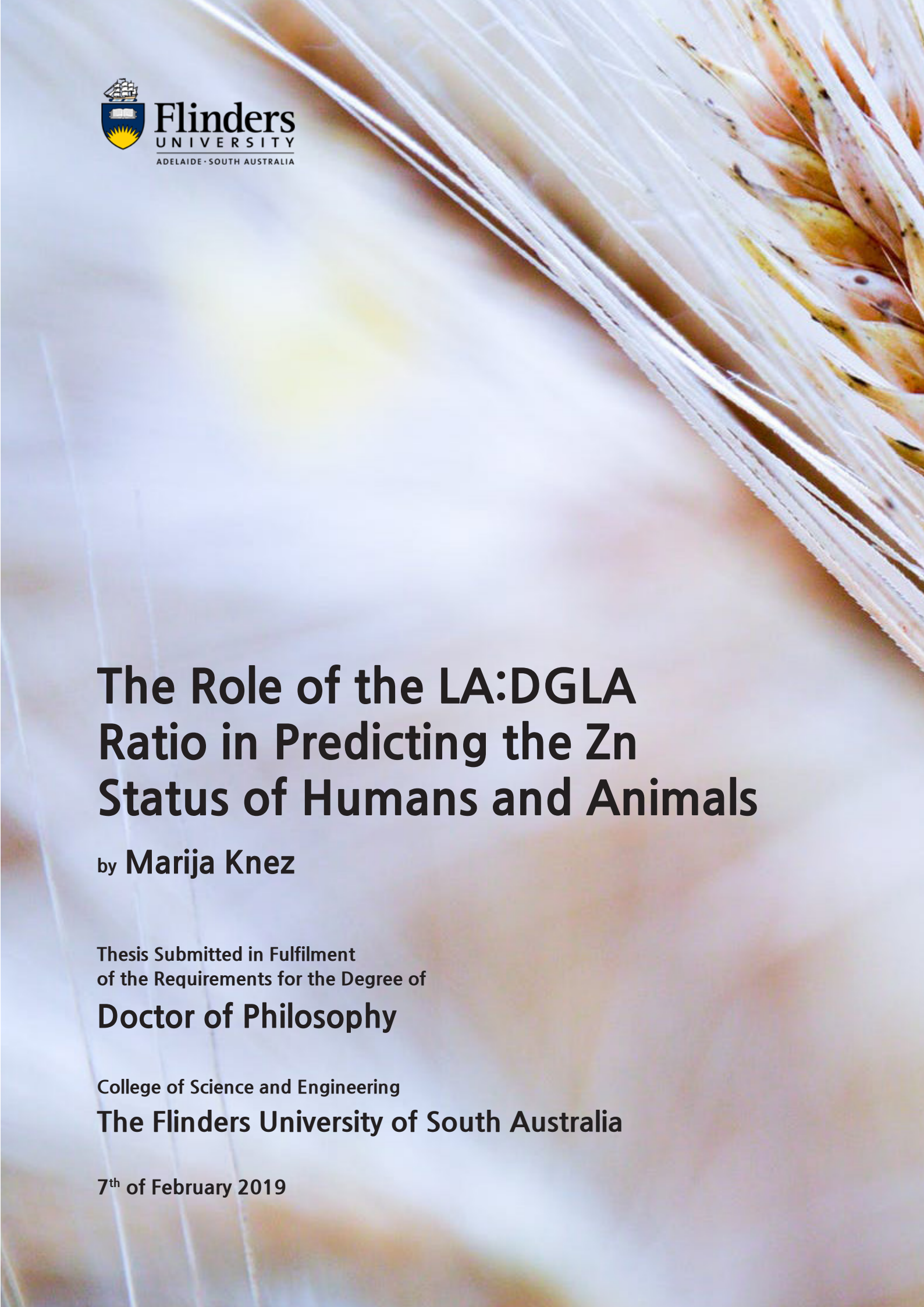

The Role of the LA:DGLA Ratio in Predicting the Zn Status of Humans and Animals

by **Marija Knez**

Thesis Submitted in Fulfilment
of the Requirements for the Degree of

Doctor of Philosophy

College of Science and Engineering

The Flinders University of South Australia

7th of February 2019

This thesis is dedicated with ultimate affection and gratitude to my parents Cveta and Ljubiša Milošević, my son Novak and my research supervisor James C.R. Stangoulis

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Note: All figures and tables presented in this thesis are produced by the candidate and are not taken from other sources.

Acknowledgments

Over the past four years I have been very privileged to be surrounded by supportive and caring people who made this thesis possible and who have made my time during this candidature a period I will treasure.
There are a number of people who I wish to thank.
Firstly, thank you to my principal supervisor, Prof. James C.R. Stangoulis for giving me the opportunity to take on my PhD under his guidance and continual support, encouragement and tremendous enthusiasm.
Thank you so much James for having faith in me. Under your guidance and encouragement I have grown immensely as a scientist. I couldn't have hoped for more supportive supervisor.
I am very grateful to Dr. Maria Glibetic for giving me the opportunity to undertake part of my PhD work in her laboratory; in the Centre of Research Excellence for Nutrition and Metabolism in Belgrade, Serbia.
Dear Maria thank you for your unreserved support and encouragement during my time in the department and for generously paving the way for my development as a research scientist. In addition, I thank all colleagues employed at the Institute.
I consider it a great honour to have been a part of the Robert W. Holley Centre for Agriculture and Health laboratory at Cornell University (USDA laboratory). I am greatly indebted to Dr. Elad Tako for giving me the opportunity to access laboratory equipment and facilities and for his expert advice, support and encouragement. I would also like to extend that thanks to technical staff, who provided a friendly environment and support during my visit to Cornell University, in particular to Nikolai Kolba for his help with PCR related work.
I am grateful to many people who contributed to the progress and publication of the work contained herein. First and foremost, I thank all my co-authors for collaboration. I would also like to thank all the men and women who took part in these studies, as participants or as researchers. Without them this study wouldn't be possible. I also appreciate the help provided by all group members of the Stangoulis laboratory team at Flinders University. I would particularly love to thank Emma de Courcy-Ireland for her help and support during the production of Zn biofortified wheat.
In addition, I acknowledge the funding from the Australian Government Research Training Program Stipend Scholarship that enabled me to undertake my PhD.
To all my friends and family members, I would love to say thank you once again for all your support and help during my PhD years. Without any names being taken, you all know who you are. Thank you for always being there for me.

