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ZINC BIOFORTIFIED WHEAT: EVALUATION OF ZINC ABSORPTION AND CONTRIBUTION TO ZINC STATUS IN INDIAN CHILDREN

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presented by

CORALIE SIGNORELL
MSc in Bioengineering, EPFL

born on 10.01.1986
citizen of Sur (GR), Switzerland

accepted on the recommendation of
Prof. Dr. med. Michael B. Zimmermann, examiner
Dr. Diego Moretti, co-examiner
Prof. Dr. Nicola Lowe, co-examiner

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Diarrhea claims close to 1.5 million infants each year in this country - one every three minutes. That is thirty thousand times the number of lives lost in the plague. The best it can get by way of space is when UNICEF's annual "State of the World's Children Report" is released. Then it makes an occasional bow in the center page. Or, in one of those anguished editorials (hastily written because the one on the stock exchange didn't turn up) asking: "Where Have We Gone Wrong?" After which, it can be packed away to be used in identical format the following year.

P. Sainath (1996), *Everybody loves a good drought – stories from India's poorest districts*, India: Penguins Books

Palagummi Sainath is a freelance journalist. In 1993, after winning a Time of India fellowship, he started to work full-time on rural poverty. "Everybody loves a good drought" is a collection of his published articles describing the life from the poorest districts of India, from 1993 to 1995. During this time, he won 12 awards and fellowships, including the Lorenzo Natali Prize, from the European Commission. In 2007, he won the Ramon Magsaysay Award for Journalism Literature and Creative Communications Arts for his "passionate commitment as a journalist to restore the rural poor to India's consciousness, moving the nation to action". http://www.rmaf.org.ph/newrmaf/main/awardees/awardee/profile/42

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TABLE OF CONTENTS

ABBREVIATIONS

A

Acrodermatitis Enteropathica: ADE · 12
Alpha-1-acid glycoprotein: AGP · 40
Atomic Absorption Spectrophotometry: AAS · 3

C

Consultative Group for International Agricultural Research: CGIAR · 62
C-Reactive Protein: CRP · 39

D

Dual Isotopic Tracer Ratio method: DITR · 24

E

Estimated Average Requirement: EAR · 25
Ethylenediaminetetraacetic acid: EDTA · 40
European Food Safety Authority: EFSA · 8
Extraction Rate: ER · 84

F

Ferroportin: Fpn · 28
Food and Agriculture Organization: FAO · 79
Fractional Zinc Asbsorption: FAZ · 17

G

Gastrointestinal: GI · 5

H

Height For Age Z Score: HAZ · 42

I

Inductively Coupled Plasma Mass Spectrometry: ICP-MS · 22
Institute of Medecine: IOM · 8
International Crops Research Institute for the Semi-Arid Tropics: ICRISAT · 94
International Zinc Nutrition Consultative Group: IZiNCG · 8
intravenous: IV · 22

L

Laser-Induced Breakdown Spectroscopy: LIBS · 49

M

Metal transcription Factor-1: MTF-1 · 29
Metallothionein: MT · 6
metal-response element-binding transcription factor-1: MTF-1 · 26
Metal-Responsive Transcription Factor-1: MTF-1 · 51

O

Orange-Fleshed Sweet Potato: OFSP · 63

P

Phytic Acid: PA · 7
Plasma Zinc Concentration: PZC · 38
Polymerase Chain Reaction: PCR · 51

Q

quantitative-PCR: q-PCR · 51

R

ribonucleic acid: RNA · 2

S

School Lunch Program: SLP · 90

T

Thermal Ionization Mass Spectrometry: TIMS · 22
Tolerable Upper Intake Level: UL · 8
total absorbed zinc: TAZ · 18

U

United Nations Children's Emergency Fund: UNICEF · 89

W

World Health Organization: WHO · 8

Z

Zinc transporter: ZnT · 25
Zinc transporter 1: ZnT1 · 51
Zrt-, Irt-like Protein: ZIP · 25

SUMMARY

Background

Low zinc status impairs immune system, proper growth and development. The tightly controlled whole body homeostasis makes the assessment of zinc insufficiency challenging, and currently, the measurement of plasma zinc concentration (PZC) is the only recommended measure for assessment of zinc status in a population. Fortification, supplementation and dietary diversifications are strategies that can be implemented to reduce the risk of zinc deficiency, but their efficacy is not well demonstrated. Biofortification, consisting of developing micronutrient-dense crops, is a new tool to tackle malnutrition. In wheat, which is the most consumed staple worldwide, it is a promising technique. Unfortunately, the breeding approach is a long-term process to improve zinc concentrations in grain in general. Applications of zinc fertilizers offer a short-term solution to the problem and has been proven successful at increasing zinc concentration in wheat grains.

Research goals

The objectives of this thesis were to determine the potential impacts of biofortified wheat on the zinc status of children at risk of zinc deficiency. This was done in a stepwise approach, including a) measurements of zinc absorption from biofortified wheat at different extraction rates, and comparison with fortified and control wheat, b) assessment of the predictors of zinc absorption in children at risk of inflammation and in adults c) an efficacy study investigating the impact of a biofortified zinc diet on biomarkers of zinc deficiency.

Experiments

Manuscript 1 Agronomic biofortification of wheat has a stronger influence on human zinc absorption than extraction rate, and can be a viable source of bioavailable zinc

We investigated with a series of 3 zinc absorption studies in adults the effect biofortification, fortification and extraction rate (EXRs) on zinc absorption. In study 1, zinc absorption by adults from a wheat porridge made from hydroponically biofortified wheat, intrinsically labeled with ^{70}Zn, was compared to that from a porridge made from an intrinsically labeled wheat of the same variety, either unfortified or fortified with $ZnSO_4$. In studies 2 and 3, zinc absorption by adults was compared at different EXRs from conventionally fortified wheat, biofortified wheat

and control wheat. Biofortified wheat was produced by foliar zinc application and meals consisted of chapattis, an unleavened flat Indian bread.

In study 1, there was no significant difference in the fractional (FAZ) or the total (TAZ) absorption of zinc from extrinsic versus intrinsic labels in either the fortified wheat, biofortified wheat or control wheat. FAZ and TAZ did not significantly differ from fortified and biofortified wheat, while the total absorbed zinc was 70-76% higher ($P < 0.01$) in the fortified and biofortified meal compared to the control meal. In studies 2 and 3, at both EXRs, FAZ and TAZ from the fortified and biofortified wheat did not differ, while TAZ was 20-48% higher from the fortified and biofortified meal compared to the control meal ($P < 0.001$).

Manuscript 2 In a pooled analysis of children and adults studies, plasma zinc and c-reactive protein are independent predictors of zinc absorption, but Hepcidin is not

A series of absorption studies in adults and children already investigated the effect of phytic acid (PA), EDTA and fortificants in zinc absorption from different staples. We pooled zinc absorption data from those performed in Switzerland and in Burkina Faso, to investigate the potential determinants of zinc absorption. We measured parameters of iron status, namely hepcidin, serum transferrin receptor, hemoglobin and c-reactive protein (CRP) as additional parameters. By a regression analysis and linear mixed model, we tested the dietary and non-dietary factors affecting zinc absorption in humans.

PZC was significantly associated with TAZ in adults ($P < 0.001$) and toddlers ($P = 0.03$). PA: Zn molar ratio and meal zinc were significant predictors in adults (both $P <0.001$ and $P <0.001$). Iron status markers were not associated with TAZ nor FAZ, and Hepcidin is not significantly associated with TAZ in adults. TAZ in toddlers was associated with height and CRP.

Manuscript 3 Biofortified wheat produced agronomically and zinc status of Indian children: a randomized controlled trial

We assessed the efficacy of zinc biofortified wheat to improve zinc status in a double-blind randomized control trial, in children from 6 to 12 years old (N = 273) from low-income settings in peri-urban settings of Bangalore. The primary outcome was PZC. Secondary outcomes consisted of hemoglobin, inflammation markers. Two potential new biomarkers were measured in a subsample of randomly selected children (N = 51). Children were randomized

to receive a daily portion of biofortified wheat, fortified wheat or control wheat. We assessed PZC at baseline, endline and at different sparse random serial samples, and analyzed the effects of time and treatment in PZC by a non-linear mixed effects model analysis. We collected a final blood sample 2 months after the end of the study, and after school holidays, to investigate the time course of PZC.

No significant time-by-treatment effect on PZC was found (P = 0.23 for fortification, 0.66 for biofortification). We detected no effect on new potential biomarkers of zinc status, namely ZnT1 transcripts and DNA integrity.

Conclusion

Zinc absorption from biofortified wheat was similar than from fortification zinc added to control wheat in the form of zinc sulphate. Both of them were higher than the one from the control wheat. This finding suggests that absorption of the biofortification zinc is similarly influenced by food components as absorption of the fortification zinc. Zinc absorption is not only influenced by dietary factors, such as meal zinc and PA concentrations, but as well by PZC. In children at risk of inflammation, CRP and height are predictors of zinc absorption. No effects were seen on PZC during a long-term efficacy trial on children who consumed fortified or biofortified wheat for 20 weeks, but this might be due to a low initial prevalence of zinc deficiency and a low bioavailability of zinc in the Indian diet. Biofortification, either via soil or foliar zinc application is a good intervention strategy to combat zinc deficiency as the conventional zinc fortification in food.

RESUMÉ

Contextualisation

Un faible statut en zinc perturbe le système immunitaire, la croissance et le développement. L'homéostasie du zinc dans le corps étant strictement contrôlée, l'évaluation des carences en zinc est complexe et, actuellement, la zincémie (PZC) est la seule mesure recommandée pour l'évaluation du statut en zinc à l'échelle d'une population. L'enrichissement des aliments, les compléments alimentaires, ou la diversification alimentaire sont des stratégies pouvant être implémentées pour réduire le risque de carences en zinc, mais leur efficacité n'a pas été clairement démontrée. La biofortification, qui consiste au développement de cultures riches en micronutriments, est une nouvelle approche pour lutter contre la malnutrition. Pour le blé, la denrée alimentaire de base la plus consommée au monde, c'est une démarche prometteuse. Malheureusement, la sélection végétale par croisements est un processus à long terme pour améliorer la concentration en zinc dans les graines en général. L'utilisation de fertilisants contenant du zinc offre une solution à court terme pour résoudre le problème et sa réussite d'augmenter la concentration en zinc dans le blé a déjà été confirmée.

Objectifs de recherche

Les objectifs de ce travail de doctorat étaient de déterminer les impacts potentiels du blé biofortifié sur le statut en zinc d'enfants à risques de carences en zinc. Cela a été fait par une démarche progressive, incluant a) les mesures de l'absorption en zinc du blé biofortifié à différents taux d'extraction, et la comparaison avec le blé enrichi b) l'analyse des prédicteurs de l'absorption du zinc chez les enfants à risque d'inflammation et chez les adultes, c) une étude évaluant l'impact d'un régime alimentaire contenant du zinc biofortifié sur les biomarqueurs de carences en zinc.

Expériences

Manuscrit 1: La biofortification agronomique du blé a une plus grande influence sur l'absorption du zinc par l'homme que le taux d'extraction et est une source viable de zinc biodisponible

Nous avons étudié par une série de 3 études d'absorption en zinc chez les adultes l'effet de la biofortification, de l'enrichissement et du taux d'extraction (EXR) sur l'absorption en zinc. Dans la 1ère étude, l'absorption en zinc par les adultes d'une bouillie de blé produit par culture hydroponique et intrinsèquement marqué par le ^{70}Zn a été comparée à celle d'un porridge intrinsèquement marqué de la même variété, non-enrichi ou enrichi avec du $ZnSO_4$. Dans les

études 2 et 3, l'absorption en zinc par les adultes a été comparées à différents EXR de blé enrichi de manière traditionnelle, de blé biofortifié ou de blé contrôle. Le blé biofortifié a été produit par application de zinc sur les feuilles, and les repas consistèrent en chappattis, une sorte de pain plat indien non-fermenté.

Dans l'étude 1, il n'y a pas de différence significative pour l'absorption fractionnelle (FAZ) ou totale (TAZ) en zinc des marqueurs extrinsèques vs intrinsèques, soit dans le blé enrichi, biofortifié ou contrôle. La FAZ ou la TAZ n'est pas significativement différente du blé biofortifié ou enrichi, alors que la TAZ était 70-76% plus élevée ($P < 0.01$) pour le repas enrichi et biofortifié, comparée au repas contrôle. Dans les études 2 et 3, à deux EXRs, la FAZ et la TAZ du blé enrichi ou biofortifié n'était pas différente, alors que la TAZ du repas enrichi et du repas biofortifié était de 20 à 48% plus élevée comparée à celle du repas contrôle.

Manuscrit 2 Lors d'une analyse combinée d'études sur les adultes et les enfants, la zincémie et la protéine c-réactive sont des prédicteurs indépendants de l'absorption en zinc, alors que l'hepcidine ne l'est pas

Une série d'étude d'absorption chez les adultes et les enfants investiguèrent l'effet de l'acide phytique (PA), de l'EDTA ou des fortifiants sur l'absorption du zinc de différents aliments de base. Nous avons combiné les données d'absorption du zinc de ces études faites soit en Suisse, soit au Burkina Faso, pour examiner les différents déterminants potentiels de l'absorption du zinc. Nous avons mesuré les paramètres du statut en fer, à savoir l'hepcidine, le récepteur soluble de la transferrine, l'hémoglobine and la protéine c-réactive (CRP) comme paramètres additionnels. Par l'analyse régressive et par un modèle linéaire mixte, nous avons testé les facteurs alimentaires et non alimentaires impactant l'absorption du zinc chez les humains.

La PZC n'est pas associé de manière significative avec la TAZ chez les adultes ($P < 0.001$) et les enfants. Le ratio PA:Zn et la concentration en zinc du repas sont des prédicteurs significatifs chez les adultes ($P < 0.001$ pour les deux). Les paramètres du statut en fer n'étaient pas associés avec ni la TAZ ni la FAZ et l'hepcidine n'est pas associée de manière significative chez les adultes. La TAZ chez les enfants était associée avec leur taille et la CRP.

Manuscrit 3: Le blé biofortifié produit agronomiquement and le statut en zinc des enfants indiens : une étude randomisée contrôlée

Nous avons évalué l'efficacité du blé biofortifié en zinc pour améliorer le statut en zinc lors d'une étude randomisée contrôlée en double-aveugle, chez les enfants de 6 à 12 ans (N = 273) de milieux à faibles revenus dans la région périurbaine de Bangalore. Le résultat principal était

la PZC. Les résultats secondaires consistèrent en l'hémoglobine, les marqueurs d'inflammation. Deux nouveaux biomarqueurs potentiels ont été mesurés dans un sous-échantillon d'enfants sélectionnés aléatoirement (N = 51). Les enfants ont été répartis aléatoirement pour recevoir soit une portion journalière de blé biofortifié, de blé enrichi ou de blé contrôle. Nous avons mesuré la PZC au début, à la fin et aléatoirement lors d'échantillonnages mensuels, et avons analysés les effets du temps et du traitement sur la PZC avec un modèle linéaire mixe. Nous avons recueilli un dernier échantillon de sang, 2 mois après la fin de l'étude, et après les vacances scolaires, pour investiguer l'évolution de la PZC par rapport au temps.

Nous n'avons pas trouvé d'effet significatif du temps par traitement (P = 0.23 pour le blé enrichi, P = 0.66 pour le blé biofortifié). Nous n'avons décelé aucun effet sur les nouveaux marqueurs potentiels du statut en zinc, à savoir les transcrits de ZnT1 and l'intégrité de l'ADN.

Conclusion

L'absorption du zinc du blé biofortifié est similaire à celle du zinc enrichi ajouté au blé contrôle sous forme de sulphate de zinc. Les deux sont plus élevés que celle du blé contrôle. Ce résultat suggère que l'absorption du zinc biofortifié est influencée de manière similaire par les composants de la nourriture que l'absorption du zinc enrichi. L'absorption en zinc n'est pas influencée seulement par des facteurs alimentaires, comme la concentration de zinc ou de PA dans le repas, mais elle est aussi influencée par la zincémie. Aucun effet n'a été vu sur la zincémie durant une longue étude d'efficacité sur les enfants mangeant du blé enrichi ou biofortifié pendant 20 semaines, mais ceci peut être dû à une faible prévalence initiale de carence en zinc et à une faible biodisponibilité du zinc dans le régime alimentaire indien. La biofortification, qu'elle soit par application de fertilisants sur le sol ou sur les feuilles, et une bonne stratégie d'intervention pour combattre les carences en zinc, aussi bénéfique que l'enrichissement conventionnel de zinc dans la nourriture.

I Introduction

The focus of this thesis is on the micronutrient zinc, its bioavailability from biofortified wheat, the predictors of its absorption in human subjects as well as the efficacy of biofortified wheat to combat zinc deficiency in school-age children. From its discovery in 1961 (Prasad, Halsted, & Nadimi, 1961), zinc deficiency has been recognized as one of the major contributors to the burden of disease in low and middle-income countries. The health consequences associated with zinc deficiency are diverse and varied, as multiple nonspecific general shifts in metabolism and function occur. They include impairment of immune function, skin lesions (King et al., 2016) and result in generalized poor growth, stunting and wasting (Golden, 1989). The tightly controlled whole-body homeostasis makes the assessment of zinc insufficiency challenging (King et al., 2016). There are currently 3 measurements for estimation of zinc status recommended by the Biomarkers of Nutrition for Development Zinc Expert Panel, namely dietary zinc intake, plasma zinc concentration and height-for-age of growing children (King et al., 2016). Recent data show the potentiality of emerging new biomarkers, such as DNA integrity and expression of zinc transport proteins, as well as hair zinc, and indices of neurological function (Lowe, 2016).

Fortification as well as supplementation are strategies that can be implemented, while dietary diversification is the only long term one (Gibson, Yeudall, Drost, Mtitimuni, & Cullinan, 1998; Shrimpton, Gross, Darnton-Hill, & Young, 2005), but the efficacy of those approaches is not well demonstrated (Brown & Baker, 2009). Biofortification is a new tool to tackle malnutrition (Bouis, Hotz, McClafferty, Meenakshi, & Pfeiffer, 2011). As the development of micronutrient-dense crops (Nestel, Bouis, Meenakshi, & Pfeiffer, 2006), biofortification through agronomic practices is a promising approach increasing zinc bioavailability in wheat (Liu, Liu, Zhang, Chen, & Zou, 2017). Wheat per capita consumption was around 67 kg/year in 2016, and is expected to remain the same, far ahead from rice (54 kg/year) and coarse grains (30kg/year) (Food and Agriculture Organization of the United Nations, 2016). Wheat is the principal food choice of Northern India, providing more than 50% of the total energy intake of people living in the North parts of the subcontinent (Khatkar, Chaudhary, & Dangi, 2016). Three of the studies presented in this thesis were carried out with funding from HarvestPlus, whose mission is to develop and scale up biofortified crops around the world and to provide evidence for

biofortification (HarvestPlus, 2017). The European Cooperation in Science and Technology funded one study.

The first part of this thesis consist of a literature review covering present knowledge of zinc metabolism, assessment of zinc status, including the review of new potential biomarkers, biofortification and a short presentation of the study site in India. This thesis was planned as a step-wise approach, whose overall goal was to determine the potential impacts of biofortified wheat on the zinc status of children at risk of zinc deficiency. Zinc absorption from biofortified wheat was compared to fortified wheat and control wheat, at different extraction rates. A regression statistical analysis was performed to assess the predictors of zinc absorption in children and in adults. Finally, an efficacy study was conducted in Bangalore, India, investigating at the impact on plasma zinc in children following a biofortified wheat diet. Two new potential biomarkers, DNA integrity and Zinc transporter 1 ribonucleic acid (RNA) transcript in whole blood cells were investigated. Results from those studies are reported in manuscripts 1, 2 and 3.

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II Zinc Nutrition and Metabolism

II.1 Properties

Zinc has an atomic weight of 65.4, an atomic number of 30 and an oxidation state of 2. Always in a divalent state, it does not exhibit redox chemistry. Within its 30 known isotopes, 5 are stable and naturally present, with the following abundances: ^{64}Zn 48.89%, ^{66}Zn 27.91%, ^{68}Zn 18.57%, ^{67}Zn 4.11%, ^{70}Zn 0.62% (Holt, Uriu-Adams, & Keen, 2012).

The overall total body zinc is around 2 g, while 60% of it is present in skeletal muscle, with a concentration of 100 - 2000 µg/g (World Health Organization, Food and Agriculture Organization, & International Atomic Energy Association, 2004). Thirty percent of zinc is found in bones, while the rest is in the skin, liver, brain, kidneys and heart. In both hair and plasma, 0.1% of total stores are found, and therefore, approximately 95% of body zinc is located intracellularly (Jackson, 1989).

As a type 2 nutrient, zinc is required for general metabolism and not for specific metabolic functions. There is no body store or reserve lasting for more than days in growing animals, and therefore general dysfunction is rapid in case of depletion (King, 2011). Zinc has an ability to form strong and flexible ligands with organic molecules, such as proteins and nucleic acids (Williams, 1989). As zinc ligands determine tertiary structure of many proteins, the integrity of cell membrane is zinc-dependent (M. Hambidge & Krebs, 2001).

The most common method to measure zinc concentration in solution is Atomic Absorption Spectrophotometry (AAS) and samples usually have to undergo either ashing or wet digestion in acid before measurement by AAS (Holt et al., 2012; King et al., 2016).

II.2 Physiological Functions

Zinc is essential for normal metabolism (King, 2011), growth and development (King, 1990) and has 3 basic biological functions: catalytic, structural and regulatory (Holt et al., 2012). It serves as a catalyst for 100 enzymes, such as RNA polymerases or alcohol dehydrogenase; it is necessary for the structure of specific proteins, such as some transcription factors, and some enzymes, such as the copper-zinc superoxide dismutase; and it an essential component of more than 300 enzymes participating in the synthesis and degradation of carbohydrates,

lipids, proteins and nucleic acids (World Health Organization et al., 2004). As a regulatory factor, it plays a role in gene expression and can influence apoptosis (*Dietary Reference Intakes: The Essential Guide to Nutrient Requirements*, 2006). By stabilizing molecular structure of cellular components and membranes, zinc contributes to the maintenance of cell and organ integrity (World Health Organization et al., 2004).

A protein classifies as a zinc metalloprotein if the removal of zinc causes a decrease in its activity without irreversibly affecting it and if adding back zinc restores its activity (King, 2011). The human genome includes more than 3000 zinc-binding proteins, including enzymes, nuclear transcription factors and even axons terminals (Sandstead, 2012). Zinc is distributed evenly throughout the body, especially as a component of zinc metalloproteins or Zinc-binding proteins, making zinc not readily detectable (K. M. Hambidge & Krebs, 2007). The cellular zinc concentration is maintained within a narrow range by a complex, extensive system for cellular zinc homeostasis (King, 2011) that will be explained in chapter II.3. Zinc metalloenzymes and zinc-dependent enzymes are involved in nucleic acid metabolism and cellular proliferation, differentiation and growth (K. M. Hambidge & Krebs, 2007). Over 3% of all identified human genes contain zinc finger domains (Maret, 2001). As zinc-finger motifs, zinc provides a scaffold that organizes proteins subdomains for the interactions with DNA or other proteins (Tapiero & Tew, 2003).

An inadequate zinc intake in experimental animals or humans causes an avid reduction of excretory losses to conserve zinc (Baer & King, 1984). Endogenous fecal losses decrease markedly within a couple days. With severe zinc depletion (<1 mg dietary zinc/day), urinary losses decline as well (King, Shames, & Woodhouse, 2000). If the decline in fecal and urinary losses fails to reestablish zinc balance, additional metabolic adjustments occur to mobilize zinc from a small pool, sufficient to support body needs for several weeks, while tissue catabolism may occur to release zinc (King, 2011). Because zinc losses are markedly reduced with severe depletion, the total amount of zinc lost is small and the response to zinc repletion is very rapid, as there is no significant loss of tissue zinc with depletion (King, 2011). A direct link between zinc deficiency and the function of an individual enzyme has not been identified, but this link is unlikely, as it would occur only if this enzyme was the rate-limiting factor in a critical pathway (King, 2011). Data from human studies show that growth retardation is an early response to zinc deficiency, confirmed in animals (King, 1990). In 2016, the effect of zinc

deficiency was proven significant on salt taste acuity, as the perception was significantly higher in the zinc deficient group. Zinc deficiency was significantly positively correlated with dietary sodium intake, suggesting the relation between zinc deficiency and possible high salt intake, leading to high dietary sodium intake (Kim et al., 2016).

II.3 Homeostasis and Metabolism

Homeostatic regulation is the balance between absorption of dietary or endogenous zinc and the excretion of endogenous zinc. Zinc absorption usually exceeds the amount utilized, and therefore, excess zinc needs to be excreted (Holt et al., 2012). As there is no zinc storage capacity, except maybe in infants (Coni et al., 1996), regular intake is required. Cellular tissue and whole-body zinc homeostasis are tightly and efficiently controlled (Jackson, 1989; King et al., 2000) to sustain cellular and tissue zinc concentrations over a wide range of zinc intakes (King et al., 2016). Around 90% of zinc turns over slowly and is therefore not readily available for metabolism (Olivares, Hertrampf, & Uauy, 2007) and the remaining zinc in the body is part of the so-called “zinc pool”. Plasma is the major route of zinc transport and within this fluid, zinc appears to be primarily bound to albumin (Jackson, 1989).

Zinc homeostasis is maintained by regulation of both Gastrointestinal (GI) absorption and GI excretion (World Health Organization et al., 2004). Total body zinc is maintained by the regulation of intestinal absorption, intracellular and tissue distribution, and the excretion of endogenous zinc pools (King et al., 2000). This is possibly achieved by changes in the expression of zinc transporters (Wang & Zhou, 2010). Plasma membrane efflux transporters are important components of cellular and whole-body zinc homeostasis as they mediate the efflux of intracellular zinc to prevent the cellular overaccumulation of the metal ion thereby saving the cell from the toxic consequences of zinc overload. In addition, zinc efflux systems are critical for the movement of zinc across polarized epithelial cell layers (Eide, 2006).

A major source of zinc losses by the body is in the intestine, by desquamation of epithelial cells, in urine and in sweat (World Health Organization et al., 2004). However, zinc is primarily excreted through the feces. Normal zinc losses range from less than 1 mg/day with a zinc-deficient diet to more than 5 mg/day with a zinc-rich diet. Only a small fraction (less than 10%) of zinc is excreted in the urine and therefore changes in the urine zinc content contribute little to the maintenance of whole-body homeostasis at normal dietary zinc intakes

(Jackson, 1989). Urinary losses increase with conditions such as trauma or starvation. Other zinc losses from the body include sweat, skin cell turnover, semen, hair, and menstruation (*Dietary Reference Intakes: The Essential Guide to Nutrient Requirements*, 2006). Normally, the higher the zinc intake, the greater the endogenous intestinal losses, while urinary and integument losses are below 1 mg/day (King & Turnlund, 1989). Additional metabolic adjustments occur if the declines in fecal and urinary losses fail to re-establish zinc balance. In conditions of bone resorption and tissue catabolism, zinc is released and may be reutilized to some extent (World Health Organization et al., 2004).

During zinc deficiency, zinc concentration in muscle is conserved, while its concentration in bone, liver and plasma falls (King, 1990) but the human body is able to respond to relatively large variations in dietary zinc to maintain a relatively constant body content of zinc (Jackson, 1989). PZC remains relatively stable during restricted or increased zinc intake, unless these changes are severe and prolonged (*Dietary Reference Intakes: The Essential Guide to Nutrient Requirements*, 2006). Zinc bound to metallothionein (MT) may be a short-term zinc pool (Holt et al., 2012).

During pregnancy, the requirements were estimated from the amount of zinc accumulated in maternal and fetal tissues, using a factorial approach (Swanson & King, 1987). PZC declines during pregnancy, and renal zinc conservation increases among women with intakes below 9 mg/day, but the net increase in zinc retention is below 0.3 mg/day. This is not sufficient to meet the increased zinc needs for pregnancy when dietary zinc is low (Donangelo & King, 2012). Milk zinc declines by ~75% over the lactation period, irrespective of the maternal zinc intake, and limited evidence shows the reduction of milk concentration with low zinc intakes. Renal conservation occurs during lactation, but the net effect on zinc conservation is small. The efficiency of absorption increases during lactation (Donangelo & King, 2012).

There is no evidence of a benefit of zinc supplementation in reducing serum bilirubin or the risk of hyperbilirubinemia (Rana et al., 2011), suggesting that the interaction between zinc and bilirubin are not of much clinical significance (Shah, 2011).

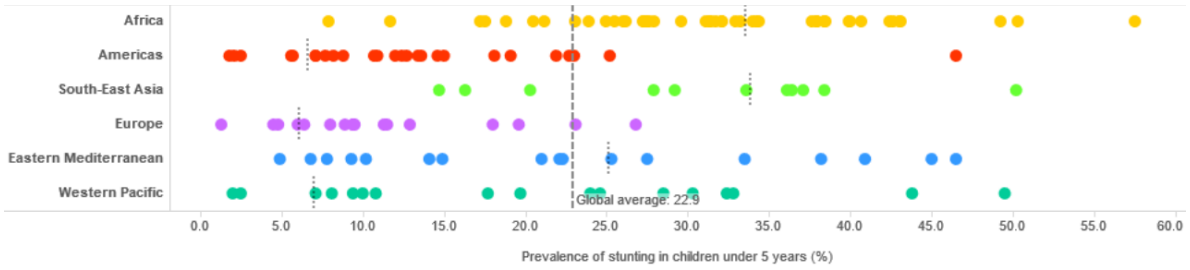

Figure 1 Prevalence of stunting in children under 5 years by WHO region 2005-2016 (World Health Organization, 2017). Dot line: regional median values: Africa: 33.5%, Americas: 6.6%, South-East Asia: 33.8%, Europe: 6.1%, Eastern Mediterranean: 25.1%, Western Pacific: 7.0%, Global median value: 22.9%. From (World Health Organization, 2017)

II.4 Dietary Sources

Food differ widely in their zinc content. The richest food sources of zinc are flesh foods, such as meat, fish and crustaceans. The major inhibitor of zinc absorption is myoinositol hexaphosphate, or Phytic Acid (PA), which binds zinc in an irreversible way in the intestinal lumen (K. H. Brown, Wuehler, & Peerson, 2001; Maret & Sandstead, 2006) as explained later in this chapter. In general, PA is present in plant foods, especially cereals and legumes but not in eggs and dairy products, items containing a small amount of zinc.

The food item with the highest content of zinc is peanut butter, with 15 mg zinc/100 g. All kind of meat, such as veal, lamb, game, and pork contain from 6 to 12 mg zinc/100 g (US Department of Agriculture, 2016). Cereals, even if they are considered good sources of zinc, contain high concentration of PA (K. H. Brown et al., 2001). The outer part of cereals are rich in zinc, such as wild rice and wheat germ, containing 5.9 and 12.3 mg zinc/100 g, respectively. In general, pulses are good sources of zinc and lentils as well as cowpeas contain around 3.5 mg zinc/100g. Fruits and vegetables are usually poor in zinc. All vegetables contain less than 0.8 mg zinc/100g, broccoli, spinach, avocado, mushrooms, containing the highest. Fruits generally contain less than 0.5 mg zinc/100 g, with raspberry, blackberries, pomegranates and durian containing 0.5 to 0.3 mg zinc/100g (Souci, Fachmann, & Kraut, 2016; US Department of Agriculture, 2016).

Diets are normally characterized depending on their PA:Zn molar ratio. Diets with a molar ratio greater than 15 have poor bioavailability while those with a molar ratio between 5 and 15 have medium bioavailability, and those with a molar ratio less than 5 have a good bioavailability. Assuming an intake of 3-5 mg per meal, about 50% of the initial zinc

concentration is absorbed from a high-bioavailability diet, 30% from a medium-bioavailability diet and 10% from a low-bioavailability diet (World Health Organization et al., 2004).

II.5 Dietary Recommendations and Requirements

Worldwide, there is a significant heterogeneity among the national recommendations of zinc intake (Doets et al., 2008). Differences exist among the estimates of the dietary zinc recommendations set by the European Food Safety Authority (EFSA), the Institute of Medicine (IOM), the International Zinc Nutrition Consultative Group (IZiNCG) and the World Health Organization (WHO), as the data used for body weight, height, and the selection of studies used for calculations are different. Due to the absence of specific and sensitive biomarkers of zinc status, and the nonspecific nature of the clinical features of mild deficiency (Gibson, King, & Lowe, 2016), the Institute of Medicine (Institute of Medicine (US) Panel on Micronutrients, 2001), the World Health Organization (FAO/WHO Expert Consultation on Human Vitamin and Mineral Requirements, 2004), IZiNCG (International Zinc Nutrition Consultative Group et al., 2004) and the European Food Safety Authority (European Food Safety Authority, 2014) used a factorial approach to calculate the dietary zinc recommendations. The required absorbed zinc is calculated on the estimation of zinc losses and additional requirements for growth, pregnancy or lactation, as well as zinc bioavailability. Zinc requirements are related to tissue turnover and growth rates, as well as variation in absorptive efficiency, which corresponds to a coefficient of variation of 25%. Recommended nutrient intakes represent the estimates of average individual requirements, with the addition of 2 standard deviations, and therefore 50% (World Health Organization et al., 2004).

The Tolerable Upper Intake Level (UL) is the highest level of daily nutrient intake that is likely to pose no risk of adverse effects for almost all healthy people (*Dietary Reference Intakes: The Essential Guide to Nutrient Requirements*, 2006). UL in general are derived from studies reporting adverse effects of high doses of zinc (Gibson et al., 2016). Requirements are more readily established than UL (Maret & Sandstead, 2006). The WHO set an UL for adults and extrapolated this to other age groups, based on differences in basal metabolic rates. The institute of medicine based their UL on the lowest observed adverse effect levels. IZiNCG based their UL on the institute of medicine for adults. While they did not set an UL for children, they set a no adverse effect level based on a study done on Indonesian children (Bertinato et

al., 2013). This study shows no impact on copper status by supplementation with 15 mg zinc daily for 4 months in healthy male children.

According to data from the Third National Health and Nutrition Examination Survey (NHANES III, 1988–1994), the highest reported dietary zinc intake, from food only, at the 95th percentile for all adults was 24 mg/day in men aged 19 to 30 years, which is lower than the UL. The 95th percentile of intake from food and supplements for adult men and nonpregnant women was approximately 25–32 mg/day; for pregnant and lactating women the 95th percentile of intake was 40 mg/day and 47 mg/day, respectively. The risk of adverse effects resulting from excess zinc intake appears to be low at these intake levels (*Dietary Reference Intakes: The Essential Guide to Nutrient Requirements*, 2006).

Different zinc requirements made by different agencies can be seen in Table 1 and Table 2. Zinc recommendations for infants are generally calculated either from breast milk zinc concentration or by extrapolating adult values (Lowe et al., 2013). There is currently a need to consider bioavailability more closely, and to account its modification on amount of dietary zinc required to meet requirements.

Table 1 Recommended dietary zinc intakes [mg] according to age and diet bioavailability. Data adapted from IZiNCG calculations (K. H. Brown et al., 2001)

		Bioavailability		
Population	**Age [year]**	**High**	**Medium**	**Low**
Infants	0.5-1	3.3	5.6	11.1
Children	1-3	3.3	5.5	11.0
	3-6	3.9	6.5	12.9
	6-10	4.5	7.5	15.0
Males	10-12	5.6	9.3	18.7
	12-15	7.3	12.1	24.3
	15-18	7.8	13.1	26.2
	18-60	5.6	9.4	18.7
Females	10-12	5.0	8.4	16.8
	12-15	6.1	10.3	20.6
	15-18	6.2	10.2	20.6
	18-60	4.0	6.5	13.1
	Pregnant	6.0	10.0	20.0
	Lactating: until 0.5 yr	7.3	12.2	24.3
	Lactating: >0.5 yr	5.8	9.6	19.2

Table 2 Estimated average requirements from IOM and EFSA(Institute of Medicine (US) Panel on Micronutrients, 2001) (European Food Safety Authority, 2014)in mg/day by life stage group and zinc dietary recommended intakes in mg/d by life-stage group

Years	EAR male	IOM EAR female	Upper limit	Years	EFSA Average Requirement male	Average Requirement female	Years	WHO UL male	UL female
0-0.5		-	4	**0-0.5**			**0-0.5**	-	
0.5-1	2.5	2.5	5	**0.5-1**	2.4	2.4	**0.5-1**	13	
1-3	2.5	2.5	7	**1-3**	3.6	3.6	**1-3**	23	
4-8	4.0	4.0	12	**4-6**	4.6	4.6	**4-6**	23	
9-13	7.0	7.0	23	**7-10**	6.2	6.2	**7-9**	28	
14-18	8.5	7.3	34	**11-14**	8.9	8.9	**10-12**	34	32
14-18 pregnant	-	10.5	34	**15-17**	11.8	9.9	**12-15**	40	36
14-18 lactating	-	10.9	34	**> 18, low PA (0.3g/day)**	9.4	7.5	**15-18**	48	38
19-50	9.4	6.8	40	**> 18, medium PA (0.9g/day)**	14.0	11.0	**>18**	45	35
19-50 Pregnancy	-	9.5	40	**> 18, high PA (1.2 g/day)**	16.3	12.7			
19-50 Lactating	-	10.4	40	**Pregnancy (additional)**	-	+1.6			
>50	9.4	6.8	40	**Lactating (additional)**	-	+2.9			

II.6 Deficiency

Zinc deficiency was discovered by Prasad in 1961 (Ananda S. Prasad, Halsted, & Nadimi, 1961) but was thought highly unlikely until then (Sandstead, 2013). The importance of the diet in the pathogenesis of the condition became obvious after Prasad studied 16 stunted males free of hookworm and schistosomiasis (A. S. Prasad, Miale, Farid, Sandstead, & Schulert, 1963). There is a contradiction between the evidence for effective homeostatic mechanisms that allow the human to adapt to low zinc intake and evidence of zinc deficiency even in circumstances where intake appears to be adequate (M. Hambidge, 2000).

In 2001, Prasad commented on his discovery, highlighting the irony that, as there is knowledge about the disease, the situation appeared to be simple, and yet implementation of a way to counteract it is difficult (A. S. Prasad, 2001). Currently, supplementation, fortification and dietary diversification are the only interventions to improve zinc intake and therefore fight zinc deficiency (Shrimpton, Gross, Darnton-Hill, & Young, 2005). As the efficacy of zinc in treating diarrhea is clear (Black, 2003), supplementation with zinc should potentially be recommended during diarrhea episodes. One shortcoming of oral rehydration therapy though, even supplemented with zinc, is that the frequency and volume of stools is not reduced (Shrimpton et al., 2005). Currently, there is no specific guidelines from the WHO for preventive zinc supplementation, only a joint statement with UNICEF on the clinical management of acute diarrhea including therapeutic zinc supplementation, along with oral rehydration therapy, of 20 mg / day for 2 weeks (World Health Organization & UNICEF, 2004). Moreover, as people have multiple micronutrients deficiencies, the consensus is that zinc should not be promoted as a single nutrient supplementation. Fortification may not be suitable in many countries, where a small-scale fortification might be easier. The only long-term way to counteract zinc deficiency is therefore by diversification of the diet (Shrimpton et al., 2005). Strategies involve reduction of PA, increase intakes of food with high bioavailable zinc or absorption enhancers. This can be done by using germination, fermentation and soaking to reduce PA (Gibson, Yeudall, Drost, Mtitimuni, & Cullinan, 1998).

II.6.1 Health Consequences

With zinc deficiency, multiple nonspecific general shifts in metabolism and function occur (King et al., 2016). In case of severe zinc deficiency, the clinical signs are bullous pustular

dermatitis, alopecia, mental disturbances, diarrhea, and immune disorders. Those are seen in patients either with acrodermatitis enteropathica (ADE), a disease due to an inborn error of zinc absorption, or in patients receiving total parenteral nutrition lacking zinc (Al Rashed, Al Shehri, & Kaliyadan, 2016; Walsh, Sandstead, Prasad, Newberne, & Fraker, 1994). ADE can become fatal (Barnes & Moynahan, 1973). Severe zinc deficiency is unusual, and when patients are adequate on other micronutrients, restoration of zinc status rapidly restores homeostasis (Sandstead, 2012).

In case of marginal zinc deficiency in adults, clinical signs are varied, less clear, and usually correctable by zinc supplementation. The signs arising after depletion are diverse and cannot be attributed to a defect in a specific function (King, 2011). They include depressed immunity with delayed wound healing, skin changes, impaired taste and smell or poor appetite, abnormal dark adaptation, impaired neuropsychological functions and memory, and decreased spermatogenesis or hypogonadism in males as well as, in children, growth retardation, delayed sexual and bone maturation (Shankar & Prasad, 1998; Walsh et al., 1994; World Health Organization et al., 2004). Reduced growth rate and impairments of immune defense are clearly demonstrated, while taste impaired and wound healing are less consistently observed (World Health Organization et al., 2004). Zinc deficiency impairs both innate immunity and acquired immunity (King et al., 2016) and urinary zinc losses decline only with severe zinc depletion of <1 mg/day (King et al., 2000).

In children from 0 to 4 yo, a meta-analysis reviewed that zinc deficiency increases the risk of incidence of diarrhea by 1.28 (1.10-1.50), pneumonia by 1.52 (1.20-1.89) and malaria by 1.56 (1.29-1.89). The estimated deaths due to those 3 diseases were then calculated to equal around 800’000, while the loss of disability-adjusted life years attributable to zinc deficiency amounts 28 million. All the considered health outcomes included diarrhea, pneumonia, malaria, measles, cognitive dysfunction, physical or visual impairment and mortality (Caulfield & Black, 2004). Zinc supplementation reduces the duration and severity of acute and persistent diarrhea by 15 and 25% respectively (Bhutta et al., 2000), showing the public health importance of zinc deficiency.

II.6.2 Prevalence

Even though the estimated worldwide prevalence of inadequate energy intake decreased from 18.6% in 1991 to 10.9% in 2016 and 23.3% to 12.9% for developing countries (Food and Agriculture Organization, International Fund for Agricultural Developement, & World Food Programme, 2015), the undernourishment trends showed progress in almost all regions, but at different rates.

