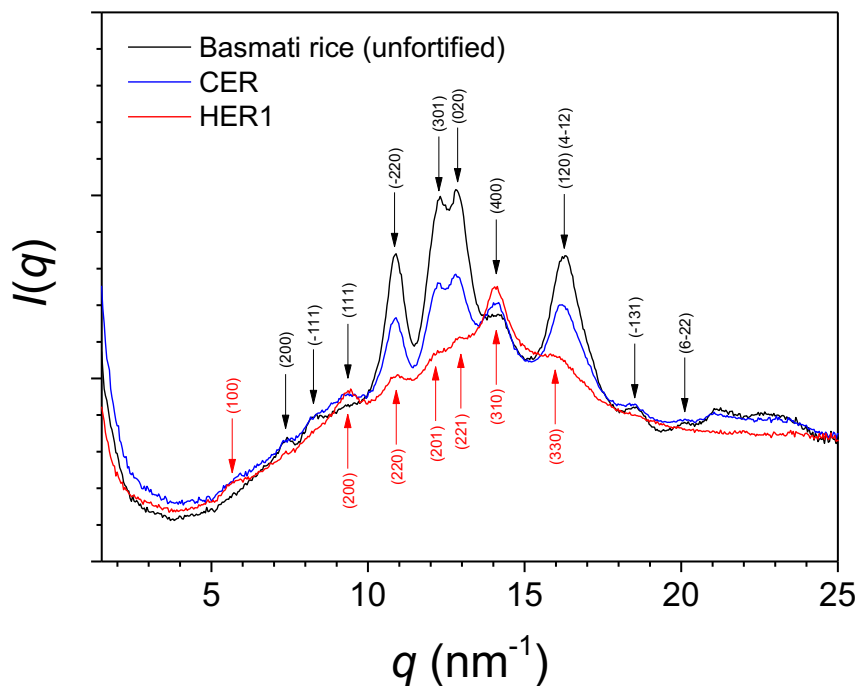
Supplemental Figure 1. 1D WAXS intensity profile for the unfortified basmati rice (black), the iron fortified cold extruded rice (CER, blue), and the iron fortified hot extruded rice (HER1, red), with the indexation of the diffraction peaks (in black and blue: A-type; in red: V-type).

Supplemental Table 1. Average degree of crystallinity (χ) polymorphism, and lattice parameters (a, b, c, and γ) evaluated from the WAXS intensity profiles.

| Sample	χ (%)	polymorphism	a (Å)	b (Å)	c (Å)	γ (deg)
Basmati rice	54	A (monoclinic)	20.5	11.4	11.9	120
Cold Extruded	49	A (monoclinic)	20.4	11.4	12.0	120
Hot Extruded	21	V (orthorhombic)	12.8	28.9	8.9	90
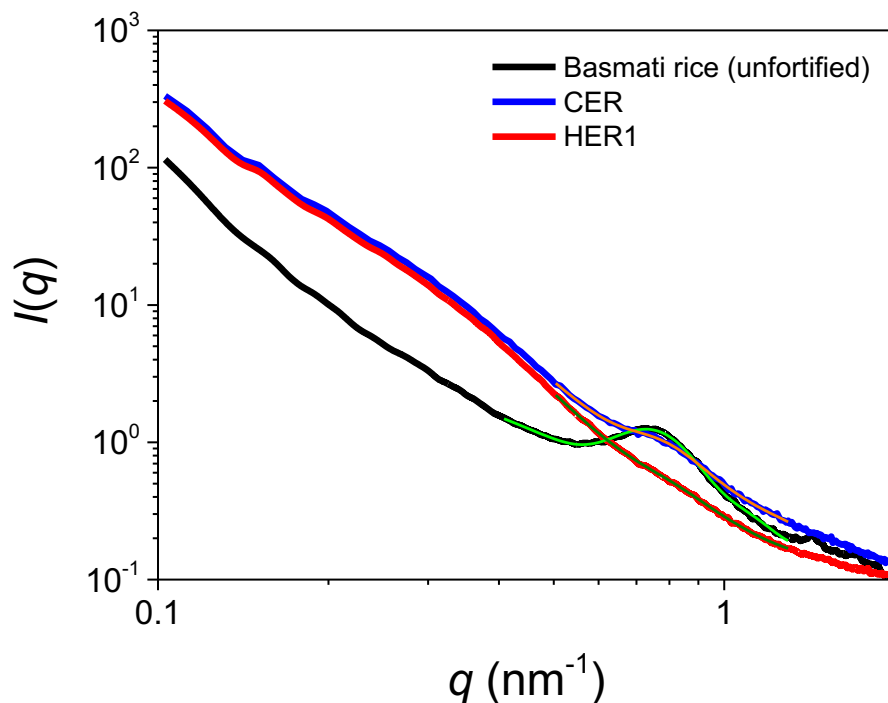
Supplemental Figure 2. 1D SAXS intensity profile for the unfortified basmati rice (black), the iron fortified cold extruded rice (CER, blue), and the iron fortified hot extruded rice (HER1, red), with the corresponding fitting curves.

Supplemental Table 2. SAXS peak (q), lamellar distance (*d*), correlation length (ξ), crystalline layer thickness ($L_{crystalline}$), amorphous layer thickness ($L_{amorphous}$), degree of crystallinity (χ) in the semi-crystalline domain evaluated from the SAXS intensity profiles.

| Sample	q (nm^{-1})	d (nm)	ξ (nm)	$L_{crystalline}$ (nm)	$L_{amorphous}$ (nm)	χ (%)
Basmati rice	0.75	8.4	21	5.8	2.7	68
Cold Extruded	0.79	7.9	19	4.0	3.9	50
Hot Extruded	0.84	7.5	21	5.6	1.9	75
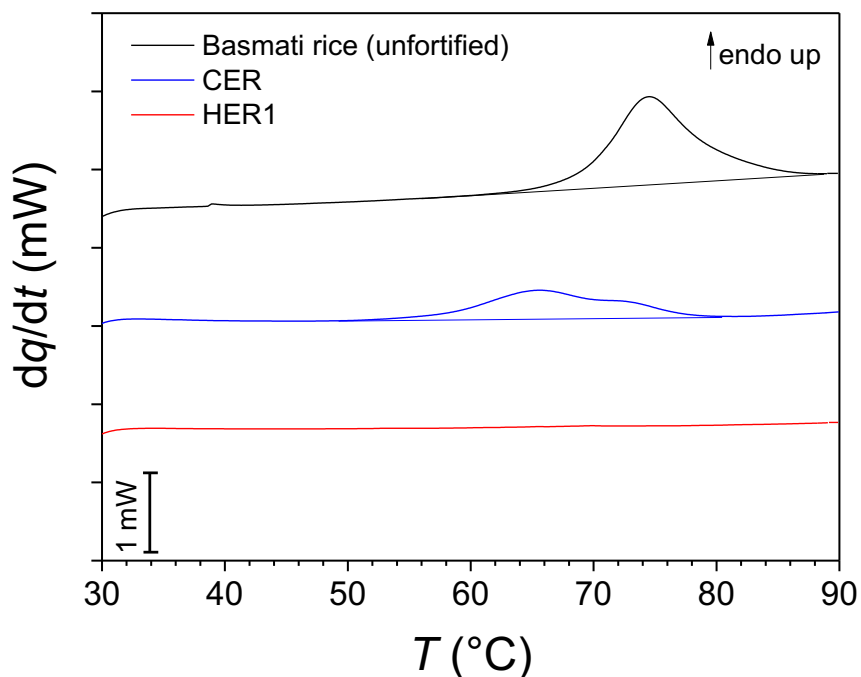
Supplemental Figure 3. Differential scanning calorimetry themograms from 25 to 90 °C at 5 K min^{-1} for the unfortified basmati rice (black), the iron fortified cold extruded rice (CER, blue), and the iron fortified hot extruded rice (HER1, red) mixtures with water (1:10).

Supplemental Table 3. Onset (T_{onset}) and maximum heat flow temperature (T_{max}) evaluated from the Differential scanning calorimetry curves.

Sample	T_{onset} (°C)	T_{max} (°C)
Basmati rice	69	74
Cold Extruded	57	66
Hot Extruded	-	not detectable
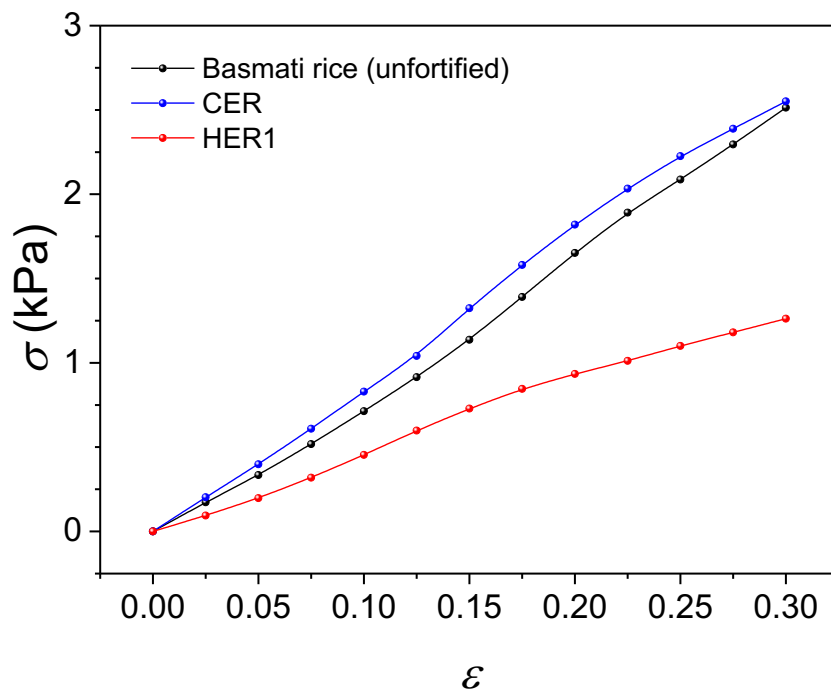
Supplemental Figure 4. Stress-strain curves in compression mode for the unfortified basmati rice (black), the iron fortified cold extruded rice (CER, blue), and the iron fortified hot extruded rice (HER1, red) after cooking the rice kernels in water (1:4).