Lastly, I would love to thank my incredible parents and my brother who always believed in me and encouraged me in all aspects of my career and life. Thanks for installing me with a strong passion for learning and doing anything and everything to put me on a path towards success. Thank you for your unconditional love and support.

I would particularly love to thank my Dad, an extremely important member in my life. Dad, it's your upbringing that set my mind to submit this work. You have encouraged and pushed me every time when I felt like giving up.

Finally, I would love to thank my son Novak who spent days and nights without me, trying to understand the importance of his mums work and dream. I thank you Novak for your understanding and patience while I was far away from home during the period of my research. I love you.

Abstract

Zinc (Zn) is a vital micronutrient for humans, being involved in multiple enzymatic, biochemical and structural processes within the body. Zn deficiency, the main cause of early childhood morbidity and mortality, is a global health problem and is estimated to affect 17% of the world's population. A precise assessment of the magnitude of Zn deficiency is challenging, as Zn intake and status data for a large number of countries are either outdated or missing. Zn deficiency has been recently recognized as possibly responsible for the expansion of iron (Fe) deficiency and Fe deficiency anemia, but there is a lack of information on the mechanism of that interaction. Zn biofortified wheat is a newly developed strategy that aims to contribute to the alleviation of Zn deficiency, yet its efficacy is still awaiting confirmation. A lack of a sensitive and specific Zn biomarker additionally limits the accurate assessment of the magnitude of Zn deficiency and the impact of nutrition intervention strategies, such as biofortification.

This PhD project aims to identify a new biomarker that can act as a reliable and sensitive indicator of Zn status in humans. Additional goals of this research project were to provide an up to date assessment of Zn and Fe intake and status data in an apparently healthy adult population, to investigate the role of Zn in the Fe absorption process and clarify the mechanism by which Zn deficiency contributes to the development of Fe deficiency and Fe deficiency anemia. Finally, the project investigates the efficacy of Zn biofortified wheat in improving Zn status of consumers.

The linoleic: dihomo-γ-linolenic acid (LA:DGLA) ratio was examined as a potential biomarker of Zn status. The content of plasma phospholipid LA, DGLA and changes in the LA:DGLA ratio were compared to the dietary Zn intake and plasma Zn status in healthy human subjects. Zn and Fe nutritional status of apparently healthy European adults (25-65 years of age) was examined by means of anthropometric, dietary, and biochemical measurements and the relation of usual Zn and Fe dietary intake to Zn and Fe status was investigated. The structure and function of major Zn and Fe transport proteins and Zn and Fe interaction at these sites is comprehensively reviewed to illuminate the mechanism of Zn involvement in the Fe absorption process. The amount of Zn absorbed from Zn biofortified wheat material was estimated using a range of physiological and molecular parameters within an in vivo (*Gallus gallus*) model of Zn absorption.

This PhD project provided several new contributions to knowledge: it demonstrated that there is a positive link between Zn and Fe for absorption and that the cellular Zn concentrations control Fe absorption processes. A comprehensive model of Zn involvement in the Fe absorption process, both at the local and systemic level, is delivered. In addition, it was revealed that marginal Zn deficiency is present in apparently healthy populations, so regular monitoring is of crucial importance. The core contribution of the work presented within this thesis is the evidence of the responsiveness of the LA:DGLA ratio to dietary Zn modifications in both animals and humans. The ratio can be used as an additional indicator of Zn status. Finally, it was confirmed that the additional Zn present in biofortified wheat is equally biologically available for absorption as Zn from conventional wheat varieties; consumption of Zn biofortified wheat improved the Zn status of consumers.

Keywords: Zinc, iron, Zn & Fe interaction, dietary intake, Zn biomarker, Zn and Fe status, LA:DGLA, Zn biofortification, wheat

Declaration of Originality

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Date:

Marija Knez

Thesis Synopsis

This PhD thesis is written in a format of a thesis that includes published material. It contains five manuscripts altogether, all of which have been published in internationally recognized peer-reviewed journals.
The list of publications and the list of presentations at international conferences are included on page 23 and 24. The candidate's contribution to this research project is explained in detail on page 22.

The thesis is divided into five sections, and the structure, as well as, the content of each of the sections is explained in detail in the segment that follows.

The thesis starts with an introductory section that provides background information related to the research that has been performed, explains rationale and major aims of this research project. This is followed by a concise literature review, five chapters (manuscripts) and a final chapter that provides a summary of research findings, explains strengths and limitations, demonstrates original contributions to knowledge and provides recommendations for further research.

Except for the first (introductory) and the last (concluding) segment, every other section within this thesis contains a preamble that explains the rationale, the order and purpose of the work that was carried out. Within a preamble the main findings of specific research papers are clarified. Some of the chapters are presented as journal article publications, contextually unchanged but formatted to follow the overall style of this thesis (not presented as reprints). The candidate is the first author on every publication included in this thesis. The contribution of each of the authors is clearly presented before bibliography sections within every single publication. Besides that, details of the candidate's contribution to individual publications and co-authorship approvals are presented in the Appendix 4.

Please note: An Editor has not been used in the construction of this thesis and the candidate is entirely responsible for the writing and the drafting of presented work. The Note System, superscripted number referencing style is used*. Photos included are taken from pexels free image stock available at: https://www.pexels.com.