Regulation of zinc homeostasis makes the diagnosis of zinc deficiency challenging, as no single measurement is sufficient for classification of zinc deficiency in individuals. Dietary zinc content and availability, the prevalence of stunting, and PZC in a population are generally used as estimates of the risk of zinc deficiency (Sandstrom, 2001). In general, data from surveys are affected by systematic reporting errors. In the case of nutrition surveillance data, another source of error is the translation of food and beverage consumption data into nutrient energy values via nutrient composition databases (Archer, Hand, & Blair, 2013), and in the case of PZC measurements, by improper collection and processing of PZC. Due to the high cost of measurements of PZC, few national surveys were conducted in developing countries assessing the risk of zinc deficiency using PZC. A potential estimate of the risk of inadequate zinc intake in populations is the amount of zinc in national food supplies, and by using food balance sheet and prevalence of stunting, the risk of zinc deficiency can be estimated in the whole population, including growing children and adults (Wessells & Brown, 2012). The estimated country prevalence of inadequate zinc intake can be seen in Figure 2.

Using indicators of stunting and low dietary zinc intake, 32 countries were identified as being at risk of zinc deficiency, as seen in Figure 3. In 2015, Kumssa and coworkers (Kumssa et al., 2015) estimated the zinc supply to be globally 16 ± 3 md/day/capita and the risk of zinc deficiency to be 16 ± 14% worldwide, as calculated by the PA:Zn molar ratio and intakes. They estimated that 39 countries are at risk of zinc deficiency in 2011, while the trend of zinc deficiency risks are decreasing between 1992 and 2011 but remain prevalent, reflecting the overall increase in global food supply. Their estimate can be seen in Figure 3.

Those two estimates are comparable, while they were produced by different methods. The main discrepancy is due to the use of different food composition data, and different data considered unusable by both groups. Kumssa and coworkers did not take into consideration

Congo, South Soudan, Eritrea and Suriname, for example, due to the lack of information. They listed Paraguay, Ethiopia and Columbia at risk of zinc deficiency while Wessells and coworkers did not. The widespread prevalence of stunting in children below 5 years old by WHO regions can be seen in Figure 1.

II.6.3 Risk Factors

Some known conditions are associated with an increased risk of zinc deficiency (King et al., 2016). Gastrointestinal and metabolic disorders, such as inflammatory bowel disease (Valberg, Flanagan, Kertesz, & Bondy, 1986) or chronic diarrhea leading to increased zinc loss (Akhtar, 2013), are known to decrease zinc absorption. Vegetarian diets are generally considered as a risk factor for zinc deficiency, as the absorption of zinc is lower, even if adverse health effects from lower zinc absorption have not been demonstrated with abundant and varied vegetarian diets in developed countries (Gibson, 1994; Hunt, 2003). Pregnant and lactating women, as explained in chapter II.5, have a higher RDA and therefore a higher risk for zinc deficiency. In general, exclusively breastfed infants of more than 6 months are believed to be more at risk of zinc deficiency than weaning infants (K. H. Brown, Peerson, Baker, & Hess, 2009). The average intake and absorption of zinc from beef by 7 month of age, with a modest intake from breast milk, are adequate to meet average dietary and physiologic zinc requirements. However, there is significant barriers to provide meat as a first complementary food in many circumstances, and they include sociocultural, economic, and household factors (K. M. Hambidge & Krebs, 2007).

II.6.4 Impacts on Cognition

The effect of zinc intake in mental and motor development in infant was reviewed in 2013 in a meta-analysis. Even though no association was found between zinc intake and mental and motor development in infants, differential effects, and therefore a reduction in heterogeneity, were seen depending on the dose of supplementation, while the intervention duration has a differential effect only on the psychomotor development (Nissensohn et al., 2013).

Another meta-analysis published in 2015 investigated zinc intake and cognitive function in adults and children. Due to the lack of studies, a meta-analysis in adults was not possible, while the heterogeneity in the study designs was a major limitation of the children meta-analysis. No significant effect of zinc supplementation on cognitive functions, as intelligence,

motor skills and executive functions was found. Small indicators of improvement on aspects of executive function and motor development could be seen though (Warthon-Medina et al., 2015). Even though no effect of zinc intake was shown on improvement of cognition, two cross-sectional studies in elderly published in 2015 showed that a lower PZC was strongly associated with poorer cognitive performances (Alghadir, Gabr, & Al-Eisa, 2015), and impaired cognitive functions (Markiewicz-Zukowska, Gutowska, & Borawska, 2015).

In a pooled data analysis, stunted school-age children had a significantly poorer performance on short-term memory, retrieval ability and visuospatial ability tests ($P > 0.03$ for all). No significant difference was found in the change in cognitive scores following a nutritional intervention of 6 months between those who remained stunted and those who were no longer stunted ($P > 0.10$). Stunting remains associated with cognitive ability in school-age children, but the reversal of these effects in this age group is difficult (Sokolovic et al., 2014). The further investigation as biomarker of indices of neurological functions, particularly assessing memory and attention, highlights the possible impacts of zinc deficiency on cognition (Lowe, 2016).

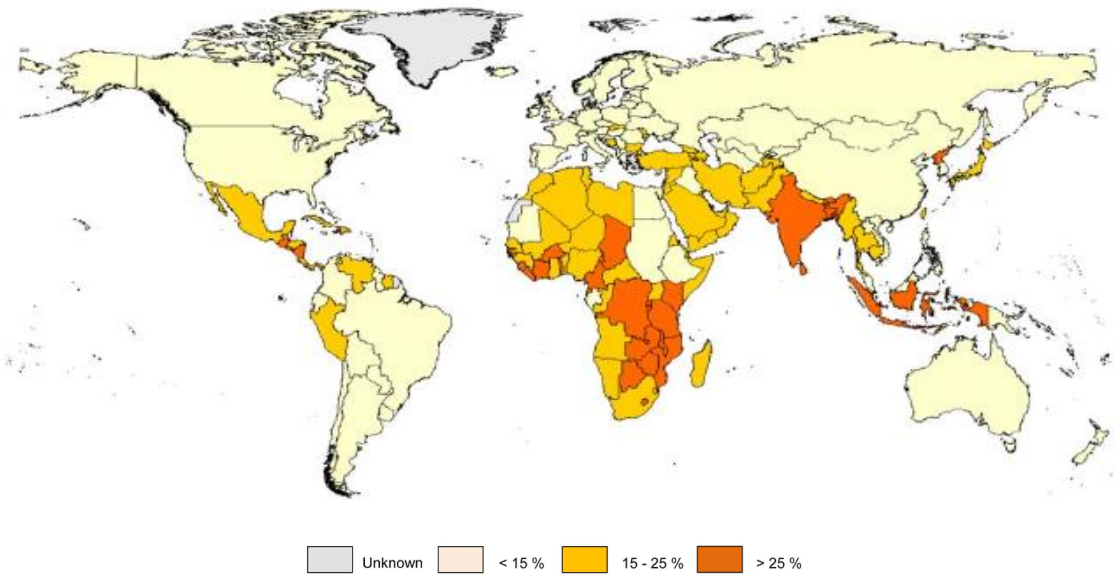

Figure 2 Estimated country-specific prevalence of inadequate zinc intake. Data are based on the composite nutrient composition database, IZiNCG physiological requirements, the Miller Equation to estimate zinc absorption and an assumed 25% inter-individual variation in zinc intake. Data are from 2003–2007. From (Wessells & Brown, 2012)

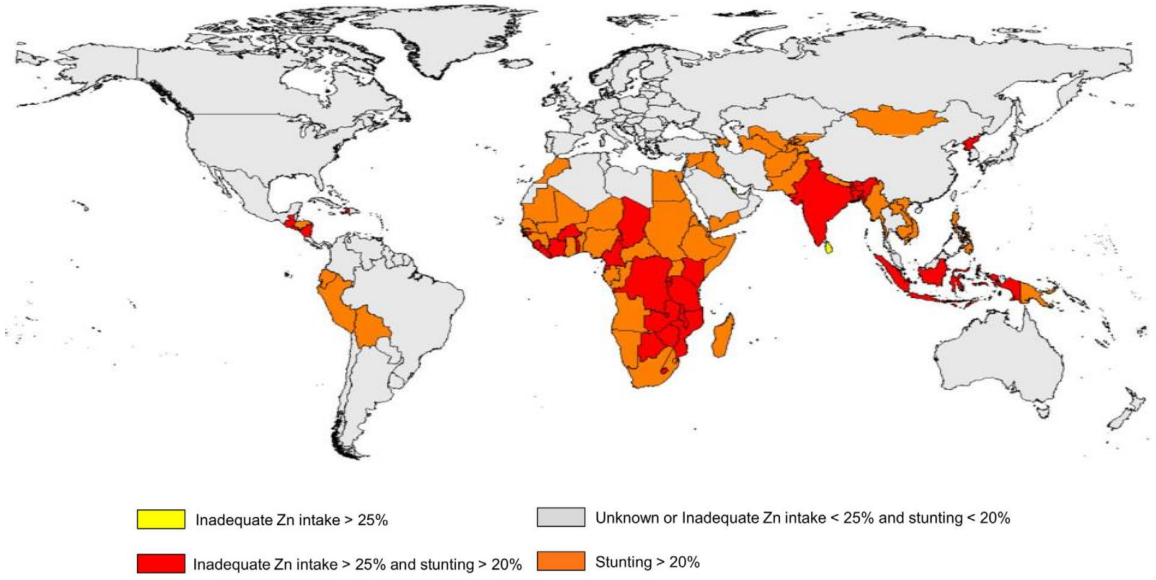

Figure 3 National risk of zinc deficiency. Data are based on the prevalence of childhood stunting and the estimated prevalence of inadequate zinc intake. From (Wessells & Brown, 2012)

II.7 Bioavailability

II.7.1 Dietary Determinants of Absorption

The bioavailability of zinc is largely a function of the presence or absence of substances in the food matrices that influence its absorption (Holt et al., 2012). The chemical environment at the absorption site determines largely zinc absorption. The efficiency at which zinc is absorbed therefore depends on the solubility of zinc, the presence of ligands and the competition between zinc and other minerals for uptake sites (Sandstrom & Lonnerdal, 1989). Iron, calcium, phosphorous, protein and PA are known dietary factors affecting zinc absorption (*Dietary Reference Intakes: The Essential Guide to Nutrient Requirements*, 2006; Lonnerdal, 2000).

The zinc content of a meal affects zinc absorption by itself, as the increasing amount of dietary zinc decreases Fractional Zinc Absorption (FAZ) (Sandstrom, Arvidsson, Cederblad, & Bjorn-Rasmussen, 1980; Sandstrom & Cederblad, 1980). There is therefore a diminishing return in the efficiency of uptake of zinc, even if the net absorption continues to increase as a dose function (Solomons, 2001). This process might be due to a saturation of the transport mechanisms (Lonnerdal, 2000; Sandstrom, 1992). In a meta-analysis done in 2014, the negative impact of a high total zinc content of the meal on FAZ was confirmed in 17 studies (Bel-Serrat et al., 2014).

Table 3 Qualitative bioavailability of zinc according to diet characteristics (Allen, de Benoist, Dary, & Hurrell, 2006; FAO/WHO Expert Consultation on Human Vitamin and Mineral Requirements, 2004)

Bioavailability	PA:Zn molar ratio	Dietary Characteristics
High	<5	Low in cereal fiber and phytic acid Adequate protein intake from meats and fish
Medium	5-15	Mixed diets containing animal or fish protein Vegetarian diets not based primarily on unrefined, unfermented cereal grains
Low	>15	High in unrefined cereal grains Animal protein intake negligible ≥ 50 percent of energy is provided by high-phytate foods

The phosphate groups in PA can form strong and insoluble complexes with cations such as zinc in the GI tract (Oberleas, Muhrer, & O'Dell, 1966) and, as no phytase activity can happen in the GI tract of humans, phytate-bound minerals will be excreted in the stool (Lonnerdal, 2000). Even though the main form of PA is usually an hexaphosphate, PA in food is a mixture of different phosphorylated forms of inositol phosphate (Sandberg & Ahderinne, 1986). The mean difference in FAZ between low and high PA diets was found to be -0.11 (-0.16, -0.07), with a heterogeneity of 94%. This heterogeneity was expected, due to the high degree of variation in the study designs included in the meta-analysis (Bel-Serrat et al., 2014). In general, meals containing a PA:Zn lower than 15 still have a significant inhibitory effect on FAZ, and therefore, zinc absorption is adversely affected by components of the vegetarian diet (Bel-Serrat et al., 2014). When the PA:Zn molar ratio is reduced from 20 to 10, the total absorbed zinc (TAZ) is significantly increased (K. M. Hambidge et al., 2017). Phytic Acid degradation can be achieved by the addition of a phytase prior to consumption, either by traditional fermentation or germination (Hotz, Gibson, & Temple, 2001), or by addition of a microbial phytase (Egli, Davidsson, Zeder, Walczyk, & Hurrell, 2004). The start of the PA degradation, either during digestion or during food preparation, improves zinc absorption in children (Brnic et al., 2017) and adults (Brnic, Wegmuller, Zeder, Senti, & Hurrell, 2014) from rice. In a secondary analysis, data from 236 children showed that an increase of 500 mg/d in PA reduced absorbed zinc by less than 0.04 mg/d, which showed no detectable PA effect on zinc absorption to raise caution about dietary PA (Miller, Hambidge, & Krebs, 2015). In general, diets are characterized depending on their bioavailability, as shown in Table 3.

As zinc binds to proteins at near neutral pH (Food and Nutrition Board Institute of Medicine, 2001), protein content of a meal is positively correlated with zinc absorption. FAZ increases linearly as protein content increases, but in general, increased dietary protein leads to increased zinc intake, as protein is food rich in protein are rich in zinc as well (Sandstrom et al., 1980). Even though zinc absorption is inhibited by some protein sources, while others have no effect, the addition of animal protein enhances zinc absorption (Bel-Serrat et al., 2014). The type of protein in a meal affect zinc bioavailability (Lonnerdal, 2000). In a meal containing 25 g of protein, of which 17 g came from the meat patty, FAZ from chicken, beef and soybean was 36, 20 and 14% respectively (Sandstrom & Cederblad, 1980). Milk, as a good protein source, given in combination with a meal, enhances FAZ compared with water. Ultra-high temperature processing does not influence FAZ. Outside of a meal though, zinc absorption

from water is higher than from milk (Talsma et al., 2017). In general, a concentration-dependent positive effect of milk solids is expected on zinc absorption (Talsma et al., 2017). Studies on the effect of various protein sources are often confounded as proteins sources contain other dietary factors that may influence FAZ (Lonnerdal, 2000). Several amino acids can form zinc complexes facilitating zinc uptake (Krebs, 2000), but in general, protein-rich food is considered to be a good source of zinc as inhibitors are not present (Holt et al., 2012).

Calcium content of the diet may affect zinc absorption from PA-containing meals (Lonnerdal, 2000), but reports are currently conflicting (Bel-Serrat et al., 2014). As such, calcium has no detrimental effect on zinc absorption (King et al., 2016), but the possible inhibitory effect of high calcium supplements made of calcium phosphate was confirmed (Wood & Zheng, 1997). This, though, was not seen with the use of calcium citrate-malate complex (McKenna, Ilich, Andon, Wang, & Matkovic, 1997) and increasing the calcium intake to 2000 mg/day did not significantly influence zinc balance and zinc losses (Spencer, Kramer, Norris, & Osis, 1984). The use of 200 mg calcium phosphate was marginally associated with lower zinc absorption ($P = 0.071$) in preschool children. There is no evidence that a prolonged supplementation with 1000 mg of calcium reduces PZC in women (Yan et al., 1996).

The impact of daily intake of iron could decrease zinc absorption (Food and Nutrition Board Institute of Medicine, 2001), as a competitive inhibition is happening between both minerals (Solomons, 1986). As explained in later chapter, it is hypothesized that comparable affinity are found for zinc with iron transporters, suggesting that absorption of zinc might be stimulated during iron deficiency (Food and Nutrition Board Institute of Medicine, 2001). In ileostomy subjects (Troost, Saris, Dainty, & Brummer, 2003) as well as lactating women (Chung et al., 2002), high doses of iron as 60 mg was been shown to decrease zinc absorption. In general, results are inconclusive, as FAZ depends on the relative levels of zinc, iron and matrix (Bel-Serrat et al., 2014)

Folic acid, copper, ascorbic acid, oxalate and tea have no significant effect on FAZ (Bel-Serrat et al., 2014). Gastric acid production, rate of gastric emptying and intestinal transit time can influence the degree of absorption, but knowledge of their importance is limited (Sandstrom & Lonnerdal, 1989). Long-term zinc intake, and therefore zinc status, was hypothesized to affect absorption of dietary zinc (Lonnerdal, 2000). Another potential determinant of absorption is genotype, as most members of zinc transporters show polymorphisms

(K. M. Hambidge, Miller, Westcott, Sheng, & Krebs, 2010). A systematic search published in 2013 highlighted the importance of this area for future research and the very small number of relevant studies (Lowe et al., 2013).

Equation 1 Miller's equation from 2013. From (Miller et al., 2013)

$$\boldsymbol{TAZ} =$$

$$0.5\left(K_t\left(1+\frac{TDPA\cdot(1-B_{Ca}TDCa}{K_p}\right)+A_{max}+TDZn\cdot(1+B_{Pr}TDPr)\right.$$

$$\left.-\sqrt{\left(K_T\cdot\left(1+\frac{TDPA\cdot(1-B_{Ca}TDCa)}{K_P}\right)+A_{max}+TDZn\cdot(1+B_{Pr}TDPr)\right)^2-4\cdot A_{max}\cdot TDZn\cdot\left(1+B_{pr}TDPr\right)}\right)$$

$where$

$TDZn = \ total\ daily\ dietary\ Zinc\ [\frac{mmol}{day}];$

$TDPA = total\ daily\ dietary\ Phytic\ Acid\ \left[\frac{mmol}{day}\right];$

$TAZ = total\ daily\ absrobed\ zinc\ \left[\frac{mmol}{day}\right];$

$TDCa = \ total\ daily\ dietary\ Calcium\ [\frac{mmol}{day}];$

$TDPr = \ total\ daily\ dietary\ protein\ [\frac{g}{day}];$

$A_{max} = 0.084, maximum\ zinc\ absorption;$

$K_T = 0.069, Zinc - transporters\ equilibrium\ dissociation\ constant;$

$K_P = 0.44, Zinc - phytic\ acid\ equilibrium\ dissociation\ constant;$

$B_{Ca} = 0.017, Calcium\ parameter;$

$B_{Pr} = 0.012, Protein\ parameter;$

Four algorithms have been proposed to predict zinc bioavailability based on the previously mentioned dietary determinants of absorption: a trivariate model by Hambidge in 2010, including PA, zinc intake and absorption (K. M. Hambidge et al., 2010); a logit regression model by IZiNCG(International Zinc Nutrition Consultative Group et al., 2004), including zinc, calcium, protein and PA content of meals; and two models based on saturation transport kinetics proposed in 2007, and 2013 by Miller and coworkers (Miller, Krebs, & Hambidge, 2007, 2013). IZiNCG and the Miller 2007 models were tested in a separate set of observations (n = 83) (Hunt, Beiseigel, & Johnson, 2008). The quality of the predictions by the IZiNCG model was not confirmed, showing an underestimation of absorption at high dietary zinc levels. The 2007 Miller model was confirmed, explaining 44% of the variance. In 2013, the 2007 Miller model,

as it included only dietary zinc and PA was improved by including calcium and protein, while a proposed iron parameter showed no further improvement in the model. The 2013 Miller equation, including PA, calcium, zinc and protein can be seen in Equation 1. It improves the explanatory power from 82% to 88% compared to the 2007 algorithm. If absorption is being predicted solely from dietary zinc and PA, the 2007 Miller model should be used, while if calcium and protein content can be estimated, then the 2013 model should be used (Miller et al., 2013). Generally, algorithms suggest that dietary factors other than PA may play a minor role in zinc nutrition (Lonnerdal, 2000).

II.7.2 Assessment of Bioavailability

One way to measure nutrient requirements is by using the stable isotope tracer methodology. The bioavailable mineral is that mineral from the food that is absorbed and retained in the body (Sandstrom, Fairweather-Tait, Hurrell, & van Dokkum, 1993). Stable isotope can be used to study bioavailability of minerals, dose effects, trace elements interactions and mineral absorption (Woodhouse & Abrams, 2001). They are nonradioactive and can be safely administered to humans (Jones & Leatherdale, 1991). Stable isotopes can be used in children as well, but the volume of blood that can be obtained from small children, the rapid rate of bone turnover in adolescents and the ethical issues linked to the inclusion of children in studies are factors limiting the use of stable isotopes in this age group (Abrams, 1999). Complications are only expected related to inappropriate isotope preparation or administration and therefore, safe and legal purchase of stable isotopes should be followed. (Abrams, Griffin, & Herman, 2002).

One of the major advantage in the use of stable isotope is the fact that there is no isotopic decay and therefore samples can be stored indefinitely. No disposal cost are associated with their use, and the cost of a specific stable isotope depends on its natural abundance and the enrichment level required (Woodhouse & Abrams, 2001). The two most frequently used methods for specimens measurements of isotopes are Thermal Ionization Mass Spectrometry (TIMS) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS). ICP-MS, even if less precise than TIMS, is the method of choice, as its cost, ease and speed of analysis are advantageous (Turnlund & Keyes, 2002). Acid digestion and ion exchange chromatography are needed prior measurement (Woodhouse & Abrams, 2001).

Mineral bioavailability from foods can be measured by labelling the food intrinsically, through biological incorporation of the isotope during food production or extrinsically, simply by mixing the isotope with the food prior to consumption (Weaver, 1985). The shift in the natural isotopic abundance can be measured and mineral nutrient uptake and elimination can be quantified (Schoeller, 2002). As explained earlier, zinc has 5 stable isotopes, three of them, ^{67}Zn, ^{68}Zn and ^{70}Zn, being of low enough abundance to be used as tracers in human research while the long half-life of ^{65}Zn makes it difficult to use in such applications (Sandstrom et al., 1993).

While assessing bioavailability, the absorption of the stable isotope label must closely match the one of the mineral it is tracing (Abrams et al., 2002). For all stable isotopes occurring in nature, an amount greater than their natural abundance is required for administration. Unfortunately, this dose can represent significant fraction of the exchangeable mineral pool, and may not function as a true tracer (Woodhouse & Abrams, 2001). Exchange of stable isotopes with native minerals takes long and the dose of isotope added depends on the expected absorption and retention, the distribution between different body tissues, its natural abundance and the degree of enrichment required (Sandstrom et al., 1993).

When applying the extrinsic approach, the main assumption is that the extrinsic isotope exchanges completely with the native mineral, and is absorbed and metabolized identically (Woodhouse & Abrams, 2001). Food can be intrinsically labelled in different manners. In case of plant food, either by adding the isotope to the nutrient solution of hydroponically grown plants, by direct injection, or by foliar application (Weaver, 1985). In case of animal foods, by adding the isotope to the diet, or by intravenous (IV) or intramuscular injection. In the edible part of the food, isotopes must be incorporated in their natural form, and the efficiency of their incorporation is of importance (Sandstrom et al., 1993). Because of the low incorporation and the large doses of isotope needed to provide detectable label in the final product, intrinsic labelling is costly (Woodhouse & Abrams, 2001).

In 2015, Robert Glahn published a commentary on an iron absorption study, made by stable isotopes. He pointed out that absorption being an indirect measure of equilibration; a similar absorption value can be obtained from fortified iron and intrinsic iron without full equilibration of the two sources (Glahn, 2015). The assumptions that the oral isotope exchanges completely with the native mineral and is absorbed alike was suggested in rice

(Brnic et al., 2016), formula milk (Egan, 1991; Serfass, Ziegler, Edwards, & Houk, 1989), beef (Gallaher, Johnson, Hunt, Lykken, & Marchello, 1988) and Turkey meat (Flanagan, Cluett, Chamberlain, & Valberg, 1985), while the extrinsic label was shown to underestimate FAZ by around 10% in beans (Donangelo et al., 2003) and chicken (Janghorbani, Istfan, Pagounes, Steinke, & Young, 1982).

Different techniques for measurement of bioavailability have been used, including whole body counting (Aamodt, Rumble, Johnston, Foster, & Henkin, 1979), fecal monitoring (Payton et al., 1982), urinary monitoring (Friel, Naake, Miller, Fennessey, & Hambidge, 1992), plasma appearance (also called deconvolution analysis) (Molokhia, Sturniolo, Shields, & Turnberg, 1980) and whole-gut technique (Zheng, Mason, Rosenberg, & Wood, 1993).

The fecal monitoring technique is used for minerals or elements with a high intestinal endogenous excretion, such as zinc, while it is of no use when a low absorption is expected, like for iron (Sandstrom et al., 1993). Fecal monitoring is not only laborious, it is sensitive to subject compliance, and may result in overestimation (Lowe, Woodhouse, Matel, & King, 2000). Moreover, as part of the absorbed isotope is excreted into the feces during the collection period, FAZ is underestimated, even if corrections can be applied (Lowe et al., 2000). The whole gut technique requires a lavage and is therefore invasive (Zheng et al., 1993). The plasma appearance technique is qualitative and well-suited for comparative studies (Sandstrom et al., 1993) but requires multiple blood drawings over several days, due to the repeated isotopes measurement in the plasma (Lowe et al., 2000)

Based on a measure of the FAZ from urine excretion of an oral and IV administration of calcium stable isotope (Yergey, Viera, & Covell, 1990), Friel and co-workers developed in 1992 a method for measuring FAZ by using the analysis of two stable isotopes in urine samples that were collected after on oral and IV dose (Friel et al., 1992). The so-called Dual Isotopic Tracer Ratio method (DITR) involved the administration of 2 zinc isotopes, one orally and one in IV. A urine sample is collected 3 days later (Woodhouse & Abrams, 2001).

The use of DITR makes the fecal collection unnecessary (Abrams et al., 2002). In this case, the oral isotope is assumed to be absorbed into a central body pool and mixes with the IV isotope, which serves to normalize for variations (Abrams et al., 2002). DITR is therefore based on 4 assumptions, mainly that both isotopes enter the plasma at the same time, that the isotope

enrichment in plasma and urine are the same after a couple of days (and therefore that the oral and IV tracer enrichment decay proportionally), that urine enrichments of both isotopes are similar from plasma and the extrinsic label isotope reflects that of the intrinsic zinc in the diet (Tran, Gopalsamy, Mortimer, & Young, 2015). FAZ in the urine is then the multiplication of the enrichment (oral/IV) by the dose (IV/oral), while the TAZ in mg/day is the multiplication of FAZ by the zinc intake in mg/day.

The isotope used in the IV must be diluted in a small amount of saline and given over a rapid period time (Abrams et al., 2002), and the solutions need to be sterile and tested for pyrogenicity. The use of a washout period between meals during crossover designs is of importance to remove the effect of the initial treatment on the results of the second treatment (Abrams et al., 2002).

DITR provides an accurate measure of FAZ and is currently the recommended method for FAZ determination (Lowe et al., 2000) but is limited when the efficiency of absorption is expected to be low, as the urine enrichment need to be higher than the levels of quantification (Fairweather-Tait, Fox, Harvey, Teucher, & Dainty, 2001). As this technique require venipuncture, it can be considered invasive (Tran et al., 2015), while high levels of laboratory expertise is needed to obtain reliable measurements (K. M. Hambidge, Krebs, & Miller, 1998) and the laboratory in which samples are measured need to have proper instrumentation. TAZ from DITR are comparable tho the one measured from the whole body metabolic balance technique (Sheng et al., 2009) and the use of zinc isotopic tracers provided data on the effects of various diet constituents on zinc retention (Sandstead, 2012).

Zinc compounds commonly used in fortification, such as zinc oxide (water-insoluble) and zinc sulfate (water-soluble), are unlikely to differ in their bioavailability in zinc fortified foods (K. H. Brown, Wessells, & Hess, 2007).

II.8 Physiology of Absorption

Zinc absorption is defined as the minimum amount of absorbed zinc necessary to equal total daily zinc losses. The dietary intake corresponding to this average minimum quantity of absorbed zinc is the Estimated Average Requirement (EAR) (*Dietary Reference Intakes: The Essential Guide to Nutrient Requirements*, 2006). Zinc absorption takes place in the small intestine in the duodenum and jejunum through a transcellular process via a saturable

carrier-mediated component and a nonspecific, unsaturable diffusion-mediated component but the absorptive capacity is not saturated at typical dietary zinc concentration (Lonnerdal, 2000; Steel & Cousins, 1985). The maximal absorption occurs in the distal duodenum and proximal jejunum, while very little is absorbed from the stomach (Krebs, 2000). Within the intestinal lumen, pH does not influence zinc uptake and digestive enzymes release zinc from food matrices (Holt et al., 2012).

The absorbed zinc is bound to albumin and transferred from the intestine via the portal system (*Dietary Reference Intakes: The Essential Guide to Nutrient Requirements*, 2006). Around 5-15% of cytosolic zinc pool is bound to MT that can incorporate 7 atoms of zinc and are important for cytosolic zinc storage, and serve as zinc acceptors and donors. Oxidation of the sulfur leads to the release of zinc, which contributes to intracellular zinc signaling (Kambe, Tsuji, Hashimoto, & Itsumura, 2015).

From pancreatic secretions, zinc is secreted into the duodenum. Bile, duodenum, mucosal cells sloughed from the gut are other endogenous sources of zinc (Holt et al., 2012). The pancreas is a conduit for endogenous zinc, but other potential routes include serosal to mucosal transport, with release into the intestinal lumen (Cousins, 2010).

The 24 zinc transporters can be divided in two classes: The Zrt-, Irt-like Protein family (ZIP), named after the yeast Zrt1 protein and the Arabidopsis Irt1 protein, and the Zinc transporter family (ZnT, also called cation diffusion facilitator – CDF). ZIP transporters work in opposition to, but in coordination with, the ZnT transporters to regulate cellular zinc homeostasis (King, 2011). The 14 transporters from the ZIP family move zinc into the cytoplasm from extracellular space, while the 10 transporters from the ZnT family move zinc from the cytoplasm into the lumen of the intracellular organelles or to the outside of the cell. ZIP proteins have 8 predicted transmembrane domains, while ZnT proteins have only 6. Zinc Transporter family use the gradient of other ions to drive the transport of zinc, but the mechanism of transport used by ZIPs is not clear, as some of them require energy to carry zinc through membranes while some do not (Eide, 2006; Lichten & Cousins, 2009). While ZIP4, ZIP11, ZIP14 and ZnT5 are found in the apical plasma membrane of intestine calls, ZnT2 and ZnT4-7 are involved in the transcellular movement of zinc in enterocytes (Cousins, 2010).

The major transporter controlling cellular zinc efflux is ZnT1 (Palmiter & Findley, 1995), localized in the basolateral membrane of the ileum (Wang & Zhou, 2010). ZnT1 is the only transporter localized in the plasma membrane and functions by exporting cytosolic zinc into the extracellular space (Kambe et al., 2015). It is essential during embryogenic development, because deletion of the ZnT1 gene in mice results in embryonic death (Andrews, Wang, Dey, & Palmiter, 2004). The metal-response element-binding transcription factor-1 (MTF-1), the only zinc-sensing transcription factor in vertebrates (Kambe et al., 2015), controls the regulation mechanism of ZnT1 (Lonnerdal, 1989).

II.8.1 Interactions of Iron and Zinc

Divalent metal ions, such as iron, can compete with zinc for mucosal cell binding sites and transporters, such as DMT1, but the significance of this interaction on zinc balance is questioned (Holt et al., 2012). The first descriptions of zinc deficiency included signs of anemia (Ananda S. Prasad et al., 1961), probably due to combined iron deficiency or the specific effect of zinc on red cell maturation (Olivares et al., 2007). The mechanisms by which zinc deficiency can induce anemia include a decrease in erythroid precursors, a decreased plasma erythropoietin level, and shortened erythrocyte life span due to oxidative stress (Olivares et al., 2007).

The inhibitory effect of zinc on iron absorption was studied in human showing conflicting results. In aqueous solution, the negative interactions was confirmed (Crofton et al., 1989; Rossander-Hulten, Brune, Sandstrom, Lonnerdal, & Hallberg, 1991). When iron and zinc are given in aqueous solution or in simple food matrix, zinc absorption decreases in a dose-dependent way when Fe:Zn molar ratio is >2:1 (Whittaker, 1998). The dose-response impact of zinc supplementation on iron absorption was confirmed in adults in a recent review (Olivares, Pizarro, Ruz, & de Romana, 2012). A decrease of iron absorption was always detected, except in pregnant women.

On more complex food matrices though, a decrease in iron absorption was detected only when Indonesian children received zinc sulphate and ferrous sulphate (Herman et al., 2002). The rest of the 15 studies involved in this review did not show any effect in hamburger meals, milk, wheat flours or ready-to-eat cereals (Olivares et al., 2012). In wheat flour cofortified with zinc, zinc sulfate was shown to decrease iron absorption in children, but not zinc oxide

(Herman et al., 2002). A high intake of zinc from formulas does not interfere with iron absorption in premature infants but high doses of zinc given between feeds inhibits erythrocyte incorporation (Friel et al., 1998). Studies involving formula-fed infants, experimental zinc-depletion diets and pregnant women taking prenatal supplements containing high iron showed growth delay for infants, and a decreased circulating zinc pool in all age groups, suggesting a determinant impact of excessively high Fe:Zn ratio in the diet (Solomons, 1986).

Inhibitory effect in complex food matrices is seen only at Fe:Zn wt/wt ration > 25:1 and therefore fortification of foods with iron at current fortification amounts has no adverse effect on zinc absorption (Whittaker, 1998). Combined iron and zinc supplement was as effective as iron supplements to control iron deficiency and anemia in Vietnamese infants (Berger et al., 2006). In contrast, the same dose and duration showed that the combined iron-zinc supplementation was less effective than iron alone in improvement of iron status in Indonesian infants (Lind et al., 2004). The discrepancy between those studies might be due to the severity of anemia and iron deficiency, which was greater in Vietnamese children compared to Indonesian children (Zimmermann, 2007). In children of less than 5 yo, joint iron and zinc supplementation has less of an effect on biochemical or functional outcomes than does supplementation with one of them alone. There is therefore no strong evidence to discourage joint supplementation (Fischer Walker, Kordas, Stoltzfus, & Black, 2005).

In rat pups, the effect of zinc supplementation on iron absorption was shown to be age-dependent, as the negative effects were seen only during early infancy and associated with increased small intestine iron retention, decrease hephaestin and increased ferroportin-1 gene expression. The reduced hephaestin levels are the reason of iron retention (Kelleher & Lonnerdal, 2006).

The mechanisms of interactions were studied in caco-2 cells where DMT1 was shown to be predominantly an iron transporter, with low affinity for zinc and that zinc upregulates DMT1 protein expression. (Yamaji et al., 2001). Iron treatment (6 µmol/L Fe2+) reduced DMT1 expression by 50% in caco-2 cells, and decreased cellular uptake of iron and zinc, while transepithelial movement of zinc were not reduced and the gene expression of DMT1 did not correlate with functional responses (Tallkvist, Bowlus, & Lonnerdal, 2000). Hepcidin reduces intestinal zinc export by post-translationally downregulating ZnT1 (Hennigar & McClung,

2016). A model of iron-uptake in caco-2 cells, comprising DMT-1 and an unknown putative transporter was derived by Iyengar and coworkers, and verified by immunoblotting. Cellular zinc status therefore influences iron uptake and interaction with zinc, while DMT-1 might not simultaneously transport iron and zinc (Iyengar, Pullakhandam, & Nair, 2009). Moreover, incubation of caco-2 cells with increasing zinc doses significantly decreases iron uptake (Arredondo, Martinez, Nunez, Ruz, & Olivares, 2006) while this dose-dependent inhibition of iron absorption by zinc was confirmed in 2009 (Iyengar et al., 2009).

The low affinity of DMT-1 to zinc ions in caco-2 cells indicates that zinc competition for DMT1 cannot be the main mechanisms for the interactions between zinc and iron. However, zinc is capable of regulating DMT-1 functioning (Bjorklund et al., 2017). Moreover, Ferroportin (Fpn) the main basolateral exporter of iron, possesses affinity to zinc (Mitchell, Shawki, Ganz, Nemeth, & Mackenzie, 2014) and the transepithelial transfer of iron is stimulated during zinc supplementation due to an increase of Fpn mRNA (Yamaji et al., 2001). Hepcidin, a systemic regulator of iron homeostasis, is affected by zinc. More specifically, zinc treatment increases hepcidin expression through the activation of MTF-1 (Balesaria, Ramesh, McArdle, Bayele, & Srai, 2010).

The critical role of Fpn in the iron-absorptive pathway and therefore iron homeostasis was discovered through the inactivation of ferroportin in the intestine of mice (Donovan et al., 2005). As the only known mammalian iron exporter (Ganz, 2005), it plays an essential role in the entry of iron into plasma (Donovan et al., 2005). Hepcidin, by binding to Fpn, induces its internalization and degradation, and regulates the plasma-membrane expression of Fpn (Nemeth et al., 2004).

Zinc was shown to activate FPN1 transcription through the Metal transcription Factor-1 (MTF-1) while Fpn transports zinc and therefore protects cells from zinc toxicity (Balesaria et al., 2010; Troadec, Ward, Lo, Kaplan, & De Domenico, 2010). Fpn, as an iron-preferring cellular metal-efflux transporter, has a narrow substrate profile that includes zinc, as its expression stimulated efflux of zinc in Xenopus oocytes (Mitchell et al., 2014). Hepcidin is also known to attenuate zinc transport in human intestinal Caco-2 cells as labile zinc decreases and ZnT1 protein was reduced by 75% in hepcidin-treated cells compared to control cells (Hennigar & McClung, 2016). Nevertheless, in mice, even if hepcidin administration resulted in a higher

hypoferremia, no effect on PZC was found and therefore no evidence that Fpn plays an important role in zinc homeostasis (Mitchell et al., 2014).

A recent review tried to clarify the role of cellular zinc status in determination of iron and zinc interactions (Knez, Graham, Welch, & Stangoulis, 2017). DMT-1 seems not to be the site of negative interactions between both minerals, while ZIP14 is an iron and zinc transporter (Iyengar, Pullakhandam, & Nair, 2012). Zinc controls expression of DMT-1 and Fpn, as well as other ZIP and ZnTs. In addition, hepcidin is coordinated by intracellular zinc levels through MTF-1.

II.9 Toxicity

Exposure to zinc can occur through the skin, by inhalation or by ingestion. Even though intoxication by excessive exposure is rare, via the inhalation of zinc-containing smoke, metal fume fever is the most common form of zinc toxicities. Effects are normally transient and signs disappear after some days (Plum, Rink, & Haase, 2010). One fatal outcome from zinc toxicity was reported in a 72 yo women who was accidentally given 1.5 g of zinc intravenously over 3 days. Zinc intoxication was confirmed by a PZC of 420 µg/dL (Brocks, Reid, & Glazer, 1977).

Most reports of zinc toxicity from food were reported to result from storage of food in galvanized containers, and sufficient zinc leached from coating to cause toxic manifestations (M. A. Brown, Thom, Orth, Cova, & Juarez, 1964). From zinc intake naturally occurring in food, no evidence of adverse effects is expected. Zinc has a low toxicity (K. H. Brown et al., 2001) but in case of severe gestational poisoning, adverse effects associated are non-specific (Brocks et al., 1977) such as: the suppression of the immune system, a decrease copper absorption, diarrhea, fever and lethargy. Doses of 225–450 mg of zinc have been estimated to cause vomiting, abdominal cramps, acute epigastric pain, nausea while GI distress has been reported at doses of 50–150 mg/day. Intake of 300 mg/day of supplemental zinc for 6 weeks has been shown to cause impaired immune function. It has been suggested that zinc intake may positively affect vitamin A status, but only in individuals with moderate to severe protein-energy malnutrition. (*Dietary Reference Intakes: The Essential Guide to Nutrient Requirements*, 2006; Fosmire, 1990; Plum et al., 2010).

The main side effect of long-term excessive ingestion of zinc supplements is a decrease copper absorption (Holt et al., 2012), and therefore secondary copper deficiency (A. S. Prasad,

Brewer, Schoomaker, & Rabbani, 1978), most likely due to the competitive interaction during intestinal absorption and therefore increase excretion (Festa, Anderson, Dowdy, & Ellersieck, 1985). A decline in erythrocyte copper-zinc superoxide dismutase activity indicates the influence on cupper status, even at dose as low as 25 mg of zinc per day during 6 weeks (Fischer, Giroux, & L'Abbe, 1984).

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III Assessment of Zinc Status

The assessment of zinc status is crucial in determining the need for zinc interventions programs and assessing their impact on health of population at risk of deficiency. Any biological specimen that indicates nutritional status, with respect to either intake or metabolism, can be a nutritional biomarker. Their interpretation can be placed into perspective as a biologic consequence of dietary intake or patterns (Potischman & Freudenheim, 2003). The lack of a consensus on appropriate biomarkers for evaluation of zinc status has made research in this field challenging. The reasons for the paucity of adequate biomarkers include the small decrements in tissue zinc that are associated with zinc deficiency, the effectiveness of homeostatic mechanisms and the widespread dependence on zinc of so many aspects of human biology (M. Hambidge, 2003). Only one biomarker, namely Plasma Zinc Concentration (PZC), is currently recommended by IZiNCG (Hess, Peerson, King, & Brown, 2007), WHO/IAEA/UNICEF (de Benoist, Darnton-Hill, Davidsson, Fontaine, & Hotz, 2007), the BOND committee (King et al., 2016), and a meta-analysis done on potential biomarkers (Lowe, Fekete, & Decsi, 2009). In this chapter, the three most used biomarkers, namely PZC, functional bioindicators and dietary intake, as well as potential biomarkers, such as hair, nail, urinary zinc, zinc transporters measurements and DNA integrity are discussed. The relative strengths and weaknesses of all biomarkers mentioned in this study is summarized in Table 7.