Supplemental Table 4. Elastic modulus (E) evaluated from the stress-strain curves.

Sample	E (kPa)
Basmati rice	6 ± 1
Cold Extruded	7 ± 1
Hot Extruded	4 ± 1

Supplemental References

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MANUSCRIPT 4

Micronutrient-fortified rice is a substantial source of bioavailable iron in pediatric populations with high infection prevalence

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Abstract

Background: Rice fortified with extruded grains is a promising approach to combat anemia, but currently-used iron formulations have low bioavailability.

Objective: To use a novel multiple stable-isotope-labeled meal design to directly quantify and compare iron bioavailability from five iron-fortification formulations for rice incorporated into the local diet consumed over several weeks by iron deficient/anemic Ghanian children.

Design: Children (aged 6-8 y; n=26) consumed 60 labelled fortified local rice-based meals (10 meals per formulation) over 30 days. Fortified meals contained 40 ppm iron as ferric pyrophosphate (FePP) labeled with ^{54}Fe, ^{57}Fe or ^{58}Fe combined with either: zinc oxide (FeZnO), zinc sulphate (FeZnSO$_4$), alone or in combination with citric acid+trisodium citrate (CA+TSC) (FeZnOCT; FeZnSO$_4$CT) or ZnO+citric acid+EDTA (FeZnOCE); compared to a reference meal fortified with FeSO$_4$. Fractional iron absorption (FAFe) was measured as cumulative erythrocyte incorporation of iron isotopes.

Results: Both zinc fortificant and CA+TSC affected FAFe ($P \leq 0.001$), but there was no significant interaction. Geometric mean (95%CI) FAFe from meals FeZnSO$_4$CT (6.3%;5.3,7.4) and FeZnOCE (6.4%;5.1,8.1) did not significantly differ from FeSO$_4$ (6.4%;5.2,7.8); FAFe from the other three formulations were significantly lower than from FeSO$_4$ ($P < 0.05$) Based on these data, children consuming 100g/d of rice fortified with FeZnSO$_4$CT and FeZnOCE would absorb 0.28 and 0.46 mg iron/day, respectively, meeting $\approx$2/3 of their daily requirements.

Conclusions: Optimized formulations for FePP-fortified rice achieve FAFe comparable to FeSO$_4$. This novel multiple-labeled meal study design provides a representative measure of iron bioavailability from the fortified food integrated into the local diet in the target population.

AA, Ascorbic acid; AGP, α-amino glycoprotein; BIS, Body iron stores; CA, Citric acid; CRP, C-reactive protein; EDTA, Ethylenediaminetetraacetic acid; FePP, Ferric pyrophosphate; ^{57}FePP, Isotopically labelled ferric pyrophosphate; ^{58}FeSO$_4$, Isotopically labelled ferrous sulfate; ^{54}FeZnO, Rice extruded with ^{54}FePP, ZnO; ^{54}FeZnOCT, Rice extruded with ^{54}FePP, ZnO, CA and TSC; ^{57}FeZnSO$_4$, Rice extruded with ^{57}FePP, ZnSO$_4$ and CA; ^{57}FeZnSO$_4$CT, Rice extruded with ^{57}FePP, ZnSO$_4$, CA and TSC; ^{58}FeZnOCE, Rice extruded with ^{54}FePP, ZnO, CA and EDTA; Hb, Hemoglobin; MN, Micronutrient mix used for rice extrusion containing vitamins A, B$_1$, B$_3$, B$_6$, B$_{12}$ and folic acid ; MR, Molar ratio; PF, Plasma ferritin; RBP, Retinol binding protein; RBV, Relative Bioavailability; Reference, Rice extruded with no iron, addition of ^{58}FeSO$_4$ solution prior consumption; sTfR, Serum transferrin receptor; TSC, Trisodium Citrate; ZnO, Zinc Oxide; ZnSO$_4$, Zinc Sulfate; ZnPP/H, Red blood cell zinc protoporphyrin/heme molar ratio

Introduction

In 2013, anemia was estimated to affect almost 2 billion people globally (1). Although the etiology of anemia is multifactorial, iron deficiency (ID) is the most common cause (1). People living in areas with a high prevalence of ID anemia (IDA) may also be at increased risk of zinc deficiency, as diets low in bioavailable iron also tend to be low in bioavailable zinc (2). Fortification of staple foods with iron and zinc can be an effective and sustainable strategy to reduce these common micronutrient deficiencies.

 Rice is a staple food for almost half the world's population and in Africa, rice consumption is expanding more rapidly than that of any other food commodity (3). There is a high prevalence of iron and zinc deficiencies in many rice-consuming countries (4, 5), but the implementation of rice fortification has been limited. Rice is mainly consumed as intact grains making fortification more technically demanding. Also, because of the white color of polished rice, only sensorially inert, poorly water-soluble iron compounds are suitable fortificants. Ferric pyrophosphate (FePP) has low (21 – 74%) relative bioavailability compared to water soluble ferrous sulfate (6), and its fractional iron bioavailability (1 – 2.9%) in humans is low (7). Because of its low bioavailability, it is recommended to double the iron fortification level of FePP in rice compared to wheat flour which is fortified with ferrous sulfate, when assuming similar consumption levels (8). These higher iron levels in fortified rice may induce increased fortification program costs, affect product appearance, and may not be without risk: unabsorbed iron from fortification may shift the colonic microbiome towards a more pathogenic profile in infants and children (9). Thus, formulations with higher iron bioavailability and yet acceptable sensory properties are needed.

Recently, we have shown that iron bioavailability is doubled from extruded FePP-fortified rice when citric acid combined with trisodium citrate (CA+TSC) is included in the extruded rice grain (10). Also, iron bioavailability from fortified rice may be reduced when co-fortified with ZnO compared to $ZnSO_4$ (11, 12). The use of other chelating agents such as ethylenediaminetetraacetic acid (EDTA) (13) increases *in vitro* solubility of FePP in rice and may be a promising approach, but this has not yet been tested *in vivo*.

Generalizability of results from previous studies using stable iron and zinc isotopes to measure bioavailability from extruded rice is limited because: 1) they were mostly found

in healthy young women in industrialized countries with generally sufficient iron and zinc status, not in target African or Asian populations with IDA and a high prevalence of infections/inflammation; 2) they used short term, single-meal study designs that may overestimate absorption effects (14); 3) they used rice fortified with a premix kernel ratio of 1:25 (compared to the 1:100 ratio used in programs); and 4) the fortified rice was not integrated into a local diet characteristic of populations in target countries.

To overcome these limitations and provide more realistic data on which to base fortification levels in rice fortification programs, our study aim was to use a novel approach to compare iron bioavailability from different formulations of fortified rice in African children with poor iron status using multiple stable isotope-labelled local meals administered over several weeks of feeding. Multiple meal studies using stable isotopes can be used to assess the potential nutritional impact of dietary interventions (15, 16) and have the advantage of more closely reflecting realistic local dietary conditions and long-term impact. Our hypotheses were: 1) co-fortification of ZnO into extruded rice grains containing FePP would decrease iron absorption compared to $ZnSO_4$; 2) CA+TSC addition would overcome detrimental effects of ZnO leading to iron absorption comparable to $FeSO_4$; 3) iron absorption would not be different from rice fortified with ZnO or $ZnSO_4$ when combined with CA+TSC; 4) EDTA-addition in combination with ZnO would show iron absorption similar to $FeSO_4$ and those meals containing CA+TSC.

Methods

Study site and participants

The study was conducted between April and June 2016 in Tamale, Northern Ghana, where the climate is tropical with one rainy season (April-September, peaking in July-August) and endemic malaria incidence (17). Most Ghanaian diets are carbohydrate based (18) and rice consumption is especially high in urban populations, whereas rural areas (9 kg annual rice consumption per capita in 2011) represent less than ¼ of the total rice consumption in the country (19). In 2014, anemia prevalence among Ghanaian children aged 6 – 59 months was higher in rural compared to urban regions (57 vs 74 %) and was highest in the Northern region (82 %) (20).