* Dodd, J.S.; Solla, L.; Bérard, P.M. The ACS Style Guide. 3rd ed.; Coghill, A.; Garson, L., Eds.; American Chemical Society: Washington. DC, USA, 2006; pp. 287-341, ISBN-13: 978-0-8412-3999-9.

The Organization of the Thesis (Thesis Summary)

This thesis is written in five main sections:

Section 1. Introduction

- Outlines the structure of the thesis and explains the candidate's contribution to the research **(Chapter 1)**
- Provides background information, explaining the rationale and major goals of this research project **(Chapter 1)**
- Contains a literature review covering the most important aspects of Zn nutrition **(Chapter 2)**

Section 2. Zn deficiency, an underlying cause for Fe deficiency

- Is devoted to the Fe and Zn interaction mechanism
- Investigates the role of Zn in the Fe absorption process and explains the potential mechanism of the interaction, both at the local and systemic level **(Chapter 3)**
- Examines the relationship between Zn and Fe intake and status data in an apparently healthy population **(Chapter 4)**

Section 3. A biomarker of Zn status, LA:DGLA ratio

- Contains studies related to a proposed biomarker of Zn status, the LA:DGLA ratio
- Describes the way the biomarker has been tested in humans **(Chapter 5)**
- Provides a summary of the structure, role and major functions of LA and DGLA. In addition, it explains the current findings related to the LA:DGLA ratio as a biomarker of Zn status and proposes guidelines for further research **(Chapter 6)**

Section 4. Zn biofortification, a solution for alleviating Zn deficiency

- Discusses Zn biofortification as a possible solution for alleviating Zn deficiency
- Presents findings of an animal study that examines the efficacy of Zn biofortified wheat in improving the Zn status of consumers **(Chapter 7)**

Section 5. Conclusion

- Summarizes the main research findings, illuminates contributions to knowledge and provides recommendations for further research **(Chapter 8)**

Candidate's Contribution to this PhD Research Project:

Main Activities	Primary Role	Involvement of Other Colleagues
Overall Study	MK	
Establishment of funds	JS, ET, MG	MK
Literature search	MK	
Conceptualization of main ideas	MK	JS, ET, MG
Formulation of various study designs	MK	JS, ET
Preparation of Ethics applications	MK	
Running of studies	MK	NK, EI, MN, MZ, JDM, ZP
Data analysis	MK	ET, NK, MN
Data interpretation	MK	ET (animal study)
Production of publications	MK	
Preparation of written work (thesis)	MK	
Submission of thesis for examination	MK	

MK: Marija Knez (the candidate); **JS:** Prof. James C.R. Stangoulis (principal supervisor); **ET:** Prof. Elad Tako (main collaborator from Cornell University, NY); **MG:** Prof. Maria Glibetic (adjunct supervisor).

NK: Nikola Kolba (laboratory technician; helped with PCR analysis); **EI:** Emma de Courcy-Ireland (research assistant, helped with the production of Zn biofortified wheat); **MN:** Marina Nikolic (statistician); **MZ:** Manja Zec & **JDM:** Jasmina Debeljak-Martacic (research assistants, assisted with fatty acid analysis); **ZP:** Zoran Pavlovic (assistance with AAS mineral analysis).

List of Publications:

Section	Chapter	Reference
2	3	Knez, M.; Graham, R.D.; Welch, R.M.; Stangoulis, J.C.R. New perspectives on the regulation of iron absorption via cellular zinc concentrations in humans. *Critical Reviews in Food Science and Nutrition* **2015**, *57* (10), 2128-2143. DOI: 10.1080/10408398.2015.1050483.
2	4	Knez, M.; Nikolic, M.; Zekovic, M.; Stangoulis, J.C.R.; Gurinovic, M.; Glibetic, M. The influence of food consumption and socio-economic factors on the relationship between zinc and iron intake and status in a healthy population. *Public Health Nutrition* **2017,** *20* (14), 2486-2498. DOI: 10.1017/S1368980017001240.
3	5	Knez, M.; Stangoulis, J.C.R.; Zec, M.; Debeljak-Martacic, J.; Pavlovic, Z.; Gurinovic, M.; Glibetic, M. An initial evaluation of newly proposed biomarker of zinc status in humans - linoleic acid: dihomo-γ-linolenic acid (LA:DGLA) ratio. *Clinical Nutrition ESPEN* **2016**, *15*, 85-92. DOI: 10.1016/j.clnesp.2016.06.013.
3	6	Knez, M.; Stangoulis, J.C.R.; Glibetic, M.; Tako, E. The Linoleic Acid: Dihomo-γ-Linolenic Acid Ratio (LA:DGLA) - An Emerging Biomarker of Zn Status. *Nutrients* **2017**, *9* (8), 825. DOI: 10.3390/nu9080825.
4	7	Knez, M.; Tako, E.; Glahn, R.P.; Kolba, N.; de Courcy-Ireland, E.; Stangoulis, J.C.R. Linoleic acid: Dihomo-γ-linolenic acid ratio predicts the efficacy of Zn biofortified wheat in chicken (*Gallus gallus*). *Journal of Agricultural and Food Chemistry* **2018**, *66*, 1394-1400. DOI: 10.1021/acs.jafc.7b04905.