III.1 Plasma/Serum Zinc

Plasma zinc is considered the best available biomarker of the risk of zinc deficiency in populations by WHO/UNICEF/IAEA/IZiNCG (de Benoist et al., 2007). Assessment of a population is used to evaluate large-scale interventions. As such, the results of a population biomarker does not need to be valid for a particular individual. It is currently the most commonly used biomarker in supplementation and depletion studies (Lowe et al., 2009) but, as a population biomarker, cannot provide certainty with regard to individuals correct zinc status (Hess et al., 2007). Both plasma and serum zinc are considered valid estimates and interchangeable (English & Hambidge, 1988) and the term “plasma” will be used in this thesis.

When a high percentage of individuals in representative sample of the population of interest have low PZC, an elevated risk of zinc deficiency occurring within the population can be

concluded (Hess et al., 2007; International Zinc Nutrition Consultative Group et al., 2004; King, 1990). Reference ranges for PZC were developed from a nationally representative sample of Americans (Pilch & Senti, 1985). For apparently healthy individuals, these were reviewed during the re-examination of the survey (Hotz, Peerson, & Brown, 2003). In adults, fasting state (morning fasting vs non fasting) increases PZC by 6%, time of day (morning vs afternoon) increases PZC by 10% while significant differences are introduced by age, sex and use of oral contraception (Hotz et al., 2003). PZC general cut-offs can be seen in Table 4, while generally healthy individuals PZC reference ranges can be seen in Table 5.

As PZC is ~50 times lower than the concentration of zinc in tissues, small differences in uptake or release zinc from muscles or liver sites can have a profound effect on PZC (Brown, 1998). Fasting PZC are maintained homeostatically within a tight range of 80 to 100 µg/dL (Hess et al., 2007; Tapiero & Tew, 2003), but PZC can vary by as much as 20% during a 24-hour period (the lowest value being at night, 4 hrs after the last meal and the highest fasting in the morning) (K. M. Hambidge, Goodall, Stall, & Pritts, 1989). There are normal circadian variations on PZC in generally healthy subjects, as well as a circadian rhythm observed in PZC in the case of fasting adults (Guillard, Piriou, Gombert, & Reiss, 1979), suggesting independence of variations from food intake. As serum glucose is related to PZC, meal consumption results in a decrease in PZC, and fasting in an increased PZC (Wallock, King, Hambidge, English-Westcott, & Pritts, 1993). Even if PZC is transiently elevated in case of acute starvation (Henry & Elmes, 1975), it diminishes within a fairly short period of time in individuals consuming a severely restricted zinc diet (Baer & King, 1984).

Several factors affect PZC, such as inflammation, age, sex, low serum albumin, elevated white blood cell counts, pregnancy, lactation, organ failure, surgery, intestinal disease and use of oral contraceptives or hormones (Brown, 1998; International Zinc Nutrition Consultative Group et al., 2004). Plasma zinc was shown to be significantly higher in older women (Kim, Paik, Joung, Woodhouse, & King, 2011), while active exercise can influence PZC (Lukaski, Bolonchuk, Klevay, Milne, & Sandstead, 1984). PZC is reduced during pregnancy, as a result of a normal physiologic adjustment (Swanson & King, 1983).

Inflammation effects on PZC were studied in different community-based surveys, in adults and children. C-Reactive Protein (CRP), an acute-phase inflammation protein, is the most extensively assessed inflammatory marker in prognostic studies, as its half-life is 19 hours and

it is neither consumed nor produced during the inflammation reaction (Biasucci, 2004; Bresnahan & Tanumihardjo, 2014). Currently, a cut-off of 10 mg/l is used, even if healthy subjects tend to have a normal CRP concentration below 5 mg/l. CRP values between 5 and 10 mg/l indicate mild inflammation (Thurnham, Mburu, Mwaniki, & Wagt, 2007). Alpha-1-acid glycoprotein is another inflammation protein. In adults, the reduction in PZC associated with inflammation was in average 10%, but varied from 17% in early convalescence to 6% in late convalescence (Mburu, Thurnham, Mwaniki, Muniu, & Alumasa, 2010). In infants, PZC is lowered by 11% if CRP is elevated, 8.3% if AGP is elevated and 11.6% if both are elevated (Wieringa, Dijkhuizen, West, Northrop-Clewes, & Muhilal, 2002). This was not confirmed in Peruvian children from 10 to 19 months, whose PZC were similar with or without infection (P = 0.16, 45.78 ± 15.04 vs 49.05 ± 13.08 µg/dL) (Brown et al., 1993). Infections in children in field settings might be less severe than in infections related studies in adults and the mean PZC of children might therefore be a useful indicator of population zinc status, despite the high prevalence of infections seen in those settings (Brown, 1998).

Because of the potential contaminations from the environment, proper collection, storage and processing of the sample is of extreme importance. Standardized procedures were already described in detail (International Zinc Nutrition Consultative Group, 2007; International Zinc Nutrition Consultative Group et al., 2004). In short, subject should remain in a seated position during the blood drawing procedure, as changes in intravascular pressure at the time of blood drawing may cause variations in PZC. Ethylenediaminetetraacetic acid (EDTA) being a potential contaminant (Pineau, Guillard, Chappuis, Arnaud, & Zawislak, 1993), trace-element free tubes should be used for blood collection. Time of blood collection and fasting status of all subjects should be recorded. In order to avoid an increase in PZC prior to removing the cells, samples should be kept cold directly after withdrawal and prior to separation (Tamura, Johnston, Freeberg, Perkins, & Goldenberg, 1994). A standardized clotting of 40 min is recommended before centrifugation at 3000 g for 10-15 min, to remove all blood cells. As they contain higher concentrations of zinc, separation time is crucial (International Zinc Nutrition Consultative Group, 2007). After separation, plasma should be kept frozen at -25°C or lower. PZC can then be measured further by AAS, either after dilution in water, aqueous acid solution or organic acids. This dilution reduces the viscosity of the samples, minimizing the matrix effect (King et al., 2016). Viscosity of the standards can also be

adjusted with glycerol (5/95 %v) (Smith, Butrimovitz, & Purdy, 1979). PZC are then expressed in either µg/dL or µmol/L (1 µg/dL being equal to 6.54 µmol/L).

The usefulness of PZC as a biomarker of severe zinc deficiency and its clear relationship with clinical signs of zinc deficiency was demonstrated in individuals following a restricted zinc diet and individuals suffering from ADE. PZC predicted clinical signs with ~80% sensitivity and ~90% specificity when using a cutoff of 50 mg/dL for both population (Wessells, King, & Brown, 2014).

The changes in total body zinc content during depletion was studied in 2004, using metabolic balance techniques (Lowe et al., 2004). A correlation between the change in total body zinc concentration and changes in PZC was found ($r^2 = 0.826$, $P < 0.001$). During short-term severe depletion, and in the absence of confounding factors, changes in PZC accurately reflect changes in whole-body zinc status. In general, severe dietary zinc restriction (< 1mg/day) in generally healthy adults produces a rapid decline in PZC, while moderate dietary zinc intake (3-5 mg/d) reduces PZC if the diet is poorly bioavailable or continued for months (de Benoist et al., 2007).

The response to supplementation of PZC as a population biomarker has been reviewed in different meta-analyses, linking zinc intake with changes in PZC and highlighting the difficulties in finding high quality studies measuring PZC. In 2007, clear evidence was found that individual and population average PZC increases during zinc supplementation, regardless of the initial PZC and that PZC responds rapidly to severe dietary zinc restriction (Hess et al., 2007). The appropriateness of PZC for population zinc status was highlighted in 2008 (Gibson, Hess, Hotz, & Brown, 2008). In 2009, the significant response of PZC to dietary zinc intake was shown (with a heterogeneity of 94%) in adults, elderly, pregnant and lactating women, men, women and mixed sex groups (Lowe et al., 2009). Only two studies with high baseline status were included in this meta-analysis, and none of them responded to an increase in zinc intake. In generally healthy children aged 1-17 years, a pooled analysis showed that a doubling of zinc intake increases PZC by around 10% (Moran et al., 2012), with an overall pooled beta-coefficient of 0.12 (0.04, 0.20) and a heterogeneity of 97.6%. In adults, the estimated effect of zinc supplementation on PZC was 0.08 (0.05, 0.11, $I^2 = 85.5\%$, $P < 0.01$). For every doubling in zinc intake, the difference of PZC is 6% in adults (Lowe et al., 2012). A very recent meta-analysis (Shah, Sachdev, Gera, De-Regil, & Pena-Rosas, 2016) showed that food zinc fortification

increases PZC in comparison with unfortified food, with a mean difference of 13 µg/dL (8.2, 19.6), in only low quality evidence, while their risk of underweight and stunting are similar. In food fortified with multiple micronutrient, no effect was seen on PZC.

Table 4 Suggested lower cutoffs of PZC, by age, group, sex and fasting status. From (International Zinc Nutrition Consultative Group et al., 2004; Pilch & Senti, 1985)

Fasting status	Children < 10 yo	Females ≥ 10 yo	Males ≥ 10 yo
Morning, fasting	-	70	74
Morning, non-fasting	65	66	70
Afternoon	57	59	61

Table 5 Lower reference range of PZC based on age group, sex, fasting status and use or contraceptive for females. From (Hotz et al., 2003)

Population	Time and fasting state	Male [µg/dL]	Female [µg/dL]	Female oral contraceptive [µg/dL]	Pregnancy 1st trimester [µg/dL]	Pregnancy 2nd, 3rd trimesters [µg/dL]
Children < 10 years	Morning fasting	-	6	-	-	-
	Morning non-fasting	65 ± 0.7	64 ± 0.8	-	-	-
	Afternoon non-fasting	56 ± 0.7	57 ± 0.7	-	-	-
Adolescent, Adults 10-64 years	Morning fasting	74 ± 0.5	70 ± 0.4	65	56	50
	Morning non-fasting	70 ± 0.6	66 ± 0.4	61	56	50
	Afternoon non-fasting	61 ± 0.4	59 ± 0.4	57	56	50
	Evening	-	-	53	-	-
Elderly > 55 years	Morning fasting	72 ± 0.8	70 ± 0.4	-	-	-
	Morning non-fasting	61 ± 1.0	66 ± 0.4	-	-	-
	Afternoon non-fasting	56 ± 1.0	59 ± 0.4	-	-	-

III.2 Functional Indicators

Stunting or Height For Age Z score (HAZ) is recommended as a functional indicator of zinc inadequacy (King et al., 2016). A z-score represents the deviation of an individual's value from the median value of a reference population, divided by the standard deviation of the reference population (de Onis & Blössner, 1997). Morbidity from diarrhea and respiratory infections are two other potential functional responses to correction of zinc deficiency (Fischer Walker & Black, 2007). As those measurements lack high degree of sensitivity, and reflect changes within specific biological systems, they are commonly referred to as bioindicators and should be always used in conjunction with other biomarkers (Raiten et al., 2015).

An impaired linear growth suggests inadequate intakes or exposure to zinc, while weight is impacted by height (King et al., 2016). As other factors may limit growth as well, a lack of growth with supplemental zinc does not rule out zinc deficiency (King et al., 2016). Linear growth is nevertheless a recommended bioindicator, as there are standardized methods for measurement of growth as well as available reference data (De Onis, Onyango, Borghi, Siyam, & Pinol, 2006). A prevalence of low HAZ higher than 20% is considered indicative of an elevated risk of zinc deficiency (de Benoist et al., 2007).

The effect of zinc supplementation and dietary zinc intake on bioindicators was evaluated in different meta-analyses. In infants, zinc supplementation increases growth parameters, with a heterogeneity of 45%, but does not affect HAZ (Nissensohn et al., 2016). During supplementation studies of maximum 20 weeks, zinc intake was positively associated with duration (β = 0.01; CI 95% 0 to 0.02), but no effect was found during a trial of more than 20 weeks (β =- 0.01; CI 95% -0.003 to 0.0002).

Zinc supplementation was shown to produce significant increments in height (effect size of 0.350 (0.189, 0.511)) in prepubertal children (Brown, Peerson, Rivera, & Allen, 2002). In children aged 1-8 years, no significant effect of zinc supplementation was found on weight gain, HAZ, or weight for height (Stammers et al., 2015). All studies included in this latter meta-analysis had low initial HAZ. HAZ is negatively related to cognition, while diarrhea is not (Fischer Walker et al., 2012). No pharmacological effects of zinc on growth in zinc-replete individuals is expected (King et al., 2016).

A meta-analysis revealed that zinc supplementation significantly reduces the occurrence of diarrhea episodes by 15% (RR: 0.86 (0.79 – 0.93)) in children receiving at least 4 weeks of zinc supplementation, while a significant 8% reduction was seen in the occurrence of respiratory illness (RR: 0.92 (0.85-0.99)) (Aggarwal, Sentz, & Miller, 2007). Stunting is currently under investigation and nutritional interventions to prevent stunting in children below 5 yo in slums is currently being looked at by the COCHRANE database (Goudet, Griffiths, Bogin, & Madise, 2015).

In the latest COCHRANE review (Mayo-Wilson et al., 2014) assessing zinc supplementation for prevention of morbidity, mortality and growth failure in children from 6 months to 12 years, zinc supplementation did not have a significant effect on all cause of mortality (RR 0.95 (0.86 1.05), I^2 = 0%). There was a 13% reduction in the incidence of diarrhea (RR = 0.87 (0.85-

0.89), I^2 = 88%) with supplementation. There was no effect on respiratory tract infection incidence (RR = 1.00 (0.94, 1.07), I^2 = 1%) and the dose, duration, formulation or age have no effect on morbidity, mortality or growth. Zinc supplementation was associated with a significant small increase in height, while age, dose and duration were significantly different. The heterogeneity of this finding was 86%. Shah et al concluded that fortification of foods with zinc makes little or no difference to the incidence of stunting in children (Shah et al., 2016)

In children older than six months with acute diarrhea, oral zinc supplementation (10-20 mg zinc/day) shortens the duration of diarrhea by around 10 hours (10.44 hrs, 95%CI -21.13 to 0.25) (Lazzerini & Ronfani, 2016). Oral zinc supplementation probably reduces the number of children whose diarrhea persists for one week. In a subgroup analysis focusing on children with signs of malnutrition, this effect is greater, as the duration of diarrhea is reduced by more than 2 days (-26.98 hrs, 95% CI -14.62 to -39.34, high quality evidence). In infants of less than 5 months, oral zinc supplementation has no effect on diarrhea duration (Brooks et al., 2005), but the mean duration of the diarrhea episode was not significantly longer among infants receiving oral zinc supplementation (Fischer Walker et al., 2006).

III.3 Dietary Zinc Intake

Dietary zinc intake provides only an estimate of zinc exposure (Gibson, 2012), as day-to-day variation in food selection and errors in estimating the total quantity of food consumed is only an approximation of an individual's usual zinc intake (King et al., 2016).

At households and individuals levels, diet history, food frequency questionnaires, 24-hr recall, 1-day weighed food records were extensively described in 2005 (Gibson, 2005). On an individual level, an estimate of the variability of the requirement and intake requiring multiple 24-hr recalls or daily food records can be implemented (Institute of Medicine (US) Panel on Micronutrients, 2001). In general, at a population level, the same protocols must be followed, but the distribution of observed zinc intakes need to be adjusted to represent usual zinc intakes by removing the variability introduced by day-to-day variation in an individual (International Zinc Nutrition Consultative Group, 2008).

Table 6 Average requirements and population reference intakes for zinc. Data assembled from (World Health Organization (WHO), Food and Agriculture Organization (FAO), & International Atomic Energy Association (IAEA), 2004). The interindividual variation of requirement is assumed to be 25% and infants are assumed to be exclusively breastfed.

Population	Timeframe	Body Weight [kg]	High bioavailability	Moderate bioavailability	Low bioavailability
Children	0-6 months	6	1.1	2.8	6.6
	7-12 months	9	2.5	4.1	8.4
	1-3 years	12	2.4	4.1	8.3
	4-6 years	17	2.9	4.8	9.6
	7-9 years	25	3.3	5.6	11.2
Adolescents	Female	47	4.3	7.2	14.4
	Males	49	5.1	8.6	17.1
Male Adults	From 19 years	65	4.2	7.0	14.0
Female Adults	From 19 years	55	3.0	4.9	9.8
Pregnant Women	1st trimester	-	3.4	5.5	11
	2nd trimester	-	4.2	7.0	14
	3rd trimester	-	6.0	10.0	20.0
Lactating Women	0-3 months	-	5.8	9.5	19
	3-6 months	-	5.3	8.8	17.5
	6-12 months	-	4.3	7.2	14.4

Dietary assessment require the quantitative estimation of zinc intake and appraisal of the likely absorption of zinc from local diet (Gibson, 1994). The percentage of the population being below the recommended intake is then a sign of a possible risk of zinc deficiency. As explained

in chapter II, the PA:Zn molar ratio of specific food items or whole diet is used as an estimate of the bioavailability of zinc (FAO/WHO Expert Consultation on Human Vitamin and Mineral Requirements, 2004), and therefore, recommended daily intake need to take this into consideration, as seen in Table 6. The proportion of a population at risk of inadequate zinc intake is then estimated as the prevalence of usual zinc intakes below the EAR. If databases information are unavailable for specific food items, then duplicate diet composites can be collected from each individual with some repeats on a subsample of the population for analysis of zinc and PA (International Zinc Nutrition Consultative Group, 2008; King et al., 2016).

III.4 Hair Zinc Concentration

The BOND committee graded hair zinc concentration as a potential biomarker in 2016 (King et al., 2016). The main advantages in using hair is that the zinc concentrations are expected to be high, and therefore easy to measure. The collection, transport and storage can be done at room temperature, without degradation of the specimen. Zinc concentration should not be subject to rapid variation. Hair and nails are formed in a short time after which they are expelled from the surface of the body. They therefore become isolated from its further metabolic activities (Hopps, 1977). Consequently, the concentration of hair in a specific trace element reflect only the events occurring during the time of its formation. Even though there is human hair material available as references, many studies fail to use standardized methods for sampling and washing (King et al., 2016).

Following standard processes for sampling and washing is essential. It is preliminary necessary to remove under a microscope nits and lice before washing the hair (King et al., 2016). As contamination from exogenous sources is a major issue (Kempson, Skinner, & Kirkbride, 2007), washing should be done with nonionic detergents, in order to not leach out bound zinc from the hair, while removing superficially adsorbed zinc (Assarian & Oberleas, 1977). While hair zinc concentration was found to be stable on the position of the head (Trunova, Parshina, & Kondratyev, 2003), only 1-1.5 cm should be retained for analysis, to represent the state of nutrition of an individuals over the past couple of months. Hair can then be dissolved in acid, before measurement by AAS.

It was shown in 2009 in a meta-analysis (Lowe et al., 2009) that the concentration of hair zinc was significantly elevated after supplementation (WMD: 13.24 ppm, 95% CI: 11.9, 14.56;

I^2 = 0%). Unfortunately, only three studies (Barrie, Wright, Pizzorno, Kutter, & Barron, 1987; Medeiros, Mazhar, & Brunett, 1987; Shaaban et al., 2005) were included in this review, and it is not certain whether hair zinc reflect zinc status in individuals who are zinc deficient.

Until now, no references ranges for interpreting hair zinc concentrations have been established (King et al., 2016) and studies show a variety of results. In a study done in healthy Polish students, average hair zinc concentration is 230 ± 142 mg/kg (Chojnacka, Zielinska, Gorecka, Dobrzanski, & Gorecki, 2010), while in Italian schoolboys, it is 150 mg/kg (Senofonte, Violante, & Caroli, 2000), for example.

Very few studies correlated PZC and hair zinc concentration. In a study done in South Korean in children presenting with malnutrition and poor growth, zinc deficiency was diagnosed in 55% of the children with a low PZC. No correlation between PZC levels and hair zinc level on those 56 children was found (Han, Lee, & Kim, 2016), even though the washing protocol was standardized and each hair was washed with acetone, water, dextran 1% v/v and HNO_3. Moreover, a hair zinc concentration below 16 mg/dL was chosen as being the threshold for deficiency, without explanations. In another study, looking at the effect of zinc supplementation during pregnancy on the newborn, a correlation at birth was studied between all parameters (Shaaban et al., 2005). Mothers received vitamins supplements, containing 10 mg/d of zinc in the supplemented group, or no zinc in the control group. No correlation was found between maternal nail, maternal hair and maternal PZC at time of birth. After 2 months of supplementation, the maternal milk zinc showed a significant positive correlation with infant hair zinc and nail zinc, but not with PZC. These studies clearly show the issue of correlation between hair, nail and PZC.

In Germany, 41 persons took part in a study looking at the zinc scalp hair and zinc pubic hair concentration (Wilhelm, Ohnesorge, & Hotzel, 1990). The first 2-3 cm of their hair was taken and washed. Zinc concentration in scalp and pubic hair were found to be 128.8 (116.4-190.1), 133.3 (105.7-168.1) mg/kg, respectively. Both of them were significantly correlated and higher in female, reaching 152 and 140 mg/kg while men hair was 139 and 116 mg/kg as an average, in scalp hair and pubic hair, respectively. Significant lower values were seen with age, and during the summer for scalp hair, but not for pubic hair. Pubic hair zinc concentration, even though it might be more difficult to sample, would be less prone to contamination, while both types of hair would reflect the same metabolic pool of zinc available for hair growth from the

blood circulation. Some main disadvantages of using hair zinc concentration were highlighted in this study, as the age, sex and season were found to affect it. Moreover, the rate of hair growth might influence hair zinc concentration as well (Bradfield & Hambidge, 1980).

III.5 Nail Zinc Concentration

As nails slowly grow and are not impacted by transient factors, they should be reliable indicators of overall mineral metabolism (Bank et al., 1981). Their use for measurement of zinc concentration has been graded as an emerging biomarker by the BOND committee (King et al., 2016).

One of the first standardized protocols for preparation of nails for trace element analysis was published in 1981 (Bank et al., 1981), but the current main issue in measuring zinc from nail samples is the lack of a sensitive measurement techniques (King et al., 2016). Moreover, a rigorous standard process for sampling and washing nails is essential (King et al., 2016). In general, the collection of nails is tedious, as they first need to be scraped and trimmed with stainless steel material (Bank et al., 1981). As fingernails have a faster growth rate and are less prone to infection (Hussein Were, Njue, Murungi, & Wanjau, 2008), they are normally preferred from toenails.

During washing, exogenous sources of zinc should be removed, first by scraping, and then washing in an aqueous nonionic detergent, followed by rinsing with high purity water and drying (Hussein Were et al., 2008). The detergent used must be effective in releasing cations bound to amphipathic molecules attached to the nail surface (Bank et al., 1981). The amount of nails to be collected depends on the sensitivity of the method that will be furtherly used, but in general, it can be expected that a dry sample of fingernails contain at least 60 ppm of zinc (King et al., 2016).

Nails samples have to undergo pre-treatment, before rapid measurement by AAS. Soaking in non-ionic liquid soap must be performed before washing from metallic debris. A subsequent soaking in acetone, rinsing with distilled water and over drying can then take place. Polished nails need to be first washed in 4-methyl pentan-2-one and then oven dried. Acid digestion followed by AAS analysis is then the recommended method (Hussein Were et al., 2008).

In German children, the normal zinc concentration in toenails and hair is 129 (83 -200) and 118 ± 45 mg/kg (Wilhelm, Hafner, Lombeck, & Ohnesorge, 1991). Even though zinc

concentrations exhibit approximately the same range in both biological media, the correlation between hair and toenails zinc was found to be not significant in this study, while it was for cadmium, copper and lead.

In Kenya, the mean nail concentration in school age children was 122.3 ± 6.9 µg/g (Hussein Were et al., 2008). This was significantly higher in rural areas compared to urban area. Moreover, the results found in those children show that lead levels influence negatively zinc levels (R = −0.256).

In a recent meta-analysis looking at the effect of zinc supplementation on different biomarkers (Lowe et al., 2009), only one study was found to look at nail zinc values (Shaaban et al., 2005). This study was already discussed in the hair zinc chapter. It clearly shows the issue in determination of hair and nail zinc, enhancing the need for further studies.

A possible new field-portable instrument, called Laser-Induced Breakdown Spectroscopy (LIBS) has the potential to determine zinc concentration accurately in the mid-nanogram range (Miziolek, Palleschi, & Schechter, 2006). A clip of nail, or even hair, could be dried directly on field and the LIBS instrument could determine the zinc content of the samples within minutes. Preliminary results obtained in 2017 (Riberdy, Frederickson, & Rehse, 2017) are encouraging, but the concentration of fingernails of malnourished populations is expected to be lower.

III.6 Urinary Zinc Concentration

As 24-hour urinary zinc excretion does not reflect day-to-day variations in zinc intake, it is relatively constant at adequate zinc intake (Sandstrom, 2001). In 2016, it was graded as a potential biomarker by the BOND committee (King et al., 2016).

Renal losses of zinc tend to be low, especially when put into perspective with the amount of zinc lost in the GI tract. Fecal losses vary from 27 to 90 micromol/day, on intakes ranging from 63 to 472 µmol/day while urinary losses range from 8 to 11 micromol/day (King, Shames, & Woodhouse, 2000). Between 0.3 and 0.6 mg/day zinc is excreted in the urine (King et al., 2016), and this can therefore be analyzed by AAS without pre-treatment. The difficulty of collecting a 24-hr sample obviously makes this biomarker more challenging, even though the collection of this potential biomarker is non-invasive.

Urinary zinc excretion is negatively impacted by muscle anabolism and positively impacted by muscle catabolism (Sandstrom, 2001). Some specific amino acids, such as histidine and

cysteine, bind zinc and increase its filterability through the renal system. In 1989, the impact of those 2 amino acids on urinary zinc was studied (Zlotkin, 1989). A significant elevation was found during either a high-dose histidine or a high dose of cysteine in infants. Therefore, the intake of specific amino acids should be determined and a proper evaluation of the complete diet should be assessed when urinary zinc is used as a biomarker of zinc status.

Moreover, zinc depletion studies showed that urinary zinc concentrations decline with time only when less than 3 mg of zinc is consumed per day (Johnson, Hunt, Milne, & Mullen, 1993). In case of a low intake, the rapid changes in endogenous fecal as well as urinary zinc excretion limit the decrease in PZC (King et al., 2000). In countries and set-up where zinc consumed is below the EAR but higher than 3 mg, the sensitivity of urinary zinc as a biomarker is therefore compromised.

Different national cross-sectional studies were performed already, but the lack of proper reference ranges make the evaluation of zinc status via urinary zinc difficult (King et al., 2016). In Canada, through the Canadian Health Measures Survey, urinary zinc was measured from 2009 to 2011 in 2980 subjects, aged between 3 and 79 yo. The reference value for an apparently healthy population was found to be 1100 µg/l (990-1200) (Saravanabhavan et al., 2017). In France, 2000 residents of Northern France were enrolled between 2008 and 2010. The representativeness of the participants was guaranteed by the quota method of the French National Institute of Statistics and Economic Studies. The 95% confidence interval found was 1039 (967-1101) µg/l, significantly higher for men than women, and significantly higher for smokers than non-smokers (1176 (1065-1302) and 955 (870-1063) µg/l respectively) (Nisse et al., 2017).

In a recent meta-analysis, a significant effect of zinc intake was seen on urinary zinc excretion during supplementation with more than 15 mg zinc/day (WMD: 0.31 mmol/mol creatinine; 95% CI: 0.20, 0.43 I^2 = 0%) as well as depletion studies (WMD: 3.89 µmol/d; 95% CI: 1.01, 6.76; I^2 = 93%) in adults and elderly (Lowe et al., 2009). All the included studies used zinc gluconate as a supplement. The effect of dietary zinc on urinary zinc concentrations was not yet studied in children and infants (King et al., 2016). Urinary zinc could therefore be a marker of dietary zinc increase in adults, providing that the prevalence of zinc deficiency can be first measured in the population (Wieringa, Dijkhuizen, Fiorentino, Laillou, & Berger, 2015). Urinary zinc excretion can provide useful information on zinc status in zinc-supplemented individuals, but

whether these reflect zinc status in depleted individuals is not certain (Lowe et al., 2009). It might be a good marker of compliance for a zinc supplementation program, if 15 mg zinc/d is given at least (Lowe et al., 2009).

III.7 Zinc-Dependent Proteins

Zinc-dependent proteins were rated as emerging by the BOND committee, while zinc-dependent enzymes were graded as not useful (King et al., 2016), as the link between zinc-dependent enzymes and zinc deficiency signs has not yet been seen (Lowe et al., 2009).

As already explained in previous chapters, zinc is a cofactor for ~3000 proteins. Even if zinc is not the only factor involved in regulating cellular protein expression, some proteins are thought to reflect cellular zinc status (King et al., 2016). The ones presented below, namely MT and Zinc transporter 1 (ZnT1) , are all measured in peripheral blood cells, such as lymphocytes, and measured by Quantitative Polymerase Chain Reaction (Q-PCR) , as described by Zyba (Zyba et al., 2017) and Cousins (Cousins et al., 2003).

Metallothionein was proposed as a potential zinc biomarker as the Metal-Responsive Transcription Factor-1 (MTF-1) is essential for MT gene expression (Heuchel et al., 1994). Moreover, the DNA-binding domain of MTF-1 is composed of six zinc fingers and it is the main activator of MT genes (Gunther, Lindert, & Schaffner, 2012). While it is known that MT has 7 potential sites for binding zinc, its synthesis increases when cellular zinc increases (Krezel & Maret, 2007). The expression of the cellular zinc efflux transporters are believed to be reduced with a decline in cellular zinc. As ZnT1 facilitates zinc transfer from the enterocyte into circulation and its expression is ubiquitous in all tissues, it has been proposed as a potential biomarker of zinc status or exposure (King et al., 2016).

In vitro studies have been successful at showing expression of MT and ZnT1 in GI cell lines. In the latest study, the expression of MT and ZnT1 was significantly higher after 120 min in presence of exogenous zinc sulfate, while it significantly decreases after 120 min of zinc deficiency in Jurkat cells (Holland, Killilea, Shenvi, & King, 2015).

One supplementation study showed that MT responds to marginal zinc intake in humans (Allan et al., 2000), even though a considerable interindividual variation in the MT concentration was found. In this study though, PZC did not respond to the 10-week marginal

intake. More recently, MT gene expression did not change in a depletion-repletion study (Zyba et al., 2017).

A negative correlation between PZC and MT mRNA levels was found in 2006 already, proposing that this transcript cannot be used to predict poor zinc nutrition (Kwon et al., 2007). This cohort study done in Korea showed a correlation of MT transcripts with the gender of the participant, but subjects were not randomly selected, as they were screened depending on their eating habits, to obtain a wide range of PA and zinc intake.

In a study in men who were experiencing acute zinc depletion (<2 mg zinc/d), the expression of ZnT1, declined rapidly in leukocytes and whole blood (Ryu, Langkamp-Henken, Chang, Shankar, & Cousins, 2011). In a recent depletion-repletion study, in which PA was used to reduce the absorption of zinc, gene expression of ZnT1 did not significantly change, even after repletion (Zyba et al., 2017). During dietary zinc depletion, no significant changes in zinc transporters, MT and ZnT1, were found in human erythrocyte membrane (Ryu, Guthrie, Maki, Aydemir, & Cousins, 2012). ZnT1 was as well not affected by taking zinc supplement of 22 mg zinc/day for 27 days (Andree et al., 2004).

In a short 10-d supplementation with 15 mg zinc/day, significant changes in MT transcripts level could be seen from blood collected on collection card as dried blood spots, in lymphocytes, monocytes and granulocytes (Aydemir, Blanchard, & Cousins, 2006). In total RNA from dried blood spots, ZnT1 mRNA levels increased to 2.5-fold compared to control subjects. This study though does not compare the baseline values from endline values after supplementation, but a negative control group with a supplemented group. In buccal cells though, the concentration of MT after 10 days of zinc depletion was significantly lower than at baseline, while this cannot be seen in ZnT1 (Ryu et al., 2011).

III.8 DNA Integrity

DNA integrity, as measured by the COMET olive tail moment (Olive, Wlodek, & Banath, 1991), is a new and emerging biomarker of zinc nutrition (King et al., 2016). Different DNA repair mechanisms are known to involve zinc, as explained in chapter 1. One example of it is the p53 protein, a tumor suppressor, mutated in half of human tumors. In case of loss of zinc, its functions are compromised. This is due to the fact that the only metal in the core domain of p53 is zinc, and p53 can therefore be considered as a metalloprotein (Pavletich, Chambers, & Pabo, 1993). Micronutrient deficiency, and therefore zinc deficiency, can mimic processes

such as radiation or chemicals damaging DNA, causing either simple or double strand breaks as well as oxidative lesions (Ames, 1999). Low zinc intake has an impact on DNA integrity and can contribute to single and double-strand DNA breaks, increasing risk for cancer development. However, its effects on DNA integrity are multilayered, involving an environment with increased oxidative stress and signaling pathways (Ho, 2004). This condition is exacerbated by an inability to adequately signal DNA repair mechanisms, which provides an environment for increased DNA damage (King et al., 2016).

Details protocols on measurement of DNA strand breaks by the COMET assay were already published (Fairbairn, Olive, & O'Neill, 1995; Olive et al., 1991). In short, a small amount of cryopreserved peripheral blood cells is applied onto microscope slides. After unwinding of DNA, slides are subjected to electrophoresis before washing and then stored. Nuclear material are then stained with SYBR green and nuclei are identified for measurement of the olive tail moment (Singh, McCoy, Tice, & Schneider, 1988).

Zinc deficient diets have led to chromosome breaks in rats' liver (Castro, Kaspin, Chen, & Nolker, 1992), while offspring of zinc-deficient rhesus monkeys have increased chromosome breaks. Those damages might be due to an increased rate of oxidative damage and/or a reduction in the rate of DNA repair (Olin et al., 1993). Interactions between zinc deficiency, DNA integrity and therefore DNA repair was confirmed in rats, as severe zinc depletion caused DNA damage in peripheral blood cells while this was normalized by zinc repletion (Song, Leonard, Traber, & Ho, 2009). In vitro studies showed that a high dose of zinc increases DNA damage in lymphocytes harvested from 10 generally healthy non-cancer participants (Harreus, Baumeister, Zieger, & Matthias, 2005).

DNA integrity being a new field of study, the short-term effect of an increased intake was studied only once in a depletion-repletion study and no supplementation study using it as a biomarker has already taken place. Dietary zinc depletion during 6 weeks was significantly associated with an increase in the DNA strand breaks in peripheral blood cells, while those changes were ameliorated by zinc repletion. Moreover, in the depletion period, PZC was shown to be negatively correlated with DNA strand breaks ($r = -0.60$, $P = 0.006$) (Song, Chung, et al., 2009). Those changes were confirmed in a recently published depletion-repletion study, in which DNA strands breaks measured in leukocytes increased during depletion and

decreased during repletion (Zyba et al., 2017). Both those studies suggest that DNA strand breaks are responsive to dietary zinc intake.

III.9 Other Potential Biomarkers: Taste Acuity, Zinc Kinetics, Erythrocyte and Leukocyte Zinc

A total of 32 potential biomarkers were inventoried in 2009 in a meta-analysis of methods used for assessment of zinc status (Lowe et al., 2009). Out of those, only 3 were graded as potential namely, hair zinc, urinary zinc and neurobehavioral functions, 5 as emerging, and 2 as not useful biomarkers by the BOND committee (King et al., 2016), as seen in Table 7. Twenty-four other biomarkers, used at least once in the literature, such as monocyte salivary zinc, exchangeable zinc pool and fecal zinc, for example, were inventoried (Lowe et al., 2009). Their efficiency cannot be calculated though, as not enough studies have been completed. One last promising biomarker was found in 2013, as the plasma proteome was analyzed to identify protein sensitive to changes in dietary zinc levels in humans. During a depletion-repletion study, Fibrin b was shown to increase 2-fold following zinc depletion and decreased to baseline values after repletion (Grider, Wickwire, Ho, Chung, & King, 2013).

Table 7 Relative strengths and weaknesses of biomarkers investigated in this literature review. BOND: Biomarkers of Nutrition for Development (King et al., 2016)

Biomarker	Advantages	Disadvantages	Analytical process	BOND grade
Plasma Zinc	Quick response (5-10 d) to supplementation Established cut-offs	High heterogeneity in supplemented studies Trace-element free material need to be used for collection and processing	Blood collection (2 ml) Plasma separation AAS measurement or ICP-OES, ICP-MS	Biomarker of zinc status or dietary zinc exposure
Functional bioindicators	Non invasive WHO growth standards available Availability of WHO anthro programs Most likely the cheapest No lab work Reasonable cut-off: 20% low HAZ, LAZ	No cut off for elevated risk of deficiency Between examiners errors and spine compresses during the day, so time should be standardized HAZ and WAZ cannot be applied for adults No pharmalogical effect of zinc on growth Morbidity is based on self-recall	Calibrated scale and stediometer for HAZ and LAZ Morbidity is self-recalled Strict adherence to standard procedures is needed	Functional indicator of zinc inadequacy
Dietary assessment	Determine the most important zinc source Determination of zinc bioavailability National food balance sheets can estimate risk of zinc deficiency No lab work required Cut-off	Time consuming data collection Tedious food-composition database search PA must be assessed Zinc fortification of cereals need to be documented Portion size is difficult to estimate	24-hr recall Food Frequency questionnaire 1-d weighed food record	Estimation of zinc exposure
Hair	Non invasive Cheap processing	Lack of standardized washing and processing protocols Lice must be removed before processing No cut-off	Standardized sampling, washing and measurement by AAS after acid digestion	Potential
Nail	Non invasive Cheap processing	Lack of standardized protocols Difficult washing process No cut-off	Standardized sampling, washing and measurement by AAS after acid digestion	Emerging
Urinary	Non invasive	Tedious collection as 24-hr recall needed No cut-off	24-hr collection AAS measurement	Potential
Zinc-dependent proteins	Highly standardized protocol	Expensive collection and processing Requires a molecular laboratory and expensive equipment Can measure only fold increase	Blood collection (2ml) RNA-stabilization Processing by q-RT-PCR	Emerging
DNA integrity	Easy lab work for processing Availability of programs to count under microscope	Long counting time under microscope Units are arbitrary, so no measure of prevalence possible	Blood collection (100 µl) Cryopreservation Electrophoresis Cells counting on microscope	Emerging

III.10 Reference

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IV Biofortification

The most effective intervention to reduce mineral malnutrition is diet diversification, including fruit, vegetables, fish and meat (Gibson, Yeudall, Drost, Mtitimuni, & Cullinan, 1998). Different strategies might include increasing the intake of food with a high content of mineral, of mineral enhancers, or decreasing the intake of mineral inhibitors. This, in general, is more culturally acceptable and sustainable that supplementation and fortification (Gibson et al., 1998) but can be impractical in many countries were such food is not widely available (Gomez-Galera et al., 2010). If infrastructure are present, supplementation, in the form of a tablet or a sachet, or conventional fortification can be applied. Fortification was proven effective at reducing risk of different nutrient deficiency disease, current fortification programs are based on low dietary intakes rather than a diagnosable condition (Dwyer et al., 2015). Moreover, due to poor funding and distribution networks, those strategies have not been largely successful (Nantel & Tontisirin, 2002). As those conventional interventions have limited impacts, biofortification was proposed as an alternative long-term approach. The definition, as well as development of biofortified crops are explained in this chapter.

IV.1 Definition and Development

Biofortification was defined in 2006 as “the development of micronutrient-dense staple crops using traditional breeding practices and modern biotechnology” (Nestel, Bouis, Meenakshi, & Pfeiffer, 2006). Since 2006, the definition of biofortification has evolved, and was simplified, as “the process of breeding nutrients into staple food crops” (Bouis, Saltzman, Low, Ball, & Covic, 2017; Hotz, 2009). Biofortification can be achieved through three different processes: namely 1) conventional breeding, conducted with specific selection of plant genotypes; 2) genetic engineering, employing recombinant DNA techniques and molecular breeding; 3) agronomic practices, such as applications of fertilizers (Hotz, 2009; Saltzman et al., 2013). Biofortification may have long-lasting impacts and is considered to be cost-effective, comparatively inexpensive and a sustainable way to deliver more micronutrients to rural poor population strata, who may have limited access to fortified foods and supplements (Bouis, Hotz, McClafferty, Meenakshi, & Pfeiffer, 2011)

HarvestPlus, part of the Consultative Group for International Agricultural Research (CGIAR), is currently one of the leading players worldwide in the development of biofortified crops. Its

goals are to improve nutrition and public health by developing and promoting crops biofortified in vitamins or minerals (HarvestPlus, 2017). The processes and definition of biofortification currently used by HarvestPlus mentions only conventional breeding strategies and agronomic practices (HarvestPlus, 2017), this most likely due to the issues of the public perception of genetic engineering.

In order for biofortification to be successful, the edible part of the crop has to be enriched with micronutrients. The movement of micronutrient from soil to edible part of the food starts with the uptake of micronutrients from the rhizosphere. In case of zinc, this process has not been not well characterized (Waters & Sankaran, 2011). The transfer from root to shoot happens via xylem transport, and is the rate-limiting step for translocation to seeds (Palmgren et al., 2008). After reaching the shoot xylem, micronutrients are carried to the leaves and seeds covering tissues thanks to transpirational tension. Physical barriers between the plant and the developing seed necessitate phloem transport to the seed tissues. Nutrients moving to the seed are then unloaded from the phloem (Broadley, White, Hammond, Zelko, & Lux, 2007; Raven, Evert, & Eichhorn, 2007; Waters & Sankaran, 2011). This process is shown in Figure 5. Biofortification can target any of those processes, increasing the uptake of a micronutrient, or reducing molecules that will inhibition its bioavailability.