Inclusion criteria were: 1) Age at screening between 6 and 8 years; 2) ID anemia [defined at screening as hemoglobin (Hb) <11.5 g/dL and erythrocyte zinc protoporphyrin/heme (ZnPP/H) >43 µmol/mol. Exclusion criteria were: 1) Severe underweight or wasting; 2) Chronic or acute illness; 3) Regular intake (>2 days) of iron-containing mineral and vitamin supplements within 2 months prior study start; 4) Blood donation or comparable blood loss in the 4 months preceding the study; 5) Severe underweight (Z-score weight-for-age < -3); 6) Severe wasting (Z-score weight-for-height < -3). We included anemic and/or ID children who were aged between 5 and 10 years **(Figure 1; Table 1)**. Informed consent was obtained from the participant's caregivers by signature or finger print. The ethical committees of the Kintampo Health Research Centre Institutional Ethics Committee, Ghana, and ETH Zurich, Switzerland, reviewed and approved the study protocol (2016-3 and EK 2015-N-73, respectively) and its amendment, the trial was registered at clinicaltrials.gov as NCT02176759.

Study design

We used a randomized, cross-over, single-blind study design. The primary outcome was fractional iron absorption (FAFe). We used a computer-generated randomization schedule and assigned eligible children to the groups based on their randomly assigned study number. Randomization took into account that different meal types with the same isotopes had to be given at least 14 days apart. Each participant consumed six different isotopically labeled test meals each provided in series of 10 servings over 5 consecutive days (2 meals/d). This resulted in a total number of 60 servings per participant, which were administered over 30 days (days 1 – 5, 8 – 12, 14 – 18, 35 – 39, 42 – 46 and 49 – 53) including a 16 day break (days 19 – 34) between the first and second half of the administered series as well as two days' breaks between administrations of the first, second and third as well as fourth, fifth and sixth series **(Figure 1)**. The meals were based on rice extruded with 1) ^{57}FePP and $ZnSO_4$ (^{57}FeZnSO$_4$); 2) ^{54}FePP and ZnO (^{54}FeZnO); 3) ^{57}FePP, $ZnSO_4$, CA and TSC (^{57}FeZnSO$_4$CT); 4) ^{54}FePP, ZnO, CA and TSC (^{54}FeZnOCT); 5) ^{58}FePP, ZnO, CA and EDTA (^{58}FeZnOCE); 6) no iron nor zinc, but fortified with ^{58}FeSO$_4$ added as solution prior to consumption (Reference meal). Each extruded rice batch also contained a vitamin premix (vitamin A, folic acid and vitamins B_1, B_3, B_6 and B_{12}) and the meals were accompanied with a bean or a tomato sauce.

On study days, the first serving was administered in the morning between 0700 and 0800 h after an overnight fast and the second serving was administered ~3.5 h after completion

of the first serving. All meal administrations were supervised. After consumption, the empty bowls were rinsed twice with a total of 20 ml water and the participants received 250 ml water; all water had to be consumed by the participants. Participants were not allowed to eat or drink between the test meals. Approximately 1.5 h after the second serving, the participants received a standardized snack (wheat flour cookies, Mass Industries Ltd, Tema, Ghana) and had to refrain from eating and drinking for another 1.5 h. Thereafter, they were allowed to eat and drink *ad libitum* until one day prior to the next meal administration.

During screening (baseline measurements), approximately 1 wk before the first meal administration, body weight and height of the participants were measured and blood samples collected to assess iron status [Hb, ZnPP/H, plasma ferritin (PF), plasma C-reactive protein (CRP), serum transferrin receptor (sTfR), body iron stores (BIS), retinol binding protein (RBP), α-amino glycoprotein (AGP)] and malaria parasitemia. Before each blood sampling, the body temperature of the children was measured with a digital thermometer (OMRON Healthcare Europe B.V., Nigeria) to help identify children who may have symptomatic malaria. All participants received anthelmintic treatment (Albendazole 400 mg), participants positive for malaria antigens received antimalarial treatment (Arthesunate Amodiaquine 100 mg/ 270 mg). Measurements for body weight and height, body temperature as well as blood collections were repeated on days 32 and 67. Iron absorption was determined as incorporation of isotopic iron labels into erythrocytes at least 14 days after the meal administrations.
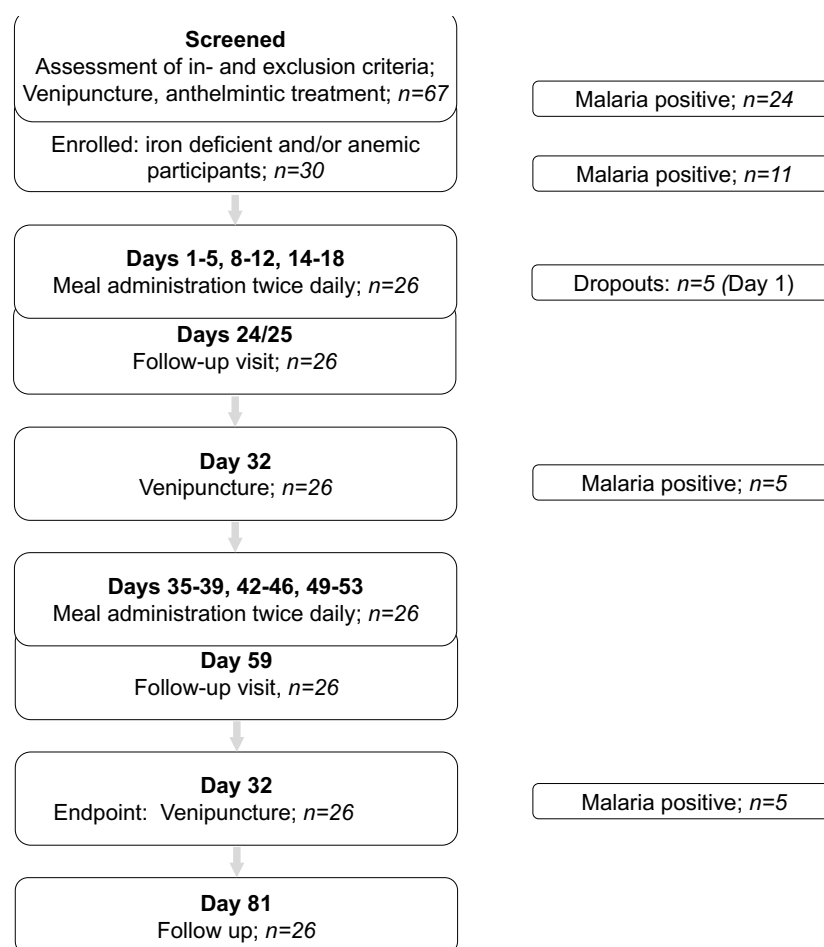
FIGURE 1. Study flowchart for human absorption study.

Preparation of isotopically labeled extruded rice grains

Six different batches were prepared for hot extrusion (~90 °C) as described earlier (21). Labeled ^{54}FePP, ^{57}FePP and ^{58}FePP were prepared in separate batches by Dr. Paul Lohmann GmbH from elemental isotopically enriched iron (Chemgas, Boulogne, France) using a scaled down process as used for the synthesis of the commercial compound. The batches were then mixed with commercial FePP (Dr. Paul Lohmann GmbH) resulting in an isotopic enrichment of 47.2 % ^{54}FePP, 19.8 % ^{57}FePP and 8.6 % ^{58}FePP, respectively.

Test meal preparation

The composite test meals were prepared at the school kitchen of SOS children's village in Tamale, Ghana and contained 49.5 g unfortified rice and about 500 mg extruded rice (433 $\pm$ 1.4, 529 $\pm$ 0.5, 508 $\pm$ 0.6, 481 $\pm$ 0.5, 509 $\pm$ 0.6 and 498 $\pm$ 2.0 mg extruded rice for meals ^{54}FeZnO, ^{57}FeZnSO$_4$, ^{54}FeZnOCT, ^{57}FeZnSO$_4$CT, ^{58}FeZnOCE and Reference, respectively).

Unfortified rice and extruded rice were mixed and cooked in a small industrial oven for 37 min at ~150 °C in separate glass bowls for each participant. After cooking the rice, 499 ± 4.0 mg Fe in the form of a labeled $^{58}FeSO_4$ solution, which was produced from enriched elemental iron (9.92 % ^{58}Fe enrichment; Chemgas), as previously described (24), were added to each Reference meal.

Tomato and bean sauces were prepared in three and two batches, respectively and kept frozen until one day prior to a meal administration when they were thawed. On each administration day, the sauces were reheated and 50 g sauce added to each cooked rice meal. Each sauce was given once per day (either with the first or second serving). The meals were transported in isolation boxes to Dungu primary school, where each meal was administered to its corresponding participant.

Test meal analysis

Iron contents in the unfortified rice, extruded rice and vegetable sauce were analyzed via atomic absorption spectrophotometry (AAS; Agilent Technologies GTA 120 or AA240FS) after mineralization by microwave digestion (TurboWave; MLS GmbH) using nitric acid. Phytic acid (PA) and polyphenol (PP) contents were determined as previously described (22) with the only difference, that fat was extracted from the sauces with petroleum ether prior to PA-determination. The ascorbic acid (AA) content in the vegetable sauce was analyzed via HPLC (Acquity H-Class UPLC System; Waters AG) after stabilization and extraction in metaphosphoric acid and reduction via dithiothreitol (23).