List of Presentations at International Conferences:

The 15th International Symposium on Trace Elements in Man and Animals, TEMA 15, Orlando, Florida, USA 2014

Poster Presentation: New Perspectives on the Regulation of Iron Absorption via Cellular Zn Concentrations in Humans

The 4th Micronutrient Forum Global Conference, MN Forum, Cancun, Mexico, 2016

Poster Presentation: An Initial Evaluation of Newly Proposed Biomarker of Zn Status in Humans - Linoleic Acid:Dihomo-γ-Linolenic acid (LA:DGLA) Ratio

The 13th Congress of Nutrition, CONU 2016, Belgrade, Serbia, 2016

Poster Presentation: The Influence of Food Consumption and Socio-Economic Factors on Zn and Fe Intake and Status in a Serbian Population

The 18th International Plant Nutrition Colloquium, IPNC 2017, Copenhagen, Denmark, 2017

Poster Presentation: The Linoleic Acid:Dihomo-γ-Linolenic Acid Ratio Predicts the Efficacy of Zn Biofortified Wheat in Vivo (*Gallus gallus*)

Note: All posters are presented in the original format in which they were presented at the conferences (Appendix 1).

Abbreviations:

AAS:	Atomic Absorption Spectroscopy
ALP:	Alkaline Phosphatase
ALT:	Alanine Aminotransferase
ATP:	Adenosine Triphosphate
AR:	Average Requirements
ARA:	Arachidonic Acid
AST:	Aspartate Aminotransferase
BMI:	Body Mass Index
BMR:	Basal Metabolic Rate
BOND:	Biomarkers of Nutrition for Development
Ca:	Calcium
CHO:	Cholesterol
Co:	Cobalt
Cre:	Creatinine
Cu:	Copper
DALY:	Disability Adjusted Life Years
Dcytb:	Duodenal Cytochrome b
DGLA:	Dihomo-γ-Linolenic Acid
DMT1:	Divalent Metal Transporter 1
DNA:	Deoxyribonucleic Acid
DPA:	DocosapentaenoicAcid
DTA:	DocosatetraenoicAcid
EAR:	Estimated Average Requirements
EFA:	Essential Fatty Acid
EFSA:	European Food Safety Agency
EU:	European Union
EURRECA:	European Micronutrient Recommendations Aligned Network of Excellence
EZP:	Exchangeable Zinc Pool
FAO:	Food and Agricultural Organization of the United Nations
FBS:	Food Balance Sheets
FCDB:	Food Composition Data Base

Fe:	Iron
FFQ:	Food Frequency Questionnaire
FPN1:	Ferroportin Transporter 1
GC:	Gas Chromatography
GLA:	γ-Linolenic Acid
Glu:	Glucose
Gra:	Granulocyte
HAMP:	Hepcidin
Hb:	Hemoglobin
HCT:	Hematocrit
HDL:	High Density Lipoprotein
HIF:	Hypoxia Inducible Factor
HJV:	Hemojuvelin
Hp:	Hephaestin
ICP MS:	Inductively Coupled Plasma Mass Spectrometry
IL:	Interleukin
IOM:	Institute of Medicine
IRE:	Iron Regulatory Element
IRIDA:	Iron Refractory Iron Deficiency Anemia
IRP:	Iron Regulatory Protein
IZiNCG:	International Zinc Nutrition Consultative Group
JAK:	Jak-kinase
LA:	Linoleic
LA:DGLA:	Linoleic: Dihomo-γ-Linolenic Acid
LDL:	Low Density Lipoprotein
Mon:	Monocyte
MT:	Metallothionein
MTF1:	Metal Transcription Factor 1
MRE:	Metal Responsive Element
MUFA:	Monounsaturated Fatty Acid
NADH:	Nicotinamide Adenine Dinucleotide
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate
NF:	Nuclear Factor

NNR:	Nordic Nutrition Recommendation
NRC:	National Research Council
NTBI:	Non Transferrin Bound Iron
PCR:	Polymerase Chain Reaction
PUFA:	Polyunsaturated Fatty Acid
PZC:	Plasma Zinc Concentration
RBC:	Red Blood Cells
RDA:	Recommended Dietary Allowance
RDI:	Recommended Dietary Intake
RNA:	Ribonucleic Acid
RNI:	Reference Nutrient Intake
RT-PCR:	Real Time Polymerase Chain Reaction
SAC HREC:	Southern Adelaide Clinical Human Research Ethics
SCFA:	Short Chain Fatty Acid
SFA:	Saturated Fatty Acid
SGLT:	Sodium Glucose Co-transporter
SE:	Standard Error
Se:	Sedimentation
SES:	Socio Economic Status
SI:	Sucrose Isomaltase
SWOC:	State of the World's Children
TE:	Total Energy
TfR:	Transferrin
UA:	Urine Analysis
UK:	United Kingdom
UL:	Upper Limit
UNICEF:	United Nations Children's Fund
USA:	United States of America
USDA:	United States Department of Agriculture
WBC:	White Blood Cells
WC:	Waist Circumference
WHO:	World Health Organization
Zn:	Zinc

The lack of minerals is the root of all disease

Dr. Gary Price Todd

Section 1 | Introduction

Chapter 1

Background Information

1.1 Background and Rationale

Zinc (Zn) is an essential micronutrient for human health and is required for numerous biological processes, including growth and development, neurological and enzyme functions, immune response, skin and bone metabolism, gene expression, cell mediated immunity, hormonal excretion and protein synthesis [1-4].
Due to its many functions, Zn deficiency can significantly disrupt the homeostatic mechanisms in the human body. Insufficient Zn intake has been associated with poor growth, depressed immune function, increased vulnerability to and severity of infections, adverse outcomes of pregnancy, and neurobehavioral abnormalities [5-7]. Inadequate Zn intake has negative consequences at all points of the human lifecycle, from the point of conception through to old age [8].

Zn deficiency was first described in humans during the early 1960s, in Middle Eastern, male adolescent dwarfs, consuming plant-based diets [9]. Subsequently, Zn deficiency was identified in many other regions of the world and nowadays Zn deficiency is a global health problem, estimated to affect 17% of the total world's population [10]. Zn inadequacy, in combination with other micronutrient deficiencies, is identified as one of the major contributors to the burden of disease in developing countries [6,11]. Zn deficiency contributes significantly to early childhood morbidity and mortality and is responsible for more than 450,000 deaths per year in children under the age of 5. This equates to 4.4% of global childhood deaths [11]. Zn insufficiency is also accountable for death and disability among other age groups [12]. There is a strong correlation between the development of chronic diseases (i.e. cardiovascular, depression, type 2 diabetes) and Zn deficiency [13-16]. Finally, deficiency of Zn increases the severity and the risk of death from other illnesses [11].