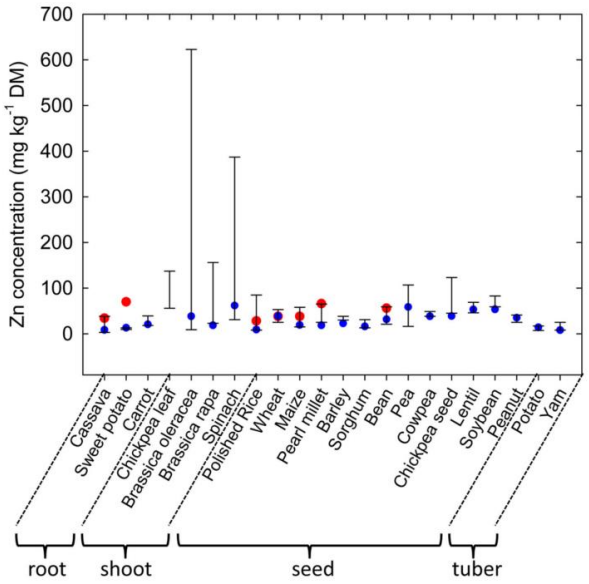

Figure 4 Variation in zinc concentrations in edible parts of crops. Bars represent maximum and minimum values obtained for large collections of cassava, sweet potato, carrot, chickpea, Brassica oleracea, Brassica rapa, spinach, rice, wheat, maize, miller, barley, sorghum, bean, pea, cowpea, chickpea, lentil, soybean, peanut, potato, yam genotypes. Blue circles indicate the theoretical average zinc concentrations. Red circles indicate target zinc concentrations proposed by the HarvestPlus program (Bouis and Welch, 2010). Taken from (White & Broadley, 2011)

IV.1.1 Conventional Breeding

The first step in conventional breeding is to determine whether sufficient genetic variation exist to breed for a particular trait of interest (Bouis et al., 2011). Parents lines with high micronutrient levels are then crossed for several generations, producing plants having the desired nutrient and agronomic traits (Saltzman et al., 2013). Another possibility in conventional breeding is to breed parents crops to remove an undesirable trait, such as a high PA concentration. Conventional breeding is normally phenotype-driven, selections being based on visible traits. The strategy used during breeding is called backcrossing, a process during which lines showing favorable phenotypes are crossed back to the one parents to regain some of the parent's superior phenotypes for other traits (Rocheford et al., 2014). In a recent review, the genetic variation in zinc of edible crops was analyzed (White & Broadley, 2011), as seen in Figure 4. The achievements already done in conventional breeding suggest that the breeding potential to increase zinc concentration for rice, maize, wheat and pearl millet is adequate (Hotz, 2009).

One example of a successful biofortified crop produced by conventional breeding is the Orange-Fleshed Sweet Potato (OFSP) , from the International Potato Center, part of the CGIAR (International Potato Center, 2017). Provitamin A from OFSP was shown to be highly bioavailable and its consumption increases vitamin A body stores (Haskell et al., 2004; Low et al., 2007).

IV.1.2 Genetic Engineering

Transgenic approaches are used when the genetic variability for a given micronutrient is too low to meet the desired target level, in the case of a very difficult to breed crop (Saltzman et al., 2013) or when conventional breeding is not applicable as a new trait needs to be introduced in a crop (Beyer, 2010). This approach can enable greater enhancements, but regulatory and safety concerns need to be addressed before it becomes widely available (Hotz, 2009). In general, transgenic approaches are used to improve the ability to translocate micronutrient to phloem, in plants for which this is a limiting factor (White & Broadley, 2011).

The current examples of genetically biofortified crops are golden rice, in which the level of provitamin A reaches more than 30 ppm (Ye et al., 2000), or the BioCassava Plus program, aiming at increasing levels of provitamin A and micronutrients in high-yield and resistant cassava (Sayre et al., 2011).

As of today, none of the transgenic biofortified crops is approved for cultivation, this most likely due partly to the regulatory barriers associated with such technologies (De Steur, Mehta, Gellynck, & Finkelstein, 2017). Moreover, golden rice is to date the only transgenic biofortified crop that underwent a randomized control trial in humans (clinicaltrials.com, NCT00680355), showing promising results (Tang, Qin, Dolnikowski, Russell, & Grusak, 2009). The only way to examine the potential benefits of other genetically modified crops on health of human populations has therefore been only through indirect assessments of dietary intake of micronutrients.

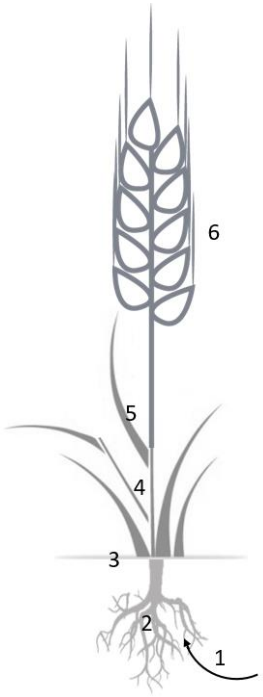

Figure 5 Model of location of Zinc uptake, showing the translocation steps to the seed : 1) uptake from the rhizosphere, 2) xylem loading in the roots, 2) root-to-shoot transfer, 4) distribution to the leaves, 5) xylem-to-phloem loading, 6) loading into the seed Translocation steps. Modified from (Waters & Sankaran, 2011)

IV.1.3 Agronomic Practices

Different micronutrient concentrations can be enhanced via the application of fertilizers, such as selenium (de Figueiredo et al., 2017), iodine (Smolen et al., 2016) or zinc (Palmgren et al., 2008). Fertilizers, as explained in chapter V.5.1, can be applied either on the soil or on the leaf, by foliar application. The downside of foliar application is that fertilizers can easily be washed off by rain and are more costly and difficult to apply (de Valença, Bake, Brouwer, & Giller, 2017).

It was shown in 1999 that the zinc concentration in many soils are sufficient to support the growth of mineral-dense crops (Graham, Senadhira, Beebe, Iglesias, & Monasterio, 1999). However, root acquisition, phytoavailability (the bioavailability in the plant) and transfer from xylem to phloem limit the uptake of zinc by plants (Broadley et al., 2007). Moreover, zinc is both an essential and a toxic element for cereals (Palmgren et al., 2008). In plants hyperaccumulating zinc, the formation of zinc-complexes and the translocation of zinc from root to shoot prevent the accumulation of toxic zinc concentration in root tissues (Broadley et al., 2007). In cereals, the xylem is discontinuous at the base of each seed, and therefore minerals must be transferred from xylem to phloem before entering the grain. The translocation of zinc from leaves contributes more to total zinc allocated to cereal grains than concurrent zinc uptake during grain filling (jan Stomph, Jiang, & Struik, 2009). In wheat, the retranslocation from leaves is important for zinc allocation to the grains (Pearson & Rengel, 1995), while this is not the case in rice (jan Stomph et al., 2009). One way of overpassing the xylem to phloem translocation issue is to deliver phytoavailable zinc directly through the application of zinc-fertilizers to foliage, or even to soil, even though the mobility of zinc in the phloem limits zinc accumulation (Cakmak, 2008; Cakmak et al., 2010).

In the case of agronomic biofortification, only a temporary micronutrient increase is achieved, only in the season it is applied. Moreover, even if the genetic variation in zinc of edible crops in Figure 4 seems to be considerable, it can be noted that most of the variation is in plants whose shoot is consumed. The zinc phytoavailability issue can clearly be seen, as plants from which seeds only are consumed have a lower variability. Agronomic practices are therefore promising for zinc biofortification. The case of wheat will be developed in chapter V.

IV.2 Implementation

The identification of a target population and their staple food consumption is the first step towards implementation of biofortification. A target level for micronutrient content of the biofortified crop is then estimated, depending on the food intake, nutrient losses, and bioavailability of the desired micronutrient. The CGIAR institutes hold global germplasms banks, available for screenings for high micronutrient contents (Consultative Group for International Agricultural Research, 2017). The first step is to determine if sufficient genetic variation is available to choose how to improve the crop (Bouis et al., 2011; Nestel et al., 2006). In case of HarvestPlus, the improvement of a crop can take up to 8 years, and consist of an

early-stage product development, involving the building of parents lines, followed by the intermediate stage, during which bioavailability retention and composition are characterized and final product development stage, during which the efficacy of a crop can be tested (HarvestPlus, 2016). Germplasms are then tested in target countries, for their release suitability, as the expression of the micronutrient should be stable through different environments (Bouis et al., 2011). Before releasing the biofortified crops, it is then necessary to test their nutrient retention, bioavailability and efficacy in human subjects (King, 2002). The release of a biofortified crop can then take place, including the facilitation of dissemination and consumer acceptance (Bouis et al., 2011).

As seen in Table 8, 28 clinical trials, as either an efficacy or an absorption study, have already taken place in humans from 2011. Even though the funding of those studies and the process of production of the biofortified crops are not always revealed at time of registration, HarvestPlus funded most of them. The Improved Nutrition through Stable Foods in Africa project funded an absorption study from biofortified rice under the funding from the European Union's Seventh Framework (NCT01633450), The European Cooperation in Science and Technology funded an absorption study from biofortified wheat (NCT01775319), while PepsiCo funded a β-cryptoxanthin biofortified maize study (NCT02800408). Only one assessment of food composition and initial status in Human was registered prior to implementation of the crop. This was the case of iron in Burkina Faso (NCT02376140). A sweet potato study, (NCT02363985) involved the promotion of biofortified sweet potatoes only, and not direct supply.

Currently, biofortification was proven successful for different micronutrients, as well as protein, pigments (such as β-cryptoxanthin) and different vitamins. Different examples can be seen in Table 9. Milk and eggs, through the increased intake of micronutrient-rich crops by cows and hens, can be biofortified as well. Funding is diverse, and biofortification does not only interests organizations such as HarvestPlus or the Bill and Melinda Gates foundation. Fertilizers-producing companies invested in studies showing the use of agronomic practices to improve nutritional quality of crops, while PepsiCo, a beverage producer, is interested in pigment biofortification of maize, most likely to produce colorful beverages without the use of colorants. Governments, through university funding, financed as well biofortification research, mainly by focusing on hydroponics production of a biofortified crop.

Table 8 Human efficacy or absorption studies with at least one arm including consumption of biofortified crops and one arm including consumption of control non-biofortified crops. Status on clinicaltrials.com as of June 2017.

Staple	Micronutrient	Population	Location	Primary outcome	NCT number	Status
Bananas	Vitamin A	Female adults	USA	Retinyl ester in the plasma triacylglycerol-rich lipoprotein	NCT02702622	completed
Beans	Iron	Adolescent	Rwanda	Iron status	NCT01594359	completed
	Iron	Female Adults	Rwanda	Iron absorption	NCT02215278	completed
	Iron	Female adults	Rwanda	Iron absorption	NCT01521273	completed
Cassava	Vitamin A	Pre-school children	Nigeria	Body retinol pool	NCT02627222	ongoing
	Vitamin A	Female adults	USA	Change in vitamin A in triacylglycerol-rich lipoprotein	NCT02210507	completed
	β-carotene	Female Adults	USA	Vitamin A absorption	NCT01381276	completed
Eggs	Vitamin D	Senior adults	Ireland	Serum total 25-hydroxyvitamin D concentration	NCT02678364	completed
Food basket	Vitamin A, Iron, Zinc	Infants < 1000 days	India	Ferritin, STFR, serum retinol, serum zinc, RBP, Hb, hepcidin, CRP AGP, growth, cognitive function	NCT02648893	not yet recruiting
Maize	Vitamin A	Mothers infants	Zambia	Vitamin A stores Breast milk retinol	NCT02804490	recruiting
	β-cryptoxanthin	Adults	USA	Serum β-cryptoxanthin	NCT02800408	ongoing
	β-carotene	Adults	Spain	Vitamin A absorption by plasma all-trans retinol response	NCT02373943	completed
	Zinc	Children 2-3 yrs	Zambia	Zinc absorption	NCT02208635	completed
	Vitamin A	Breastfeeding women	Zambia	Breastmilk retinol	NCT01922713	completed
	β-carotene	Children	Zambia	Serum retinol	NCT01695148	completed
Milk	Vitamin E Selenium	Elderly	Brazil	Serum α-tocopherol Serum selenium Fatty acid profile	NCT02980094	completed
Pearl millet	Iron Zinc	Children 12-18 months	India	Ferritin, STFR, total body iron, immune function, cognitive functions, physical grown	CTRI/2015/11/006376 NCT02233764	recruiting
	Iron Zinc	children 2 yrs	India	Iron absorption	NCT01783067	completed
	Iron	Adolescents 12-16 yrs	India	Iron status	NCT02152150	completed
	Iron	Female adults	Benin	Iron absorption	NCT01634932	completed
Rice	Zinc	Children	Bangladesh	Plasma zinc Growth Hair, nail zinc Microbiota	NCT03079583	not yet recruiting
	Zinc	Adults	Switzerland	Zinc absorption	NCT01633450	completed
	Zinc	Children 3-5 yrs	Bangladesh	Zinc absorption	NCT01346722	completed
	B-carotene	Adults	USA	Vitamin A absorption	NCT00680355	completed
Sweet Potato	Vitamin A	Children 3.5 yrs	Ethiopia	Serum retinol Total vitamin A	NCT02363985	unknown
Wheat	Zinc	School-age children	India	Serum zinc	CTRI/2015/06/005913 NCT02241330	completed
	Zinc	Preschool children, women	India	Zinc status and morbidity	CTRI/2014/04/004527	completed
	Zinc	Adults	Switzerland	Zinc absorption	NCT01775319	completed

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Table 9 Examples of biofortification already achieved in different crops and staples. References of either production or human study with biofortified crop, when the production was not published. NA: not available.

Staple	Target	Funding Agencies	References
Banana	Provitamin A	HarvestPlus	(Andersson, Saltzman, & Pfeiffer, 2017)
Beans	Iron	HarvestPlus	(Andersson et al., 2017)
	Selenium	NA	(de Figueiredo et al., 2017)
	Zinc	NA	(de Figueiredo et al., 2017)
Cabbage	Zinc	PAI program (Plan Andaluz de Investigación, Grupo de Investigación AGR161)	(Barrameda-Medina et al., 2017)
Carrots	Iodine, Selenium	NA	(Smolen et al., 2016)
Cassava	Provitamin A	HarvestPlus	(Andersson et al., 2017)
	Provitamin A	Biocassava Plus	(Sayre et al., 2011)
	Iron	Bill and Melinda Gates Foundation	(Leyva-Guerrero, Narayanan, Ihemere, & Sayre, 2012)
	Protein	Bill and Melinda Gates Foundation	(Leyva-Guerrero et al., 2012)
Eggs	Vitamin D	NA	NCT02678364
Finger Millet	Calcium	Program Support for research and development in Agricultural Biotechnology at G. B. Pant University of Agriculture and Technology	(Sharma, Jamra, Singh, Sood, & Kumar, 2016)
Lentil	Zinc	HarvestPlus	(Andersson et al., 2017)
Lettuce	Iodine	PAI programme (Plan Andaluz de Investigacio´n, Grupo de Investigacio´n AGR161)	(Blasco et al., 2008)
Maize	Provitamin A	HarvestPlus	(Andersson et al., 2017)
	Zinc	Acharya N. G. Ranga Agricultural University	(Subbaiah et al., 2016)
	B-cryptohanthin	PepsiCo	CT02800408
Pea	Zinc	NA	(Poblaciones & Rengel, 2016)
Pearl Millet	Iron	HarvestPlus	(Andersson et al., 2017)
Rice	Zinc	HarvestPlus	(Andersson et al., 2017)
	B-carotene	Golden rice	(Tang et al., 2009)
	Iodine	NA	(Mackowiak & Grossl, 1999)
	Folate	Ghent University, Belgium	(Blancquaert et al., 2015)
	Iron	Asian Development Bank, The Danish Trust Fund, Micronutrient Initiative of Canada, International Food Policy Research Institute	(Haas et al., 2005) (Chen et al., 2017)
	Zinc and Iron	Transgenic approach	(Vasconcelos et al., 2003)
	Thiamin	Bill & Melinda Gates Foundation	(Dong, Thomas, Ronald, & Goyer, 2016)
Spinach	Folate	NA	(Watanabe et al., 2017)
	Iodine	‘‘Recruiting Out- standing Overseas Chinese Scientists’’ scheme of the Chinese Academy of Sciences, China.	(Zhu, Huang, Hu, & Liu, 2003)
Sweet potato	Provitamin A	HarvestPlus	(Andersson et al., 2017)
Tomato	Vitamin C	Ministry for Science and Innovation New Zealand	(Bulley et al., 2012)
Wheat	Zinc	HarvestPlus	(Andersson et al., 2017)
	Selenium	Fertilizer’s companies (Carr’s, Yara, Marks abd Spencer, Velcourt)	(Broadley et al., 2010; Wu, Salisbury, Graham, Lyons, & Fenech, 2009) (Poblaciones, Rodrigo, Santamaria, Chen, & McGrath, 2014)

IV.3 Advantages and limitations

As already discussed in this chapter, biofortification is believed to be sustainable, especially in the case of conventional breeding. As its benefit is higher than cost of breeding, testing and disseminating (when compared to the price paid for each case of deficiency) (Bouis et al., 2011), biofortification is also believed to be cost-effective. It can reach malnourished populations easily (Saltzman et al., 2013) and it does not require the food vehicle to be centrally processed (Hotz, 2009). In the case of zinc deficiency in India, biofortification of rice and wheat can reduce the annual burden of disability-adjusted life years by 20-50% (Stein et al., 2007). Moreover, for a general acceptance from farmers, biofortification has to preserve yield, as well as disease-resistant traits. The strategy to implement biofortification includes breeding micronutrient-dense traits in the most profitable, high-yielding varieties (Bouis et al., 2011), providing thus an additional incentive for adoption, in the case yield of the crop can be increased. Consumers research has also shown that consumers feel positively about the idea of biofortification, believing the nutritional and health benefits are credible (Uchitelle-Pierce & Ubomba-Jaswa, 2017).

Nevertheless, agronomic approach of zinc biofortification can theoretically increase zinc bioavailability in wheat flour (Liu, Liu, Zhang, Chen, & Zou, 2017) but the ability of zinc biofortification to demonstrate an impact on zinc status is a challenge that still needs to be addressed (Hotz & McClafferty, 2007).

Even though it seems that there is no adverse effects expected from consumption of biofortified crops, the evaluation of the impact of biofortification in human nutrition requires laboratory and community-based trials (King, 2002). Studies are required to quantify the burden of micronutrient deficiency as well to determine the cost-effectiveness of a biofortification program (Ortiz-Monasterio et al., 2007). By breeding, random mutations can happen and a crop can express unexpected abilities. In a recent study, beans with low PA concentrations caused transient adverse digestive side effects in nearly all participants; this most likely due to the presence of residues of phytohemagglutinin L in the cooked beans (Petry et al., 2016).

The time required to develop the crops and the available natural variation of certain micronutrients in crops may not permit as great an increase in micronutrient content as fortification (Hotz, 2009) or supplementation. Therefore, biofortification only complements

existing interventions to sustainably provide micronutrients (Saltzman et al., 2013), but no single intervention is likely to solve the problem of malnutrition. Biofortification can also target only populations already consuming the staple food vehicle and its efficacy depends on the amount consumed. It is known that its benefits are not equivalent throughout the life cycle (Bouis et al., 2011), and therefore the level chosen for biofortified crop has to be adequate for all population groups.

A foreseen cost of biofortification is also recurrent expenditures for monitoring and maintaining traits (Bouis et al., 2011). The dissemination by itself can also be costly, as it depends on the effectiveness of seed systems, which are limited in some countries (Hotz, 2009). The success of biofortification depends mainly on public investment in agricultural research and extension, seed supply and consumer education. Finally yet importantly, biofortification success depends only on consumer acceptance, which can be altered if the biofortified crop phenotype is impacted during biofortification.

IV.4 Dissemination of Biofortified Cereals

HarvestPlus has already released 115 varieties from 10 different crops (Banana, bean, cassava, cowpea, lentil, maize, pearl millet, sweet potato and wheat), focusing on biofortification of Vitamin A, iron and zinc, in 26 countries (Andersson et al., 2017). The focus of HarvestPlus has lately been iron-biofortified beans, released in 11 countries since 2004, and Provitamin-A biofortified maize, released in 9 countries since 2012. The release of zinc-biofortified rice has been done only in Bangladesh, incrementing the zinc content by around 6 ppm only (Andersson et al., 2017). This shows the practical challenges of producing biofortified rice. In case of wheat, out of 5 released varieties in Pakistan and India, 2 are no longer commercialized. The current widely commercialized wheat in both countries had a zinc concentration of 40 ppm, around 10% more that the target initial concentration (Andersson et al., 2017). It is challenging to quantify the release of biofortified crops currently under research. It is clear that hydroponic culture can be done only in laboratory setting, and should be used only as a proof of concept as implementation research is needed.

There is a real need for effective, sustainable delivery systems for biofortified crops in each countries with a high burden of micronutrient deficiencies. This, however, necessitates a successful partnership between research institutions, private sector stakeholders, national governments as well as non-governmental organizations, but implementation science

research is still needed to understand how to achieve a successful and sustainable adoption of biofortified crops (MacDonald, Hilton, & Dove, 2017). One key consideration for a fruitful dissemination might be to avoid the competition between the biofortified and traditional varieties of crops, especially by breeding the biofortified varieties into all new high yield varieties released in each country of interest. Consumer research, promotion and marketing are also needed, to develop sustainable markets and to create demand for biofortified crops (Uchitelle-Pierce & Ubomba-Jaswa, 2017).

Another potential way of using biofortification is to combine it with phytoremediation. Fossil fuel combustion, mine waste, and phosphate fertilizers release zinc into the environment (Zhao, Yuan, Zhangmin, Tianyu, & Yin, 2012). Soil contamination can be remediated by phytoremediation, which used plants to take up, accumulate, store or degrade organic as well as inorganic contaminants, while taking advantage of the natural abilities of the plants (Vassilev, Schwitzguebel, Thewys, Van Der Lelie, & Vangronsveld, 2004). Unfortunately, phytoremediation linked with zinc biofortification is still currently limited by the plant species (Zhao et al., 2012). In Switzerland, mutants sunflowers and tobacco plants, produced by conventional breeding, were used in a field test to remove zinc contamination from alpine soils. The labile zinc pool in topsoil was shown to be lowered by 45-7% over 5-year and plant zinc intake was confirmed (Herzig et al., 2014).

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V Wheat

V.1 Origin and Production

The evolution of bread wheat through domestication happened around 10'000 years ago (Lev-Yadun, Gopher, & Abbo, 2000). Domestication, involved morphological, physiological and genetic changes, the two most important traits being an increase in grain size and number (Zohary, Hopf, & Weiss, 2000) and the development of non-shattering seed, preventing natural seed dispersal (Purugganan & Fuller, 2009). This increase of weight and size is due to an increase in starch content, while wheat protein, fiber and mineral contents slowly decreased over domestication (Friedman & Atsmon, 1988; Hebelstrup, 2017). Drought tolerance characteristics, even though they are complicated by interactions with the environment (Budak, Kantar, & Kurtoglu, 2013), were lost during cultivation of modern lines (Ashraf, Ozturk, & Athar, 2009). Wild emmer wheat (*Triticum turgidum* ssp *dicoccoides*) is the progenitor of domesticated tetraploid durum wheat (*Triticum turgidum* ssp *durum*) and hexaploid bread wheat (*Triticum aestivum* L.) (Peleg, Fahima, Korol, Abbo, & Saranga, 2011). Domestication of wild emmer, a relatively smaller grain with a long and thin shape, resulted in a uniform larger grain with a short wide shape (Eckardt, 2010) and a significant reduction of phenotypic variation in grain shape in the modern germplasm pool (Gegas et al., 2010).

During the past 50 decades, the production of wheat worldwide has more than tripled, reaching 730 million tons in 2014, while the area used for production increased by 40%. In India, wheat production was multiplied by 9 and the area dedicated to it only doubled, as seen in Figure 6. Wheat is currently grown on more land than any other crop, reaching 220 million hectares in 2014, while rice and maize are grown on 160 and 180 million hectares respectively. Despite this, though, the yield is largely superior for maize (5.6 tons/ha), than rice (4.5 tons/ha) and wheat (3.3 tons/ha) (Food and Agriculture Organization of the United Nations, 2017). In India, 6 states represent more than 85% of the total area dedicated for wheat production in 2015, namely Uttar Pradesh, Punjab, Madhya Pradesh, Haryana, Rajasthan and Bihar. Wheat is mainly a winter season crop in India and is usually planted in October and harvested in April (X. Zhang, Obringer, Wei, Chen, & Niyogi, 2017). More than 90% of their area dedicated to wheat production is under irrigation. The yield varies considerably, ranging from 1.8 tons/ha in Bihar to 4.6 tons/ha in Haryana (Government of India: Ministry of Agriculture and Farmers Welfare, 2015). This shows the potential for

improvement of wheat production in India, as well as the inherent difficulties of implementing more efficient agricultural practices.

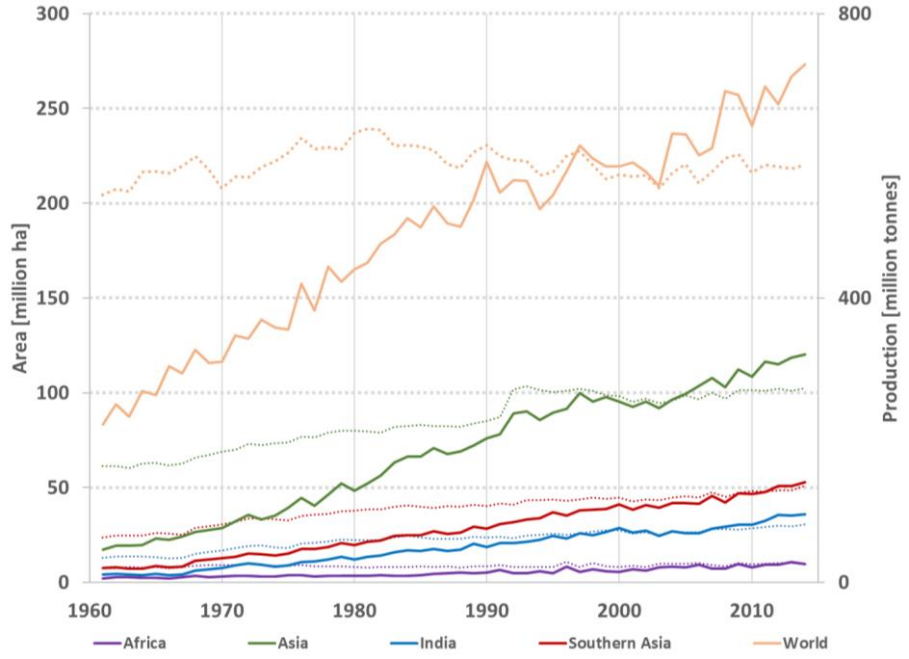

Figure 6 Wheat production in million tons and million hectares. Dashed lines represent the area, solid line the production. Data from the FAO Agriculture Production Data (Food and Agriculture Organization of the United Nations, 2017)

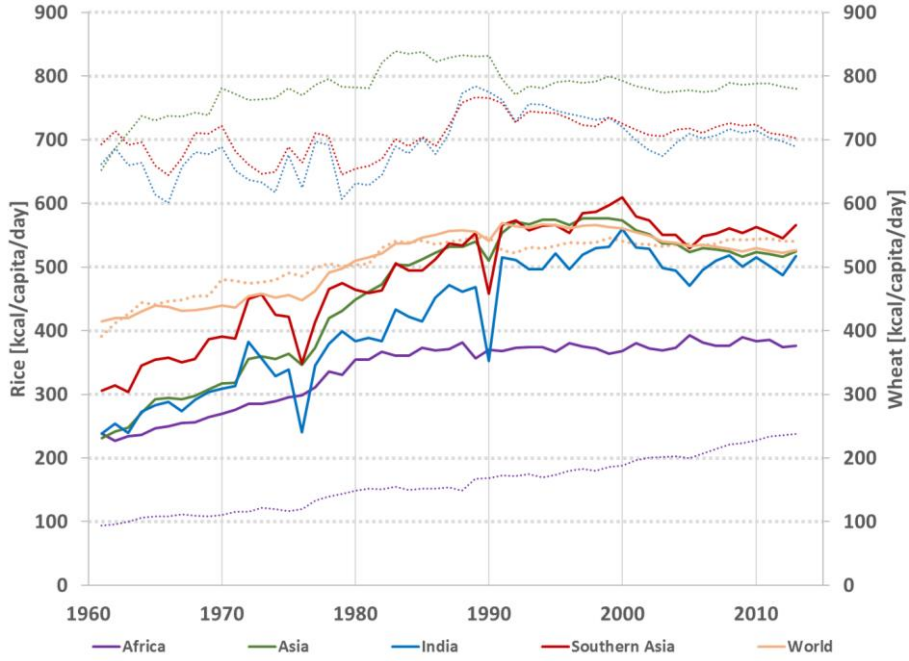

Figure 7 Wheat and rice consumption in kcal/capita/day. Dashed lines represent the rice, solid line the wheat. Data from the FAO Food balance sheet Data (Food and Agriculture Organization of the United Nations, 2017)

V.2 Consumption

It can be seen in Figure 6 that wheat and rice, both provide nowadays approximately 550 kcal/day/capita worldwide. In case of India, the average wheat consumption follows over time the approximate worldwide consumption. In 1976 and 1990, the consumption of wheat drastically declined in the subcontinent, and therefore in Southern Asia and Asia as well (Food and Agriculture Organization of the United Nations, 2017). This was due to the slight decrease in the production of wheat the years just before and to the adoption of neoliberalism by India at the end of the 80s. Moreover, India suffered from 2 severe meteorological droughts during wheat growing season in 2004, 2006 and 2007 (X. Zhang et al., 2017). The pattern of production of wheat, though, does not necessarily reflect the pattern of consumption, as wheat is a global commodity, subject to trade (Shewry & Hey, 2015). The trade in the past reported by the Food and Agriculture Organization (FAO) decades accounted for approximately 150 million tons per year (Food and Agriculture Organization of the United Nations, 2016). Wheat is consumed in North India, while the diet of Southern part is predominantly made of rice (National Institute of Nutrition & Ministry of Health and Family Welfare, 2015). This is reflected in the average consumption per capita, seen in Figure 7.

Wheat consumption is forecast to decrease in 2017 and 2018, due to increased local availability of maize in Europe and China. Wheat production is expected to stagnate in 2017, mainly due to reduced planting in Europe and dry weather in Africa (Food and Agriculture Organization of the United Nations, 2016). In India, and to protect the interior market, the 25% tax on wheat imports was extended in 2016 (Food and Agriculture Organization of the United Nations, 2016).

Figure 8 shows the trend of cereals consumption in India and worldwide. Only 19 countries consume more than 1000 kcal/capita/day of wheat, all of them being in central and Western Asia as well as North Africa with an exception of Italy. Countries eating nearly no wheat are Viet-Nam, Thailand and Cambodia, as their main source of calories is rice (Food and Agriculture Organization of the United Nations, 2017).

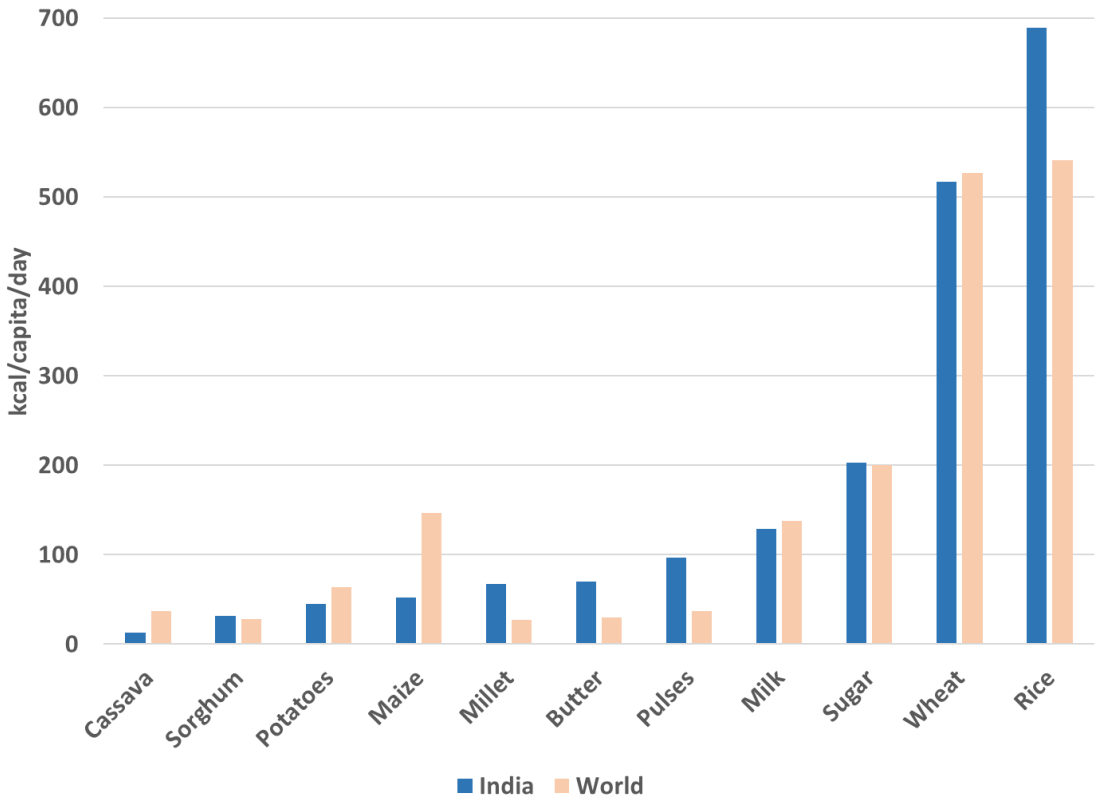

Figure 8 Main crops consumption worldwide and in India in 2013, in kcal/capita/day. Data from FAO food balance database (Food and Agriculture Organization of the United Nations, 2017)

Wheat quality can be defined from the farmer, miller and consumer points of view, and is thus a broad concept. Yield and resistance to stress are obvious qualities needed to grow wheat, while protein content, flour yield and baking performances are needed to produce flour. From the consumer's viewpoint, taste, nutrition and safety are clear requirements (Varzakas, 2016). Protein content of wheat varies from 7 to 22%, but is generally around 12% of dry weight (Vogel, Johnson, & Mattern, 1976). The only limiting amino acid in wheat grain is lysine, while other essential amino acids are present in adequate amounts for adults (Shewry & Hey, 2015).

The two major wheat species used for consumption is *Triticum aestivum*, called "common" or "bread" wheat, while "pasta wheat" refers to *Triticum turgidum* var *durum*, a species adapted to Mediterranean weather. Einkorn (*T. monococcum* var *monococcum*), emmer (*T. turgidini* var *dicoccum*) and spelt (*T. aestivum* var *spelta*) are cultivated on small areas and differ from the previous 2 species as they are hulled (Shewry & Hey, 2015). Even though more than 200 varieties have been released in India for cultivation in the last 40 years, a maximum of 15 varieties accounts for 80% of the crop, while 2 of them cover 30% of area. India has around 1.5 mio ha under durum wheat (Nagarahan, 2006).

V.3 Wheat Use in Nutrition

The worldwide use of wheat can be explained by its gluten protein that allows the processing of it to produce goods, such as bread, noodles and pasta (Shewry & Hey, 2015), a process that cannot be obtained with other cereals, such as rice and maize, for example.

Worldwide, wheat is consumed in varied forms, from bread and pasta to sweets and biscuits. In India, it is consumed commonly in the whole country in the forms of biscuits, cakes, halwa, bread, and puffs. Locally, it is consumed in all kind of sweets, such as maalpua and pinni in the Northern region, khazoor and paadarpeni in the Southern region, and different kind of savouries, such as chaat, roti, puri, paratha and naan in the Northern region. In the Southern regions, where the diet is mainly based on rice, wheat flour is added to rice recipes, such as bhaath, as well as dosa, iddli, vadai and pongal (National Institute of Nutrition & Ministry of Health and Family Welfare, 2015).

V.4 Grain Characteristics

Besides having low levels of zinc, wheat grain is rich in substances limiting utilization of iron and zinc, such as PA. Micronutrients are largely co-localized in the aleurone cells and embryo with PA, the main storage compound of phosphorous in grains. Zn-PA complexes are therefore the main molecular species of zinc in cereals (Gibson, Bailey, Gibbs, & Ferguson, 2010). Seed PA is of great importance for seed germination though (Guttieri, Peterson, & Souza, 2006). Most of the zinc located in the seed is present in the embryo and aleurone layer (150 ppm), while very low zinc concentration (15 ppm) are found in the endosperm (Ozturk et al., 2006). This can be seen in Figure 9, where the protein pattern in wheat is shown to follow the same way. A high correlation between iron and zinc concentrations has been shown in wheat (Welch & Graham, 2004). The nutritional quality of 10 Indian wheat varieties were characterized in 2013 (Mallick, Azaz, Gupta, Sharma, & Sinha, 2013). In general, even if their phosphorous and zinc concentrations can vary by more than 50%, their iron concentration is relatively constant throughout the varieties. One of the specie, namely PBW-550, has an average calcium concentration of 48.5, 63.9 phosphorous, 4.36 iron and 3.52 zinc mg/100g dry weight. In the 10 varieties studied, determinants of absorption vary greatly; from 0.35 to 1-20 mg PA/100 g. PBW-550 contain 0.65 mg PA/100g grain.

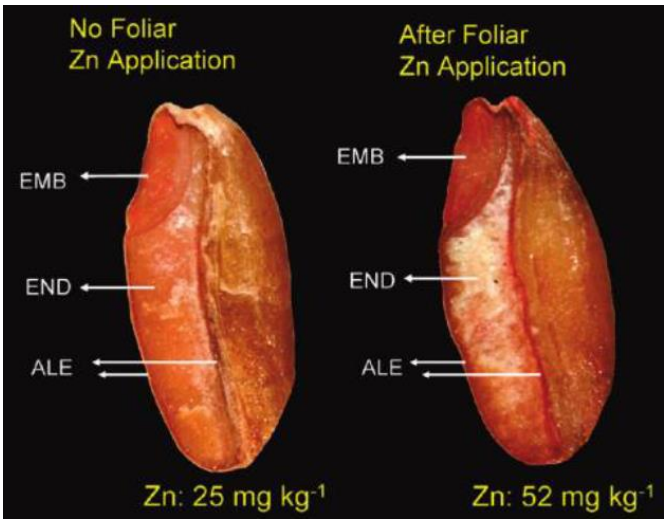

Figure 9 Localization of zinc in bread wheat grain without (left) and with foliar zinc application (right). Red color formation indicates zinc localization. Color density indicates zinc concentration. EMB: embryo, ALE: Aleurone, END: Endosperm. From (Cakmak, Kalayci, et al., 2010)

V.5 Zinc Concentration

In general, wheat contains 25-30 ppm of zinc. The genetic variation currently found for zinc in bread wheat is very narrow and unfortunately cannot be useful for the exploitation in breeding programs (Cakmak et al., 2004). Among wild wheat species, emmer shows a wide range of genetic variation, having a concentration of zinc up to 190 mg/kg, iron up to 109 mg/kg and protein 380 g/kg (Cakmak et al., 2004). However, high grain concentration of micronutrients can be associated with low grain yield (Cakmak, 2008). Spelt, which carries high levels of resistance and is suitable for growing in challenging environment, has been shown to be a promising source of genetic variation for grain protein and mineral, as genotypes containing zinc up to 70 mg/kg and iron up to 60mg/kg have been identified (Gomez-Becerra et al., 2010).

Table 10 Wheat zinc and phytic acid concentrations, depending on extraction rates. ER: Extraction rate, PA: Phytic acid

Wheat variety	ER [%]	Zinc content [ppm]	PA content [mg/g]	Reference
Whole wheat grains	100	26.9	-	(Souci et al., 2016)
Flour type 405	50	10.5	-	(Souci et al., 2016)
Flour type 550 (all purpose flour)	72	12.3	-	(Souci et al., 2016)
Flour type 630	79	13.6	-	(Souci et al., 2016)
Flour type 812	80	16.3	-	(Souci et al., 2016)
Flour type 1050	83	20.1	-	(Souci et al., 2016)
Flour type 1700	100	26.1	-	(Souci et al., 2016)
CIMMYT combined wheat biofortified	100 80	41.3 23.8	11.1 3.3	(Rosado et al., 2009)
CIMMYT combined wheat control	100 80	23.6 14.4	8.9 3.4	(Rosado et al., 2009)
Appio (Durum wheat)	100 66	43.8 15.3	-	(Cubadda et al., 2009)
Duilio (Durum wheat)	100 66	37.3 11.8	-	(Cubadda et al., 2009)
Simeto (Durum wheat)	100 66	33.0 11.8	- -	(Cubadda et al., 2009)
Bezostaya (Bread wheat)	65 75 85 100	- - - -	0.88 1.18 2.24 8.34	(Turksoy et al., 2010)
Gun-91 (Bread wheat)	65 75 85 100	- - - -	1.05 1.11 2.34 8.90	(Turksoy et al., 2010)
Dagdas-94 (Bread wheat)	65 75 85 100	- - - -	0.92 1.16 2.67 9.56	(Turksoy et al., 2010)
Gerek-79 (Bread wheat)	65 75 85 100	- - - -	0.95 1.37 2.56 10.55	(Turksoy et al., 2010)
Kirgiz-95 (Bread wheat)	65 75 85 100	- - - -	0.72 1.17 2.46 9.44	(Turksoy et al., 2010)
Ikizce (Bread wheat)	65 75 85 100	- - - -	0.73 1.32 2.44 8.29	(Turksoy et al., 2010)

The loss of zinc in wheat upon milling obviously depends on the Extraction Rate (ER) used for the production of flour. The common all-purpose white flour contains around 70% ER. Even though the loss of zinc during milling was already studied, the PA loss is still unclear. Review of different findings can be found in Table 10. The only published study showing the zinc and PA concentration in the same wheat with different ER shows a 40% loss of zinc due to milling, compared to a 60% loss of PA for the same ER (Rosado et al., 2009). Those results are somehow consistent with other findings, showing approximately 70% decrease in PA (Turksoy, Ozkaya, & Akbas, 2010) and a 40% loss of zinc with 80% ER (Souci, Fachmann, & Kraut, 2016) while a 65% decrease of zinc is achieved with a 66% ER (Cubadda, Aureli, Raggi, & Carcea, 2009). A very low ER will lead to endosperm only, which contains only around 15 ppm of zinc.

V.5.1 Increasing Zinc Concentration

As explained in Chapter IV, biofortification can be successful with cereals only after enhancing the root to shoot transfer of zinc, as well as the transfer from xylem to phloem, as, in cereals, xylem is discontinuous at the base of each seed (Olsen & Palmgren, 2014; Palmgren et al., 2008). This last issue, though, can be overcome in rice, as its xylem is continuous. However, in rice, endosperm loading is low because of limited uptake capacity in the starch-filled endosperm cells (jan Stomph, Jiang, & Struik, 2009).