Determination of *in vitro* iron solubility

In vitro solubility was assessed from the same extruded rice kernels as in the human absorption study using amylase and pepsin enzymes as previously described (21), modified from Miller et al. (24); and relative iron solubility was calculated with $FeSO_4$ as a reference sample (11).

Blood analysis

Hemoglobin was measured in venous blood samples on the collection day using HemoCue 301 (HemoCue AB, Angelholm, Sweden). ZnPP/H was measured using a hematofluorometer (206d, Aviv, Belgium) on the day of collection after washing erythrocytes with a physiologic saline

solution. Both devices were calibrated prior measurement using controls supplied by the manufacturer. Serum samples were aliquoted, frozen and shipped to VitMin Lab (Freiburg, Germany) to determine SF, sTfR, RBP, CRP and AGP via sandwich ELISA as described elsewhere (25). Hepcidin was measured in serum samples using ELISA testkits (Hepcidin 25 bioactive HS DRG, DRG Instruments, Germany). Malaria parasitemia was determined in whole blood samples via parasite counting by an experienced microscopist. We used rapid malaria diagnostic test kits (SD Bioline) to support the results of the blood smears.

As it was not possible to perform ELISA essays in the field, for screening purposes only, we defined anemia as Hb <11.5 g/dL, ID as ZnPP/H >43 µmol/mol or ID anemia as Hb <11.5 g/dL and ZnPP/H >43 µmol/mol (26-28). However, in the subsequent analyses and for reporting SF <30 µg/L or sTfR >8.3 mg/L were used for definition of ID and, in combination with Hb <11.5 g/dL, ID anemia. The expected high sensitivity CRP concentrations for healthy individuals were <5 mg/L (29) and ≤1 g/L for AGP. The isotopic composition in the blood samples and the calculation of iron absorption were determined as previously described (23). The amount of absorbed isotopic label (= FAFe) in the blood was assessed by a shift of the isotopic iron ratio in red blood cells, to calculate total isotopic label using the participant's blood volume (30) and assuming 80% of the absorbed iron to be incorporated into red blood cells (31). The calculation was conducted using the principles of isotopic dilution and taking into account that isotopic labels are not mono-isotopic (31-33). We corrected for previously incorporated ^{54}Fe, ^{57}Fe or ^{58}Fe by using the isotopic ratio value at least 14 days after a test meal administration as a new baseline value for the following test meal administration (33).

Statistical analysis

Based on the primary outcome of FAFe, we estimated that a sample size of 16 would be sufficient to detect an intra-subject difference in FAFe of 30 % between the administered meals with a β of 0.8, α of 0.05 and a standard deviation of 0.5 units in the log-transformed FAFe. To compensate for an anticipated high drop-out (due to the length and demands of the study), we recruited 30 participants. Data were analyzed using SPSS (version 22.0, 2013; SPSS Inc, Chicago, IL) and Microsoft Excel (2013; Microsoft Corporation, Redmond, WA). FAFe from the different meals within the same participant was compared by linear mixed models followed by Bonferroni correction for multiple comparisons; RBV was

compared with repeated measures ANOVA followed by Bonferroni correction for multiple comparisons. Linear regression was used with iron absorption from ^{54}FeZnO, ^{57}FeZnSO$_4$, ^{54}FeZnOCT, ^{57}FeZnSO$_4$CT, ^{58}FeZnOCE and the Reference as dependent variables and Hepcidin, ZnPP/H, Hb, SF, sTfR, BIS, RBP, CRP and AGP as independent variables. Univariate ANOVA was used to measure the effects of the zinc source, CA+TSC and CA+EDTA addition. Results for age, anthropometric measurements, Hb, PF and CRP were presented as means $\pm$ SDs if normally distributed, otherwise the results were presented as geometric means (95% CI). Calculated iron and zinc contents in composite meals were based on the means from the analysis of single components (regular Basmati rice, extruded rice, average mineral contents from the two different sauces; *n = 3*), SDs for iron and zinc contents of composite meals were adapted by calculating the square root of the squared and summed SDs from each single component. *In vitro* solubility was analyzed with univariate analysis of variance (ANOVA) and multiple comparisons were conducted using Bonferroni correction. Non-normally distributed data were logarithmically converted for statistical analysis and reconverted for reporting. RBV from each meal was calculated based on FAFe [%] respective to FAFe from the reference meal for each subject. Differences were considered as significant at *P < 0.05*.

Results

Participants' characteristics

Four participants discontinued participation after the first meal administration as they could not comply with the study protocol, 26 participants completed the study and were included in the analysis; their baseline characteristics are shown in **Table 1**. At baseline, eleven participants were positivie for malaria parasitemia, 3 participants had elevated CRP concentrations and 6 participants had elevated AGP concentrations. Two participants remained positive for malaria parasitemia throughout the whole study period and one additional participant was malaria positive at the endpoint.

Test meal composition

The iron and zinc concentrations and the Zn / Fe molar ratios in the different test meals and the reference meal are summarized in **Table 2.** The targeted fortification levels per fortified rice batch

were 400 mg/100 g iron and 600 mg/100g zinc, respectively. The mean±SD iron concentrations in the isotopically labelled extruded rice grains from meals ^{54}FeZnO, ^{57}FeZnSO$_4$, ^{54}FeZnOCT, ^{57}FeZnSO$_4$CT and ^{57}FeZnOCT were 395±9.1, 331±5.2, 331±1.8, 368±12.3 and 339±3.3 mg/100 g, respectively. The zinc concentrations in the same grains were 595±1.8, 558±36.5, 591±14.9, 598±6.9 and 570±2.4 mg/100 g from meals ^{54}FeZnO, ^{57}FeZnSO$_4$, ^{54}FeZnOCT, ^{57}FeZnSO$_4$CT, and ^{58}FeZnSOCE, respectively. The extruded rice grains in the reference meal contained 6±3 mg/100 g iron and 8±0.6 mg/100 g zinc. The native iron content was 1.5±0.06 mg iron in each composite test meal, which also contained 1.3±0.25 mg zinc and 0.1±0.02 g PA. The PA and AA contents in the bean or tomato sauce per each serving were 68±1.8 or 6±0.32 mg PA and 0.7±0.02 or 0.7 ±0.06 mg AA, respectively. The polyphenol contents in each serving of sauce were 11.1 mg±0.66 and 12.3 mg±2.2 as gallic acid equivalents in bean and tomato sauce, respectively. AA and PP contents were determined only in the vegetable sauce as their contents in rice were assumed to be negligible.
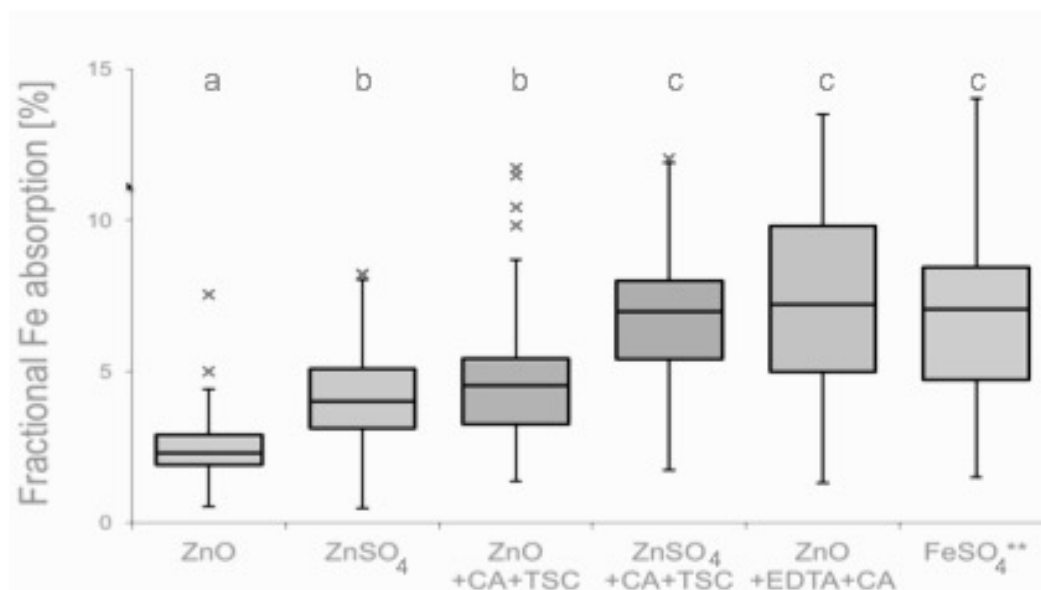
Iron absorption

Type of meal as well as the zinc co-fortificant and the presence of CA and TSC significantly affected FAFe (for all three, $P \leq 0.001$), but there was no significant interaction between CA/TSC and the zinc compound. Geometric mean (95% CI) FAFe from ^{54}FeZnO (2.3%;1.9,2.8) significantly differed from ^{57}FeZnSO$_4$ (3.5%; 2.7,4.5), ^{54}FeZnOCT (4.5%; 3.6,5.5), ^{57}FeZnSO$_4$CT (6.3%; 5.3,7.4),^{58}FeZnOCE (6.4%; 5.1,8.1) and the Reference (6.4%; 5.1,8.1) (for all, $P \leq 0.033$) (Table 2 and **Figure 2**). ^{57}FeZnSO$_4$ significantly differed from all other conditions ($P \leq 0.001$) except ^{54}FeZnOCT. There was no significant difference between ^{54}FeZnSO$_4$CT, ^{58}FeZnOCE and the Reference meal, however, they all significantly differed from ^{54}FeZnOCT (for all, $P \leq 0.037$). A similar pattern was seen for RBV: mean RBV from ^{54}FeZnO (44%) significantly differed from ^{57}FeZnSO$_4$ (66%), ^{54}FeZnOCT (85%), ^{57}FeZnSO$_4$CT (113%), ^{58}FeZnOCE (124%) and the Reference (for all, $P \leq 0.004$). ^{57}FeZnSO$_4$ differed from all other conditions ($P \leq 0.015$), except for ^{54}FeZnOCT ($P > 0.05$). There was no significant difference between ^{54}FeZnSO$_4$CT, ^{58}FeZnOCE and the Reference, however, ^{54}FeZnOCT, ^{58}FeZnOCE significantly differed from ^{54}FeZnOCT ($P \leq 0.002$), which did not significantly differ from the Reference.