Knowledge of Zn nutrition has advanced considerably over the last 60 years. Remarkable advances in both clinical and the basic science of Zn metabolism in humans has been observed [17]. Zn deficiency was acknowledged as a public health problem on the World Health Organization list [10,18] and progress in our understanding of Zn homeostasis, cellular Zn absorption and excretion mechanisms, the identification of and in some cases the functioning of numerous Zn transporters has been made [19,20]. The Zn intake for various study populations in a number of industrialized countries has also been reported and phytate as a limiting factor for Zn absorption has been acknowledged and a significant research effort has been undertaken to identify a physiological biomarker that predicts Zn status [4,21-23].
Over recent years, there have been ground breaking events in the history of Zn nutrition but still many unknowns exist for this critical micronutrient.

Zn deficiency does not occur in isolation and it is very often followed by deficiencies of other micronutrients; the most important being Fe deficiency, reported to affect around 30% of people worldwide [24].
The simultaneous occurrence of Zn and Fe deficiencies in humans has been known since the discovery of Zn deficiency [25-28], however for a long period of time, Zn and Fe absorption processes were seen as competitive. Fisher-Walker et al. (2005) [29] were first to publish a review article providing support for non-competitive absorption mechanisms between Zn and Fe. There were also hypotheses made that a significant proportion of Fe deficiency anemia in humans is due to an underlying Zn deficiency [30]. Additionally, a positive link between Fe and Zn for absorption and a strong influence of Zn on Fe absorption and Fe status has been confirmed by a number of studies [31-38]. However, the potential mechanism that could explain this causality, and clarify the Zn and Fe interaction at the absorption sites hasn't been elucidated and requires further research.
In order to address the concurrent problem of Zn and Fe deficiency effectively, one needs to elucidate the absorption mechanisms that control the uptake and transport of these nutrients. This would help not only in the more appropriate treatment of Zn deficiency, but also in the alleviation of Fe deficiency that is caused by insufficient cellular Zn concentrations.

The burden of Zn deficiency is most common in low income countries, however inadequate intake of Zn is also becoming an important public health problem in developed countries. Low dietary intake of Zn is a recognized health issue in the USA, the UK and Australia, especially among women, children and elderly people [39-42]. Recent results from the nutritional surveys conducted in the United States, Great Britain and Germany indicate that the recommended intake of Fe and Zn is not always achieved [43,44]. Inadequate Zn intakes have been documented among healthy people of various age and sex groups [40-49]. The prevalence of inadequate intakes of Fe and Zn is not consistent between studies performed across developed countries. For certain countries, the relevant data for Zn and Fe intake, and status are either limited or outdated, therefore needing further investigation. Regular monitoring of dietary Zn intakes of various populations is needed for obtaining an accurate picture on the magnitude of Zn deficiency, not only of severe etiology but marginal insufficiencies that usually pass unrecognized. Finally, the relationship between Zn and Fe intake and status should additionally be examined and explained not only in Zn deficient but in apparently healthy population cohorts too.

An additional factor that hinders the appropriate assessment of the magnitude of Zn deficiency is the lack of an accurate biomarker of Zn status. An indicator that accurately represents Zn status under various physiological conditions in humans is still missing, particularly for individuals affected by mild to moderate Zn deficiency [22]. It is generally accepted that currently there is no specific, reliable biomarker of Zn status [8].

As set by the World Health Organization, the development of a sensitive Zn biomarker is still a high priority [10]. A more profound and specific biomarker is needed to provide a more objective assessment of Zn status. Out of 32 prospective biomarkers investigated over the years, serum/plasma Zn concentrations, hair Zn concentration and urinary Zn excretion are the only three biomarkers identified as potentially useful [21]. The BOND (Biomarkers of Nutrition for Development) Zinc Expert Panel recommended the following Zn biomarkers for use: dietary intakes, plasma/serum Zn concentrations and stunting [22]. However, there are reservations in terms of the reliability of these biomarkers as all of the currently accepted and commonly used biomarkers of Zn status have certain limitations. Serum/plasma Zn, hair Zn concentration and urinary Zn levels decrease in severely Zn depleted individuals [8]. However, the concentration of Zn in the serum or plasma is perceived as an unresponsive index of Zn nutritional status over an extended period of time; due to effective homeostatic regulation that acts to maintain plasma Zn concentration within the physiologic range, by maintaining losses via GI tract and kidneys [8]. Similarly, dietary Zn intake very often is not correlated with plasma Zn status and does not realistically reflect the nutritional state of an individual [22,50-52]. For example, unchanged plasma Zn concentrations were observed with the intakes as low as 2.8 mg/kg to as much as 40 mg/kg, showing the limitation of plasma Zn status to reliably represent the dietary Zn intake [53,54].
Plasma Zn levels have a tendency to rise and then drop after a meal [55]. The concentration of Zn in the plasma can fall as a result of factors not related to Zn status or dietary Zn intake, i.e. infections, inflammation, trauma and stress [8,22]. The reliability of all currently used biomarkers under infection and inflammatory conditions is ambiguous [8,22].
In 2014, Reed et al.[56] discovered a significant negative correlation between dietary Zn deficiency and the ratio of two essential fatty acids, linoleic (LA) and dihomo-γ-linolenic acid (DGLA). Using supplemental Zn in the chicken (*Gallus Gallus*) model, the authors clearly demonstrated that the LA:DGLA ratio is able to differentiate Zn status between Zn adequate and Zn deficient subjects, showing that the LA:DGLA ratio can be used as an effective indicator to detect an early stage Zn deficiency, before the onset of any symptoms [56].