The most sustainable approach to enrich cereal grains with zinc is plant breeding. Emmer and spelt could be good candidates for agronomic breeding into bread wheat, as their zinc concentration is higher than the one of common wheat. Unfortunately, this is a long-term process, as explained in the biofortification chapter. Moreover, the pool of zinc available to plants in soil is a limiting factor for the accumulation of a sufficient amount of zinc in grains (Cakmak, Kalayci, et al., 2010). Furthermore, newly bred crops must adapt over a range of crop and soil managements practices, while soils might have adverse soil chemical factors, such as high pH or low moisture that could potentially decrease the zinc absorption (Cakmak, 2008). A promising short-term solution to the problem is the foliar application of zinc fertilizers. This process could be used as a complementary approach to plant breeding strategies (Cakmak, 2008).

The first evidence showing a positive impact of zinc fertilizer in improving wheat grain zinc concentration and productivity was done in Turkey in 1997. During this trial, emmer and

common wheat were subjected to fertilization either through soil application of 23 kg Zn/ha as $ZnSO_4 \cdot 7H_20$ before planting, or through seed application with 1 liter of 30% $ZnSO_4 \cdot 7H_20$ on 10 kg of seed or by foliar application, where 400 ml 0.4% $ZnSO_4 \cdot 7H_20$ solution (440 g Zn/ha) per plot was sprayed twice directly on the leaves. The different method of fertilization were also combined. The grain zinc concentration increased with all fertilization methods used, but the highest increment was found with soil and foliar zinc application (3.5 fold), while grain yield in all cultivars was increased irrespectively of the method (Yilmaz et al., 1997).

Since then, foliar zinc application has been widely examined by different groups and proven to significantly increase zinc concentration in wheat seeds (Persson et al., 2016; Saifullah, Javed, Naeem, Rengel, & Dahlawi, 2016; Yang, Tian, Lu, Cao, & Chen, 2011; Y. Zhang, Shi, Rezaul, Zhang, & Zou, 2010; Zhao, Tian, Cao, Lu, & Liu, 2014). The importance of the timing at which foliar application has to be applied was first studied in 2010 (Cakmak, Kalayci, et al., 2010). The increase in zinc concentration was more pronounced when foliar zinc application was performed in the late compared to the early growth stages. $ZnSO_4$ and ZnO were both studied, showing that a small amount of zinc in the form of 0.5-2% of zinc -enriched urea significantly increased yield and grain zinc concentration in spring wheat, while zinc sulphate and zinc oxide were equally effective in increasing grain yield. A 1% zinc enrichment of urea with either ZnSO4 or Zn0 was therefore recommended (Shivay, Prasad, & Rahal, 2008). The colocalization of protein and zinc within grains were shown in 2010, and the fertilization of nitrogen and zinc showed a synergistic effect on zinc concentration in grains (Kutman, Yildiz, Ozturk, & Cakmak, 2010). As seen in Table 11, foliar and soil zinc fertilization does not increase PA content, and therefore divides PA:Zn molar ratio by around 4, as grain zinc concentration is multiplied by 3. In 2016, in a study analyzing the combined effects of zinc and nitrogen on the molecular speciation and compartmentation of zinc, zinc was found not to be associated with PA or any other ligands containing phosphorous in the soluble part of the endosperm (Persson et al., 2016).

Currently, $ZnSO_4$ is the recommended form for fertilizer use. In India, a recommendation of 25 kg/ha of $ZnSO_4 \cdot 7H_20$ is advocated for every year, or alternate years for soil application, while 0.5% $ZnSO_4$ is advocated for foliar application (Das & Green, 2013). The lack of awareness of the farmers and workers, as well as the unavailability of zinc fertilizers at the time of need and their low quality mean that fertilization is not widespread in India. Consequently, large fertilizer companies are coming forward into zinc fertilizers

manufacturing (Das & Green, 2013). Tata chemicals, for example, offer now new fertilizers containing zinc (Tata Chemicals Limited, 2017).

Table 11 Effects of Various Zinc application Methods on Bread wheat and Durum wheat done in Turkey under field conditions Modified from (Cakmak, Pfeiffer, & McClafferty, 2010), but originally published by Erdal, I, 1998, PhD thesis (In Turkish) Ankara University. PA:Zn: phytic acid, zinc molar ratio

Fertilization method	Cultivar	Grain Zn [mg/kg]	Grain P [g/kg]	Phytic Acid [g/kg]	PA:Zn
Control	Durum wheat	11	4.2	11.8	112
	Bread wheat	8	4.3	12.0	140
Soil	Durum wheat	17	3.3	8.7	53
	Bread wheat	16	3.3	9.5	62
Leaf	Durum wheat	19	4.0	10.3	56
	Bread wheat	28	3.5	9.8	35
Soil+Leaf	Durum wheat	33	3.9	9.7	30
	Bread wheat	36	3.8	10.0	29

V.6 Reference

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VI India

VI.1 Country Profile

With more than 1.2 billion inhabitants (Ministry of Home Affairs, 2011), India faces a variety of public health challenges, as varied as 12% under-five mortality rate, 22% probability of dying before 15 years old and less than 40% of population using improved sanitation facilities (World Health Organization and UN partners, 2015).

Bangalore, with an estimated population of nearly 8.5 million in 2011, is the capital of Karnataka State. Around 11% of its population is below 6 years old and a recent census estimates that Bangalore literacy rate is ~88% (Ministry of Home Affairs, 2011). In 2006, and in the whole state of Karnataka, 88% of children 5-14 years were attending school (International Institute for Population Sciences & Government of India Ministry of Health and Family Welfare, 2016a). Around 63% of the population has no access to sanitation facilities, while this is reduced to 26% of its urban population (UNICEF, 2011).

There are 29.4% of children below 5 in the urban population of Karnataka who are stunted, while 25.7% of them are wasted, 11.1% severely wasted and 28.0% underweight. In the total population of the state, these values are 28.1, 28.9, 11.7 and 26.8, respectively (International Institute for Population Sciences & Government of India Ministry of Health and Family Welfare, 2016b, 2016c), suggesting that the difference in the nutritional status of children between urban and rural area are similar as only 38% of the population of Karnataka live in an urban area.

The stunting prevalence in India has linearly decreased during the past twenty years, as shown in Figure 10. This linear decrease is comparable with the linear increase of the overall population being able to access improved water and sanitation facilities. It was already shown that tackling open defecation could be part of the answer to saving children from stunting (Bhandari, 2016) and the United Nations Children's Emergency Fund (UNICEF) has been advocating the Indian State Governments for the need to generate demand for better health and sanitation facilities (UNICEF, 2017). Open defecation, which is exceptionally widespread in India, can account for much of the excess stunting in India (Spears, 2013). Despite intrapartum and preterm complications, only 2 diseases, such as respiratory infections and diarrhea account for around 50% of under-5 deaths in India (UNICEF, 2011, 2016).

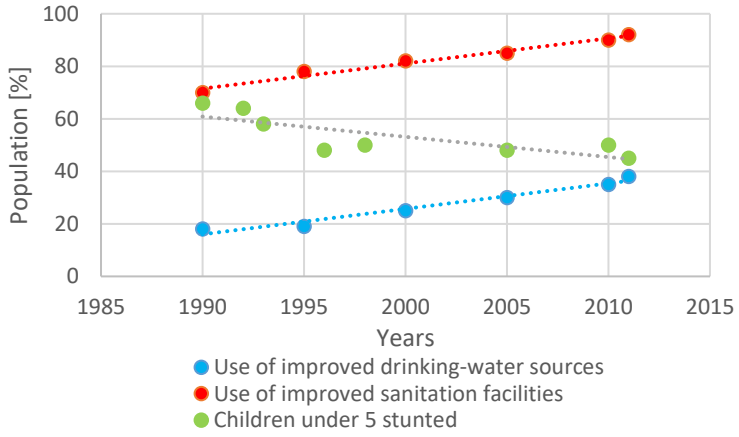

Figure 10 Population of India having access to improved water and sanitation facilities, children under 5 stunted. Data adapted from (World Health Organization and UN partners, 2015)

VI.2 State of Nutrition

As poverty restricts access to food and malnutrition affect educational and economic attainments, poverty and nutrition are inter-related. In India, the coexistence of those two factors lead to a high burden of malnutrition (Varadharajan, Thomas, & Kurpad, 2013). Malnutrition is known to play a role in more than half of under-five deaths in India (Pelletier, 1994; Pelletier, Frongillo, Schroeder, & Habicht, 1994; Schroeder & Brown, 1994). Most of Indians follow a monotonous and vegetarian diet, based on cereals, while the dietary consumption vary across geographical areas and cultural contexts. In general, food intake is lower than RDA (Vecchio et al., 2014). In Karnataka rural areas, the dietary adequacy of children up to 6 years was assessed and their diet is predominantly cereal based. While the nutrient intake was found to be inadequate, irrespective of the season, no gender inequality was found (Lakshmi, Khyrunnisa, Saraswathi, & Jamuna, 2005). In school-age children living in Bangalore, dietary patterns is influenced by the socio-economic status of the family and the frequency of eating out (Swaminathan, Thomas, Kurpad, & Vaz, 2007). Around 75% of those urban school children were non-vegetarian.

The School Lunch Program in India (SLP) was started in 1995, keeping into consideration the requirements of the children and local food habits (Chutani, 2012). In 2012, it was estimated that around 140 million children benefited from the SLP (Chutani, 2012). In Karnataka, SLP has improved the school enrollment, attendance and reduced dropout rate. A marginally higher growth performance was also found in children going to school that beneficiated from the SLP compared to children going to school in which SLP was not implemented (Laxmaiah et al.,

1999). Even though many other strategies and policies have been proposed to counter undernutrition in India, access to food is particularly susceptible to poverty and inequality (Varadharajan et al., 2013). Female illiteracy, and therefore lack of knowledge about food, with unscientific cooking methods and cultural eating patterns (Gupte, 2001), add up to poverty and contribute to the lack of nutrient security in India.

VI.3 Zinc Status

It is well known that a substantial population segment is predisposed to zinc deficiency in South Asian developing countries (Akhtar, 2013), but there is no reliable national-level data to really demonstrate the extent of the prevalence of Zinc deficiency in India. The magnitude of Zinc deficiency was studied as a primary outcome of prevalence studies only in specific areas and in subgroups of the population, namely children, adolescents and nulliparous or pregnant women. Only one of those studies, though, was done with rigorous blood collection.

In a survey published in 2011, the overall prevalence of zinc deficiency in Karnataka was estimated to be 36.2%, the lowest compared to 44% in all five states surveyed (range 36% - 51%) (Kapil & Jain, 2011). This, however, was done with an acid-washing protocol not meeting the required standards, as material was rinsed with tap water after acid washing. The same group, based in the All India Institute of Medical Sciences, had previously published three surveys: one pilot study in Delhi with pregnant women, and two studies in Haryana, with pregnant and nulliparous women. The Delhi pilot study showed that 55.5% of pregnant mothers had zinc deficiency, noting a decline of PZC over the gestational age (Kapil, Pathak, Singh, & Singh, 2002). In Haryana, 64.6% of pregnant women and 41% of nulliparous women were found to have zinc deficiency (Pathak, Kapil, Dwivedi, & Singh, 2008; Pathak, Kapil, Kapoor, Dwivedi, & Singh, 2003). Unfortunately, blood for those surveys was collected directly in polypropylene tubes and no mention of acid washing any material was made. Even though a high contamination would be expected from the lack of proper acid washing in those 4 studies, PZC values are still found to be very low. This brings an interrogation on how accurately the measurements were performed.

PZC was found to be low in 72.4% of female adolescents of Western India (Kawade, 2012). Regrettably, the method used for blood collection is poorly described in this study and there is no mention of use of any trace-element free tubes for blood collection and acid-washed material for processing.

In a rigorous and conscientious study conducted in a slum of New Delhi, 73.3% of children between 6 to 35 months had a PZC below 70 µg/dl, while only 7.3 % of them were stunted (Dhingra et al., 2009). This shows the difficulty in assessing stunting in pre-school children, and that zinc deficiency is not the only parameter affecting stunting in this population.

Prevalence of zinc deficiency was investigated as a secondary outcome of a study assessing risk of micronutrient deficiencies in relation to weight status in South Indian women. The average PZC was 114, 117 and 116 µg/dl in normal weight, overweight and obese women, respectively (Herter-Aeberli, Thankachan, Bose, & Kurpad, 2015). In this study however, blood was not collected in trace element free tubes. The high PZC values are therefore more likely due to contamination during collection and further processing. Even though the values seem physiologically too high, no differences can be detected between the groups, showing that, in this case, obesity is not linked with an increased zinc deficiency.

The scarcity of PZC information show the need for prevalence studies on zinc deficiency in India. As explained in earlier chapters, one of the main outcomes of zinc deficiency is stunting. Stunting, considered as a predictor of high risk of zinc deficiency, has been widely studied. A recent review compiling all studies done on malnutrition in India found that existing evidence shows a high prevalence of under-nutrition throughout the subcontinent, with a stunting rate of 15.4 (in Bihar) to 74% (in Delhi), depending on the state studied (Sahu et al., 2015).

VI.4 Wheat Production and Consumption

Wheat is the staple food for North Indians, consuming it as chapattis or rotis, a kind of unleavened, unfermented flat bread. The diversification happening currently in the Indian diet is due to the growth in the economy. Mainly urban households are slowly reducing cereals consumptions, while increasing fruits, dairy products, meat and processed foods (Singh, 2016).

Indian wheat, *Triticum aestivum*, produced all over India, is a soft/medium hard, bread wheat. The country produces as well durum wheat, in 3 states only. During the past decades, a shift from durum wheat to high yield non-durum varieties has been noticed, due to the government's policy of steady increase in minimum supported price (Singh, 2016).

Wheat production in India during the last two years declined due to adverse weather conditions, specifically rains during the harvest season and dryness during the growing season

of 2016. In northwest India, where the majority of wheat is produced, declining water table and soil salinity are a concern. Overexploitation of ground water and flood irrigation depleted resources or water irrigation, putting pressure on areas under wheat cultivation. Over the past 10 years, wheat production in India has been approximately equal to rice production. Indian wheat production, equaling 100 million metric tons, is more than sufficient for India's consumption, equaling around 90 million metric tons per year. India continues to export around 10 times more wheat than it imports (Department of Agriculture Cooperation and Farmers welfare, 2017; Government of India: Ministry of Agriculture and Farmers Welfare, 2015; Singh, 2016; Zhang, Obringer, Wei, Chen, & Niyogi, 2017). Over the past decade, the government of India has been selling wheat at subsidized prices through the public distribution system. Requisitioning only 13% of the wheat production in 2006, the Government of India requisitioned 32% of the whole wheat produced in 2015/2016 (Singh, 2016). This can be seen in Table 12.

Indian wheat is consumed either as refined wheat flour, called maida, or as whole-wheat flour, called atta, both of them containing around 1400 kJ/100 g edible portion. Generally, maida contains 0.9 mg zinc/100g flour, 1.7 mg iron /100g flour and 123 mg PA/100g flour, while atta contains 2.9 mg/100g, 4.1 mg/100g and 632 mg/100 g respectively (Longvah, Ananthan, Bhaskarachary, & Venkaiah, 2017). The total subsidy of wheat has increased by 5 over the past decades, while the price at which the government sell wheat has been left unchanged.

Table 12 Government Wheat Procurement and PDS operation. Modified from (Singh, 2016). PDS: public distribution system, GOI: Government of India. PL: Poverty line

Marketing year [Apr-Mar]	Production [million tonnes]	GOI procurement [million tons]	Market Price [INR/ton]	PDS Above PL [INR/ton]	PDS Below PL [INR/ton]	PDS Poorest of the poor [INR/ton]	Food subsidy [Billion INR]
2006/2007	69	9	7'000	6100	4150	2000	240
2010/2011	81	23	11'000	6100	4150	2000	640
2012/2013	94	38	12'850	6100	4150	2000	850
2015/2016	87	28	15'000	6100	4150	2000	1240

VI.5 Biofortification and Fortification Implementations

With more than 45 partners, mainly located in North India, HarvestPlus has been focusing on the development and growth of iron-biofortified millet and zinc-biofortified wheat in India. The 2 currently available pearl millet varieties from HarvestPlus provides up to 80% of daily iron needs, while the available wheat variety should provide 50% of daily zinc need, and is disease-resistant (Cherian & Virk, 2016). The International Crops Research Institute for the

Semi-Arid Tropics (ICRISAT) commercialized in 2014 one high iron (71 mg/kg) pearl millet, already adopted by farmers in north-west India, and since then continued to collaborate with HarvestPlus to release one similar variety per year (Andersson, Saltzman, & Pfeiffer, 2017). A high-iron and high-zinc sorghum is currently being grown by ICRISAT for trials in Maharashtra, providing 50% more iron and 50% more zinc than usual sorghum (International Crop Research Institute for the Semi-Arid Tropics, 2017). HarvestPlus has now released 5 varieties of high-iron cowpeas in India, the most successful one having double the iron content as the normal cowpea. It released 2 zinc biofortified lentils, reaching a 75 ppm zinc content. Zinc biofortified wheat was first released by HarvestPlus in 2012, but only with a slight improvement of the zinc concentration (30-35 ppm versus 25 ppm). The latest variety released by HarvestPlus, in 2014, reaches 42 ppm of Zinc (Andersson et al., 2017), so an increase of nearly 70% of the zinc content.

The Indian government published draft standards for food fortification at the end of 2016 (Varzakas, 2016). Mentioning salt fortification with iodine, the documents also promote the fortification of milk and vanaspati, a hydrogenated vegetable oil, with vitamin A and vitamin D. Cereals fortification focuses on iron, at 20 g/kg, folic acid and vitamin B12. All cereals, including atta and maida, may also be fortified in combination with zinc, at a minimum level of 30 g/kg. Zinc sulphate is the only fortificant suggested for wheat fortification, while zinc oxide is recommended for rice, with no further explanation. Currently, the Indian government as well as several state governments supports financially flour fortification. Therefore, the government’s welfare system distributes most of the fortified flour (Food Safety and Standards Authority of India, 2016).

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VII Manuscript 1

Agronomic biofortification of wheat has a stronger influence on human zinc absorption than extraction rate, and can be a viable source of bioavailable zinc

Coralie Signorell[1], Michael B. Zimmermann[1], Rita Wegmüller[1], Christophe Zeder[1], Erick Boy, Richard Hurrell[1], Ismail Cakmak[2], Seher B Aciksoz[3], Fabian Tay[4], Emmanuel Frossard[3], Diego Moretti[1]

[1] Laboratory of Human Nutrition, Institute of Food, Nutrition and Health, Department of Health Sciences and Technology, ETH Zurich, Switzerland
[2] Faculty of Engineering and Natural Sciences, Sabanci University, Istanbul, Turkey
[3] Plant Nutrition Laboratory, Institute for Agricultural Sciences, Department of Environmental Systems Science, ETH Zurich, Lindau, Switzerland
[4] Clinical Trials Center, Center for Clinical Research, University Hospital of Zurich, Zurich, Switzerland

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Abbreviations: **AAS**, Atomic Absorption Spectrophotometer; **BFW**, biofortified wheat; **CW**, control wheat; **FW**, fortified wheat; **CRP**, C-reactive protein; **EXR**, extraction rate; **FAZ** fractional zinc absorption; **PA**, Phytic acid; **PZC**, plasma zinc concentration; **TAZ**, total zinc absorption

Abstract

Background Agronomic biofortification of wheat may be a promising strategy to counter the global burden of zinc deficiency, but its impact on zinc nutrition has not been quantified in humans. zinc biofortification of wheat through foliar zinc application can increase zinc concentration in wheat, but its effect on fractional and total zinc absorption is uncertain. zinc bioavailability is typically measured using extrinsic isotopic labels but whether absorption from the extrinsic label reflects native (intrinsic) zinc absorption is unclear.

Objectives Our study aims were: 1) to compare zinc absorption from simultaneously intrinsically and extrinsically-labeled biofortified wheat and conventionally grown wheat, 2) to compare zinc absorption at different milling extraction rates (EXR) from conventionally fortified wheat versus biofortified wheat produced by foliar zinc application.

Methods Fractional and total absorption of zinc (FAZ, TAZ) were measured in adults using the double-isotopic tracer method. In study 1, conventionally fortified and biofortified wheat-based meals (FM, BFM) were labelled both intrinsically and extrinsically with ^{70}Zn and ^{67}Zn, and FAZ and TAZ from the two labels compared to a nonfortified intrinsically labeled control meal (CM). In the studies 2 and 3, we compared FAZ and TAZ from meals from foliar BFM to FM and CM, using flour with 100% and 80% EXR.

Results In study 1, there was no significant difference in FAZ or TAZ from extrinsic versus intrinsic labels in either FM or BFM; FAZ and TAZ of FM and BFM did not significantly differ, while TAZ was 70-76% higher ($P < 0.01$) in FM and BFM compared CM. In studies 2 and 3, at both EXRs, FAZ and TAZ of FM and BFM did not differ, while TAZ was 20-48% higher from FM and BFM compared to CM ($P < 0.001$).

Conclusion An extrinsic zinc label accurately quantifies native zinc absorption from fortified wheat. Consumption of foliar biofortified wheat significantly increased FAZ and TAZ compared to unfortified wheat, while varying EXR had only a limited impact FAZ and TAZ**.**

Introduction

Zinc deficiency is estimated to be responsible for 4% of the disease and disability burden in children under-five years of age in low income countries (1). It is common in populations eating monotonous cereal-based diets with low zinc and high phytic acid (PA) content. Wheat is widely consumed worldwide (2) and its zinc content is only 20-25 ppm. Biofortification is the development of micronutrient-dense staple crops via traditional plant breeding, agronomic techniques or genetic engineering (3). Application of zinc fertilizers in agriculture has been recommended to increase crop yield in areas where soils are depleted in zinc (4). Soil zinc fertilization can increase zinc concentration in grains up to ≈40 ppm (5, 6). Even greater increments are possible when soil zinc fertilization is combined with foliar zinc application; this can achieve concentrations of 50-60 ppm zinc. Notably, agronomic biofortification leaves the PA level in the crop unchanged, resulting in a lower grain PA:Zn ratio (4, 6) which favors human zinc absorption (6, 7). Thus, zinc biofortification of wheat may be a sustainable strategy to address zinc deficiency, particularly in populations with limited access to commercially fortified foods (3, 8, 9).

The native zinc in foods can be intrinsically labeled by adding zinc isotopes during plant growth in preparation for human absorption studies, but this is challenging and expensive. Therefore, zinc bioavailability is typically measured using isotopic labels added extrinsically to the labeled test meal just before consumption (extrinsic labelling) (10) under the assumption that zinc isotopes homogeneously mix with native zinc in the meal and are then identically absorbed and metabolized. However, there is little direct evidence to support this assumption. Two previous studies have compared zinc absorption from intrinsic and extrinsic labels: a study in beans (11), reported zinc absorption was 10% lower from extrinsically labelled beans compared to intrinsically labeled beans; in brown rice there was no significant difference (12). Whether native wheat zinc introduced through biofortification is differently absorbed than zinc fortification added to the milled wheat (extrinsic labeling) has not been tested. This difference may be important, because native forms of organically-bound zinc, associated with various food components in cereals (13, 14) may not exchange completely with the extrinsically added zinc in the common zinc pool in the gastrointestinal tract during digestion (15). Also, zinc is associated with low molecular sized proteins rich in cysteine in wheat, in particular in the endosperm (14), and proteins in general and sulfur containing amino acids in

particular are reported to enhance zinc absorption (7). Thus, whether milling affects zinc absorption differently from biofortified wheat compared to conventionally fortified wheat is also unclear.

Therefore, our study aims were: 1) to compare zinc absorption from simultaneously intrinsically and extrinsically-labeled biofortified wheat and conventionally grown wheat, 2) to compare zinc absorption at different milling extraction rates (EXR) from conventionally fortified wheat versus biofortified wheat produced agronomically by foliar zinc application.

Methods

Study design

We performed three randomized, single-blind, crossover zinc absorption studies, in different subjects, in series. In study 1, we compared zinc absorption from intrinsically (^{67}Zn) and extrinsically (^{70}Zn) labeled biofortified (BFW) and conventionally-fortified wheat (FW), as well as from intrinsically labelled, conventionally produced, non-fortified wheat (CW) (**Figure 1**). The wheat cultivars were all of the same species (Triticum aestivum cv. Fiorina) and biofortification was achieved by mimicking soil zinc fertilization by growing the plants in hydroponic nutrient solutions with different ^{67}Zn content. The zinc level of the biofortified test meal (BFM) and fortified test meals (FM) was matched by fortification at point of use with a $ZnSO_4$ solution. All test meals consisted of a whole wheat porridge.

We then compared zinc absorption from BFW (Triticum Aestivum L. cv,, Esperia) produced with foliar zinc application versus zinc absorption from CW and FW when the wheats were milled at 80% extraction rate EXR (study 2) and at 100% EXR (study 3). In these studies, conventional fortification with $ZnSO_4$ was done to match the zinc content of the BFM. Test meals consisted of chapattis and potato cauliflower curry.

In all 3 studies, zinc absorption was measured with the double isotopic tracer ratio technique applied to spot urine samples and expressed as fractional and total zinc absorption (FAZ and TAZ, respectively) (16, 17).

Subjects

Subjects were recruited among the students and staff of the ETH Zurich and the University of Zurich (Switzerland). Inclusion criteria were: 1) apparently healthy; 2) for the women, nonpregnant and nonlactating; 3) age between 18 and 45 years; 4) BMI between 18.5 and 25

kg/m^2; 5) not taking chronic medication (except oral contraceptives); 6) no intake of vitamin/mineral supplements 2 weeks prior and during the whole duration of the study; and 7) no participation in any other research study for the past 30 days. Eligibility to participate and written informed consent from all participants were obtained at the ETH Zurich before the start of the studies. For study 1, 18 men and women were recruited, while for studies 2 and 3, 55 women were recruited and randomly allocated to either study 2 or study 3. The studies were approved by the Ethics Committee of the Canton of Zurich (KEK Zürich, KEK-ZH-NR-2012_0483) and registered on clinicaltrials.com (NCT01775319).

Study procedures

In study day 1, the participants provided the first baseline spot urine sample and had a venous blood sample drawn after an overnight fast (no food after 9 pm and no drink after 12 am) for the analyses of plasma zinc (PZC) and C-reactive protein (CRP). Participants received the first test meal after an overnight fast, labeled with the extrinsic oral isotopic tracer (extrinsic ^{70}Zn combined with wheat intrinsically labelled with ^{67}Zn for study 1; extrinsic ^{67}Zn for study 2 and 3). Immediately afterwards another stable isotopic tracer was administered intravenously over 5 min (^{68}Zn for study 1, ^{70}Zn for study 2 and 3). No foods or liquids were allowed during the following 3h (for study 1) or until lunch (for study 2 and 3). For study 2 and 3, the same test meal was administered for lunch. No foods or liquids were allowed during the following 3h. An afternoon snack (consisting of a banana and a chocolate), as well as dinner (vegetable lasagna, vanilla yoghurt) were given to the participants of study 2 and 3, in order to control zinc intake over the whole day in all participants who were asked to eat only these items during the day and reported the quantity. Thereafter, each of the subjects resumed their usual dietary habits until the evening before the next meal administration. Four days after the tracer administration, the first enriched urine sample was collected. The fourth day from isotope administration was identified in a pilot study as being the most suitable time for collection because of the proportional decline of both isotopes in urine (12, 18-20). The second and the third test meals were administered each after a minimum 4-week washout period, on days 29 and 57, respectively, by using the same procedure and the same tracers. New baseline samples were collected before each test meal to account for residual urine enrichment.

Wheat production

Study 1

Intrinsically labeled wheat with ^{67}Zn was grown in a continuously aerated greenhouse in hydroponic nutrient solutions and under controlled climatic conditions. Two different types of wheat were produced, a low zinc-intrinsically labeled wheat and a zinc-biofortified intrinsically labeled wheat. Wheat was harvested and thrashed with a small scale laboratory thrasher sorted by hand, and milled at the Human Nutrition Laboratory, ETH Zürich, by using the centrifugal mill (Retsch GmbH) using a titanium sieve (0.25 mm mesh).

In studies 2 and 3, bread wheat (Triticum Aestivum L. cv,, Esperia) was produced under field conditions in Central Anatolia. Wheat plants were sprayed 3 times with 0.5% $ZnSO_4 \cdot 7H_2O$ at the following growth stages: booting stage, early milk stage and early dough stage as described previously (21). The CW was not subjected to foliar zinc application.

For study 2 and 3, grains were milled to flour using a downscaled laboratory roller mill equipped with sifting sieves (Bühler, Uzwil). Water content of wheat was adjusted from 10% to ~18% for milling purpose. 35 kg of CW were milled first followed by 18 kg of BFW, after thorough cleaning of the mill and discarding the first 2 kg of milled grains of each type of flour. zinc and Iron were measured and compared to smaller samples previously milled with a centrifugal mill (Retsch GmbH) using a titanium sieve (0.25 mm mesh) to check that no contamination occured during milling. Bran and germ fractions were milled with a rotor mill (Max Lüscher AG, 30694) to reduce size and reintroduced in the flour. To make whole wheat flour, and therefore 100% EXR, all sieved parts were mixed to the same initial proportion. Wheat of 100% EXR was composed of 65% endosperm (white flour), 25% bran and 10% germ. For the 80% EXR, 6% of germ, 13% of bran and 81% of endosperm were mixed.

Test Meals

For study 1, test meals consisted of 50 g of whole wheat flour (100 EXR), water and sugar. Meals were prepared one by one, quickly heated until boiling and then maintained at a temperature higher than 80°C for 30 min. They were then stored at -20°C until consumption, when meals were thawed overnight (5°C) and re-heated prior to consumption using a microwave oven.

For study 2 and study 3, test meals consisted of chapatti, accompanied by 100 g of cauliflower-potato sauce. Each chapatti consisted of 50 g wheat (dry weight) and water, was rolled to a 5 mm thickness and cooked on an induction plate, for 3 min on each side. Participants were asked to eat 2 chapattis per meal and each meal was served both in the morning and at lunch so that 200g of wheat flour per treatment were consumed by each subject

For all studies, extrinsic label and fortification zinc were added immediately prior to consumption. Demineralized water (18 MΩ.cm) was served as a drink (3 dl).

Preparation of stable isotopes labels

Stable isotopes of zinc ^{67}Zn and ^{70}Zn (90.6% and 95.4% enrichment, respectively), were purchased in form of zinc oxide (Chemgas, Boulogne, France).

$ZnSO_4$ for oral administration was prepared from ^{70}ZnO by dissolution in stoichiometric amounts of diluted H_2SO_4. Doses for intravenous administration were prepared from ^{67}ZnO in a sterile environment at the Cantonal Pharmacy of the University Hospital Zurich as previously described (20).

Analytical Methods

Freeze-dried meals, wheat and all labeled and unlabeled zinc solutions, were analyzed for their zinc content by flame Atomic Absorption Spectrophotometer (AAS) (AA240FS; Varian) after microwave digestion (MLS-ETHOS plus; MLS GmbH) by using an HNO_3/H_2O_2 mixture. The PA content was determined in the freeze-dried porridges by the modified Makower method (22) in which iron was replaced by cerium in the precipitation step. After the hydrolization of the precipitate in concentrated H_2SO_4 (Digest Automat K-438; Büchi Labortechnik AG), inorganic phosphate was determined according to Van Veldhoven and Mannaerts (23) and converted into PA concentrations.

Blood samples, drawn into trace-element free tubes, were kept at room temperature and serum was separated into aliquots in acid-washed plastic vials, and frozen at -25°C until analysis. PZC concentration was measured by flame AAS (AA240FS; Varian) after dilution with HNO_3 10% by using commercial aqueous standards (Titrisol; Merck) for external calibration. Accuracy was checked by analysis of commercially available serum controls (Seronorm Trace Elements Serum L-1 and L-2; Sero). CRP was measured on an Immulite 2000 automatic system (Siemens Healthcare Diagnostics). Mild zinc deficiency was defined as 74 mg/dL for men and

70 mg/dL for nonpregnant women for morning fasting blood samples in adults (4). Inflammation was defined as an elevated CRP value (>5 mg/L) (24).

Spot urine samples, collected in polyethylene/polypropylene containers, were stored at -20°C and concentrated by freeze drying. Urine samples and intrinsically labelled grains were mineralised by microwave digestion (MLS UltraWave microwave digestion unit; MLS GmbH) by using HNO_3 and zinc isolated from the matrix by ion-exchange chromatography (20). Preparatory steps were performed in duplicate under chemical blank monitoring and isotopic ratio was assessed with a high-resolution double-focusing magnetic sector field multicollector ICP-MS (Finnigan Neptune; Thermo Electron). All acids used for the preparation of samples were ultrapure. ^{70}Zn:^{66}Zn, ^{67}Zn:^{66}Zn and ^{68}Zn:^{66}Zn isotopic ratios were measured to determine ^{70}Zn, ^{67}Zn and ^{68}Zn enrichment in the urine samples.

Calculation of FAZ

The fractional absorption of the zinc dose from the test meal was calculated by using the oral to intravenous. tracers ratio method applied to spot urine samples, according to the method described by Friel et al (17). Enrichment observed in the baseline samples from the second and third test meal were used as the new natural abundance in subsequent calculations. FAZ and TAZ values are presented as geometric means and SDs.

Statistical methods

We required 18, 24 and 24 eligible subjects for studies 1, 2 and 3 respectively to detect a difference in FAZ of 40%, with 80% power at a 0.05 significance level (2-tailed), taking into account a 20% dropout rate and based the sample size calculation on the pooled results of 5 previous zinc absorption studies that we performed at the ETH (SD of the log-transformed differences between pairs = 0.20). For study 2 and 3, a higher percentage of drop-out was expected due to the additional number of appointments involved in participation. Data were analyzed with SPSS version 23.0 (IBM) and Microsoft Excel. When the data were not normally distributed, the values were logarithmically transformed before the statistical analysis. The average difference between the absorption of intrinsic and extrinsic wheat labels (study 1) was expressed as a geometric mean ration of the differences from each test meal and subject (25). To assess the effect of meal on FAZ and TAZ linear mixed-effects models were fitted for each study with FAZ and TAZ as dependent variables, different meal compositions as the fixed effects and subjects as random components (intercept). An overall analysis was conducted

combining both study 2 and 3 by fitting a linear mixed-model, with TAZ or FAZ as dependent variables, and study and meals as fixed factors and subjects as random components (intercept). Significance was defined at the P = 0.05 level. Unpaired t tests were used to compare differences in age, BMI, and zinc status between study participants. One-way ANOVA followed by a post hoc Bonferroni's test was used for comparisons of zinc concentration among the different zinc concentrations in the test meals.

Results

Study 1

In study 1, all 18 subjects completed the study and all data were included in the analysis (**Table 1**). The mean ± SD zinc concentration in intrinsically labeled CW and BFW was 20.16 ± 4.52 ppm and 60.00 ± 11.50 ppm, respectively (**Table 2)**. The PA concentration (g/meal) was 205.6 ±8.2 for CM and FM and 188.3 ± 22.6 for the BFM. The iron (g/meal) concentration was 4.30 ± 0.19 for the CM and FM and 5.45 ±0.55 for the BFM. The PA concentrations did not significantly differ between the BFW and CW or corresponding test meals (**Table 2**). The zinc concentration in the BFM was 3.21 mg higher per meal than in the CM. The intrinsic dose of ^{67}Zn was 43.59% ± 0.17 of total zinc in CM and FM, and 26.67% ± 0.31 for the BFM.

In the absorption study, there was no significant difference in FAZ or TAZ from extrinsic and intrinsic labels in both the FM or BFM (Table 2). For FM the mean (95%CI) difference (extrinsic – intrinsic labels) in FAZ was 0.22 % (-0.466, 0.897), while for TAZ it was 0.01 mg (-0.020, 0.0394). For BFM the mean difference (95% CI) in FAZ was 0.03% (-1.228, 1.284) while for TAZ it was 0.001 mg (-0.062, 0.064), (**Table 2, Figure 2, Panel A**). Thus, both TAZ and FAZ of FM and BFM did not differ (**Table 2**). In contrast, TAZ was 70% and 76% higher in FM and BFM, respectively, compared CM (both $P < 0.01$). The regression line between zinc absorption from the extrinsic and intrinsic label for the FM was $y = 0.9722x + 0.0276$, $R^2 = 0.963$, $P < 0.001$ while it was $1.0321x – 0.2315$, $R^2 = 0.964$, $P < 0.001$ for the BFM.

Studies 2 and 3

Of the 55 subjects recruited for studies 2 and 3, 7 subjects in study 2 and 4 subjects in study 3 discontinued participation at the first meal administration and 7 were replaced. Out of the 51 participants who consumed at least 1 meal, 41 participants completed studies 2 and 3.

There were no significant group differences in subject characteristics comparing study groups 2 and 3 (Table 1).

With 100% and 80% EXR, the CW zinc concentration was 25.9 ± 0.4 and 18.1 ± 0.2 ppm while the PA concentration was 0.83 ± 0.04 and 0.49 ± 0.03 g/100 g, respectively. The PA:Zn molar ratio in CW was 31 at 100% EXR and 27 at 80% EXR. The BFW zinc concentration was 43.5 ± 0.6 and 31.0 ± 0.2 ppm, while the PA concentration was 0.80 ± 0.03 and 0.50 ± 0.01 g/100 g. This resulted in a PA:Zn molar ratio of 18 and 16 for 100% and 80% EXR, respectively. Test meals contained a mean (±SD) 0.17 ± 0.01 mg/100 g Zn, 5.8 ± 0.2 mg/100 g PA, 24 ± 0.25 mg/100 g ascorbic acid (n = 3) (**Table 3**). With 100% EXR, the amount PA (g/day) was 1.66 ± 0.08 and 1.61 ± 0.06 for CM and BFM respectively. With 80% EXR, the amount of PA (g/day) was 1.00 ± 0.06 and 1.00 ± 0.02 for CM and BFM respectively.

At both EXRs, and for both FAZ and TAZ, we found no significant differences in zinc absorption between the BFM and the FM (Table 3). With 100% EXR, the TAZ from BFM and FM was 48% higher than from CM (both, P <0.001), while FAZ did not differ. Similarly, at 80% EXR, where TAZ compared to CM was 40% higher in BFM (P <0.01) and 27% higher in FM (both P < 0.05). In the overall model for FAZ, there was a significant effect for study (P < 0.001), meal (P = 0.003) and study by meal interaction (P = 0.036). In the overall model for TAZ, there was a significant meal effect (P < 0.001), but no study effect (P = 0.105) and no study by meal interaction (P = 0.420).

Comparing between studies, in both fortified and biofortified wheat, 69% higher FAZ was found at lower EXR (P < 0.05); in contrast, EXR did not significantly affect TAZ. When EXR was reduced from 100% to 80%, FAZ for the CM, FM and BFM increased by 83% (P < 0.001); 55% (P = 0.001) and 44% (P = 0.001), respectively; however there was no significant increase in TAZ from the 3 types of wheat.

Discussion

Our main findings are: 1) in study 1, FAZ and TAZ of FM and BFM did not significantly differ, while TAZ was 70-76% higher in FM and BFM compared CM; 2) in studies 2 and 3, at both EXRs, FAZ and TAZ of FM and BFM did not differ, while TAZ was 20-48% higher from FM and BFM compared to CM. To our knowledge our study is the first to label the same meal both intrinsically and extrinsically with two distinct zinc isotopes, thus directly comparing zinc

absorption of an intrinsic and extrinsic label not only in the same subject, but also on the same eating occasion and from the exact same food serving.

There are two previous reports comparing zinc absorption from intrinsically versus extrinsically labeled staple crops (11,12). In rice, no significant difference was found between the FAZ and TAZ of the extrinsic or intrinsic label (12). In beans, extrinsic labeling was shown to underestimate intrinsic zinc absorption by ≈10% (11). Other studies in milk and meat products compared zinc absorption from intrinsic and extrinsic labels. Studies in young women consuming milk (26) and infants consuming formula (27) reported no significant differences in absorption from the two labels. In turkey meat (28), there was an absorption ratio of 1.16 ± 0.33 comparing FAZ from extrinsic versus intrinsic labeling; in contrast, this ratio varied from 0.79 to 0.92 in chicken (29) and the ratio was not significant in beef (30). In the present study, we administered different amounts of label relative to the total zinc content in BFM and CM, but this did not result in any noticeable differences in FAZ and TAZ. Thus, the quantity of tracer relative to the native amount of zinc does not seem to play a major role in zinc absorption from wheat. Our findings also suggest that native zinc speciation in wheat and the presence of zinc cysteine rich moieties (14) in wheat have negligible effects on zinc absorption. Because intrinsic labeling is more time consuming, expensive and labor intensive, extrinsic labeling has practical and methodological advantages and simplified logistics. Our findings confirm the validity of extrinsic labelling for the determination of human zinc absorption from biofortified and conventionally fortified wheat. Our data support the assumption that the extrinsic label mixes with the native zinc in the wheat to form a common zinc pool in the gastrointestinal tract (31).

The only previous study of zinc absorption from BFW used an extrinsic label (8). The BFW was produced by plant breeding (6) with no additional use of zinc fertilization and had a zinc content of 40.5 ppm. Test subjects received an equivalent of 300 g of wheat flour/day which provided 2.1 and 2.0 mg of absorbed zinc per day from the biofortified wheat, and 1.6 and 1.5 mg/day from the control wheat in 95% and 80% EXR, respectively. FAZ was 0.20 and 0.38 for the control wheat and 0.15 and 0.31 for the biofortified wheat, at 95 and 80% EXR, respectively. Extrapolated to our study with 200 g of wheat, this would correspond to 1.3 mg of absorbed zinc per day. Differences in TAZ and FAZ reported in this previous study to our findings may be due to several factors, including: 1) different timing of oral and intravenous doses; 2) differences in test meal composition; 3) differences in data presentation (arithmetic

means versus geometric means); and/or 4) differences in zinc status (zinc status was not reported in the previous study), as it has been reported that zinc status is a predictor of zinc absorption (32).

Comparing our PA:Zn molar ratios for study 2 and 3 with the WHO standards, an absorption of 15% was expected in study 2 and 30% in study 3. Our results are at least 50% lower, showing that even if milling is known to reduce PA concentrations in wheat grains (33), its inhibitory effect is not overcome by milling, as zinc concentrations are reduced as well. One way to overcome this could be by addition of phytase prior to consumption (18, 34, 35) or fermentation (33).