Regression analyses indicated ZnPP and hepcidin as predictors for FAFe in subjects with ID and IDA ($n = 14$; both, $P \leq 0.02$), whereas AGP and Hb were significant predictors for

FAFe (both, *P ≤ 0.03*). In subjects without ID or IDA (*n = 12)* hepcidin was the only significant predictor for FAFe (*P ≤0.02*).

From meals [54]FeZnO, [57]FeZnSO$_4$, [54]FeZnOCT, [57]FeZnSO$_4$CT and [58]FeZnOCE and the reference meal, respectively, children with ID or IDA (*n = 14*) showed geometric mean (95% CI) FAFe of 2.3% (2.5,2.8), 3.9% (3.5,4.7), 5.4% (4.3,7.3), 7.0% (5.2,8.5), 6.7% (6.3,9.7) and 6.7% (6.1,7.7) compared to children without ID (*n =12*) where FAFe was 2.7% (1.0,4.1), 3.3% (2.6,5.6), 3.1% (3.7,5.2), 5.2% (5.4,8.0), 5.4% (4.3,8.8) and 5.6% (6.0,9.2) in the same meals.

FIGURE 2. Boxplots for the fractional iron absorption [%] from six different rice meals in iron deficient and/or anemic Ghanaian children. Horizontal bars show the median fractional iron absorption for each meal; each box represents the first to third quartile, the whiskers represent the lowest and highest data points regardless of outliers. Asterisks indicate outliers 1.5 interquartile ranges above the 3rd quartile. Different letters indicate significant differences, Bonferroni corrected linear mixed model (P<.05); *n = 26*. [54]FeZnO: rice extruded with [54]FePP, ZnO and a vitamin premix; [57]FeZnSO$_4$ rice extruded with [57]FePP, ZnSO$_4$ and a vitamin premix; [54]FeZnOCT: rice extruded with [54]FePP, ZnO, a vitamin premix, citric acid (CA) and trisodium citrate (TSC); [57]FeZnSO$_4$CT: rice extruded with [54]FePP, ZnO, a vitamin premix, CA and TSC; [58]FeZnOCE: rice extruded with [54]FePP, ZnO, a vitamin premix, CA, TSC and EDTA; Reference: rice extruded with a vitamin premix, [58]FeSO$_4$ added prior consumption.

In vitro solubility

In vitro iron solubility from [54]FeZnO (3.6%) significantly differed from [54]FeZnOCT (19.8%), [57]FeZnSO$_4$CT (27%), [58]FeZnOCE (24%) (for all, *P<0.01*), but not from [57]FeZnSO$_4$ (4.7%), which differed from all other conditions (for all, *P<0.01*). Solubility did not significantly differ between [54]FeZnOCT, [54]FeZnSO$_4$C and [58]FeZnOCE.

Discussion

Our main finding is, in Ghanaian children eating their local diet, iron from fortified rice containing ^{54}FeZnO, ^{57}FeZnSO$_4$, ^{54}FeZnOCT was significantly less bioavailable compared to FeSO$_4$, while iron from ^{57}FeZnSO$_4$CT and ^{58}FeZnOCE was as bioavailable as from FeSO$_4$ (Figure 2). These differences in bioavailability were not only statistically significant, but nutritionally relevant: iron from the formulation currently widely-used to fortify rice (FeZnO) was absorbed at only 30-40% of that from the ^{57}FeZnSO$_4$CT and ^{58}FeZnOCE (Table 2). Based on our FAFe data, iron-deficient rural African school-age children consuming 100 g rice per day fortified with FeZnSO$_4$CT and FeZnOCE would absorb 0.25 and 0.28 mg of iron per day, providing ~35-40% of the median iron requirements for children aged 7 – 10 years (34). In the future, use of these formulations may allow revision of rice-fortification guidelines towards lower, yet equally effective, iron fortification levels which are closer to those levels recommended for wheat flour fortification.

To our knowledge, this is the first study to directly quantify and compare iron bioavailability from iron fortificants by labelling multiple meals representing the local diet that are consumed over several weeks by the target population. We provided 10 meals per condition, and a total of 60 labelled fortified meals per subject. This novel approach has several advantages over traditional stable iron isotope studies (15, 16). First, it measures the cumulative iron absorption from different meals characteristic of the local diet: we included traditional sauces with the rice meals, to mimic the local balance of iron absorption inhibitors (such as PA and polyphenols) and absorption enhancers (such as vitamin C). The multiple meal approach allows testing a rice kernel premix level of 1 : 100, which is commonly used in rice fortification programs (11). Moreover, using this design over several weeks in African children with poor iron status and common infections integrates the 'human factor': iron bioavailability reflects not only dietary factors but also the countervailing effects of anemia driving higher iron absorption while infection/inflammation reduce absorption and utilization through elevated plasma hepcidin. Together, this provides a representative estimate of iron bioavailability from the fortified food as an integral part of the local diet in the targeted population. We feel this novel study design could also be used to measure iron bioavailability from other iron

fortificants and in other food vehicles. It could supplant efficacy studies as an approach to gauge potential impact of iron fortification, because it requires fewer subjects, lower costs and avoids the dependence on iron biomarkers that are often confounded by inflammation; it directly and quantitatively measures the amount of fortification iron that is absorbed and used to build new red blood cells of the recipients.

Our findings clearly show that CA+TSC addition to FePP-fortified extruded rice improves iron absorption regardless of the zinc fortificant used in the rice kernels. We recently reported the enhancing effect of CA+TSC on iron absorption from FePP in extruded rice in healthy Swiss women and attributed this effect to an *in situ* generation of soluble FePP during extrusion (10). We also reported an inhibitory effect of ZnO on iron absorption from FePP-fortified rice in Swiss women with poor iron status, whereas $ZnSO_4$ showed no such effect (11). However, in both studies, the PA content of the meals was low (12,13). Findings from this study show that addition of CA+TSC to the extruded rice does not fully overcome the inhibitory effect of ZnO relative to $ZnSO_4$: about 30% less iron was absorbed from the FeZnOCT than from $FeZnSO_4CT$ (Table 2). A recent review on the effect of zinc on iron bioavailability from foods containing both minerals concluded there is no significant effect at zinc / iron molar ratios below 2 : 1, whereas higher molar ratios decrease iron absorption (35). In the current study, the molar ratio of zinc / iron per fortified kernel was 1.3 : 1. Potential drawbacks of using $ZnSO_4$ instead of ZnO in extruded rice containing vitamin A are increased vitamin A degradation during storage (36); however, rice may not be the preferred vehicle for vitamin A fortification (37). Compared to ZnO, $ZnSO_4$ is more expensive. However, the estimated cost for a rice-premix is around USD 1 – 2/kg premix and a theoretical cost-comparison study concluded that the final cost of rice-premixes would be comparable regardless of the number and type of micronutrients added (38).

EDTA is an effective iron chelator that binds to iron at gastric pH protecting it from binding to inhibitory dietary ligands, but releases iron for absorption at near-neutral pH in the duodenum (39). Studies of the effects of EDTA on dietary iron absorption have mainly used NaFeEDTA or Na_2EDTA and consistently show increased iron absorption in meals rich in iron absorption inhibitors, such as PA (40, 41). However, in lipid-based nutrient supplements iron absorption was higher in the presence of AA (an absorption enhancer) and $FeSO_4$ compared to those containing NaFeEDTA and $FeSO_4$ (42). Also, EDTA may not

be able to overcome the inhibitory effects of polyphenols on iron absorption (39, 43). EDTA / iron molar ratios of 1 : 2 to 1 : 1 have been suggested for iron fortification (39), in the current study, the ratio used was ~0.5 : 1. The amount of EDTA administered to our study children in the rice meals was well below the acceptable daily intake (ADI), and would remain below the ADI even if the children had met all their caloric requirements with fortified rice (44) or if younger children (1 – 6 years) had been included. Nevertheless, CA may have advantages over EDTA as a chelator in extruded rice because it has no ADI and is generally recognized as safe (GRAS) (45).