The same association may be seen in humans and the LA:DGLA ratio may possibly be a novel, effective, non-invasive, sensitive and reliable biomarker of Zn status; yet, this hypothesis certainly requires further examination. Initially, the correlation of the LA:DGLA ratio with dietary Zn intake and plasma Zn status in humans should be tested. Similarly, it is important to first determine the efficacy of prospective biomarker in predicting Zn status of consumers on traditional diets rather than supplements.

In Zn deficient populations where cereals are a primary staple in a diet, up to 75% of the daily calorie intake comes from cereal-based foods with very low Zn concentrations and low Zn bioavailability [57]. In many developing countries (i.e. North Africa and the Eastern Mediterranean) nearly 50% of the daily food energy is derived from wheat [5]. Wheat contains substantial amounts of phytate, a compound known to inhibit Zn absorption [58]. In addition, after significant extraction, the remaining white flour of modern wheat cultivars becomes inherently poor in Zn [59].

The widespread occurrence of Zn deficiency in developing countries, low consumption of animal products, and a large number of people dependent on wheat as a major food source, stimulated the development of biofortified wheat varieties with enhanced Zn concentrations. Biofortification, the delivery of Zn via staple food crops accomplished through the use of conventional plant breeding techniques, has been proposed to complement existing intervention efforts for the alleviation of Zn deficiency [60]. Agronomic biofortification (application of Zn fertilizers) represents a quick approach to the problem and wheat has been found to be the most promising cereal crop for increasing Zn in grains through foliar Zn fertilization (an average of about two-fold increase in grain Zn concentration after foliar application of Zn) [57]. While these conventional agronomic initiatives can be exceptionally beneficial to poor rural populations [61-64], quantitative data on efficacy of the biofortified products is still limited. Welch et al. (2005) [61] and Rosado et al. (2009) [62] indicated that newly developed Zn biofortified varieties of wheat may be useful in improving the Zn status of consumers. Nevertheless, a more precise measurement of Zn bioavailability from Zn biofortified products is still needed. The efficacy of consumption of Zn biofortified wheat varieties in improving Zn status of consumers' needs to be verified. A protocol for a proposed Cochrane review (intervention) [65], to assess the benefits and harms of biofortification of staple crops on vitamin and mineral status, health and cognitive function in the general population, should be implemented. In conclusion, the effectiveness of various other Zn nutrition intervention strategies in improving Zn status should additionally and more explicitly be examined and compared, in order to identify the most suitable approach for treating and reducing the number of people currently affected by dietary Zn deficiency.

1.2 The PhD Project Aim, Research Goals and Study Objectives

The main intention of this PhD work was to address previously discussed gaps in our knowledge of Zn nutrition. Several specific goals and objectives were set in order to achieve this broad general aim:

GOAL 1. To explore the extent of the impact of inadequate Zn intake on the development of Fe deficiency by examining the role of Zn in Fe metabolism.

Objectives:

- To elucidate the mode of action in the Zn and Fe interaction and explain the mechanism by which cellular Zn concentrations may control the Fe absorption process.
- To provide an up to date data set on Fe and Zn dietary intake and status for an apparently healthy population with no recent data on the intake of these nutrients.
- To explore the link between dietary Zn and Fe intake and status in humans, both in apparently healthy and in Zn deficient population cohorts.

GOAL 2. To test the efficacy of a newly proposed biomarker (LA:DGLA ratio) in predicting Zn status in both humans and animals.

Objectives:

- To assess the correlations among the concentration of linoleic (LA, 18:2n-6), dihomo-γ-linolenic acid (DGLA, 20:3n-6) and the LA:DGLA ratio, with plasma Zn status and dietary Zn intake in humans.
- To evaluate the efficacy of the LA:DGLA ratio in predicting the Zn status of subjects consuming a cereal based diet rather than a supplement.
- To describe the structure and main functions of the LA:DGLA ratio, summarize the current knowledge related to this biomarker and pinpoint the most important areas for further research.

GOAL 3. To examine the efficacy of Zn biofortified wheat in improving Zn status of consumers.

Objectives:

- To examine the bioavailability of Zn from Zn biofortified wheat plants.
- To complete an in vivo study to demonstrate the effectiveness of Zn biofortified wheat in improving the Zn status of consumers.

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Chapter 2

Literature Review

2.1 Introduction

This literature review provides a concise summary of the most important aspects of Zn nutrition. It starts by explaining the importance of Zn, its main biochemical and physiological functions, Zn homeostasis, absorption and excretion mechanisms, food sources of Zn and human physiological requirements. This is followed by an overview of dietary Zn intake and the up to date research related to the role of Zn in the Fe absorption process; with the major focus on the mechanism of the Zn and Fe interaction. Research efforts undertaken to identify a sensitive biomarker of Zn status are extrapolated, concentrating mainly on the discoveries presented in the last ten years. The last section of this review explains the current magnitude, causes, clinical manifestations and consequences of Zn deficiency. Finally, Zn biofortification of wheat, as a potential strategy for increasing Zn bioavailability of plant based diets, and its efficacy in improving Zn status of consumers and alleviating Zn deficiency states are reviewed.

The review is not covering every single aspect of Zn biology, but is providing a brief general overview of the most relevant information required for understanding the material presented within this thesis.
The interaction of Zn with micronutrients other than Fe is not included as it is not the focus of this work.
The review summarizes the information related to the link between Zn and Fe absorption processes, more so the research related to the mechanism of this interaction. Out of all the Zn nutrition intervention strategies presented over the years, the emphasis in this review is exclusively on the efficacy of Zn biofortified wheat plant foods.

The material presented within this literature review is, in some instances, intentionally shortened to avoid the repetition of information presented in subsequent chapters of the thesis, which, when this is done, is clearly indicated.