Our results in studies 2 and 3 confirm that foliar zinc application significantly increases TAZ from both 100% and 80% EXR wheat; TAZ was 40-48% greater from the BFM compared to the CM (Table 3). The data also show that milling EXR (100% versus 80%) has only a limited effect on TAZ from CM, FM or BFM. This may be due to the fact that changing the EXR from 100% to 80% significantly decreased Zn content but also decreased the PA:Zn ratio resulting in increased FAZ; overall these changes resulted in no significant difference in TAZ from the meals. The physiological zinc requirement for adult women proposed by the WHO is 1.47 mg/day in diets of low bioavailability (36), and the corresponding requirement for zinc intake is 9.8 mg/d. Our test meals with 200 g of BFW provided 45% and 56% of the daily zinc requirement at 100% and 80% EXR flour, respectively. In contrast, consumption of 200 g of CW provided only 32% or 40% of the zinc requirement in 100 and 80% EXR flour, respectively. Thus, biofortified wheat produced using foliar zinc application may be a good source of bioavailable zinc in humans.

Table 1- Subject characteristics in the zinc absorption studies[1]

Study	Participants (male)	Age	BMI	Plasma zinc concentration		C-reactive protein	
	n	y	kg/m^2	µg/dL	% <70 µg/dL	mg/L	% <5 mg/L
1	18 (11)	22.91±3.23	22.43±1.52	87.01±12.10	11.11	1.71±4.24	5.56
2	22 (0)	23.87±3.84	20.86±1.84	78.94±17.33	20.69	2.87±2.84	17.24
3	19 (0)	22.49±2.53	21.77±1.65	76.58±10.57	19.23	2.19±2.29	7.69

[1] Values as mean ± SD. There were no significant differences in values between study groups

Table 2. Total Zn content, PA: Zn molar ratio, amount of intrinsic and extrinsic Zn labels, fractional and total Zn absorption and the absorption ratio of extrinsic to intrinsic label absorption from wheat porridges prepared with conventional wheat flour with an intrinsic Zn label (control), and biofortified or conventional but fortified wheat flour with both an intrinsic and extrinsic Zn label.[1]

	Total Zn [mg][2]	PA:Zn [mol/mol][2]	Zn dose [mg/meal][3]		FAZ [%][2, 4]		TAZ [mg][2, 4]		Ratio
			IL	EL	IL[4]	EL	IL	EL	EL/IL
Control wheat	1.01	20.2	1.01 ^{67}Zn±0.05	0	8.93 (7.78, 10.23)[a]		0.17 (0.14, 0.19)[a]		
Fortified wheat	3.60	5.7	1.01 ^{67}Zn±0.05	1.00 ^{70}Zn + 1.59 Zn	6.27 (5.27, 7.44)[b]	6.44 (5.45, 7.58)[b]	0.29 (0.24, 0.33)[b]	0.29 (0.24, 0.33)[b]	1.03 (0.99, 1.07)[a]
Biofortified wheat	4.00	4.7	3.00 ^{67}Zn±0.35	1.00 ^{70}Zn	5.68 (4.37, 7.32)[b]	5.76 4.51, 7.36)[b]	0.30 (0.22, 0.39)[b]	0.30 (0.23, 0.39)[b]	1.02 (0.97, 1.07)[a]

[1] PA: Phytic acid; IL: Intrinsically label; EL: Extrinsically label; FAZ: Fractional zinc absorption; TAZ: Total zinc absorption

[2] Values within a column with different superscripts differ significantly, $P < 0.01$

[3] Values are means ± SDs (n = 3 for all components).

[4] Values are geometric means (95% CIs) n = 18.

Table 3- zinc content, PA: Zn molar ratio, and fractional and total Zn absorption from control, biofortified wheat-based meals (200g flour equivalent) obtained with foliar zinc application, and conventionally fortified wheat.[1]

Extraction rate [%]	Meal	zinc [2] [mg/day]	PA:Zn molar ratio	FAZ[3,4] [%]	TAZ[3,4] [mg]
100	Control	6.54 ± 0.04[a]	25	8.08 (6.80-9.56) [a]	0.48 (0.40-0.56)[a]
	Fortified	10.14 ± 0.04[b]	16	7.46 (6.44-8.63) [a]	0.71 (0.61-0.81)[b]
	Biofortified	10.06 ± 0.12[c]	16	8.37 (7.16-9.76) [a]	0.71 (0.61-0.82)[b]
80	Control	4.96 ± 0.08[d]	20	14.83 (12.55-17.50) [b]	0.59 (0.49-0.69) [ab]
	Fortified	7.55± 0.08[e]	13	11.55 (10.21-13.04) [c]	0.75 (0.66-0.85) [b]
	Biofortified	7.54 ± 0.0.04[f]	13	12.26 (10.41-14.40) [c]	0.83 (0.70-0.97) [b]

[1] PA: Phytic acid; FAZ: Fractional zinc absorption; TAZ: Total zinc absorption

[2] Values are mean ± SDs. (n = 3 for all components).

[3]Values are geometric means (95% CIs), n = 22 in each study

[4]Values within a column with different superscript letters are significantly different, P < 0.01

Figure 1. Study 1 design. Fasting subjects (n = 18) received BFM, CM and CFM in the morning and were randomly allocated to start the study with one of the meals (crossover design). Subjects acted as their own controls. PZC and CRP were assessed in the first morning, prior to meal consumption.

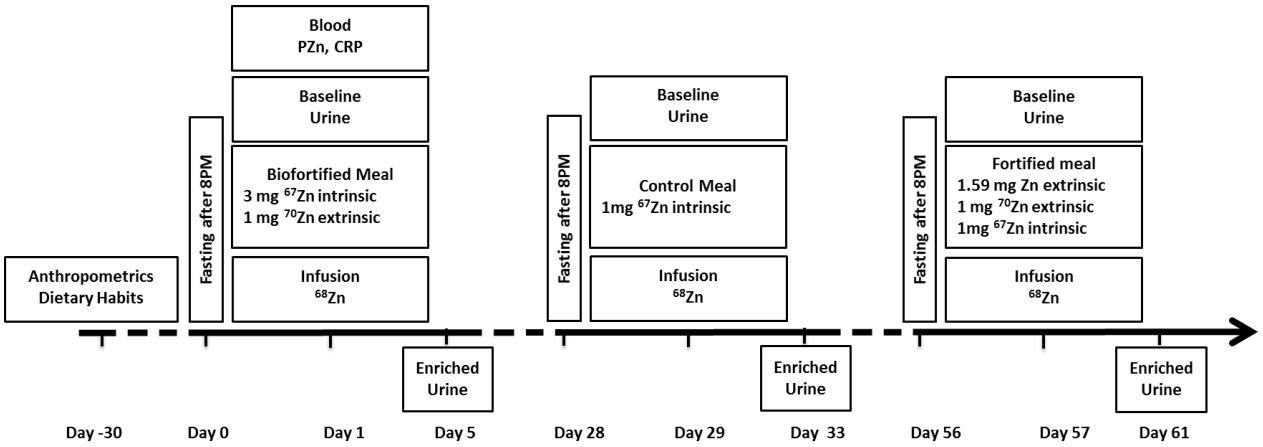
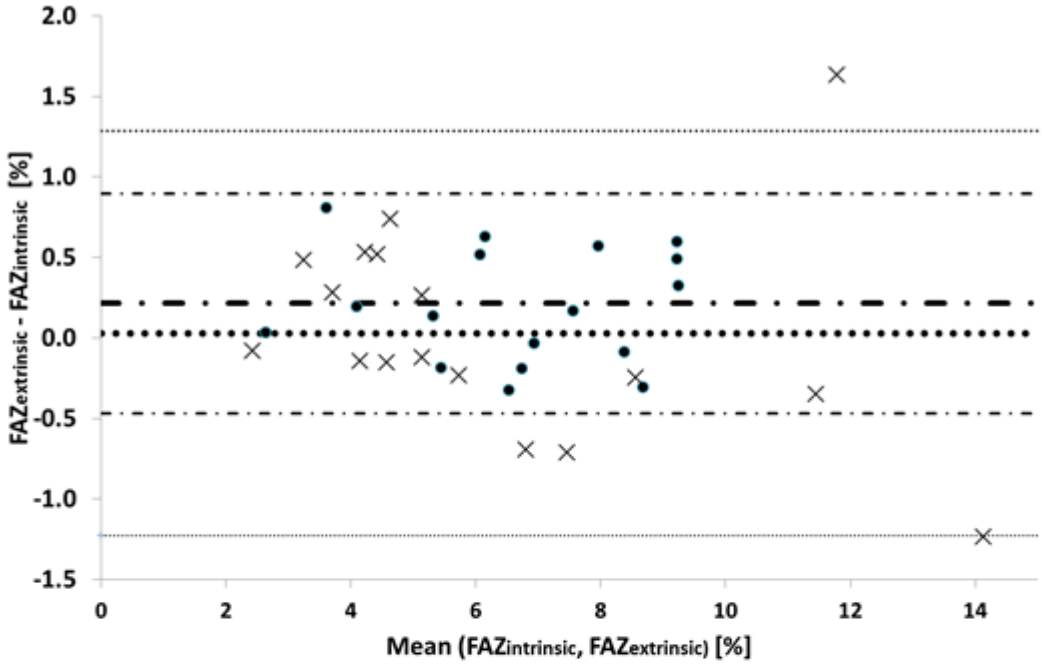
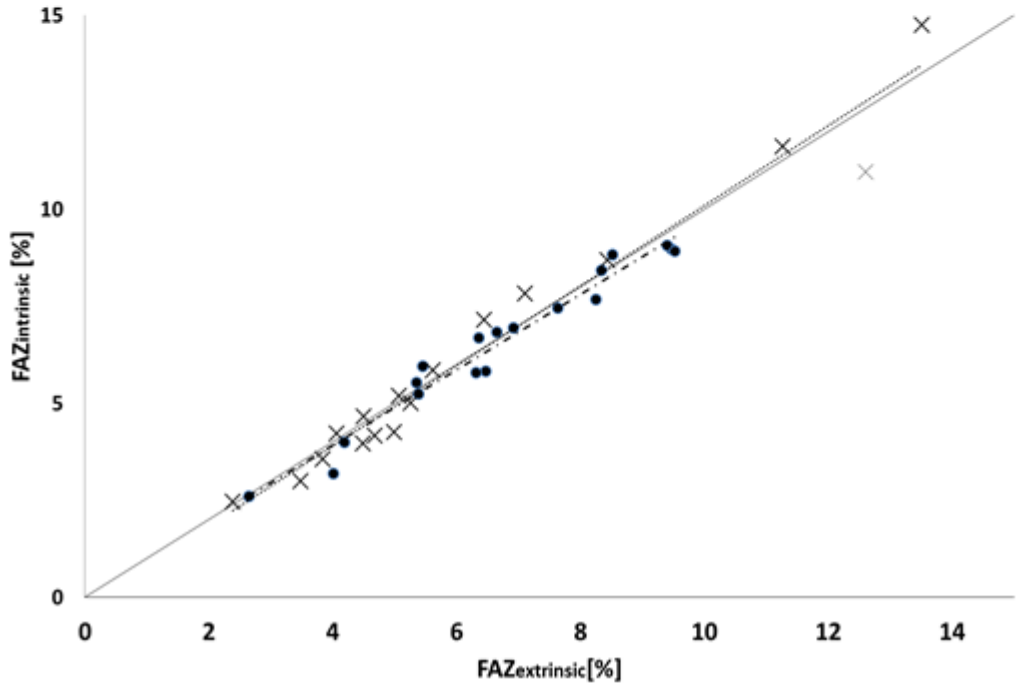


Figure 2 (A) Correlation plot for FAZ between the intrinsic and extrinsic tags (n = 18). Data points are plotted on the original scale. Crosses: biofortification values. Circles: conventional fortification values. Dash-line: fortification, dot- line: fortification. Conventional fortification: R^2 = 0.963, y = 0.9722x + 0.0276. Biofortification: R^2 = 0.964, y = 1.03212x – 0.2315. (B) Bland–Altman plot for fractional zinc absorption comparing the intrinsic and extrinsic label (n=18). Cross : biofortification; circle: conventional fortification; Dash-line : fortification, dot-line: fortification; Bold line: mean; thin line: 95% CI.





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VIII Manuscript 2

Plasma hepcidin is not a significant predictor of zinc absorption measured by zinc stable isotope studies in infants and adults

Coralie Signorell[1], Michael B. Zimmermann[1], Marica Brnic[1], Valeria Galetti[1], Dorine W. Swinkels[2], Diego Moretti[1]

[1] Laboratory of Human Nutrition, Institute of Food, Nutrition and Health, Department of Health Sciences and Technology, ETH Zurich, Switzerland
[2] Hepcidinanalysis.com and Department of Laboratory Medicine, Translational Metabolic Laboratory, Radboud University Medical Centre, Nijmegen, The Netherlands

Abbreviations: **AAS**, Atomic Absorption Spectrophotometer; **CRP**, C-reactive protein; **DMT1**, dimetal transporter 1; **EDTA**, Ethylenediaminetetraacetic acid; **Fpn**, ferroportin; **FAZ** fractional Zn absorption; **PHep**, Hepcidin; **MTF-1**, Metal transcription Factor-1; **PA**, Phytic acid; **PF**, plasma ferritin; **PZC**, plasma Zinc concentration; **sTfR**, soluble transferrin receptor; **TAZ**, total Zn absorption; **ZnO**, Zn oxide; **ZnSO4**, Zn sulfate

Abstract

Background Zinc and iron may share common absorptive pathways. In cell and animal models, hepcidin, the iron regulatory protein, may affect enterocyte zinc transport. Whether plasma hepcidin (PHep) predicts zinc absorption in humans is unclear.

Methods In a cross-sectional analysis, we investigated determinants of total and fractional zinc absorption (TAZ, FAZ) in Burkinabé infants (n = 41) and Swiss adults (n = 183) from previous zinc absorption studies using stable isotopic labels. Iron, inflammation and zinc status were assessed at time of isotopic administration by measuring PHep, plasma ferritin (PF), soluble transferrin receptor (sTfR), C-reactive protein (CRP) and plasma zinc (PZC). To identify predictors of zinc absorption, we fitted regression models and mixed effects models on zinc absorption with potential predictors as fixed factors and subject as a random factor.

Results Meal zinc, phytic acid:zinc molar ratio and PZC were significant negative predictors of TAZ in adults and infants; in addition, height and CRP were significant positive predictors in infants (for all, $P < 0.05$). PHep and iron status were not significant predictors of TAZ.

Conclusion Our data suggest PHep is not an important determinant of zinc absorption in humans. In contrast, zinc status, meal profile, height and inflammation are significant determinants.

Keywords zinc, stable isotope technique, total absorbed zinc, iron status, zinc status, hepcidin, ferroportin, ZnT, ZIP, infection, C-reactive protein, plasma zinc.

Introduction

Zinc deficiency is common worldwide (1) and can adversely affect growth, immune status and the nervous, reproductive and gastrointestinal systems (2, 3). Iron and zinc deficiencies often coexist in populations: in 2005, 93 countries were at moderate-to-severe risk of both zinc deficiency (1) and anemia (4). Zinc and iron share common dietary sources (whole cereals, pulses, animal source foods) and absorption inhibitors (phytic acid). Zinc and iron may also share common gastrointestinal absorption pathways. Zinc is absorbed through a saturable mechanism at intakes up to the EAR, and the total absorbed zinc (TAZ) increases linearly past these intake levels, consistently with non-saturable mechanism (5). On the apical membrane of enterocytes, ZIP4 is primarily responsible for zinc uptake (5). Also on the apical membrane, the dimetal transporter 1 (DMT1) mediates non-heme iron absorption; its physiological role as an active transporter of zinc is uncertain. Whether dietary zinc modulates the expression of DMT1 and thereby affects the uptake of non-heme iron is unclear (6-9).

The zinc transporter 1 (ZnT1) is believed to be the primary transporter of zinc across the enterocyte basolateral membrane into the circulation (5). Ferroportin (Fpn), the only known mammalian iron exporter from enterocytes, is also located on the basolateral membrane (10). Fpn has a narrow substrate profile but may transport zinc, as its expression stimulated cellular zinc efflux *in vitro* (11). Furthermore, zinc was shown to activate Fpn1 transcription through the Metal Transcription Factor-1 (MTF-1); Fpn can transport zinc and thereby protect cells from zinc toxicity (12, 13). Plasma hepcidin (PHep), by binding to Fpn, induces its internalization and degradation and acts as a systemic regulator of iron absorption and metabolism (14). Hep has been reported to attenuate zinc transport in human intestinal Caco-2 cells by inducing a 75% reduction in ZnT1 compared to control cells not treated with PHep (15). However, the potential links between PHep and zinc absorption in humans, as measured by stable zinc isotopes, has not been investigated.

Several reviews have reported on the *in vivo* inhibition of iron absorption and bioavailability by zinc (16-18) and this interaction implies a common transport pathway in the GI tract, possibly through DMT-1 on the apical membrane and/or Fpn on the basolateral membrane. Therefore, our study objective was to investigate dietary and physiological determinants of zinc absorption, with a focus on potential interactions with iron status, and specifically, PHep. We tested the hypothesis that PHep is a predictor of zinc absorption in humans, after

controlling for iron status and inflammation. To test these relationships, we combined data from 11 zinc absorption studies that we previously performed in infants and adults where iron, zinc and inflammation markers had been determined and methods were standardized across studies, and then tested associations by fitting predictive models on the fractional and total absorbtion of zinc (FAZ and TAZ, respectively), as measured quantitatively using zinc stable isotopes.

Material and Methods

Study sites and subjects

We conducted a secondary analysis of pooled data from 11 human studies measuring zinc absorption in infants and adults from different test meals to investigate predictors of zinc absorption. We performed studies 1-10 in the clinical trials center of the University Hospital of Zurich, and study 11 in the pediatric ward of the University Hospital Yalgado Ouédraogo, Ouagadougou, Burkina Faso. In Burkina Faso, the subjects were generally healthy infants, aged 1 to 2 years. In the adult studies, subjects were generally healthy Swiss adults with normal BMI, they were not smokers and were not taking chronic medications (expect oral contraceptives). Participants were asked to refrain from vitamin and mineral supplements at least 2 wk prior the beginning of the studies. We obtained written informed consent from all participants, or, in case of infants, their parents. All studies were approved by the Ethics Committee of the ETH Zurich and the Cantonal Ethic commission of the Canton Zürich (KEK) in Switzerland for studies 1 to 10 and by the Ethics Committee for Health Research in Burkina Faso, for study 11. The trials were registered as clinical trials (www.ClinicalTrials.gov; NCT01775319 studies 1-3, NCT01636583, study 4, NCT01506648, study 5, NCT01210794 study 6-10, NCT01633450 study 11).

Study design

In all 11 studies, which had as their primary objective to assess human zinc absorption (**Table 1**), a similar, standardized double isotopic tracer ratio technique applied to spot urine samples was used to measure FAZ (19, 20). Two or 3 test meals were served per study in a crossover design and in a random order. On study day 1, participants provided the first baseline spot urine sample and had a venous blood sample drawn after an overnight fast to determine plasma zinc (PZC) and iron status markers: plasma ferritin (PF) soluble transferrin receptor (sTfR), PHep as well as C- reactive protein (CRP). Fasting subjects (no food after 9.00

pm and no drinks after 12 pm on the evening before) received the first test meal labeled with ^{67}Zn or ^{64}Zn as ZnSO4 or ZnO. Immediately after that an intravenous saline solution containing 0.1 (infants) or 0.2 (adults) mg Zn as $ZnCl_2$ labelled with stable isotopes (^{70}Zn or ^{67}Zn) was administered. No foods or liquids were allowed during the following 3 h. Five days after the tracer administration, a urine sample was collected for determination of the zinc isotopic enrichment and calculation of FAZ and TAZ. We analyzed data for which a blood sample collected at the time of isotopic administration was available.

Description of the included studies

Meal composition, primary study hypothesis, zinc concentrations, phytic acid (PA) and ethylenediaminetetraacetic acid (EDTA) molar ratios are shown in **Table 1**. Study 1 meals consisted of water wheat porridges, prepared with intrinsically labeled wheat with varying zinc levels obtained via fortification or biofortification. Test meals in Studies 2 and 3 consisted of chapattis with a cauliflower potato sauce, prepared with flour with different milling extraction rates with either biofortified, fortified or nonfortified wheat flour. The aim of study 4 was to assess zinc absorption from zinc fortified water or fortified maize. In study 5, zinc absorption was assessed from fortified and biofortified rice. Study 6 focused on the effect of phytase administered at point of use in maize porridge. Studies 7 and 8 assessed absorption from maize porridge fortified with Zn sulphate ($ZnSO_4$) or ZnO without or with EDTA (EDTA:Zn molar ratio of 1:1 or 2:1). Effects of polyphenols was investigated in study 9 and 10, were zinc absorption from low polyphenol white sorghum porridge, was compared high polyphenols red sorghum porridge and to high polyphenols red sorghum porridge with and without dephytinazation and addition of EDTA at an EDTA:Zn molar ratio of 1:1. Study 11 was conducted in Burkinabé infants and aimed to compare zinc absorption from a fortified millet-based complementary food with and without phytase added at point of consumption. The prevalence of stunting in this population was 20%. In test meals where phytase was added at time of meal consumption, an assumption of 50% PA degradation was postulated, and PA:Zn ratio for the test meal calculated accordingly.

Biochemical analysis

All meals and flours used were analyzed for zinc and PA content after being milled by using a centrifugal mill (Retsch GmbH), water served was high-purity water and all testmeals were cooked with high-purity water (18 MΩ cm). Freeze-dried meals, and all labeled and unlabeled

zinc solutions, were analyzed for their zinc and PA content following methods previously described (21). In short, zinc content was measured by flame AAS, PA content by the modified Makower method (22), followed by inorganic phosphate was determined according to Van Veldhoven and Mannaerts (23).

Blood samples were drawn into Zn-free heparinized tubes. PZC was measured by AAS as previously described (21). CRP was measured on an Immulite 2000 automatic system (Siemens Healthcare Diagnostics). PHep was quantified by a c-ELISA method as described by Kroot et al (24). PF was quantified by a solid-phase, 2-site chemiluminescent immunometric assay (Immulite 2000 XPI, Siemens Medical Solution Diagnostics, Deerfield, USA). Serum concentration of sTfR was measured immunonephelometrically (Behring Nephelometer II Analyzer; BNII, Dade Behring Marburg GmbH, Marburg, Germany).

Spot urine samples, collected in polyethylene containers, were stored at -25°C until analysis. Their zinc isotopic composition was analyzed in duplicate under chemical blank monitoring. Urine samples were concentrated by freeze drying, then mineralized by microwave digestion (MLS-ETHOS plus; MLS GmbH) by using a mixture of HNO_3 andH_2O_2 (studies 4-11) or by microwave digestion (MLS-turboWAVE 1500; MLS GmbH) by using HNO_3 alone (studies 1-3). Zinc was isolated from sample matrix by ion-exchange chromatography (25). All acids used for the preparation of samples were ultrapure. Isotope ratios were measured to determine enrichment in the urine sample by using a high-resolution double-focusing magnetic sector field multicollector inductively coupled plasma mass spectrometer (Finnigan Neptune; Thermo Electron).

Statistical analysis

Statistical analysis was conducted with SPSS (SPSS statistics,Version 23, IBM) and Excel (2016; Microsoft, Seattle, WA). Normally distributed data were expressed as means ± standard deviation. Non-normally distributed data were expressed as geometric means (95% CIs) and log-transformed for comparisons. We calculated Pearson correlations between the assessed variables and computed multiple regression models to test for associations. As more than one zinc absorption measurement was available in some studies from the same subjects for different meals, each of the model included a subject variable. The dependent variables were TAZ and FAZ while independent variables were ID, markers of iron status (PHep, PF, sTfR), zinc status (PZC), inflammation (CRP), sex, height and weight. To achieve the minimal adequate

model of predictors of TAZ, we applied a stepwise backward deletion procedure by first including all physiological variables and discarding variables that, when removed, did not significantly decrease R^2. Regression models are characterized by R^2 from regression analysis for goodness of the fit. We defined initial models based results from the correlation analysis. Including either 1) all variables correlated with TAZ or FAZ, or 2) all variables correlated with TAZ with the addition of PHep and markers of iron status 3) replaced variables by variables correlating with each other on a hypothesis driven iterations (such as for example replacing hepcidin with inflammation and iron status markers and investigating changes in R^2).

Because there was a marked difference between the two population (Swiss adults and Burkinabé infants), we computed separate models for these two population groups.

In addition to regression models, linear mixed effect model were fitted using the same selection procedure as for the regression models in which dependent variables were TAZ and FAZ. Independent variables were defined based on the results of the correlation analysis and to test the hypothesis of an association of PHep and zinc absorption. The subject variable was used as a random factor in the linear mixed effect models (slope). For all models and correlations, significance was set at $P < 0.05$.

Results

Table 2 shows the characteristics of the infant and adult subjects. Mean ± SD age of the infants (n=41) was 1.49 ± 0.24 y; mean PZC was 81.15 ± 13.19 mg/dL (8.86% were zinc deficient) and 20% of them were stunted. Nearly all the infants were iron deficient, with a median (95% CI) PF of 6.500 (5.619, 12.851), but there was little inflammation (only 6.85% had an elevated CRP) and as a result, median (95% CI) PHep was low, at 0.385 (0.3926, 3.848). Mean± SD age of the adults (n=182) was 22.56 ± 2.66 y and mean PZC was 88.34 ± 11.19 mg/dL (10.75% were zinc deficient). The adults were mainly iron sufficient (only 13.98% were iron deficient based on a low PF) and median (95% CI) PF was 38.645 (34.007, 43.916), as a result, median (95% CI) PHep was non significantly higher than in the infants, at 1.570 (1.371, 1.785). Comparing the two groups, CRP was higher and sTfR was lower in the adults ($P < 0.05$ for both) (**Table 2**).

Adult studies

In the simple correlations (**Supplemental Table 1**), the EDTA:Zn ratio and PZC were negatively correlated with TAZ (r = 0.436, $P < 0.001$ and r = -0.200, $P = 0.007$ respectively) while the

concentration of zinc in the meals was positively correlated (r = 0.436, *P* < 0.001). Men had higher PZC than women (r = -0.206, *P* = 0.005) and CRP was negatively correlated with PZC (r = -0.414, *P* < 0.001). Height and weight were both positively correlated with PZC (r = 0.395, *P* < 0.001; r = 0.321, *P* < 0.001 respectively), and men had a higher height and weight (r = -0.646, *P* < 0.001; r = -0.640, *P* < 0.001 respectively). PHep was significantly correlated with gender, with higher values in males (r = -0.392, *P* < 0.001), as well as with height, weight, PF and sTfR (r = 0.176, *P* = 0.016; r = 0.243, *P* < 0.001, r = 0.714, *P* < 0.001, r = -0.318, *P* < 0.001, respectively), sTfR was correlated with weight, PF (r = 0.189, *P* = 0.013, r = - 0.318, *P* < 0.001), and PF was correlated with gender, height, weight (r= -0.581, *P* < 0.001; r = 0.276, *P* < 0.001; r = 0.308, *P* < 0.001) (**Supplemental Table 1**).

In the multiple regression analyses with PZC as the dependent variable, PZC was associated with CRP (β= -0.173, *P* = 0.015), gender (β= -0.419, *P* < 0.001) and sTfR concentrations (β = 0.162, *P* = 0.020). In total, these variables explained 23% of the variance in PZC. PF was the only independent predictor of PHep (β= 0.717, *P* < 0.001), explaining 51% of the variance. When PF was replaced by sTfR (β = -0.301*P* < 0.001,), gender became a significant predictor (β -0.311 *P* <0.001), with the model explaining only 20% of the model variance.

In the multiple regression analyses with TAZ as the dependent variable (**Tables 3** and **4**), TAZ was associated with the PA:Zn ratio (β = -0.299, *P* < 0.001), PZC (β = -0.237, *P* < 0.001), and the concentrations of zinc in the meal (β = 0.366, *P* = 0.015). These variables explained 35% of the variation in TAZ. When PHep was added to the model (β = 0.106, *P* = 0.083), the explanatory power of the regression model increased to 36%, but this change in explanatory power was not significant (*P* = 0.111). Replacing PHep with CRP (β = 0.005, *P* = 0.944) did not change the explanatory power of the regression model. Iron status markers were not significant predictors of TAZ. In regression analysis, FAZ was associated with the PA:Zn ratio (β = -0.275, *P* < 0.001), PZC (β = -0.281, *P* < 0.001), and the concentrations of zinc in the meal (β = -0.522, *P* < 0.001). These variables explained 26% of the variations in FAZ.

Consistent with the regression analysis, in the linear mixed effect models (**Table 5**), meal zinc (*P* < 0.001) and PZC (*P* < 0.001) were significant predictors of TAZ. However, PA:Zn ratio was not. PHep was a non-significant positive predictor of TAZ (*P* = 0.106).

Infant studies

In Burkinabé infants (n = 41), TAZ was negatively correlated with PA:Zn and PZC (r = -0.400, *P* < 0.001 and r = -0.327, *P* = 0.004 respectively) and positively correlated with CRP, age, height and weight (r = 0.322, *P* = 0.004; r = 0.282, *P* = 0.013; r = 0.327, *P* = 0.004 ; r = 0.298, *P* = 0.008 respectively). Only CRP was negatively correlated with PZC (r = -.305, *P* = 0.006) (**Supplemental Table 2**). PHep was correlated with gender, height, weight, PF and sTfR (r = -0.427, *P* < 0.001; r = 0.210, *P* = 0.004; r = 0.273, *P* < 0.01, r = 0.724, *P* < 0.001, r = -0.237, *P* < 0.01). PF was correlated with gender, height, weight, PZC and sTfR (r = -0.622, *P* < 0.001; r = 0.345, *P* < 0.001; r = 0.369, *P* < 0.01; r = 0.214, *P* = 0.005; r = -0.293, *P* < 0.01). sTfR was correlated with weight (r = -0.293, *P* < 0.001) (**Supplemental Table 2**).

In regression analysis CRP (β = -0.315, *P* = 0.005) was the only significant predictor of PZC in Burkinabé infants, explaining 10% of variance. Independent predictors of PHep were PF (β = 0.688, *P* < 0.001) and gender (β= 0.167, *P* = 0.045), the explanatory power of the regression model was 53%. In the regression analysis (**Tables 3 and 4**), the positive predictors of TAZ in Burkinabé infants were height (β= 0.311, *P* = 0.001) and CRP (β= 0.209, *P* = 0.023), while the negative predictors were PA:Zn (β = -0.452, *P* < 0.001) and PZC (β = -0.208, *P* = 0.024). These variables explained 49% of the variations in TAZ. If CRP was replaced by PHep, the model explanatory power decreased to 45%, while PHep was not a significant predictor of TAZ (β = -0.060, *P* = 0.505). Iron status markers were not predictors of TAZ.

In the regression analysis the positive predictors of FAZ in Burkinabé infants were height (β= 0.334, *P* < 0.001), while the negative predictors are PA:Zn (β = -0.507, *P* < 0.001) and PZC (β = -0.249, *P* = 0.005). These variables explained 48% of the variations in TAZ. If CRP is added to the model, the model explanatory power was unchanged, while CRP was not a significant predictor of FAZ (β = 0.145, *P* = 0.110). Iron status markers were not predictors of FAZ.

In the linear mixed model analysis, height, PA:Zn, PZC and CRP significantly predicted TAZ (**Table 6**). Variables that were not significant independent predictors of TAZ, PZC or PHep did not significantly increase the explanatory power in the models.

Discussion

The main findings of this exploratory study are: 1) in adults, higher total zinc absorption from labeled test meals was predicted by lower phytic acid: zinc ratios and lower total zinc contents

in the meal, and lower PZC in the subject; however, these 3 variables explained only ≈ 30% of the variation in zinc absorption; 2) in infants, higher total zinc absorption was predicted by lower meal PA:Zn ratios and lower PZC in the subject, and also by greater height and higher CRP; these variables explained ≈50% of the variation in zinc absorption; and 3) there were no significant associations between iron status (PF, sTfR) or PHep and zinc absorption.

Previous studies in cell cultures and animal models have suggested a possible role for PHep in regulation of zinc absorption. Ferroportin in Xenopus oocytes has some affinity to zinc as a substrate (11). An *in vitro* study applying physiologic hepcidin concentrations of 1 μM for 3-24h on Caco-2 cells reported decreased zinc transport from the apical to the basolateral cell side, a change which was associated with a marked decrease in ZnT1 expression. However, although knockdown of ZnT1 inhibited the PHep-mediated reduction in zinc transport, this did not occur in the cells when Fpn was knocked down (15). In mice, Fpn knockdown resulted in marked hypoferremia but no change in plasma zinc (11). Our data from this pooled analysis in human zinc absorption studies suggests that PHep is unlikely to be a major physiological determinant of zinc absorption in humans.

In this study, PZC was a significant negative predictor of TAZ in both adults and Burkinabé infants. A previous study of dietary zinc depletion and repletion reported no correlation between PZC and FAZ, but PZC was not assessed at the same time as meal isotopic zinc administration (26). In contrast, and consistent with our data, a recent study in Dutch women found PZC, assessed at the same time as isotopic zinc administration, was a significant predictor of FAZ (27). Several authors have suggested zinc stores and zinc pool size to play a minor role in regulating zinc absorption (28), and it is generally accepted that zinc homeostasis is chiefly maintained by modulating zinc losses instead of directly regulating gastrointestinal absorption (5). Our data indicates a relatively strong association between PZC and TAZ, which may or may not be related to body zinc stores for which PZC is not considered to be a very reliable measure. PZC is affected by infection/inflammation and by gender, and although we attempted to correct for these effects by including these factors in our models, we cannot rule out a residual effect being present in our final calculations. In another study of zinc absorption in children <5y (29), there were no significant associations between CRP, PZC and TAZ, but PZC and CRP concentrations were not measured in all the children.

In our study, the PA:Zn molar ratio was a significant negative predictor of zinc absorption, independent of PZC. The inhibitory effect of PA on zinc absorption is well established in adults (30, 31). However, a comprehensive study of determinants of zinc absorption in children <5 y identified zinc intake, weight, age and zinc pool size as determinants of TAZ, but not the content of PA in the meal (29). In our study, PA:Zn ratio was associated with TAZ in infants, but this was based on a study employing a phytase at point of consumption. Therefore, further studies should investigate the putative effect of PA on zinc absorption in infants and young children.

In our infants, height was a positive independent predictor of TAZ. This is consistent with a recent analysis indicating that age, body weight, and length/height, all of which are associated with small intestine length, were significant predictors of zinc absorption (29). In their comprehensive analysis in children between 8 months and 5y of age, age was the best predictor of TAZ, and when age was controlled for, weight was a stronger predictor of zinc absorption than height (29).

Among the infants in this study, 38% had inflammation as defined by a CRP > 1 mg/L at least 1 meal administration, indicating common infections (32). CRP was a significant negative predictor of PZC in the infants; in addition, it was a positive predictor of TAZ. The effect of inflammation on TAZ appears to be distinct from its effect on PZC. In animal studies, endotoxin administration enhanced the absorption/retention of an orally administered isotopically labelled zinc (33, 34), possibly associated with an increase in ZIP14 along the entire gastrointestinal tract (33), suggesting that proinflammatory cytokines may positively affect gastrointestinal zinc absorption. However, in contrast to our data in infants, in our adult subjects, who had CRP levels in the normal range, sTfR and gender were the only predictors of PZC. Gender has previously been described as a predictor of PZC (35). A lower PZC is also expected during hemodilution, which may be caused by oral contraceptive use (36), which was common in our study population.

Some of the findings in our dataset can be explained by the design of the included studies: in infants, the meal zinc content was constant, which explains it not being a predictor of TAZ. In adults, the prevalence of elevated CRP was low (9.7%), making it unlikely to be an important determinant of zinc absorption. Studies 8-12 focused on the ability of EDTA at different EDTA:Zn molar ratios to enhance Zn absorption from water-soluble and water-insoluble zinc

compounds and its interaction with PA and other meal ingredients, such as polyphenols. Generally, the presence of EDTA, a well know enhancer of iron absorption (37) was not a predictor of zinc absorption in our data.

To our knowledge this is the first study examining potential links between PHep and zinc absorption, controlling for iron and inflammation, measured with zinc stable isotopes in infants and children. Strengths of our study include: 1) all studies assessed zinc absorption by the dual isotope tracer method, implemented in the same laboratory using the same standardized methodology; 2) the test meals included a range of different cereals, such as wheat, rice, maize, millet and sorghum and a variety of different zinc content levels, resulting in a broad range of PA:Zn ratios; 3) our plasma samples were free from potential confounding from diurnal variation (38) as all samples were collected in the morning, after an overnight fast; and 4) importantly, all biochemical markers were assessed at the same time as test meal and isotopic administration. However, our study also has limitations: 1) the analysis in infants was done in a relatively small number of subjects (n = 41); 2) hemoglobin was not measured in the studies and thus could not be included in the analysis; 3) the study was a secondary analysis and this may have introduced bias into our findings; and 4) while we attempted to correct for known confounders of the potential relationship between PHep and zinc absorption, such as iron status and infection/inflammation, we cannot fully exclude a residual effect in our models. Despite these limitations, our human data support previous data from animal models and suggests that PHep is unlikely to be a significant predictor of zinc absorption in humans.

Table 1. Summary and key characteristics of included studies

			Meal 1				Meal 2				Meal 3			
Study	Investigated effect	Ref.	Description	Zn content	PA:Zn	EDTA:Zn	Description	Zn content	PA:Zn	EDTA:Zn	Description	Zn content	PA:Zn	EDTA:Zn
1	Intrinsically labeled wheat	(39)	Control wheat	5.7	32	0	Fortified wheat	9.3	18	0	Biofortified wheat	8.3	17	0
2	Biofortified wheat, 100% ER	(39)	Control wheat	3.8	24	0	Fortified wheat	6.4	10	0	Biofortified wheat	6.6	13	0
3	Biofortified wheat, 80% ER	(39)	Control wheat	1.5	20	0	Fortified wheat	4.0	6	0	Biofortified wheat	4.5	5	0
4	Zn fortified water	(40)	Fortified Maize	2	16	0	Maize + fortified water	2	16	0	Fortified water	2	0	0
5	Intrinsically labeled biofortified rice	(32)	Biofortified rice	1.1	12	0	Millet + phytase	1.1	12	0	-	-	-	-
6	Phytase during digestion	(21)	$ZnSO_4$ Fortified Maize porridge	2.8	9	0	$ZnSO_4$ Fortified Maize	2.8	4.5	0	$ZnSO_4$ Fortified Dephytinized maize	2.8	0	0
7	EDTA at 2 ratios with soluble fortificant	(21)	$ZnSO_4$ Fortified Maize porridge	2.8	9	0	$ZnSO_4$ Fortified Maize + EDTA	2.8	9	1	$ZnSO_4$ Fortified Maize + 2 EDTA	2.8	9	2
8	EDTA at 2 ratios with insoluble fortificant	(21)	ZnO Fortified Maize porridge	2.8	9	0	ZnO Fortified Maize + EDTA	2.8	9	1	ZnO Fortified Maize + 2 EDTA	2.8	9	2
9	Polyphenols and EDTA	(21)	$ZnSO_4$ Fortified Dephytinized white sorghum	2.8	0	0	$ZnSO_4$ Fortified dephytinized red sorghum	2.8	0	0	$ZnSO_4$ Fortified dephytinized red sorghum + EDTA	2.8	0	1
10	Polyphenols, PA and EDTA	(21)	$ZnSO_4$Fortified white sorghum	3	11.6	0	$ZnSO_4$ Fortified red sorghum + EDTA	2.8	9.2	0	$ZnSO_4$ fortified red sorghum	2.8	9.2	1
11	Phytase in children	(41)	Millet	1.4	7.7	0	Millet + phytase	1.4	3.5	0	-	-	-	-

EDTA:Zn, EDTA zinc molar ratio; ER: extraction rate; PA:Zn, phytic acid zinc molar ratio; ZnO, Zn oxide; $ZnSO_4$, Zn sulfate; EDTA administered in the form of Na_2EDTA.

Table 2. Characteristics of study participants, by group [1]

	Children	Adults	p-value
n (males)	41 (21)	183 (75)	
Age, y	1.49 ± 0.24	22.56 ± 2.66	< 0.001
Height, cm	76.8 ± 3.9	174.08 ± 7.89	< 0.001
Stunting prevalence [%]	20	-	
Weight, kg	9.2 ± 0.93	66.64 ± 8.99	< 0.001
BMI, kg/m2	15.42 ± 0.96	22.10 ± 1.80	< 0.001
PZC, mg/dL	81.15 ± 13.19	88.34 ± 11.19	0.197
CRP, mg/L	0.649 (0.543, 4.415)	0.749 (0.583, 0.934)	0.037
PF (µg/L)	6.500 (5.619, 12.851)	38.645 (34.007, 43.916)	0.500
PHep (nM)	0.385 (0.3926, 3.848)	1.570 (1.371, 1.785)	0.181
PHep < 1 NM [%]	68	36	
sTfR (mg/L)	3.275 (2.909, 3.925)	1.141 (1.100, 1.183)	< 0.001
Body iron (mg/kg)	1.149 ± 4.084	11.254 ± 3.475	< 0.001

[1] Values are means ± SDs or median (95% CIs).

CRP, C-reactive protein; PHep, plasma hepcidin; PZC, plasma Zn; PF, plasma ferritin; sTfR, soluble transferrin receptor.

Table 3. Summary of different linear regression models with total absorbed zinc (TAZ) as the dependent variable in Swiss adults (n = 183).