Supporting our *in vivo* findings, our *in vitro* solubility data clearly show the enhancing effects of CA+TSC and CA+EDTA addition on iron solubility. However, in contrast to our *in vivo* findings, our *in vitro* experiments showed no difference in iron solubility from $^{57}FeZnSO_4$ or $^{54}FeZnO$, which confirms previous data (11). *In vitro* iron solubility may oversimplify highly complex physicochemical and physiological events during human digestion and therefore provide qualitative rather than quantitative information on human iron bioavailability (46, 47).

Our study had several strengths: 1) the highly precise and accurate measures of iron bioavailability provided by the stable isotope technique; 2) as discussed above, the fortified rice was integrated into multiple meals including sauces based on local Ghanaian recipes fed over several weeks; 3) we used the 1 : 100 rice premix blending ratio which is commonly used for large-scale rice fortification programs; and 4) we studied young children with poor iron status and our study design using multiple stable isotope labels allowed for within-subject comparisons of all five rice formulations compared to a reference meal. Our study also had limitations: 1) we did not assess zinc absorption from the fortified rice and the different formulations may affect zinc absorption; 2) we judged visual differences in sensory properties of the different fortified rice grains as minimal, both before and after cooking, but we did not perform thorough sensory testing or storage stability studies; and 3) the short study duration limited our ability to detect potential improvement in biomarkers of iron status.

In conclusion, our findings show that iron bioavailability in Ghanaian children from FePP-fortified rice can be sharply increased by optimizing fortification formulations. We feel the best formulation, considering the results of this study and others (10, 11), is the use of $ZnSO_4$ as co-fortificant in combination with CA+TSC for FePP-fortified extruded rice. We

recommend that future studies determine whether 1) the enhancing effect of EDTA is also present in composite meals of lower PA-contents; 2) fortification with $ZnSO_4$ instead of ZnO combined with EDTA would lead to an increased iron absorption; and 3) zinc absorption would be influenced differently by EDTA depending on the zinc source (ZnO or $ZnSO_4$).

Acknowledgments

Conflict of Interest

The authors of the study have no conflicts of interest to disclose.

Authorship Contributions

LH, DM, MZ, ARA and CC designed the studies. LH, DM, ARA, HZ and CS conducted the experiments. LH and CZ analyzed data. LH, DM, MZ and ARA wrote the paper. LH, DM and MZ had primary responsibility for the final content. All authors read and approved the final version of the paper.

Table 1. Anthropometric, iron and inflammatory variables of the Ghanaian children (*n = 26*), assessed prior to first meal administration[1]

Sex (female), %	42
Age, y	7 (7,8)
Weight, kg	22.1 ± 3.6
Height, cm	114.7 ± 9.3
Weight for age, Z-score	-1.3 ± 0.81
Height for age, Z-score	-1.2 ± 0.96
BMI for age, Z-score	-0.7 ± 0.65
ZnPP/H, µmol/mol heme	51.5 (44.6,59.5)
Hb, g/L	112 ± 8.7
Plasma ferritin, ng/ml	43.6 (32.5,58.4)
Hepcidin, mM	12 (7.4,19.1)
Iron deficient[2], non-anemic, *n*; %	*4*; 15
Anemia[3], *n*; %	*15*; 58
Non-iron deficiency anemia	*5*; 19
Iron deficiency anemia[4]	*10*; 39
CRP > 5 mg/L, *n*; %	*3*; 12
AGP > 1 g/L, *n*; %	*6*; 23
Positive malaria by microscopy, *n*; %	*11*; 42

[1] AGP: α- amino glycoprotein; CRP: C-reactive protein; Hb: Hemoglobin; ZnPP/H: Zinc protoporphyrin : heme molar ratio. Values are means ± SD or geometric means (confidence interval). [2] Iron deficiency was defined as sTfR >8.3 mg/L and/or plasma ferritin <30 µg/L. [3] Anemia was defined as hemoglobin <115 g/L. [4] Iron deficiency was anemia defined as sTfR >8.3 mg/L and/or plasma ferritin <30 µg/L in combination with hemoglobin <115 g/L.

Table 2. Composition of extruded rice, administered study meals, human iron absorption and *in vitro* iron solubility.[1]

	Iron [mg/g][2]	Zinc [mg/g][2]	Fe : Zn : CA : TSC : EDTA [mol/mol][3]	Fe : Zn : PA : AA [mol/mol][4]	Fractional iron absorption [%][5]	RBV [%][5,6]	Relative iron solubility [%][7]
[54]FeZnO	3.5 ± 0.50	4.2 ± 0.08	1 : 3.8 : 0 : 0 : 0	1 : 1.0 : 0.8 : 0.0	2.3 (1.9, 2.8) [a]	45 [a]	3.6 ± 0.5 [a]
[57]FeZnSO₄	3.2 ± 0.50	4.1 ± 0.19	1 : 4.8 : 0 : 0 : 0	1 : 1.1 : 1.0 : 0.0	3.5 (2.7, 4.5) [b]	67 [b]	4.7 ± 0.5 [a]
[54]FeZnOCT	3.2 ± 0.50	4.2 ± 0.1	1 : 4.2 : 0.3 : 6.0 : 0	1 : 1.1 : 0.9 : 0.0	4.5 (3.6, 5.5) [b]	85 [b]	19.8 ± 2.7 [b]
[57]FeZnSO₄CT	3.4 ± 0.50	4.1 ± 0.19	1 : 4.5 : 0.3 : 5.5 : 0	1 : 1.1 : 0.9 : 0.0	6.3 (5.3, 7.4) [c]	113 [c]	27.1 ± 4.0 [b]
[58]FeZnOCE	3.3 ± 0.50	4.1 ± 0.19	1 : 4.1 : 0.3 : 0 : 0.6	1 : 1.1 : 0.9 : 0.0	6.4 (5.1, 8.1) [c]	122 [c]	24.1 ± 3.1 [b]
Reference	3.6 ± 0.10	1.3 ± 0.01	N/A	1 : 0.3 : 0.8 : 0.0	6.4 (5.2, 7.8) [c]	N/A	N/A

[54]FeZnO: Extruded Rice containing [54]FePP, ZnO and a micronutrient mix (MN); [57]FeZnSO₄: Extruded Rice containing [57]FePP, ZnSO₄ and MN; [54]FeZnOCT: Extruded Rice containing [54]FePP, ZnO, MN, citric acid (CA) and trisodium citrate (TSC), [57]FeZnSO₄CT: Extruded Rice containing [57]FePP, ZnSO₄, MN, CA and TSC; [58]FeZnOCE: Extruded Rice containing [58]FePP, ZnO, MN, CA and EDTA. Reference: Extruded Rice containing MN, [58]FeSO₄ solution added prior consumption. RBV: Relative bioavailability.

[1] Each rice meal serving contained 49.5 g unfortified rice, 0.5 g fortified rice with 50 g of either bean or tomato sauce. Values are means ± SDs or geometric means (95% CI). Labeled means in a column without a common letter differ, $P < 0.05$; Bonferroni corrected linear mixed model. Designated micronutrient composition of fortified kernels can be found in the appendix. MR: Molar Ratio; RBV: Relative bioavailability. [2] Calculated iron and zinc contents in composite meals were based on the means from the analysis of single components (49.5 g regular Basmati rice, 0.5 g extruded rice, average mineral contents from the two different sauces [50 g sauce]; $n = 3$), SDs for iron and zinc contents of composite meals were adapted by calculating the square root of the squared and summed SDs from each single component. [3] MR in fortified rice. [4] MR per serving of rice – average mineral, PA and AA contents from the two different sauces were calculated. [5] $n = 26$. [6] RBV calculation based on fractional iron absorption [%] respective to fractional iron absorption from the reference meal for each subject. [7] Iron solubility was assessed in fortified rice kernels. For details on the calculation, please refer to the text, $n = 3$.

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GENERAL DISCUSSION

The overall aims of this thesis were to:

∂ Investigate how iron absorption enhancers and inhibtors influence iron absorption from FePP-fortified extruded rice meals;

∂ assess whether observed inhibiting/enhancing effects from single meal administrations can also be found upon multiple meal administrations;

∂ investigate whether the technology used for rice fortification impacts human iron and zinc absorption; and

∂ to suggest a nutrient formulation that provides optimal iron absorption from FePP-fortified extruded rice in an iron depleted, vulnerable population in rural Africa.

Overview on the main findings

Manuscript 1

Addition of CA+TSC prior to extrusion of FePP-fortified rice drastically enhanced iron absorption causing a relative iron bioavailability of 83 % (compared to the highly bioavailable FeSO$_4$-reference) in healthy Swiss women and iron absorption was almost doubled compared to either the addition of CA+TSC after cooking or no CA+TSC addition. We attributed this effect to an *in situ* generation of soluble FePP in the extruded rice. The combination of extruding FePP with CA+TSC thus reduced the amount of unabsorbed iron in the gut, an important consideration as an increase in unabsorbed dietary iron may shift the gut microbial composition from a beneficial towards a potentially pathogenic profile (Jaeggi, Kortman et al. 2015).

Manuscript 2

In this study, we could show, that not zinc per se but rather the zinc source, affects iron absorption from FePP-fortified rice in iron-depleted, but otherwise healthy, Swiss women. Thus, iron absorption from FePP-fortified extruded rice was lower, when rice was co-

fortified with ZnO rather than $ZnSO_4$ at molar ratios Fe / Zn of 1 : 1.3. We speculated ZnO to counteract the acid-driven dissolution of FePP due to its high acid-binding capacity.