2.2 Overview of Zn Nutrition

2.2.1 Zinc: Importance and Main Biological Functions

Zinc (Zn) is an important trace element required for a number of biochemical, immunological, and clinical functions. It is involved as a structural metal in at least 3000 proteins and in over 300 enzymes as a catalytic metal [1-4]. Zn is the only metal that is associated with all six classes of enzymes: oxido-reductases, hydrolases, transferases, isomerases, lyases and ligases [5,6]. Zn has an important role in numerous biological processes including growth, cell division and differentiation [7]. Zn regulates a variety of genes involved in nucleic acid metabolism, cell signaling and apoptosis [8-11], and has a crucial role in immune system functioning [12-18]. Moreover, Zn is responsible for enzyme activity; performing as an activator or inhibitor ion [19].

Zn is a bluish-white metallic element categorized as a group IIB post-transition metal with an atomic weight of 65.37 and an atomic number of 30 [20,21]. It is the second most abundant trace element in humans (after Fe) [22]. It is found in all tissues and fluids in the body, but is predominantly an intracellular ion. In biological systems, Zn exists as the Zn^{2+} cation. The human body contains 1.5-2.5 g of Zn, of which 60% is in skeletal muscle, 30% is in bone, and 5% is in liver and skin [1,23,24]. Only about 0.1% of the body Zn is replaced daily by the diet [25] (**Figure 2.1**).

There are two major Zn reservoirs in the body, one with a short and another with a long half-life [26,27]. The short-term pool of Zn, also known as exchangeable pool of Zn (EZP) or 'functional pool' has a turnover rate of about 12.5 days and represents only 10% of the total body Zn [26,28,29]. This pool contains the most metabolically active form of Zn in plasma, intestine, extracellular fluid, kidney and liver and can be easily mobilized for Zn-dependent functions [28]. The exchangeable Zn pool is linked to endogenous fecal excretion of Zn and to total daily absorption of Zn [30]. The size of the EZP is between 1.5-3 mmol (100-200 mg) [31] and is related to habitual dietary intake [28]. A larger exchangeable pool leads to a larger endogenous excretion [30]. When EZP becomes depleted, Zn deficiency is manifested [32].

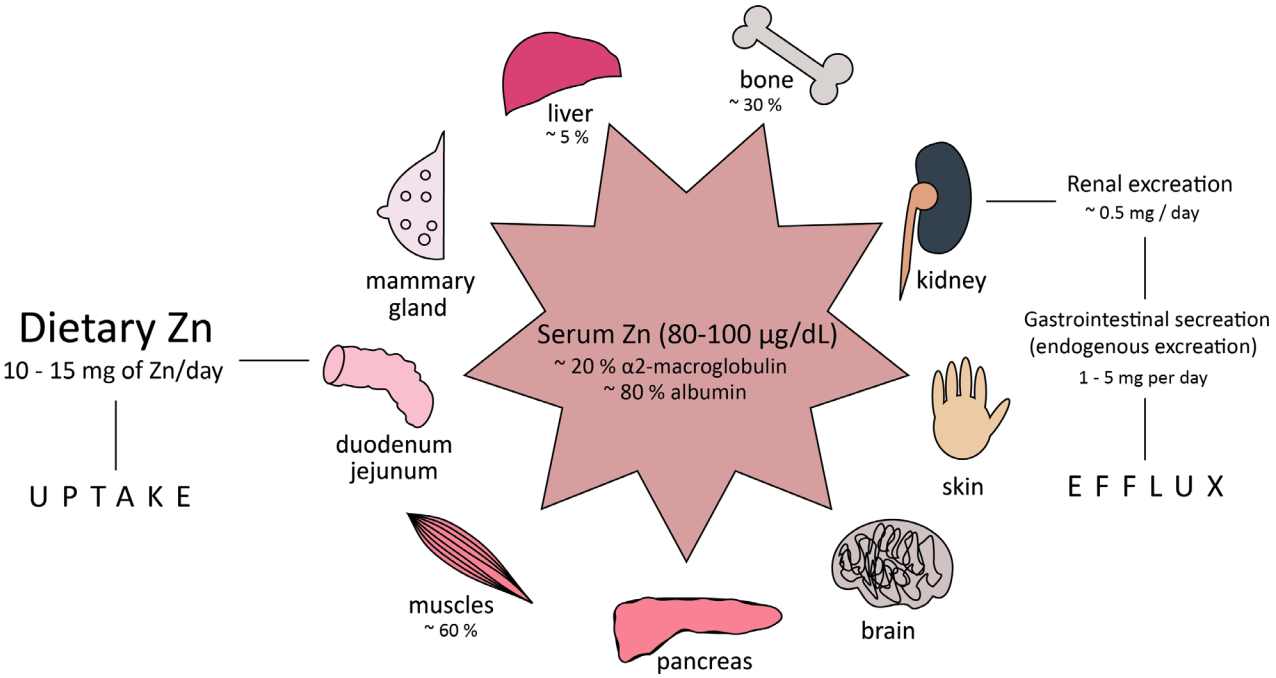

Figure 2.1 Zn homeostasis and distribution of Zn in the body

Zn homeostasis is tightly regulated by the harmonized regulation of the uptake, distribution, storage and efflux of Zn.
The gastrointestinal tract is the major site for regulation of Zn homeostasis. An insufficient Zn intake stimulates Zn absorption in the small intestine Zn is absorbed and distributed to the organs (bones and skeletal muscles are major reservoir tissues). At very low Zn intakes absorption can increase between 60-84% and urinary and faecal losses decrease accordingly. The absorbed Zn is bound to either albumin (lightly, exchangeable pool) or α2-macroglobulin (tightly, non-exchangeable) or chelated by amino acids [40,41]. Low dietary Zn intake induces the mobilization of amino acids from muscle to liver for gluconeogenesis and releases Zn into circulation to retain constant serum Zn levels [37]. The additional (extra) Zn is taken out mainly via gastrointestinal secretion and through sloughing of epithelial mucosal cells.