	ID		Meal Zn		EDTA:Zn		PA:Zn		Gender		Log(Age)		Log(Height)		Log(Weight)		Log(PZC)		Log(CRP)		Log(PHep)		Log(PF)		Log(sTfR)	
	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p
1	-.270	.012	.367	<0.001	-.115	.097	-.322	<0.001	-.051	.649	.032	.637	.043	.704	-.089	.453	-.233	.002	.021	.767	.095	.307	.059	.611	.051	.521
2	-.266	.012	.369	<0.001	-.117	.089	-.316	<0.001	-.050	.659	.034	.614	.036	.745	-.084	.473	-.236	.002			.094	.310	.060	.604	.051	.520
3	-.268	.011	.367	<0.001	-.118	.086	-.314	<0.001	-.058	.595	.034	.611			-.061	.511	-.232	.002			.092	.318	.060	.608	.051	.520
4	-.273	.010	.365	<0.001	-.120	.080	-.316	<0.001	-.072	.494					-.074	.403	-.238	.001			.095	.301	.055	.633	.057	.469
5	-.272	.010	.365	<0.001	-.119	.082	-.314	<0.001	-.095	.311					-.075	.394	-.238	.001			.122	.086			.042	.557
6	-.231	.021	.387	<0.001	-.102	.117	-.292	<0.001	-.062	.489					-.042	.602	-.243	.001			.084	.205				
7	-.232	.020	.392	<0.001	-.099	.128	-.289	<0.001	-.039	.614							-.247	<0.001			.084	.205				
8	-.232	.020	.383	<0.001	-.099	.126	-.297	<0.001									-.233	<0.001			.097	.113				
9	-.275	.004	.368	<0.001			-.300	<0.001									-.249	<0.001			.106	.083				
10	-.269	.005	.366	<0.001			-.299	<0.001									-.237	<0.001								

ID, subject ID; EDTA:Zn, EDTA:zinc molar ratio; PA:Zn, phytic acid : zinc molar ratio; PZC, plasma zinc concentration, CRP, C-reactive protein, PHep, plasma hepcidin; sTfR, soluble transferrin receptor.

Models R^2 : 0.341, 0.345, 0.349, 0.352, 0.355, 0.344, 0.347, 0.349, 0.344, 0.337 for models 1-10 respectively

Table 4. Final linear regression models obtained describing the variation in total absorbed zinc (TAZ) in African infants and Swiss adults[1]

	Infants		Adults	
Variable	Standardized β	P value	Standardized β	P value
Intercept	0.248[1]	0.023	0.661[1]	<0.001
ID	.169	0.054	-0.269	0.005
Log(PA:Zn)	-.452	<0.001	-0.299	<0.001
Meal Zn	-	--	0.366	<0.001
Log(Height)	.311	.001	-	-
Log(PZC)	-.208	.024	-0.237	<0.001
Log(CRP)	.209	.023	-	-

[1] unstandardized β. R^2 = 0.491 and 0.506 for children and adults, respectively. ID, subject ID; CRP, C-reactive protein; PZC, Plasma zinc concentration; PA, phytic acid.

Table 5. Linear mixed effect models for total absorbed zinc (TAZ) in Swiss adults (N = 183) and Burkinabe infants (N = 41)

Parameter	Estimate (95% CI)	P values
Estimates of fixed effect for adults		
Intercept	.50 (0.22, 0.79)	0.001
Zinc concentration in meal	.12 (0.09, 0.16)	<0.001
PA:Zn	-.000125 (-0.001, 0.0009)	0.823
PZC	-.22 (-0.37, -0.76)	0.003
PHep	.038 (-0.008, 0.850)	0.106
Estimates of fixed effects for infants		
Intercept	-.662039 (-1.19, -0.13)	0.015
PA:Zn	-.004449 (-0.0066, -0.0023)	<0.001
Height	.490301 (0.23, 0.75)	<0.001
PZC	-.090652 (-0.178, -0.004)	0.040
CRP	.017794 (0.001, 0.35)	0.038

CRP, C-reactive protein; PA:Zn, phytic acid zinc molar ratio; PHep, plasma hepcidin; PZC, plasma zinc concentration. Models used total absorbed zinc as the continuous response variable, indicated parameters as fixed factors, and subject ID as random effects on the intercept.

Table 6. Summary of different linear regression models with total absorbed zinc (TAZ) as the dependent variable in Burkinabe infants (n = 41).

Model	ID		PA:Zn		Gender		Log(Age)		Log(Height)		Log(Weight)		Log(PZC)		Log(CRP)		Log(PHep)		Log(PF)		Log(sTfR)	
	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p
1	.179	.101	-.401	<.001	.031	.775	.128	.316	.299	.118	-.065	.697	-.199	.062	.244	.029	-.020	.889	-.130	.506	-.076	.640
2	.183	.080	-.400	<.001	.028	.790	.125	.317	.302	.109	-.067	.683	-.200	.058	.246	.025			-.145	.372	-.076	.634
3	.181	.080	-.402	<.001			.136	.247	.290	.109	-.059	.713	-.205	.047	.241	.025			-.135	.390	-.068	.663
4	.183	.075	-.400	<.001			.143	.217	.240	.042			-.200	.049	.239	.025			-.137	.378	-.076	.621
5	.168	.084	-.398	<.001			.133	.239	.251	.030			-.201	.046	.225	.027			-.077	.423		
6	.166	.072	-.392	<.001			.142	.192	.247	.025			-.212	.027	.212	.028						
7	.183	.047	-.389	<.001					.324	.001			-.207	.032	.219	.024						

R^2 = 0.361, 0.372, 0.381, 0.389, 0.397, 0.405 and 0.399 for models 1-7, respectively.

ID, subject ID; PA:Zn, phytic acid : zinc molar ratio; PZC, plasma zinc concentration; CRP, C-reactive protein; PF, plasma ferritin; PHep, plasma hepcidin; sTfR, soluble transferrin receptor.

Supplemental Table 1: Pearson Correlations coefficients for Adults with log-transformed data

		ID	Meal Zn	PA:Zn	EDTA:Zn	Gender	Log(Age)	Log(Height)	log(Weight)	Log(PZC)	Log(CRP)	Log(Phep)	Log(PF)	Log(sTfR)
ID	Pearson Correlation	1	-.740**	-.430**	.300**	-.259**	-.047	.177*	.243**	-.006	-.079	.072	.147	.112
	Sig. (2-tailed)		.000	.000	.000	.000	.524	.015	.001	.940	.285	.333	.054	.143
Meal Zn	Pearson Correlation	-.740**	1	.300**	-.159*	.319**	.027	-.252**	-.303**	-.035	.072	-.071	-.162*	-.057
	Sig. (2-tailed)	.000		.000	.031	.000	.716	.001	.000	.636	.329	.341	.036	.462
PA:Zn	Pearson Correlation	-.430**	.300**	1	-.120	.308**	-.007	-.210**	-.284**	-.120	.316**	-.041	-.137	-.010
	Sig. (2-tailed)	.000	.000		.104	.000	.920	.004	.000	.105	.000	.582	.075	.892
EDTA:Zn	Pearson Correlation	.300**	-.159*	-.120	1	-.073	-.045	.007	-.014	.138	-.121	-.057	-.012	.162*
	Sig. (2-tailed)	.000	.031	.104		.327	.540	.924	.853	.062	.101	.446	.872	.035
Gender	Pearson Correlation	-.259**	.319**	.308**	-.073	1	-.037	-.645**	-.639**	-.422**	.223**	-.392**	-.581**	.065
	Sig. (2-tailed)	.000	.000	.000	.327		.615	.000	.000	.000	.002	.000	.000	.397
Log(Age)	Pearson Correlation	-.047	.027	-.007	-.045	-.037	1	-.086	-.171*	-.095	.057	-.003	-.045	.059
	Sig. (2-tailed)	.524	.716	.920	.540	.615		.242	.019	.200	.444	.970	.555	.445
Log(Height)	Pearson Correlation	.177*	-.252**	-.210**	.007	-.645**	-.086	1	.797**	.384**	-.305**	.176*	.276**	.146
	Sig. (2-tailed)	.015	.001	.004	.924	.000	.242		.000	.000	.000	.016	.000	.057
log(Weight)	Pearson Correlation	.243**	-.303**	-.284**	-.014	-.639**	-.171*	.797**	1	.321**	-.174*	.243**	.308**	.189*
	Sig. (2-tailed)	.001	.000	.000	.853	.000	.019	.000		.000	.018	.001	.000	.013
Log(PZC)	Pearson Correlation	-.006	-.035	-.120	.138	-.422**	-.095	.384**	.321**	1	-.262**	.111	.158*	.122
	Sig. (2-tailed)	.940	.636	.105	.062	.000	.200	.000	.000		.000	.134	.039	.112
Log(CRP)	Pearson Correlation	-.079	.072	.316**	-.121	.223**	.057	-.305**	-.174*	-.262**	1	-.017	-.096	-.017
	Sig. (2-tailed)	.285	.329	.000	.101	.002	.444	.000	.018	.000		.824	.212	.828
Log(Phep)	Pearson Correlation	.072	-.071	-.041	-.057	-.392**	-.003	.176*	.243**	.111	-.017	1	.714**	-.318**
	Sig. (2-tailed)	.333	.341	.582	.446	.000	.970	.016	.001	.134	.824		.000	.000
Log(PF)	Pearson Correlation	.147	-.162*	-.137	-.012	-.581**	-.045	.276**	.308**	.158*	-.096	.714**	1	-.450**
	Sig. (2-tailed)	.054	.036	.075	.872	.000	.555	.000	.000	.039	.212	.000		.000
Log(sTfR)	Pearson Correlation	.112	-.057	-.010	.162*	.065	.059	.146	.189*	.122	-.017	-.318**	-.450**	1
	Sig. (2-tailed)	.143	.462	.892	.035	.397	.445	.057	.013	.112	.828	.000	.000	

**. Correlation is significant at the 0.01 level (2-tailed)

*. Correlation is significant at the 0.05 level (2-tailed)

Supplemental Table 2: Pearson Correlations coefficients for Infants with log-transformed data

		PA:Zn	Gender	Log(Age)	Log(Height)	Log(Weight)	Log(PZC)	Log(CRP)	Log(Phep)	Log(PF)	Log(sTfR)
PA:Zn	Pearson Correlation	1	-.023	.061	.077	.046	.105	-.166	-.134	-.116	.017
	Sig. (2-tailed)		.841	.593	.508	.690	.359	.144	.238	.323	.888
Gender	Pearson Correlation	-.023	1	.268*	-.017	.108	-.161	-.004	.175	.083	.060
	Sig. (2-tailed)	.841		.017	.883	.343	.157	.969	.124	.485	.610
Log(Age)	Pearson Correlation	.061	.268*	1	.558**	.394**	-.034	-.006	-.103	-.138	.200
	Sig. (2-tailed)	.593	.017		.000	.000	.769	.960	.369	.240	.087
Log(Height)	Pearson Correlation	.077	-.017	.558**	1	.801**	-.113	-.047	-.179	-.137	.059
	Sig. (2-tailed)	.508	.883	.000		.000	.329	.684	.119	.253	.623
Log(Weight)	Pearson Correlation	.046	.108	.394**	.801**	1	-.202	.033	-.150	-.147	.106
	Sig. (2-tailed)	.690	.343	.000	.000		.075	.771	.186	.211	.369
Log(PZC)	Pearson Correlation	.105	-.161	-.034	-.113	-.202	1	-.316**	.000	-.073	.014
	Sig. (2-tailed)	.359	.157	.769	.329	.075		.005	.999	.537	.904
Log(CRP)	Pearson Correlation	-.166	-.004	-.006	-.047	.033	-.316**	1	.080	.176	.038
	Sig. (2-tailed)	.144	.969	.960	.684	.771	.005		.485	.133	.749
Log(Phep)	Pearson Correlation	-.134	.175	-.103	-.179	-.150	.000	.080	1	.690**	-.474**
	Sig. (2-tailed)	.238	.124	.369	.119	.186	.999	.485		.000	.000
Log(PF)	Pearson Correlation	-.116	.083	-.138	-.137	-.147	-.073	.176	.690**	1	-.726**
	Sig. (2-tailed)	.323	.485	.240	.253	.211	.537	.133	.000		.000
Log(sTfR)	Pearson Correlation	.017	.060	.200	.059	.106	.014	.038	-.474**	-.726**	1
	Sig. (2-tailed)	.888	.610	.087	.623	.369	.904	.749	.000	.000	

**. Correlation is significant at the 0.01 level (2-tailed)

*. Correlation is significant at the 0.05 level (2-tailed)

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IX Manuscript 3

The efficacy of postharvest fortified wheat and biofortified wheat produced through foliar application on zinc status of Indian children: a randomized, controlled trial

Coralie Signorell[1], Anura V Kurpad[2], Maria Pauline[4], Swapna Shenvi[3], Arpita Mukhopadhyay[2], Janet C King[3], Michael B. Zimmermann[1], Diego Moretti[1]

[1] Laboratory of Human Nutrition, Institute of Food, Nutrition and Health, Department of Health Sciences and Technology, ETH Zurich, Switzerland
[2] Divisions of Nutrition and St. John's Research Institute, St. John's National Academy of Health Sciences, Bangalore, India
[3] Nutrition and Metabolism Center, Children's Hospital Oakland Research Institute, Oakland, California
[4] Department of Physiology, St. John's Research Institute, St. John's National Academy of Health Sciences, Bangalore, India

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Abbreviations: **AAS**, Atomic Absorption Spectrophotometer; **BFW**, biofortified wheat; **CW**, control wheat; **CRP**, C-reactive protein; **HAZ**, height for age Z-score; **Hb**, hemoglobin; **mRNA**, messenger ribonucleic acid; **LMM**, linear mixed-effects models; **PHFW**, fortified wheat; **PA**, Phytic acid; **PZC**, plasma zinc concentration; **SRSS** sparse random serial sampling; **WAZ**, weight for age Z-score; **ZnT1**, Zinc transporter 1

Abstract

Background: Biofortification by foliar application of zinc can increase zinc content of wheat but its efficacy to improve zinc status is uncertain. New putative zinc biomarkers are available but their response to long-term increase of zinc intake has not been tested.

Objective: Our objectives were to test: 1) the efficacy of biofortified wheat (BFW) produced by foliar zinc application or post-harvest fortified wheat (PHFW) in Indian schoolchildren; and 2) the response of two zinc biomarkers, leukocyte DNA strand breaks and zinc transporter 1 (ZnT1) transcripts, to these interventions.

Methods: We conducted a 20-week double-blind intervention trial in Indian school-age children (n = 273) who were randomly assigned to receive a daily meal made from either control unfortified wheat (CW), PHFW, or BFW (mean ± SD zinc concentrations in the wheats were 21.17 ± 0.38, 45.05 ± 1.10 and 47.96 ± 8.12 ppm, respectively). Primary outcome was plasma zinc concentration (PZC); secondary outcomes included anthropometric measures, leukocyte ZnT1 transcripts and DNA strand breaks as assessed by the Comet assay. We applied sparse serial sampling and analysis was by intention-to-treat using mixed-effects models.

Results: At baseline, mean ± SD age of children was 8.2 ± 1.5 years and the prevalences of zinc deficiency, underweight and stunting were 12.3%, 28.6% and 14.7%, respectively. Accounting for compliance and a transient batch labeling error for the BFW, mean daily zinc intakes from the CW, PHFW and BFW meals during the trial were 2.6, 5.9 and 4.4 mg Zn/day, respectively. We found no time-by-treatment effect of zinc fortification on PZC (P = 0.23 for fortification; P = 0.66 for biofortification) or on any of the secondary outcomes. DNA strand breaks were not significantly correlated with PZC (n = 51; r = 0.004, P = 0.945).

Conclusions: Consumption of post-harvest zinc fortified and zinc biofortified wheat by Indian children did not significantly effect PZC, growth or zinc biomarkers. The trial was registered at: clinical trials NCT02241330 and clinical trials registry of India: CTRI/2015/06/005913

Keywords: biofortification, zinc, wheat, zinc deficiency, school-age children, India

Introduction

Zinc deficiency is common in low-income countries, mainly due to consumption of monotonous plant-based diets low in bioavailable zinc (1). Children, pregnant women and infants are at increased risk of zinc deficiency, because their zinc requirements are increased by growth (1). Cereal seeds are the major food staples and increasing their mineral content may help prevent mineral deficiencies (2, 3). Wheat is a major cereal crop worldwide (4), but provides limited zinc (20-25 ppm). The zinc content of wheat can be increased by the soil application of zinc-containing fertilizers (5) and/or foliar application of zinc to the wheat plants (6, 7).

To assess zinc status, measurement of plasma zinc concentration (PZC) is recommended (8-10). PZC responds to zinc supplementation (9): in children less than 12 years of age, zinc supplementation, with a dose as low as 1 mg, for at least 8 weeks, increased height, weight and PZC (11). PZC is most useful as a population biomarker of zinc status. But at the individual level, the association between PZC and zinc intakes is weak, as PZC can be influenced by health status and inflammation (12); dietary zinc bioavailability varies and accurate individual estimates of habitual dietary zinc intake is challenging (10). Thus, additional biomarkers of zinc status in humans would be valuable (10).

Low dietary zinc intake increases risk for single and double strand DNA breaks as well as oxidative modifications to DNA (13). The negative relationship between low zinc intake and DNA integrity has been confirmed in rat hepatocytes and white blood cells (14, 15) and rhesus monkey hepatocytes (16). A study in men investigating DNA integrity as a biomarker of zinc status found that zinc depletion was associated with an increase in DNA strand breaks of peripheral white blood cells, and these adverse changes that were ameliorated by subsequent zinc repletion (17). A recent human depletion-repletion study confirmed that leukocyte DNA strand breaks increased during zinc depletion, while they decreased with an additional 4 mg per day of dietary zinc (18).

The expression of the cellular zinc efflux transporters may decrease if cellular zinc levels fall (10). In a study in men who were experiencing acute zinc depletion (<2 mg dietary zinc/d), the expression of the zinc transporter 1 (ZnT1), which facilitates zinc transfer from the enterocyte into circulation, declined rapidly in leukocytes and whole blood (19). Because the expression

of ZnT1 is ubiquitous in all tissues, DNA integrity and expression of ZnT1 are putative zinc biomarkers (10).

Therefore, the objectives of this study were to test: 1) the efficacy of biofortified wheat (BFW) produced by foliar zinc application or post-harvest fortified wheat (PHFW) in Indian school-age children; and 2) the response of two zinc biomarkers, leukocyte DNA strand breaks and zinc transporter 1 (ZnT1) transcripts, to these interventions.

Material and Methods

Study design

We conducted a 20-wk double-blind randomized controlled trial from November 2014 to March 2015 to investigate the impact of BFW or PHFW compared with control wheat (CW) to improve zinc status. We randomly allocated around 70 children to 1 of 4 possible midpoint sampling days, between week 4 and week 16, by applying a randomized, arm-based stratified selection, so that at each midpoint the 3 arms were equally represented. We served each child lunch 6 days a week consisting of 3 chapattis prepared with the wheats and 7 sauces, served on a rotating basis to maintain interest. We assessed the response to the intervention with sparse random serial sampling (SRSS) with 3 observations per subject, as previously described (20, 21). Baseline and endline observations were fixed timepoints. Figure 1 shows study overview. Our primary outcome was PZC from baseline to endline. Two months after the end of the intervention, we collected a final blood sample.

Study site and participants

We conducted the study at the two neighboring primary schools located in Silvepura, a rural, low income setting on the northern outskirts of the Bangalore metropolitan area. We explained the study to the teachers, who invited parents of all children below 12 years of age to the school for informed consent in individual meetings, during which we explained the study procedures in the local language (Kannada). The two schools are in the same school district, but the language of instruction is Kannada in one, and English in the other.

All parents gave written informed consent. The inclusion criteria were: 1) less than 12 years of age; 2) generally healthy; 3) no chronic medications; and 4) no recent or current zinc supplementation. We enrolled a total of 284 children. The study protocol was approved by

the ETH Ethics Commission (EK 2013-N-52), by St John's National Academy of Health Ethics Commission and by the Ministry of Health of India (CTRI/2015/06/005913).

Randomization and masking

Using spreadsheet software (Microsoft Excel), we individually randomized children at enrollment and assigned them to 1 of 3 treatment arms (Figure 1). Randomization was masked, and we used a color code to identify the 3 groups. The code was held concealed by a person not involved in the fieldwork, laboratory measurements and data analysis.

Wheat preparation

Control wheat and BFW were produced by zinc foliar application in the Himalaya region of northern India by HarvestPlus and milled by Arti Flour (Punjab, India). The wheat variety PBW550 was sprayed 3 times with 0.5% ZnSO4·7H2O at the following growth stages: booting stage, early milk stage and early dough stage, as described earlier (22). Control wheat was grown in the same conditions, without spraying. We performed fortification on field, in batches for 4 days, to increase the PHFW concentration by 40 ppm, to the level of BFW. For a final amount of 50 kg of wheat and an increase of 40 ppm, 2 g of pure zinc was needed. The tolerance limit for the final fortification level was set to be 38.8 to 51.2 ppm. Fortification was carried out in two steps, first by making a concentrated premix, by diluting 5.48 g of H_20_5SZn (515059002, Dr. Paul Lohmann GmbH KG) (and therefore 1.97 g of elemental zinc) into 3 kg of CW and mixed. We added this premix to 47 kg of CW. We mixed the flour in a rotation mixer (Kirloskar) and then divided it into 12 kg packages. We collected samples of PHFW daily and measured zinc concentrations prior to consumption for the first half of the study. Control wheat and BFW were repacked in new 12 kg packages, and to ensure masking, a person not involved in the study was in charge of replacing the arm tags on the package with color tags according to the defined code. After masking, we transported wheat to the study kitchen, and collected flour samples for later analysis every 4 days. Wheat was weighed in a scale up to 100 g (Weighman AJN, 06332). During the school lunch break, we handed out color-coded identity cards to the study children, who, according to group assignment, collected their chapatti serving. Servings were consumed ad libitum under direct supervision. At each administration, we recorded any full, partial, or missing chapatti consumption. Compliance of 100% was defined as 120 g of wheat intake, equivalent to 3 chapattis.

We measured weekly samples from fortification before consumption. We analyzed all wheat samples, BFW, CW and PHFW, as well as cooked chapattis and sauces after the end of the study in ETHZ in Switzerland. Freeze-dried meals and wheat flour were analyzed in Zurich for their zinc content by flame atomic absorption spectrophotometry (AAS) (AA240FS; Varian) after microwave digestion (MLS-ETHOS plus; MLS GmbH) by using an HNO_3/H_2O_2 mixture and acid-washing of all material. The relative standard deviation of the analytical duplicates was 2%. We determined the phytic acid (PA) content by the modified Makower method (23) in which iron was replaced by cerium in the precipitation step. After the hydrolization of the precipitate in concentrated H_2SO_4 (Digest Automat K-438; Büchi Labortechnik AG), inorganic phosphate was determined according to Van Veldhoven and Mannaerts (24) and converted into PA concentrations.

Secondary outcomes

At all timepoints, we measured PZC, and because inflammation is a confounder of PZC, we measured α-1-acid glycoprotein (AGP) and C-reactive protein (CRP) (25). We performed anthropometric measurements at baseline and endline. We measured at each timepoint leukocyte DNA strand breaks and ZnT1 RNA transcripts in a random subsample children (n = 51) who had completed all timepoints and who had the midpoint blood sample within the sparse serial sampling scheme after 1 month of intervention. We measured height to the nearest 0.1 cm using a stable stadiometer and weight to the nearest 0.1 kg using a calibrated scale while the subjects were wearing their school uniform and no shoes (26). We calculated height-for-age, weight-for-age z scores (HAZ and WAZ respectively) using the WHO AnthroPlus program 2007.

Blood sampling and biochemical analysis

We followed standardized protocols for blood collection for zinc analysis from the International Zinc Nutrition Consultative Group (27). For the biochemical analyses, we collected morning fasting venous whole-blood samples from each study participant directly into trace element–free lithium heparin tubes (for PZC analysis, AGP and CRP) (Sarstedt, S-Monovette for Trace Metal Analysis (7.5mL; Lithium Heparin for Trace Metal Analysis; 15 x 92mm)) and EDTA tubes (for analysis of all other biochemical indicators) (Sarstedt S-monovette Potassium-EDTA 2.7ml). We assessed hemoglobin (Hb) with a portable HemoCue 201+ photometer (HemoCue AB). We recorded the time of blood withdrawal, plasma

separation and aliquoting. Samples from the trace element-free tubes were immediately stored in a refrigerated cool box and centrifuged within 40 min to 1 h (3000 g for 10 min at room temperature) in a portable centrifuge.

We aliquoted plasma in acid-washed test tubes (Eppendorf AG) and transported in a refrigerated cool box to the laboratory unit, where it was stored at -80°C within 6 h. We measured PZC by flame AAS (iCE3000, Thermo Scientific) using a commercial aqueous standard (Fixanal 02761-1EA-R; Fluka, Sigma) for external calibration, and Seronorm Trace Elements Serum L-1 (Sero AS) as reference material, which delivered values within acceptable ranges as specified by the manufacturer. Instrumental parameters were set at 213.9 nm for wavelength and no background correction. We analyzed CRP and AGP by immunoassay on a Hitachi 902 Analyser (Roche Diagnostics).

We directly aliquoted samples from the EDTA tubes in either a cryopreservation solution (15% DMSO, Sigma, 85% media DMEM, Gibcolife tech) for analysis of DNA strand breaks, or transferred in PAXGene tubes (Becton-Dickson) for analysis of RNA transcripts. Within 6 hours, we stored both samples at -80°C. We measured DNA single-strand breaks in peripheral blood cells, in a subsample of children, with the use of a Comet assay as described initially by Singh et al. (28) and recently by Zyba et al (18). Briefly, cryopreserved cells underwent electrophoresis after rapid thawing, slides application and DNA unwinding. After neutralization and washing, slides were dried and stored at room temperature. Nuclear material was stained with SYBR Green (Life Technologies) and images of randomly selected nuclei (of 2 replicate slides) were analyzed. The olive tail moment was used to indicate DNA damage.

We measured ZnT1 transcripts from PAXgene tubes frozen at -80°C until analysis, following a method previously described (29), for a subsample of 51 children. Briefly, RNA extraction was completed with the use of a PAXgene Blood RNA kit (Qiagen). The concentrations and purities of the extracted RNA samples were evaluated by measuring absorbance at 260 and 280 nm wavelengths following Quant-iT Ribogreen reagent kits (Invitrogen). Complementary DNA was synthesized from 1 mg total RNA with the use of a High capacity cDNA Reverse transcription kit (Applied biosystems) according to the manufacturer's protocol. Relative messenger RNA (mRNA) concentrations of specific ZnT1 and BACT genes were measured with the use of a quantitative real-time polymerase chain reaction (PCR). All quantitative real-time PCR assays

were conducted with SYBR Green dye (Invitrogen) on a Quantstudio 6 Flex (Applied biosystems). The specificity of each primer pair was confirmed with the use of melting curve analyses, and the relative quantity was determined by normalization with respective housekeeping genes with the use of the ΔΔ threshold cycle method.

We analyzed all samples without knowledge of study arm assignment and analyzed all timepoints of each participant within the same analytical run. All values represent the mean of an independent duplicate measurement; we reran the analysis if the CV exceeded 5% or if PZC was higher than 100 µg/dL. We calculated the prevalence of zinc deficiency by using sex- and age-specific PZC lower cutoffs (30). Subclinical inflammation was defined as CRP>5mg/L and/or AGP >1 g/L. Severe anemia was defined as hemoglobin < 70 g/L (31).

Statistical analysis

We estimated that 90 children were needed in each group, based on 80% power to detect a PZC difference of 5 mg/dL with a 0.05 significance level (2-tailed) and anticipating a dropout rate of 20%. We based the sample size calculation on the SD of PZC measured in previous studies in school-age children from rural settings conducted by the ETH (32). We conducted data analysis with the SPSS software (version 23) and the R statistical programming environment (version 3.0.3) using packages nlme and lme4 (33). When data were not normally distributed, values were logarithmically transformed before statistical analysis. Values in the text and in the tables are represented as means ± SDs for normally distributed data, medians (IQRs) for non-normal data, and percentages (95% CIs) for prevalences.

In the absence of the time variable, we tested group differences by using one-factor ANOVA with Bonferroni test for multiple comparisons for continuous variables and Pearson chi-square test for binary outcomes. We assessed the intervention effect over time (time-by-treatment interaction) by fitting linear mixed-effects models (LMMs) and logistic regression LMMs for all 3 timepoints for continuous and binary outcomes, respectively. Midpoint values were used only for the linear mixed model. Fixed effects of the variance were time, which was defined as the day from intervention start to account for unequal sampling intervals (subject-specific), and treatment, defined as the intervention arm. Subjects were the random component. For the PZC data only, and to best fit the correlation structure of the data, we modeled the fixed effect as a(t +1)21 function of time t, as known from previous work (34). As covariates of the model, we included variables that correlated with the dependent variable at baseline or that

were reasonably thought to predict the dependent variable. Statistical dependence was tested with the Pearson product-moment correlation coefficient (r) for normally distributed data and with Spearman's rank correlation coefficient (rs) for nonnormal data. To achieve the minimal adequate model, we applied a stepwise backward deletion procedure.

Significance was set at $P < 0.05$.

Results

Thirty-six PHFW flour samples were analyzed in duplicate for zinc concentration for the first 36 days of the study. The median (IQR) zinc concentration of the PHFW flour was 57.09 (54.7, 59.5) ppm. Due to a labeling mistake at the wheat mill, the first batch of BFW delivered to the study had an incorrect zinc concentration. The children were therefore fed for ~3 weeks BFW with zinc content comparable to CW. During that time, the median (IQR) zinc concentration of the CW, PHFW and BFW was 23.1 (22.6, 23.5) ppm, 57.0 (55.4, 59.4) mg/kg, and 24.29 (23.89, 24.55) respectively. For the rest of the study, the mean ± SD zinc concentration was 21.17 ± 0.38, 45.05 ± 1.10 and 47.96 ± 8.12 ppm for the CW, PHFW and BFW respectively. The mean ± SD PA concentration was 0.77 ± 0.03, 0.79 ± 0.09 and 0.79 ± 0.04 mg/100g respectively.

Overall compliance was 87 ± 25%. In the Kannada school, compliance was 92 ± 12, 98±22 and 95 ± 12, while it was 82 ± 16, 83 ± 26 and 81 ± 25 in the English school for the control, fortified and biofortified groups, respectively. Taking into account compliance, the zinc intake over the intervention period compliance to the test meal administration was 2.61 mg Zn/day in the CW group; 5.88 mg Zn/day in the PHFW group and 4.38 mg Zn/day in the BFW group. The results for the zinc content of the accompanying curry sauces ranged from 1.50 ± 0.52 to 6.20 ± 0.25 ppm and the PA content from 0.019 ± 0.005 and 0.101 ± 0.029 g/100g, and therefore a zinc : phytic acid molar ratio of 4 to 7.

In total, 284 children came to the informed consent with either a parent or a local guardian. 273 children enrolled in the study and blood could be collected and processed at baseline from 270 children. 257 children completed the study, resulting in a drop-out rate of 4.8%. Due to the fact that a new school year had started, blood was collected during the final time point from only 236 children, resulting in a drop-out rate of 8.9% from endline (Figure 1).

Baseline characteristics of the population can be found in Table 1. Mean ± SD age of the children was 8.2 ± 1.5 years. There was no significant group differences at baseline, except Hb

was significantly higher in the PHFW compared to CW and BFW (P = 0.035). At baseline, PZC positively correlated with weight (r = 0.134, P = 0.03) and study site (r = 0.219, P < 0.01), height correlated with Hb (r = 0.341, P < 0.01) and study site (r = -0.176, P = 0.04), and Hb negatively correlated with study site (r = -0.161, P = 0.008). The prevalence of zinc deficiency was higher in the Kannada school than in the English school (18.66% vs 5.93%). Of all drop-outs, 7% were from the Kannada school, while the remaining 93% were from the English school. At baseline, the prevalence of stunting was 14.65%, while 1.83% of the study population was severely stunted (HAZ <-3SD). The prevalence of underweight was 28.57%, while 1.83% were severely underweight. Around 29% of the study population was stunted and underweight.

The response of PZC by group during the intervention is shown in Table 1 and Figure 2. PZC was not significantly different between groups at baseline. We found no significant time-by-treatment effect of zinc fortification on PZC (P = 0.23 for fortification; P = 0.66 for biofortification). When AGP, CRP, gender and compliance were added in the model, only compliance was a predictor of PZC (P = 0.006). Compared to the endline measurement, PZC was significantly lower in CW and PHFW (P = 0.018 and 0.017 respectively) but not in BFW group (P = 0.900).

There was no time by treatment differences (P = 0.306) on HAZ or WAZ (P = 0.808) (Table 1). There was a significant group differences for WAZ to be lower in the control group compared to PHFW (P = 0.049) but not BFW (P = 0.070). There was a significant time differences for WAZ (P < 0.001), with a lower endline compared to baseline.

Olive tail moments (DNA single-strand breaks) as measured by the COMET assay by group during the intervention are shown in Figure 3; there were no significant group differences (P = 0.364). Olive tail moments negatively correlated with AGP (r = -0.167, P = 0.003) and height (r = -0.124, P = 0.038), but not with PZC (r = 0.004, P = 0.945). Group differences in ZnT1 transcripts during the intervention are presented in Figure 4, as a fold increase from baseline. No significant differences were found (P < 0.225 for all).

Discussion

The main finding of this study is that a 120% increase in daily zinc intake by Indian children through consumption of biofortified wheat or post-harvest fortified wheat for 20 weeks has no significant impact on PZC. Previous studies conducted with zinc stable isotopes in Swiss women indicate that ≈0.7 mg of zinc could be absorbed from ≈120 mg of consumed BFW (C.

Signorell, personal communication), and would provide ≈70% of the daily zinc requirement for children of this age group (0.97–1.12 mg/d). PZC is the only zinc biomarker recommended by IZiNCG (35), WHO/IAEA/UNICEF (8) and the biomarkers of nutrition for development committee (10) for assessment of zinc status, and PZC is a useful biomarker of severe zinc deficiency (36). However, the PZC response to zinc fortification is equivocal (37, 38). In school-age children, randomized trials of zinc fortification varying in length from 4 to 5 months have shown modest positive effects on PZC (21, 32, 39). There was a significant time by treatment effect on PZC and on zinc deficiency during a 20 week trial in which 2.8 mg of zinc was given daily in zinc-fortified water to Beninese children (20); ≈40% of children were zinc-deficient at baseline and, from stable isotope absorption studies, the bioavailability of zinc from the fortified water was estimated to be ≈7x higher than from fortified maize (21). In a study in Thai children, PZC increased in response to consumption of ≈9 mg of zinc daily in the form of extruded rice grains incorporated into a school lunch program (32); children were included in the trial only if they were zinc-deficient. In South African children, ≈50% who were zinc deficient at baseline, consumption of a micronutrient powder containing 2.5 mg of zinc daily in porridge for 23 weeks significantly decreased the prevalence of zinc deficiency (39). One important difference between our study and these previous studies is that the prevalence of baseline zinc deficiency was much lower in our study, and this may have biased our study toward a null effect. In our study, because of logistical challenges, we did not screen and include children who were zinc deficient. We anticipated that zinc status would be poor in the selected schools because of the area's low socioeconomic status, poor dietary quality and the relatively high prevalence of stunting, but this was not the case. Another difference between our study and previous studies was that the meals we provided contained a higher PA:Zn ratio (ranging from 4 to 7 in the meals), which is inhibitory to zinc absorption. The Benin study provided zinc as fortified water away from meals, the Thai study provided the additional zinc as extruded rice low in phytic acid, and the South African study provided the zinc fortificant with an exogenous phytase. Although the PA in our chapattis was likely modestly degraded through the use of lukewarm water to produce the dough, the phytic acid: zinc ratio remained high, ranging from 15 for the BFW and PHFW meals to 36 for the CW meal. Another factor which may have limited the impact of the BFW in this study was the labeling error at the mill which resulted in the children in the BFW arm of the study receiving unfortified wheat for the

first 3 weeks of the 20 week study. However, there was no increase in PZC in the PHFW group, despite receiving the fortified wheat for the full intervention period.

Foliar zinc application to produce the BFW for this study effectively increased zinc concentration in wheat, even in a scaled-up production setting. Wheat provides more than half of energy intake in India (40) but its zinc content is relatively low. The genetic variation of zinc content in bread wheat is narrow (41) and the pool of zinc available to plants in soil is a limiting factor for the accumulation of a sufficient amount of zinc in grains (22). Foliar application of zinc fertilizers thus may be a promising approach to improve zinc intakes in India (42).

Because zinc homeostasis in humans is tightly controlled, assessment of zinc deficiency and response to zinc interventions is challenging (10). Thus, in this study we explored the potential of two new putative biomarkers of zinc status, DNA integrity and ZnT1 expression, and, to our knowledge, this is the first report of their response to a long-term zinc fortification intervention. Randomized short-term controlled trials have examined the impact of zinc supplementation on gene expression of zinc transporters and metallothionein (43, 44), and the expression of zinc efflux transporters of the ZnT family may be reduced when cellular zinc is low (10). However, zinc supplementation does not appear to modify expression of zinc transporters in the absence of preexisting zinc deficiency, as gene expression likely reflects long-term zinc intake (45). Two recent studies of DNA integrity (18, 44) have reported that in zinc-deficient individuals, zinc supplementation reduces DNA damage, irrespective of changes in PZC (45). Thus, the surprisingly low prevalence of baseline zinc deficiency in our study may have contributed to the lack of effect on these two zinc biomarkers. Another challenge to the use of these new biomarkers is the difficulties in optimal collection and storage of samples for DNA integrity in field studies in low-resource settings.

Inflammation biases the use of PZC as an indicator of zinc status; the reduction in PZC associated with inflammation is generally accepted to be ≈10% in adults (12). This decrease in PZC associated with inflammation was also reported in Indonesian children (46), while Peruvian children had similar PZC with or without infection (47). In our study, few children exhibited inflammation at baseline, and thus their PZC was likely an accurate indicator of their zinc status, without any correction (48).

In summary, the strengths of our study include: 1) its three-arm, double-blind, randomized controlled design; 2) the direct supervision of each child during meal consumption to assess compliance; 3) rigorous procedures to avoid consumption of other foods and beverages around the time of test meal consumption that might affect zinc absorption; 4) careful procedures to avoid zinc contamination of plasma samples for the assessment of plasma zinc concentration; and 5) the use of sparse serial sampling to assess changes in PZC. Limitations of our study include: 1) the duration was relatively short at 20 weeks; 2) the high PA:Zn molar ratios in the test meals likely limited zinc absorption; 3) the prevalence of zinc deficiency at baseline was lower than expected; 4) the limited number of children that were included in the assessment of the new zinc biomarkers; and 5) the batch labeling error reduced overall zinc delivered to the BFW group compared to the PHFW group, limiting direct comparisons of impact between these two arms.

Although this study was negative, foliar application of zinc appears to be a promising approach to biofortify wheat to high zinc concentrations. Therefore, future studies should test the potential of BFW in longer interventions in target populations with higher rates of zinc deficiency, such as preschool children. In this explorative study in a subsample of participants, the putative zinc biomarkers, DNA strand breaks and ZnT1 transcripts, did not significantly respond to zinc fortification. However, we were able to accurately measure these biomarkers in a field study in a low-resource setting, despite the challenges of field collection and processing. Additional studies of their performance as zinc biomarkers should be done in larger populations with more severe zinc deficiency.

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The authors' responsibilities were as follows—CS, DM, and MBZ: designed the research; CS: conducted the efficacy trial, analyzed the data, and had primary responsibility for the final content of the manuscript; AVK, DM, MP: supervised the efficacy trial; AM: supervised zinc transporters measurements; SS and JCK: were responsible for the new biomarkers concept, design and supervised DNA integrity and zinc transporters experiments, CS, DM, and MBZ: wrote the manuscript; and all authors: read and approved the final manuscript. The funder provided the wheat and had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. None of the authors declared any competing interests.

Figure 1 : Study Design After the informed consent done during a face-to-face interviews of 284 children and their parents, 273 children were randomly allocated to three different arms and received either biofortified wheat, fortified wheat or control wheat meals for 20 weeks. Baseline and Endline measurements happened on the 1st day of the feeding and on the last day of the feeding respectively. We assessed the response to the intervention with a sparse random serial sampling technique with 3 observations per subject. We randomly allocated 35 children to either 1 or 8 possible midpoint sampling days, between week 2 and week 13. At each midpoint, the 3 arms were equally represented. We assessed the timecourse of plasma zinc 2 months after the ending of the intervention, for a final blood sample, at week 28.