Manuscript 3

In the first part of this study, we demonstrated that the extrusion conditions affect iron absorption from FePP-fortified rice. Cold extruded rice showed higher iron absorption than hot extruded rice in healthy Swiss women of child-bearing age and we attributed this effect to differences in the starch architecture of the extruded kernels. In contrast, as shown in the second part of the study, fortification via coating showed no significant differences in iron absorption in healthy Swiss women compared to hot extrusion. However, mineral retention upon different cooking procedures was generally higher in hot extruded compared to cold extruded or coated rice. Regarding fortification programs, both aspects should be considered: bioavailability of the administered nutrients, but also the nutrients' resistance towards different preparation procedures.

Manuscript 4

In this study, we aimed to translate findings from our previous human absorption studies and *in vitro* findings to more closely resemble realistic conditions for a fortifiation program while still being able to accurately quantify iron absorption. We conducted this study in iron-depleted school-age children in Northern Ghana, a potential target population for a fortification program and, for the first time, we used the common 1 : 100 (fortified / unfortified rice) premix approach.

Within the study, six different meal combinations with fortified rice were administered, where each combination was administered twice daily over five consecutive days. The combined approach of administering fortified foods intrinsically labelled with stable isotopes over several days allows us to draw conclusions on absorption patterns outside a controlled study environment.

The enhancing effect of CA+TSC could not only be shown in combination with ZnO as co-fortificant, but also in combination with $ZnSO_4$. The combination of $ZnSO_4$ with CA+TSC even exhibited higher iron absorption than ZnO with CA+TSC. Furthermore, we showed enhanced iron absorption from FePP-fortified rice in combination with ZnO, EDTA+CA. Results from this study not only provide evidence for future fortification

recommendations, but the novel study design may also be used as rapid and yet accurate future alternative for efficacy studies.

Context of the research

Despite a global reduction in the prevalence of micronutrient deficiencies, micronutrient malnutrition remains among the leading forms of nutritional deficiencies worldwide with ID (together with vitamin A and zinc deficiencies) being among the leading three causes. Dietary diversification, mineral supplementation and food fortification have been suggested as strategies to avoid micronutrient malnutrition (Bhullar and Gruissem 2013). Rice as a staple food for more than half the world's population is a major source of calories particularly in countries of a lower socio-economic status, however, its micronutrient content is low in its typically consumed form. Fortified rice has the potential to substantially and sustainably reduce certain vitamin and mineral deficiencies and can fill a gap in the current fortification landscape (de Pee 2014, Muthayya, Sugimoto et al. 2014).

Unlike other fortification vehicles such as salt, flours and condiments, which are all ingredients in a final product, fortified rice is a final product for the customer (Milani, Carnahan et al. 2017), thus, producing rice kernels with an optimal fortification formulation and characteristics very similar to natural rice, is required (Alavi, Bugusu et al. 2008). Due to their relatively inert sensory characterisitcs, iron phosphates, such as FePP, are most widely used for rice fortification, however, iron bioavailability from FePP is comparatively low. The commonly used '*premix approach*' in rice fortification causes high local nutrient concentrations per fortified kernel, potentially rendering nutrient interactions within a kernel more likely. Additionally, rice fortification requires special equipment for its production and blending with unfortified rice. Furthermore, in certain populations, such as the Philippines and China, 40% respectively 60% consume rice processed by small local mills, impeding the accessibility to fortified rice for those populations [23].

Given that micronutrient deficiencies rarely occur in isolation, foods are often co-fortified with multiple nutrients. Data on iron and zinc interactions in foods are somewhat contradictory and depend on the food matrix in which iron and zinc are administered, the respective compounds used as well as the amounts and ratios in which both minerals are present. Several food vehicles have been intensively studied regarding this issue, but fortified rice had not been subject to such investigations before.

The data presented in this thesis were generated in clinical trials conducted in Switzerland and Northern Ghana in women of child-bearing age and iron-depleted children, respectively. Employing stable iron isotopes in the studies allowed for an accurate quantification of iron absorbed from the fortified rice meals. Women of child-bearing age and children are particularly prone to ID due to menstrual iron losses and enhanced iron requirements during rapid growth and development, respectively. Complementary foods in developing countries often provide insufficient bioavailable iron and are often bulky cereal-based porridges with low energy density (Gibson et al. 1998). Especially in the Asia Pacific region, where rice and rice products, which typically provide insufficient micronutrient quantities, are the most common first solid foods for infants, fortified rice could be beneficial to improve micronutrient supply in such populations (Inoue and Binns 2014).

Critical evaluation of the findings

We could successfully show that FePP-fortified rice can substantially contribute to iron requirements in women and children by overcoming its low bioavailability through different approaches. Our efforts to enhance iron bioavailability from FePP-fortified rice included the use of chelating agents such as CA in combination with TSC or EDTA and both formulations almost doubled iron absorption in our studied populations compared to no addition.

Our findings further suggest the future use of $ZnSO_4$ instead of ZnO as a zinc co-fortificant to ensure maximal iron abosption.

While ZnO is advantageous in terms of price and due to its color-masking ability, it is arguable whether those two aspects would justify its use given its impact on iron bioavailability from FePP-fortified rice. In combination with CA+EDTA, nevertheless, iron absorption from FePP-fortified rice co-fortified with ZnO was as high as from rice fortified with FePP, $ZnSO_4$, and CA+TSC. Whether CA+EDTA combined with $ZnSO_4$ would outperform the combination with ZnO in terms of iron absorption remains to be investigated. However, it is questionnable, whether an iron absorption can be triggered to a higher extent than that of $FeSO_4$. A reduced vitamin A stability upon storage has been suggested for FePP-fortified rice co-fortified with $ZnSO_4$, however, it has been questioned, whether rice is a suitable vehicle for vitamin A delivery (Kuong, Laillou et al. 2016).

Cold extruded rice seems advantageous over hot extruded rice in terms of iron delivery, nevertheless, it showed a poorer performance regarding mineral retention and cold extruded kernels seemed to disintegrate more easily upon cooking. We considered the visual properties of both cold and hot extruded rice as being acceptable, however, regular rice consumers may be deterred by the mushy texture, which is more pronounced in cold rather than hot extruded rice. Due to the premix approach, however, only a small fraction of the kernels would be affected, which would likely not affect its consumers. When comparing hot extruded and coated rice, no differences in iron nor zinc absorption were found, but again, hot extruded rice outperformed coated rice regarding micronutrient retention upon different preparation and cooking procedures.

Limitations

Single meal studies
A major limitation of the four single meal studies conducted in Swiss women was that the rice was served with a standardized vegetable sauce containing negligible PA amounts. In a target population for fortified rice, a typical rice meal would likely contain more PA, thus potentially hindering iron absorption to a greater extent. Furthermore, all single meal studies were conducted in a relatively healthy population, hence, not fully representative of a target population for a fortification program.

Due to technical limitations, we used a premix approach of 1 : 24 – 25 in the single meal studies, which is considerably different from the generally used 1 : 100 – 200 approach. This approach was used given the unavoidable product losses at the beginning and end of each extrusion run as well as the required minimal batch size, which was dictated by the minimal feeding volume and size of the extruder barrel, which forced us to produce labelled fortified batches of at least 160 g while our supply of the costly stables isotopes was limited. This caused a lower nutrient density per fortified kernel and, consequently, less discoloration and potential nutrient interactions in the kernels.

In all single meal studies, the rice meals were prepared on the day before each adminstration and refrigerated over night for ~14 – 17 hours. This may have influenced the rice starch architecture, which in turn may have reduced iron absorption due to the formation of resistant starch.

Multiple meal study
In the multiple meal study, we intended to account for those earlier limitations and administered rice meals that were fortified with the 1 : 100 premix approach along with condiments, that were based on local recipes and contained considerable PA amounts. Furthermore, the meals were generally cooked on each administration day.

Nevertheless, limitations in the multiple meal study occurred and include the use of EDTA in combination with CA, whereas investigating the performance of EDTA without CA would have clarified the extent to which EDTA contributes in improving iron absorption. Furthermore, we did not test the performance of EDTA and CA in combination with $ZnSO_4$ instead of ZnO, which may have increased iron absorption even further, or the performance of the rice containing EDTA in meals with lower PA amounts.

In the study, we did not observe an improvement in participants' iron status, which was not expected due to the relatively short intervention time. However, it can be assumed, that the extra amount of absorbed iron from the meals would eventually increase iron stores when administered over an extended period. If the suggested multiple meal design should replace future efficacy studies in rice and other food vehicles, more evidence has

to be generated about the validity and safety of this design and it has to be appreciated, that a change in certain parameters may not be measurable after a short intervention time.

Rice fortification technology studies

The two studies investigating the effect of the different fortification technologies also bear limitations. Hot and cold extruded rice had the same composition of nutrients and additives, however, hot extruded rice was produced several weeks before the cold extruded rice, which may have contributed to the differences that we found in the starch architecture. Furthermore, hot and cold extruded rice from the first part of the study had a different composition compared to hot extruded and coated rice in the second part. We have not directly compared iron absorption from cold extruded versus coated rice but based on our results, it may be possible, that cold extruded rice would outperform coated rice in terms of iron absorption.