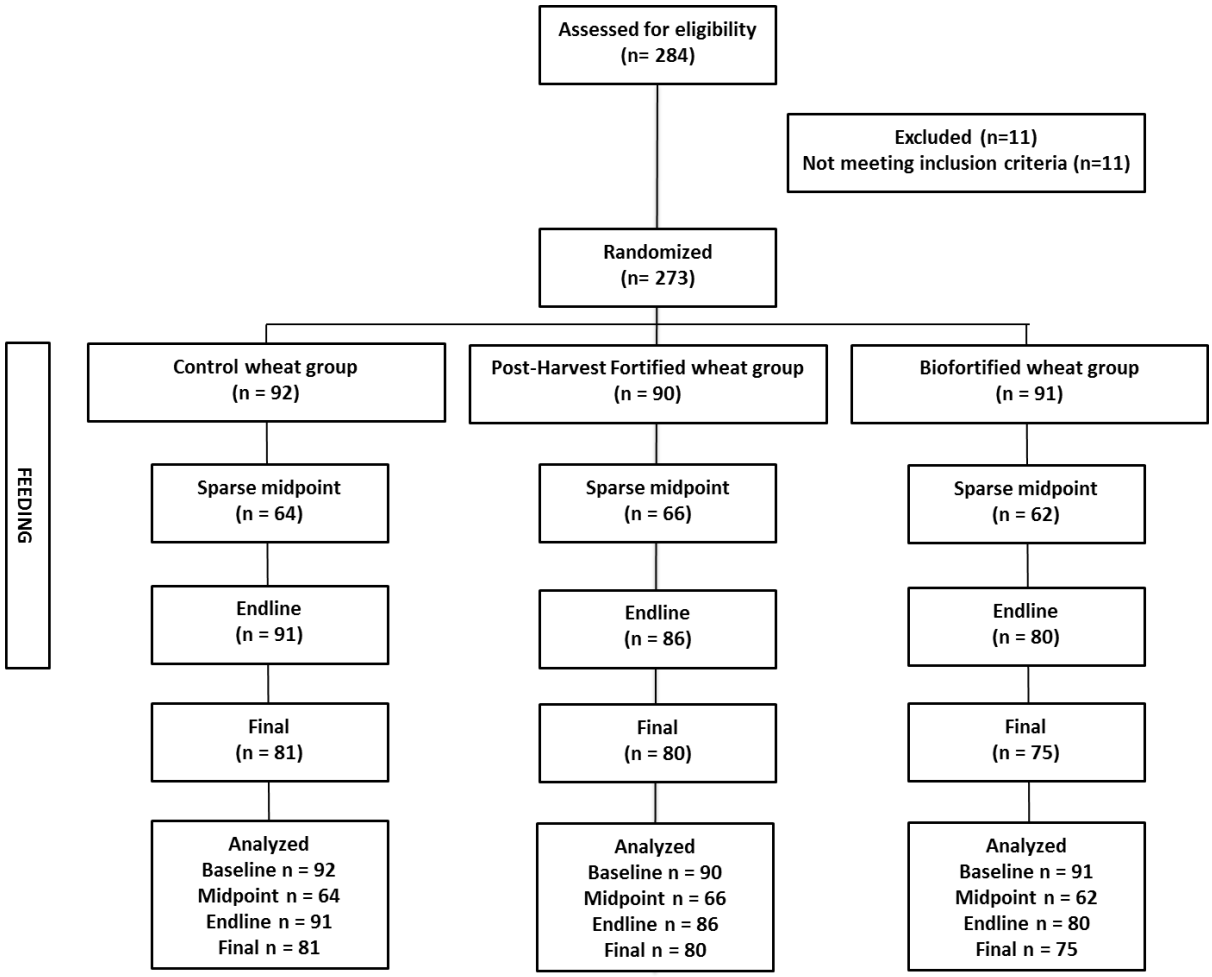


Table 1: Subject characteristics, anthropometric variables, plasma zinc concentration and the prevalence of zinc deficiency, biomarkers of inflammation and hemoglobin of Indian children participating in the efficacy trial of control, post-harvest fortified and biofortified wheat, by treatment group.

	Timepoint	Total	Control wheat group	Post-Harvest fortified wheat group	Biofortified wheat group
No of subjects (male)	Baseline	273 (139)	92 (44)	90 (48)	91 (47)
	Endline	257	91	86	80
	Final	236	80	80	75
Age [y]	Baseline	8.04 (5.51, 10.57)	8.32 (5.82, 10.82)	8.53 (6.09, 10.97)	8.12 (5.60, 10.63)
Height [cm]	Baseline	120.4 (105.0, 135.2)	119.6 (103.9, 135.3)	120.5 (108.2, 132.8)	120.5 (107.6, 133.4)
	Endline	121.3 (106.8, 135.8)	121.3 (106.1, 136.5)	122.0 (109.5, 134.5)	120.2 (106.7, 133.8)
Stunted [%]	Baseline	14.65	14.13	14.44	15.38
	Endline	15.56	14.81	17.44	16.25
Weight [kg]	Baseline	19.6 (13.8, 25.4)	19.5 (12.1, 26.9)	20.6 (15.3, 25.9)	18.9 (13.8, 24.1)
	Endline	20.3 (14.6, 26.0)	20.2 (12.9, 27.5)	21.0 (15.6, 26.5)	19.9 (14.6, 25.1)
Underweight [%]	Baseline	28.57	28.26	28.89	28.57
	Endline	24.90	23.46	29.00	25.00
Plasma Zinc [µg/dL]	Baseline	79.00 (64.77, 93.23)	80.90 (64.38, 97.42)	78.76 (66.72, 90.80)	77.92 (61.66, 94.18)
	Endline	79.96 (67.44, 92.48)	80.72 (69.96, 91.48)	82.36 (68.29, 96.43)	77.60 (66.43, 88.77)
	Final	77.30 (63.70, 90.90)	77.04 (63.86, 90.22)	76.62 (64.18, 89.06)	77.88 (63.12, 92.64)
Zinc deficiency [%]	Baseline	12.3	11.1	8.9	16.9
	Endline	8.4	3.6	9.3	12.5
	Final	13.6	11.1	16.3	13.3
CRP [mg/L]	Baseline	0.10 (-0.50, 0.70)	0.10 (-0.50, 0.70)	0.10 (-0.70, 0.90)	0.10 (-0.30, 0.50)
	Endline	0.40 (-0.20, 1.00)	0.30 (-0.10, 0.70)	0.40 (-0.35, 1.15)	0.30 (-0.38, 0.98)
AGP [g/L]	Baseline	0.72 (0.42, 1.02)	0.70 (0.40, 1.00)	0.74 (0.36, 1.13)	0.70 (0.45, 0.95)
	Endline	0.90 (0.53, 1.27)	0.92 (0.57, 1.28)	0.90 (0.46, 1.34)	0.89 (0.54, 1.24)
Elevated CRP [%]	Baseline	3.3	5.4	1.1	3.2
	Endline	5.1	3.3	5.8	6.3
Elevated AGP [%]	Baseline	16.5	13.0	21.1	14.3
	Endline	35.4	35.2	38.4	31.3
Hb [g/L]	Baseline	132 (112, 152)	132 (110, 153) [a]	137 (120, 154) [b]	131 (107, 155) [ab]
	Endline	125 (111, 139)	124 (108, 140) [a]	125 (111, 140) [b]	125 (111, 139) [ab]

Normally distributed variables are presented as medians; IQRs in parentheses, whereas prevalences are presented as percentages.

Normally distributed data were analyzed by using a mixed-effects model

Values in a row with different superscript letters are significantly different, $P < 0.05$

Figure 2. Plasma zinc concentrations in Indian children (n=273), by group. Time-by-treatment interaction effect based on linear mixed-effects model. The colored lines show the time course of PZC, the grey shaded areas show the confidence interval

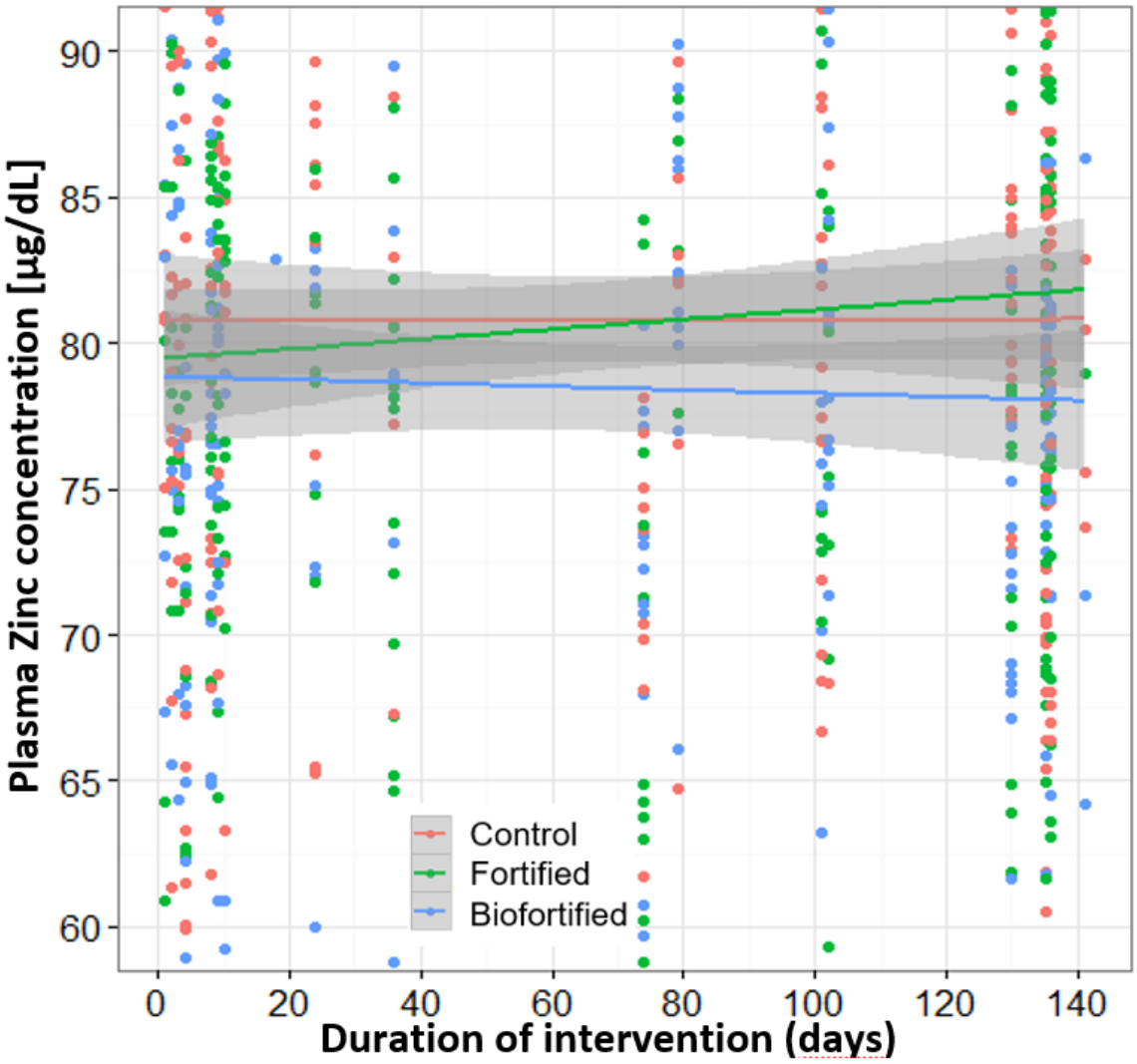


Figure 3. Mean ± SD DNA strand breaks with the use of comet assay. Olive tail moments (arbitrary units) are depicted with a red bar for the control group, green bar for the fortified group and blue bar for the biofortified group. Total n = 51. Significant differences between means were determined with the use of a linear mixed model. Bars with the same letter or symbol represent means that are not significantly different from each other.

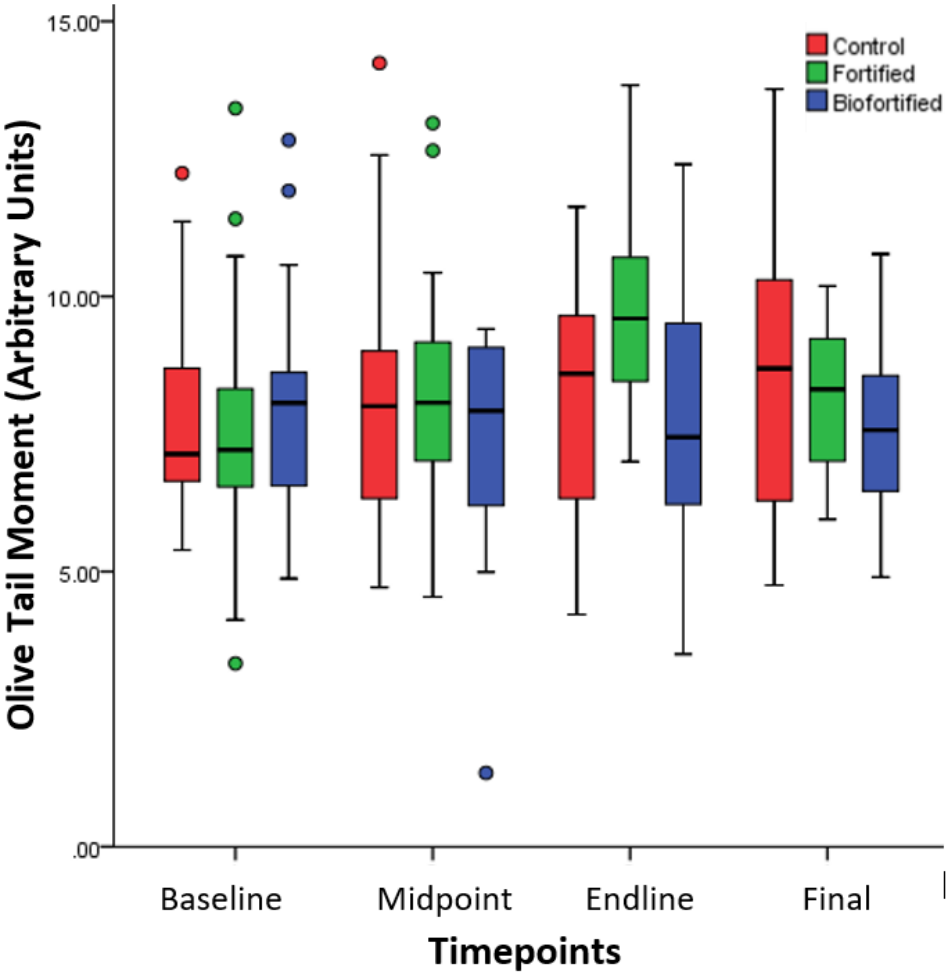
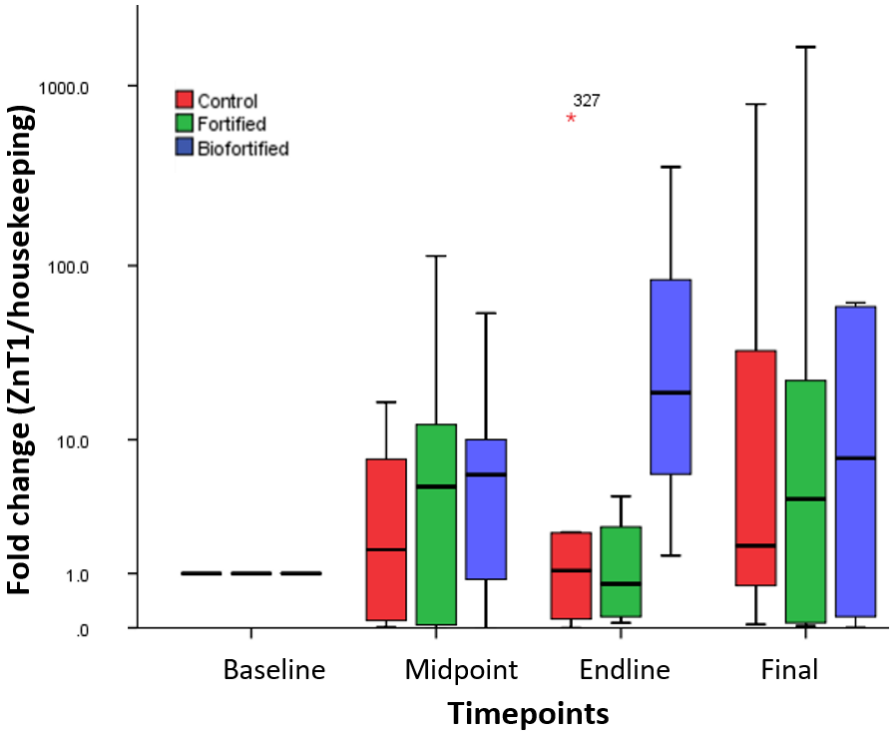


Figure 4. Gene expression analysis of zinc transporters expressed in peripheral blood cells. All measurements represent n = 2. Significant differences between means were determined with the use of a linear mixed model. Bars with the same letter or symbol represent means that are not significantly different from each other.



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X Discussion

The overall aims of this thesis were to investigate the bioavailability of zinc from biofortified wheat produced by foliar zinc application; identify determinants of zinc absorption in children and generally healthy adults; and determine the efficacy of biofortified wheat on zinc status and markers of zinc metabolism. In the following section, the project rationale is described first, followed by the interpretation, contextualization, limitations and public health relevance of the main findings.

X.1 Public Health Relevance of Zinc Deficiency and Biofortification

Recent investigations have confirmed the link between dietary zinc intake and stunting: a meta-analysis shows that zinc intake increases growth parameters of infants (Nissensohn et al., 2016) while the estimated prevalence of inadequate zinc intake is correlated with the prevalence of stunting in children under 5 years (Wessells & Brown, 2012). Impacts of zinc deficiency in children are not only related to their development and height, but their cognitive scores are predicted by stunting (Fischer Walker et al., 2012). Unfortunately, the effect of early stunting in childhood on cognition are long lasting, as a catch-up growth does not associate with cognitive development (Sokolovic et al., 2014). The long-term consequences of stunting on cognition show the public health challenges faced in countries at risk of zinc deficiency. Currently, 100 countries are classified as at risk of zinc deficiency and 23% of low and middle income countries are classified as at high-risk of inadequate zinc intake (Wessells & Brown, 2012).

Reducing zinc deficiency through dietary diversification and food interventions remain an important challenge though (Kumssa et al., 2015). Food interventions, such as fortification, cannot always reach rural areas of developing countries, have to be closely monitored, and require a relatively high initial investment. In our efficacy study in India (**manuscript 3**), we implemented small-scale fortification and this required one full-time equivalent throughout the study. Fortification has already been successful at reducing the prevalence of inadequate micronutrient intakes, but countries where the food energy supply is adequate show a very large variation in dietary quality (Beal, Massiot, Arsenault, Smith, & Hijmans, 2017). In countries affected by zinc deficiency, a greater proportion of micronutrient-dense foods is needed.

X.2 The Need for Biofortification, and Future Perspectives

Increasing the content of a micronutrient in the staples directly, or improving its bioavailability, ensure that rural population can be reached. Recent evidence shows that biofortification is considered highly effective, except if the consumption of the crop is low and its substitution would not reach 25% (De Steur et al., 2012; Garcia-Casal, Pena-Rosas, & Giyose, 2017; Ma et al., 2008).

Even if genomic selection holds great potential in biofortification to enhance grain zinc in bread wheat (Velu et al., 2016), breeding requires time. Even though they are not long lasting, as they have to be added every time wheat is grown, agronomic practices are short-term answers to improve rapidly micronutrient concentration in grains. The bioavailability of zinc from biofortified wheat produced by foliar zinc application had not been studied before and we show in **manuscript 1** that biofortification from foliar fertilization is a viable source of zinc, reaching 50% of absorbed daily zinc requirements and that zinc from biofortified wheat is not distinguishable in its bioavailability from fortified wheat. Foliar application of zinc was shown not to reduce yield, which positively correlated with zinc concentration in Pakistani wheat grains (Chattha et al., 2017). A combination of soil and foliar zinc application appears to be the most successful way of increasing grain zinc concentration (Chattha et al., 2017; Kutman, Yildiz, Ozturk, & Cakmak, 2010; Zhang, Shi, Rezaul, Zhang, & Zou, 2010). During the wheat production for our efficacy study, described in **manuscript 3**, though, we faced the inherent difficulties of constant biofortified wheat supply. Wheat is a winter crop in India, produced from October to May. Fertilization has to be planned early, and fertilizers have to be distributed to the field before the flowering stage. Additionally, we could notice during our study that some farmers were initially unenthusiastic about foliar zinc application, as this would require extra work force in case spray fertilizers were not already in used in the specific field. Moreover, by walking in the field, farmers must be careful not to break their crops and reduce their yield.

A potential future perspective for biofortification through agronomic practices is the use of zinc complexed chitosan nanoparticles that are suitable for the use in foliar zinc application (Deshpande, Dapkekar, Oak, Paknikar, & Rajwade, 2017). Insoluble zinc comprises more than 90% of soil zinc and is unavailable for plant uptake (Broadley, White, Hammond, Zelko, & Lux, 2007), showing the need of fertilizers in general. Nanotechnology in general has the potential

to achieve biofortification at lower doses of fertilizers, overcoming the effects that are associated with excessive fertilizer applications, such as drain-off of the majority of the fertilizer and potential contamination of ground water. A fertilization with encapsulated formulations which possess controlled release of zinc could allow farmers to spray foliar solutions only once during a growing season, and nanoparticles in general show a high rate of penetration in plants, due to their higher surface reactivity allowed by their greater surface area (Panwar, Jain, Bhargaya, Akhtar, & Yun, 2012).

Currently, plant biotechnology, which as a development strategy is more targeted than breeding, offers various technologies for biofortification. As gene editing tools are being developed, new perspectives can be opened for biofortification. Transgenes, once incorporated in a genome, are stable and this biofortification via biotechnology is therefore long lasting. However, there are currently ethical and acceptance issues concerning testing of biofortified crops produced by biotechnology. Moreover, inhibitors of zinc absorption, such as phytic acid, provide structural benefits to the plant. Decreasing phytic acid concentration in grains may affect plant fitness (La Frano, de Moura, Boy, Lonnerdal, & Burri, 2014).

Presently, 15 million people in more than 30 countries grow and consume biofortified crops (Saltzman et al., 2017) while, since 2012, more than 4 million farming households have been reached by HarvestPlus with biofortified planting material. Even though this can be considered a success, the effectiveness of biofortification programs must be investigated. A combination of isotopic human studies, efficacy and effectiveness studies is the most appropriate strategy to investigate the effectiveness of zinc and iron biofortification programs (Dias, Costa, Nutti, Tako, & Martino, 2017). This is one of the major strengths of this thesis: after measurement of zinc bioavailability from wheat, we performed an efficacy trial in a population at risk of zinc deficiency.

Biofortification, as well as fortification, are the most effective and sustainable strategies to combat zinc deficiency (Abbaspour, Amini, Hurrell, & Schulin, 2015). Regulatory considerations are nonexistent for biofortification by conventional plant breeding, or fertilization (Garcia-Casal et al., 2017). It has been suggested that Chinese consumers are generally willing to pay around 20% more for biofortified crops (De Steur et al., 2012), but information on health benefits of the biofortified crops have to be disseminated. Biofortification is nevertheless not a stand-alone solution. The supply and the demand for

biofortified staple crops need to be strengthened (Saltzman et al., 2017) and it can be successful in reducing malnutrition only as a part of a more complete and sustainable approach, including research on food insecurity, poverty, education and social injustices.

X.3 Zinc Bioavailability Assessment

In our **manuscript 1**, we assessed the zinc bioavailability from wheat using the double isotope tracer ratio method, published in 1992 for the first time (Friel, Naake, Miller, Fennessey, & Hambidge, 1992). Bioavailability can be studied either *in vitro*, with the use of caco-2 cells, or *in vivo*, in either animal models or humans. In general, caco-2 cells are not work intensive, and therefore not time-consuming, but require experienced personnel and good quality cell culture facilities. Relatively low cost, they are considered better than solubility method, as they simulate gastric and intestinal digestion of food. Animal models can simulate metabolic responses, but no animal model exactly simulate humans homeostasis. There is currently no biomarkers of zinc uptake *in vitro*.

Human studies are the only way to assess the true absorption, and provide accurate measurement and direct assessment of effectiveness. They are however costly and labor intensive, while social and ethical concerns have to be taken into consideration. In **manuscript 1**, the bioavailability of zinc from biofortified wheat was measured in human subjects. For a wide range of composite diets, extrinsic labeling is considered valid (Sheng et al., 2009). Nevertheless, the effect of processing on extrinsic labels is unknown. In the case of iron, contaminants are responsible for an incomplete exchange with the added tag and a greater absorption is indicative of a partial or incomplete isotopic exchange (Consaul & Lee, 1983). In general, an extrinsic label must be soluble as well. As results from different human studies are inconclusive in general for zinc, we tested and validated the hypothesis of extrinsic labeling is not different from intrinsic labelling in the first study presented in **manuscript 1**. For the extrinsic label to give an accurate estimation of absorption, the isotopic label added to the meal must equilibrate with the native food mineral during the digestive process and be absorbed and used to the same extent (Petry & Hurrell, 2015). Our study was the first study in wheat showing that the absorption from extrinsic label is similar to the intrinsic label and this at different concentrations, having two different isotope on the same meal. This proves that the extrinsic-to-intrinsic zinc absorption ratios are equal to 1 for a variety of concentrations. Extrinsic labeling may therefore be used to screen various wheat varieties for

zinc bioavailability in humans. The single meal method though is known to overestimate the potency of enhancers and inhibitors compared with multiple meal studies for iron (Cook, Dassenko, & Lynch, 1991), but this could be the case for zinc as well. Moreover, the native iron from rice grains during digestion is known to be released slowly (Bjornrasmussen, Hallberg, & Walker, 1973). This brings concerns about the validity of our finding for grains consumed as whole. Wheat grains were milled prior to consumption in our study, helping the digestion. This issue can be seen with the only two reports of zinc absorption from intrinsically labeled cereal, beans (Donangelo et al., 2003) and rice (Brnic et al., 2016). In beans, the extrinsic label absorption was underestimated by 10%. The same trend can be seen in rice, although this difference was not significant. Even though zinc transporters are found throughout the digestive tract, the median half-gastric emptying time for a solid meal is around 2 hrs (Hellmig et al., 2006), which might not be enough for a complete digestion of big cereal grains. In **manuscript 1**, we ensured that the extrinsic and intrinsic labels behave the same by labeling the same meal intrinsically and extrinsically, an approach that had never been used before.

X.4 Zinc Bioavailability from Wheat-based Meals

Bioavailability from biofortified foods is related to the food matrix structure and composition (Garcia-Casal et al., 2017). In **manuscript 1**, we investigated the bioavailability of zinc from wheat, either biofortified, fortified or unfortified, at different ER. Bioavailability was already studied in 4 studies from biofortified foods *in vitro*, 3 focusing on biofortified rice (Jou, Du, Hotz, & Lonnerdal, 2012; Y. Wei et al., 2012; Wei, Shohag, & Yang, 2012) and 1 on biofortified beans (Vaz-Tostes, Verediano, de Mejia, & Brunoro Costa, 2016). In rats, 1 study investigated zinc absorption from rice (Jou et al., 2012) and 1 from beans (Welch, House, Beebe, & Cheng, 2000). All results show an enhanced absorption of zinc from biofortified foods. In pigs though, the low zinc intake did not allow the calculation of zinc bioavailability (Carlson, Norgaard, Torun, Cakmak, & Poulsen, 2012). To our knowledge, no animals study were done with other staple foods.

In humans, bioavailability of zinc from biofortified foods was studied in rice, pearl millet, maize and wheat. The total and fractional absorbed zinc was similar in biofortified and conventionally fortified rice (Brnic et al., 2016), while the total zinc absorbed from biofortified rice was not different from control rice but was significantly lower from conventionally

fortified rice in preschool children (Islam et al., 2013). In pearl millet (Kodkany et al., 2013) and maize (Chomba et al., 2015), biofortification with zinc was shown to improve the total absorbed zinc. The fractional absorbed zinc in biofortified pearl millet is 17% higher, and as the meal prepared from the biofortified millet contained 2.5 mg of zinc more than the control millet-based meal, the zinc absorbed exceeds the physiological requirement for this age group. After 18 weeks of consumption of biofortified beans (Vaz-Tostes et al., 2016), no change could be seen on PZC.

Only one study investigated the bioavailability of zinc from biofortified wheat (Rosado et al., 2009). At 95% ER, the total zinc intake was 72% higher from the biofortified wheat and 68% higher at 80% ER compared with the corresponding control wheat. During extraction from 95 to 80%, 60% and 35% of zinc and phytic acid were conserved. Those values though come from single measures. Wheat used in this study was initially produced by plant breeding, while no agronomic practices were used. Due to limitations in low amounts of seeds available at planting, enough wheat grains were obtained for the study from several wheat lines. As the milling, and therefore the ER is succinctly described, no explanation is given as to why phytic acid concentrations decreased during milling more dramatically than zinc concentrations.

In **manuscript 1**, the main difference from this study is the way our wheat was produced. The bioavailability of zinc from biofortified wheat produced by foliar zinc application had not been studied before and we proved that biofortification from foliar fertilization is a viable source of zinc, reaching 50% of absorbed daily zinc requirements and that zinc from biofortified wheat is as bioavailable as the one from fortified wheat. Interestingly, our study suggests that the agronomic techniques (foliar zinc application or conventional), more than the ER at milling is a key determinant of bioavailable zinc from wheat. In general then, the quality of a meal and its nutritional content is then determined prior to processing, during growing of the crops and prior to harvesting. Fighting malnutrition, and specifically micronutrient deficiencies, a wide array of sectors should be brought together. Not only nutrition researchers. Policy-makers, program implementers and private sectors are needed. Involving agricultural research is the only way for a sustainable increase in food quality.

X.5 Zinc Efficacy Trials

Our results, presented in **manuscript 3**, show no time by treatment effect on PZC, when children consume biofortified, fortified or control wheat for 20 weeks. Three recent studies

showed a significant effect on PZC during an increased zinc intake in school-age children. A significant time by treatment effect on PZC of Beninese children and on zinc deficiency was seen during a 20 weeks trial in which 2.8 mg of zinc was given daily in water outside a meal in a population where there was around 40% of zinc deficiency (Galetti et al., 2015). Plasma zinc concentration was shown to increase to a greater extent in zinc deficient Thai children who received extruded rice grains providing 10 mg of Zn /day incorporated into a school lunch program (Pinkaew, Winichagoon, Hurrell, & Wegmuller, 2013). When micronutrient powders were added to porridge during 6 months, providing 2.5 mg of zinc daily together with phytase, a significant decrease in prevalence of zinc deficiency was seen in South African children, in which a prevalence of 50% of zinc deficiency was measured (Troesch et al., 2011).

There are 2 main reasons for our results to be different from those 3 studies: 1) the zinc bioavailability was enhanced in two of those studies, 2) the low prevalence of zinc deficiency in our study population. The bioavailability of zinc from water is 7x higher than from fortified maize (Galetti et al., 2015) and the consumption of water by Beninese children was done outside a meal. Supplements in solutions taken between meals significantly increase PZC while the same amount of zinc provided as a fortificant generally do not (Brown, de Romana, Arsenault, Peerson, & Penny, 2007; Lo et al., 2011). Phytase, by breaking down phytic acid, increases zinc bioavailability (Brnic et al., 2017). The micronutrient powder used in South Africa contained a phytase. Our study was not designed to involve the use of a phytase, and even though fermentation might have decreased PA concentrations, we decided to prepare lunch following local recipes. This choice was made primarily to obtain a better compliance throughout the study, and to test biofortified wheat prepared with local dietary practices. In addition, our cooking team was not used to prepare fermented items and the meal cooking time would have increased, increasing the risk of contamination, mislabeling, and the workforce need.

In the study by Pinkaew *et al*, children were selected to take part of this study based on their low initial PZC, therefore baseline data show a 100% zinc deficiency. As the initial plasma zinc may predict treatment outcome (Brown, Peerson, Rivera, & Allen, 2002), a selection of children based on low PZC would have increased our chance of detecting the relatively subtle changes expected from biofortification or fortification on PZC. In our study though, a screening was logistically impossible to perform prior to the start of the study, and assessment of zinc status relied on the fact that schools were of low socio-economical status and had a relatively

high prevalence of stunting. Another location was initially chosen in North India, but the study site had to be relocated after permission denial from our academic partner, postponing our study start. Many schools were initially approached around Bangalore to take part of the study. This trial being a long-term study, during which children were asked to eat the food we provided 6 days per week, the majority of schools declined. By default, long school holidays had to be avoided and therefore the timing of the start of the study was bound by logistical constraints. Moreover, PZC measurements turned out to be more challenging than expected, and optimization of measurements were achieved only after the end of the study.

Several other studies have found minimal or no impact on PZC following zinc interventions. A study investigating micronutrient powders with 10 mg of zinc or with no zinc in weaning infants showed no impact on PZC after 6 months and, interestingly, an increase number of days with diarrhea for both groups compared to the group not receiving micronutrient powder (Soofi et al., 2013). The lack of positive impact of zinc on diarrhea episode could be explained by the high usage of zinc supplement against diarrhea throughout the study, reducing the difference in zinc intake between the groups. The negative PZC results of this study in infants show that despite a high intake of zinc throughout the study, far higher than the upper limit of zinc intake for this age group (5 mg/day for children from 6 to 12 months and 7 mg/day for children between 1 and 3 years old), no effect was seen on PZC.

Our efficacy study results, in **manuscript 3**, showing no impact on PZC from consumption of fortified or biofortified flour is therefore not inconsistent with the current literature. The association between PZC and zinc intakes is known to be poor (King et al., 2016) and as previously explained, infants PZC did not increase while given a high dose of zinc in a micronutrient powder (Soofi et al., 2013). Even if not significant, in our study PZC increases in the population at more risk of zinc deficiency. With a longer trial duration, an effect might have been seen. It would have been interesting to include in our study schools teaching only in the local language, where no fees are generally asked, to target a population more at risk of zinc deficiency, or stunted children only. Moreover, a logistical issue was faced during biofortified wheat delivery, resulting in a lower zinc intake during ~20% of the study. Nevertheless, biofortified crops, if involved for example in a school meal program by a government, will reach the general population and not only the deficient one. Our study is a realistic representation of a population leaving in peri-urban areas around Bangalore.

X.6 Plasma Zinc as the Biomarker of Choice

Plasma zinc is currently the only recommended biomarker of zinc exposure or zinc status (King et al., 2016). The reference range for zinc deficiency as measured by PZC was set already a decade ago (International Zinc Nutrition Consultative Group, 2007) but clinical signs do not appear directly below this range, as a cutoff of 50 mg/dL predicts clinical signs among individuals with either restricted dietary zinc intake or AE with 80% sensitivity and 90% specificity (Wessells, King, & Brown, 2014). In general then, the clinical manifestations of zinc deficiency would not be seen with the cut-off used in our study, and only a couple of children had a PZC below 50 mg/dL.

Our efficacy study described in **manuscript 3** highlights the inherent difficulties of using PZC as a population biomarker. The correlation between stunting, which was nearly 20% in our population, and PZC was not shown. A higher zinc deficiency prevalence was expected in our efficacy study site. The two schools selected were part of the same congregation, both of them being private. Clinical studies were not allowed in government schools, in Karnataka at the time our study was planned. Our study site was located in the peri-urban outskirt area of Bangalore, in a village called Silvepura, accessible by car. As the schools had no school bus, children going to both schools came from the surrounding villages, from a walking distance and we therefore expected both schools to be representative of the same population. This added a logistical challenge to our study. As food had to be provided every day in big quantity, we only included schools located close to each other.

Other limitations of PZC as a biomarker are related to the difficulty of avoiding contaminations from the environment. Contamination of material, during either collection or processing, have a significant impact on measurements. One of our study strengths is the thorough protocols followed to avoid contamination from the environment, at time of collection, processing and measurement. No contamination, as assessed by investigating outliers visibly deviating from the distribution of PZC, could be identified in plasma samples.

Research has been going on about other potential biomarkers of zinc deficiency. Recently, 2 randomized controlled trials examining the impact of zinc supplementation on gene expressions of zinc transporters and metallothionein were published (Chu et al., 2015; Sharif, Thomas, Zalewski, & Fenech, 2015). In general, the expression of zinc efflux transporters from the ZnT family is believed to be reduced with a decline in cellular zinc (King

et al., 2016). However, after zinc supplementation, changes in the expression of zinc transporters are not seen in the absence of preexisting zinc deficiency, gene expression reflecting long-term zinc intake (Lowe, 2016). Two investigations of DNA integrity were done recently (Sharif et al., 2015; Zyba et al., 2017). In zinc-deficient individuals, a reduction of DNA damage following zinc supplementation can be measured, irrespective of a PZC increase (Lowe, 2016).

In our efficacy study, in **manuscript 3**, we investigated the changes of ZnT1 and DNA integrity over 20-weeks of higher zinc intake. Unfortunately, as our prevalence of zinc deficiency at baseline was lower than expected, no change in those two biomarkers were expected. We believe that the negative results are not an issue of a sample size for DNA integrity measurement, as results were seen with a lower sample size in other studies. The study highlights the difficulties in measuring zinc transporters adequately in a challenging environment, as well as the difficulty of collection and storage of samples for DNA integrity. While the samples used for measurements of transcripts can be stored easily, their processing is more challenging. Our sample size decreased by half due to difficulties faced during laboratory measurements. The collection of samples for measurement of DNA integrity was challenging as well, as a small quantity is needed, and samples need to be store in liquid nitrogen, which was unavailable in our Indian partner institute. The amount of blood collected for DNA integrity measurement could have been doubled, for example. Samples for RNA measurements were initially collected for all participants, but analyzed only in 51. Instead, it would have been better to randomly select at the beginning of the study the subsamples of children involved in new biomarkers measurements, and to collect duplicate samples from each. Due to the high cost of the material needed for collection, and the hope of a more efficient analysis, we decided to collect samples from each children.

X.7 Determinants of Zinc Absorption

Determinants of zinc absorption were investigated in manuscript 2, in healthy adults living in Switzerland and Burkinabé toddlers. All studies were done with the same dual isotope tracer method. Our manuscript suggests that the determinants of zinc absorption are not only dietary, but PZC plays a role in absorption in general, and CRP and height in growing children. Zinc status was hypothesized to influence zinc absorption in the 90s already (Sandstrom, 1992), but the current agreement is that zinc absorption is influenced by dietary zinc intake,

not status (King, 2010). As some studies suggested that the zinc status of the person may affect the fractional absorption of zinc (Wada, Turnlund, & King, 1985), results were believed to be biased because the zinc-depleted subjects were consuming low-zinc diets during the studies of zinc absorption, so the zinc intake may have affected zinc absorption independently of the subjects' zinc status (Chung et al., 2008). The fractional absorption of zinc was shown to be inversely related to current zinc intake, without any detectable effect of longer-term dietary zinc (Chung et al., 2008). Modification of fractional absorbed zinc would occur then only after severe zinc depletion. Due to the presence of a saturable zinc transporter in the enterocyte, efficiency of absorption is enhanced if the current intake is low (King, 2010).

Our findings show that zinc deficiency is resolvable, as deficient individuals absorb more zinc from their diet. As the impact of inflammation on PZC was already shown (Mburu, Thurnham, Mwaniki, Muniu, & Alumasa, 2010), it is not surprising that inflammation markers, such as CRP, impact zinc absorption in an indirect way, as it lowers PZC. Our data suggests an independent effect of CRP on PZC though. In the case of acute inflammatory stress where serum zinc are depressed, more zinc-free plasma transport proteins might be available enhancing zinc absorption (Pekarek & Evans, 1975). As CRP is an acute phase protein, one potential perspective of this study is the investigation of another protein of inflammation, namely AGP. AGP is a marker of long-term inflammation, and was shown to have a significant impact on our model from **manuscript 3**, while CRP has not. It can be hypothesized that long-term inflammation might have a greater effect on zinc absorption, as CRP is a transient marker only. Our manuscript also shows that height is a determinant of zinc absorption in growing children. While the fractional absorption of zinc was already shown to be proportional to the length of the intestine (Hambidge, Krebs, Westcott, & Miller, 2006), this holds promises for catch-up growth in children and potential improvement in zinc deficiency prevalence, after an increase zinc intake.

Recently, a pooled data analysis showed that in infants and young children, zinc absorption is not related to phytic acid intake (Miller, Hambidge, & Krebs, 2015). However, the degradation of phytic acid is known to improve zinc absorption from millet as calculated by stable isotope measurements (Brnic et al., 2017). This was proven in the same staple *in vitro* earlier (Lestienne, Besancon, Caporiccio, Lullien-Pellerin, & Treche, 2005) and phytic acid is considered to be a dietary determinant of zinc absorption (Bel-Serrat et al., 2014; Lonnerdal, 2000). In **manuscript 2**, we show a negative correlation between phytic acid concentration

and zinc absorption and therefore the benefit of phytic acid degradation to improve zinc bioavailability in adults. In children, our sample size is unfortunately small and phytic acid was only theoretically calculated, as a phytase was added just prior to consumption.

Iron and zinc have been considered as potential competitors for absorption for more than 2 decades (Lonnerdal, 2000). Iron has a negative effect on zinc absorption, if given together in a supplement but not as fortificants (Lonnerdal, 2000), suggesting an impact of iron status on zinc absorption, and not dietary iron. As hepcidin reduces intestinal zinc export by post-translationally downregulating ZnT1 (Hennigar & McClung, 2016) and much of the massive extent of iron deficiency anemia in the world may be due to an underlying zinc deficiency (Knez, Graham, Welch, & Stangoulis, 2017), we investigated the potential impact of iron status on zinc absorption for the first time in **manuscript 2** and we found no correlation between zinc absorption and iron status parameters. The close to significant p-value for hepcidin shows that our study results might change with a larger sample size.

X.8 Conclusive Remarks

Biofortification is a promising approach to deliver crops with improved micronutrient content in population at risk of micronutrient deficiencies. Even though we found no time by treatment effect on plasma zinc concentration in school-age children consuming biofortified wheat, we believe that our absorption study, first validating the extrinsic labelling approach and secondly showing a higher zinc absorption from biofortified wheat compared to control wheat, is an important proof of concept for biofortification of wheat. The absorption from biofortified wheat similar to the one of fortified wheat. The effect of the efficacy study, done in India, can be explained by the low prevalence of zinc deficiency in the population, as well as the low bioavailability of zinc in the Indian diet in general. These results highlight the inherent difficulties in measuring a change in plasma zinc concentrations. Pooled data analysis showed that dietary factors are not the only determinants of zinc absorption, but plasma zinc concentration in general, CRP in population at risk of zinc deficiency, and height in growing children affect zinc absorption. This confirms that a lower plasma zinc concentration will result in a higher zinc absorption and therefore a better response to long-term fortification or biofortification. Stunting can have long-term consequences on cognitive functions of children. Our results show that stunted children are expected to respond better to an increase in dietary zinc, no matter their iron status, resulting in a positive impact on their zinc status, and their height.

X.9 Reference

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CURRICULUM VITAE

Coralie Signorell

Born on January 10, 1986 in Vevey, Switzerland

Education

2012–2017 **Doctoral student**, ETH Zurich (Swiss Federal Institute of Technology, Zurich)
Switzerland
Laboratory of human nutrition, Prof. M. B. Zimmermann

2009–2011 **Master of Science in Bioengineering**, Ecole Polytechnique Fédérale de Lausanne (EPF)
Switzerland

2004–2009 **Bachelor of Science in Life Sciences and Technology**, Ecole Polytechnique Fédérale de Lausanne (EPFL)
Switzerland

Professional Experiences

2011–2012 **Assistant accountant and Human Resources collaborator**, TUSA SA, Vevey, **Switzerland**

2010-2011 **Master Thesis** in Synthetic Metabolic Engineering, Osaka University
Japan
Laboratory of Biochemical Engineering Laboratory, H. Ohtake

2008 **Volunteer** for the Nutrition Consortium of New York State, Troy, New York
USA

2006-2007 **Nursing Assistant,** La Maison du Pèlerin, Chardonne
Switzerland