Overall limitations

A major limitation of all presented studies is, that we did not scrutinize the sensory properties of the fortified rice nor the consumer acceptability of the fortified rice in its uncooked form. Addition of CA, TSC and/or EDTA caused slight discolorations upon extrusion, however, we considered the cooked fortified rice as being acceptable for consumption. The cooked meals the participants received in our studies were all served with sauce, which was either mixed with or added on top of the rice. Thus, it would have been difficult for the participants to distinguish the regular from the fortified rice in its administered form.

Another limitation, that applies to all presented studies, is that the reference dose of $FeSO_4$ was added as a solution to the rice and not incorporated in the kernel, unlike in those conditions, were FePP-fortified rice was administered, whereas the iron fortificant was integrated in the kernel. Thus, the rice matrix may have impaired iron absorption from the iron that was incorporated in the rice kernel, whereas this was not the case in the $FeSO_4$ conditions. Thus, relative bioavailability from the FePP-conditions presented in the studies, may have actually been higher than reported. A vitamin premix was incorporated

in all types of fortified rice, however, we did not assess vitamin bioavailability in any of the presented studies. This information would, however, be important, to assess what types of vitamins are viable for fortification.

Open questions and future directions

Questions remain regarding the ideal ratio of Fe / Zn / CA / TSC / EDTA that warrant maximal iron and zinc uptake. Furthermore, it remains unanswered, whether EDTA is also beneficial in diets of low PA contents and whether its enhancement is only given in combination with CA as well as the potential effect of $ZnSO_4$ co-fortified rice containing EDTA as an iron absorption enhancer. The optimal extrusion conditions for achieving maximal iron delivery have yet to be defined.

Consumer acceptance of fortified rice has to be assured prior to the implementation of a fortification program, as acceptability determines the use of the fortified food and the subsequent success of a fortification program. A study in Brazil suggested awareness, trust and relevance as key barriers to consumer trial and adoption of fortified rice and identified middle- to low-income mothers who value family nutrition and health as typical purchasers (Milani, Carnahan et al. 2017). Therefore, appropriate marketing strategies have to be developed and tailored to those regions,where fortified rice is supplied in order to ensure consumer's acceptance.

The ideal combination of ingredients and extrusion conditions should achieve a product that 1) Delivers high amounts of iron even when administered with condiments of a wide variety of absorption inhibitors; 2) Does not destroy other micronutrients or vitamins in the product; while still being 3) Resistant towards pre-cooking preparation methods and 4) Sensory acceptable. Furthermore, the 5) Storage stability and 6) Consumer acceptance of the fortified rice needs to be assured – both in its raw and cooked form. These parameters have to be accounted for prior to the implementation of a fortification

program. Therefore, future research should investigate approaches, where all those parameters are taken into account.

As outlined in earlier chapters, biofortification may be a possibility for rice fortification in the future, however, most recent effort achieved iron concentrations in the rice endosperm of only 10.46 µg iron/g (Boonyaves, Wu et al. 2017), which is well below the suggested target content of 15 µg/g (Bouis, Hotz et al. 2011). It can be assumed, that considerably more time is required until sufficiently high iron concentrations can be achieved.

New possibilities to enhance absorption of fortification iron may lie within nanotechnology, however, decreased costs and an increased scalability of the production procedures need to be achieved for its successful application (Perfecto, Elgy et al. 2017). Furthermore, it is questionnable, whether rice fortified with nanotechnology could outperform our proposed fortification formulation in terms of iron absorption, which is already as high as absorption from $FeSO_4$.

From a technical point of view, it has been proposed that rice could even be fortified with vitamins D and K or with the amino acid lysine (Steiger et al. 2014) and, despite the limited experience of fortifying rice with these nutrients, it may be considered where deficiencies are likely (De Pee 2014).

Public health impact

The results generated within this thesis provide a solid evidence base for future rice fortification recommendations. The suggested formulations may also be used for other fortification vehicles upon thorough evaluation. Furthermore, the presented design for a multiple meal study with stable iron isotopes may be an alternative for efficacy studies in the future.

Based on the findings from our first study (Manuscript 1), WFP amended its recommendation guidelines for rice fortification. The guidelines now suggest the use of a

chelating agent and a minimum iron fortification level of 4000 mg/kg fortified rice (Holden 2015), which is similar to recommended fortification levels for wheat flour, whereas earlier 7 000 – 12000 mg/kg were suggested (De Pee 2014). Currently, WFP recommends ZnO as zinc co-fortificant for fortified rice (Holden 2015), however, considering our findings presented in Manuscript 2 and Manuscript 4, $ZnSO_4$ may be the future compound of choice.

Our results further show that both extrusion and coating are viable fortification methods, however, it is indicated to implement hot extrusion, where possible as hot extruded rice outperformed coating and cold extrusion in terms of nutrient retention upon cooking. The evident impact of the extrusion conditions on iron absorption demands that producers of fortified rice are encouraged to share detailed specifications about their production methods.

Based on our findings, 100 g fortified rice meal (dry weight) containing 4 mg iron as FePP and 6 mg zinc as $ZnSO_4$ or ZnO in combination with CA and TSC or CA and EDTA, respectively, could meet ~1/3 of the daily iron requirements in school-age children in rural Africas, whereas 200 g of that meal could cover 1/3 of the requirements of adolescent females (Joint 2002). In severely ID populations, this coverage may even be higher.

Conclusions

In conclusion, I sincerely hope that the work presented in this thesis will contribute to further investigate and enhance the potential of rice fortification. I believe that fortified rice can improve the nutrition status of many populations and I hope that it will not remain a niche product but rather become a widely used and accepted fortfied staple, such as wheat flour, in deficient rice-consuming populations. We could show that fortified rice can substantially contribute to iron requirements and with the emergence of further evidence, fortified rice will hopefully be successful in combating ID and other nutrient deficiencies. The main recommendations based on the presented work are the use of FePP-fortified rice, co-fortified with $ZnSO_4$ and absorption enhancers such as CA+TSC. The current

amounts recommended by WFP of 4 g iron/kg fortified rice and 6 g zinc/kg fortified rice (Holden 2015) seem to be efficient, but further work on the optimal ratios of added nutrients and additives should be conducted. Furthermore, in settings where more than one staple food is fortified, a combined intake should be used to define fortification levels (De Pee 2014). The use of EDTA and CA caused higher RBV compared to CA+TSC, however, given the considerably higher price of EDTA, but especially due to potential reservations regarding its safety, the former formulation should be used until further evidence emerges. Where possible, rice should be fortified via hot extrusion – coating or cold extrusion should be used, where hot extrusion is not possible. However, further investigations should be targeted towards finding optimal extrusion conditions in terms of iron delivery, resistance upon different cooking procedures and sensory acceptibility of the extruded product.

Regarding the co-fortification with vitamins and other nutrients, more information is needed in terms of their resistance upon extrusion and their bioavailablity in humans.

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CURRICULUM VITAE

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Education and professional experience

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Publications

Hackl, L., Speich, C., Zeder, C., Sanchez-Ferrer, A.S., Adelmann, A., De Pee, S., Zimmermann, M. B., & Moretti, D. (2017) *Cold Extrusion but Not Coating Affects Iron Bioavailability from Fortified Rice in Young Women and Is Associated with Modifications in Starch Microstructure and Mineral Retention during Cooking.*
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Hackl, L., Zeder, C., Parker, M., Johns, P., Zimmermann, M. B., & Moretti, D. (2017) *Iron bioavailability from ferric pyrophosphate in extruded rice cofortified with zinc oxide or zinc sulfate in a human stable isotope study*.
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The American Journal of Clinical Nutrition, 103(5), 1252-1259.

Parma, V., Coutanche, M., Seubert, J., Fondberg, R., Hackl, L., Åhs, F., & Lundstrom, J. N. (2015) *Anxiety-dependent modulation of olfactory fear conditioning: a multidimensional approach.*
Chemical Senses, 40 (7), 536-536.

Presentations

07/2017   Recent developments in mineral fortification of rice
Oral presentation at Global Rice Fortification Strategy Meeting organized by WFP, Rome, Italy

04/2017   *A novel, high precision multiple-meal stable isotope method to compare iron absorption from extruded FePP-fortified rice containing different zinc compounds, citric acid/trisodium citrate and EDTA in Ghanaian children*
Oral presentation at *Experimental Biology*, Chicago, US

10/2016   *Evaluation of iron and zinc bioavailability from fortified rice using coating, hot and cold extrusion: human stable isotope studies* – Oral presentation

Iron bioavailability from ferric pyrophosphate in extruded rice co-fortified with zinc oxide or zinc sulfate: a human stable isotope study – Poster presentation

Micronutrient Forum, Cancun, MX

10/2015   *Enhancing human iron bioavailability through in situ generation of soluble ferric pyrophosphate citrate complexes*
Poster presentation at *FENS*, Berlin, DE

08/2015   *Iron bioavailability from extruded fortified rice*
Oral presentation at the *Kansas State University Rice Fortification Symposium*, Kansas, US

Awards

04/2017   *American Association for Nutrition's Emerging Leader Poster Award*
Experimental Biology, Chicago, US
10/2017   *NLP Young Scientist Award* – 3rd place
International Congress of Nutrition, Buenos Aires, AR