2.2.2 Zn Homeostasis

The maintenance of cellular and systemic Zn homeostasis is essential for human health [33,34]. Zn homeostasis in humans is managed through an equilibrium between intestinal absorption and excretion including adaptive mechanisms regulated by the dietary Zn intake levels [29,35,36]. The modifications in gastrointestinal Zn absorption and endogenous Zn excretion are synergistic. Changes in the endogenous excretion occur rapidly with modifications in dietary intake. On the other hand, the absorption of Zn ensues gradually, and it has the capacity to manage large variations in dietary intake [2,34]. During exceptionally low Zn intakes or with persistent marginal intakes, secondary homeostatic alterations may complement the gastrointestinal changes. These secondary modifications include variations in urinary Zn excretion, changes in plasma Zn turnover rates and a conservation of Zn release from certain tissues (i.e. bone, muscle) (**Figure 2.1**).
Homeostatic mechanisms keep serum Zn concentrations in a healthy person within a narrow range (12-15 µmol/L; 80 to 100 µg/dL) even in the presence of noticeably varying Zn levels [28,35,37,38]. The mean serum Zn concentration is 1 mg/L [39].

2.2.3 Zn Absorption and Excretion Mechanisms

Zn is absorbed in the small intestine, predominantly in the proximal jejunum by passive diffusion and on the brush border of enterocytes via a carrier-mediated process [42]. The absorption occurs within the whole small intestinal tract with the maximum rate of absorption in the jejunum [43]. Zn is released from food as free ions throughout digestion. The ions attach to endogenously secreted ligands before they get transported [44].
Within the enterocytes, absorbed Zn is bound to several molecules, including metallothionein (MT) and cystein-rich intestinal protein which are important in transmucosal transport [45]. Intestinal concentrations of Zn are controlled by the activity of Zn transporters (mainly Zip4, Zip5, ZnT1 and ZnT5) and Zn binding proteins such as metallothioneins (MTs) [19,46].

The gastrointestinal tract is the main site for regulation of Zn homeostasis [35,36]. Zn absorption is mainly influenced by dietary Zn intake and to a lower extent by Zn status [29]. Fractional Zn absorption is inversely related to dietary Zn intake, whereas intestinal absorptive capacity is high at low levels of Zn intake. During periods of increased Zn intake, there is a decrease in absorptive efficiency but an increase in the total amount of Zn absorbed. With high intakes, Zn is also absorbed via a passive paracellular route (absorbed through a non-saturable process or passive diffusion) [36,47]. 15%-60% of dietary Zn intake is absorbed.

At intakes under 9 mg/day, Zn absorption occurs mainly by a saturable process including Zip4, ZnT1, and other transporters. There is no evidence that past Zn intakes, or status, affects Zn absorption. The latest Zn intake is the principal determinant of Zn absorption [48,49].
An upper limit exists where increasing Zn intake >20 mg in solution does not result in greater Zn absorption [50]. The portal system transports absorbed Zn straight to the liver and after that Zn is taken into systemic circulation for delivery to other tissues. Approximately 60%-70% of Zn in the circulation is bound to albumin, so any condition that changes serum albumin levels can consequently have an effect on plasma Zn levels [44,51,52]. Even though, serum Zn presents only 0.1% of the whole body Zn, the circulating Zn turns over quickly to meet tissue requirements [44].

Zn excretion is also an essential process in Zn balance maintenance. Zn is excreted from the body through the intestine, kidneys and skin. The endogenous intestinal losses fluctuate from 7 mmol/day (0.5 mg/day) to more than 45 mmol/day (3 mg/day), depending on Zn intake [53]. Half of all Zn eliminated from the body is lost through the gastrointestinal tract [1]. Minor amounts of Zn are also excreted via urine and surface losses (i.e. skin, hair) [35]. Urinary and skin losses are less affected by the usual variations in Zn intake, being between 7-10 mmol/day (0.5-0.7 mg/day) [53]. Zn is predominantly excreted through the biliary and intestinal secretions, most of it being reabsorbed [36,46].

2.2.4 Estimated Physiological Requirements for Zn

An adequate estimation of physiological requirements for Zn is crucial for several reasons: for understanding human Zn nutrition, Zn homeostasis, Zn status of populations, and for providing an indication on how to best prevent and treat human Zn deficiency [54]. Additionally, accurate estimates provide critical reference points for bioavailability studies of Zn in biofortified crops [55].

The estimations of physiological Zn requirements were set by various expert groups: US IOM (2001) [57], IZiNCG (2004) [58], WHO (2004) [62] and EFSA European Food Safety Agency (2014) [63]. All four groups used a factorial approach* to estimate Zn requirements, however there were certain discrepancies in the recommendations as different body weights, various ways for estimation of external Zn losses, and different nomenclature were used. The recommendations are based on the frequency distribution of requirements of a group or an individual and the resultant values are presented differently: EAR (Estimated Average Requirement), RDI (Recommended Dietary Intake), in Australia and New Zealand, RDA (Recommended Dietary Allowance) in US and Canada and RNI (Reference Nutrient Intake) in UK [64].

The estimated average requirement (EAR) is the median intake level of Zn for a particular sex and life stage group of healthy individuals at which the needs of 50% of the population will be met [33,38]. Assessment of requirements was based on estimates of the minimal amount of absorbed Zn necessary to match total daily excretion of endogenous Zn [32,33]. Estimates were made using factorial approach that includes both intestinal and non-intestinal losses [61,66]. However, as the needs of the other half of the population will not be met by the EAR, the EAR is enlarged by 20% to rich the Recommended Dietary Allowance (RDA). Since, currently there is no sufficiently sensitive biomarker of Zn status the RDA for Zn is based on a number of different indicators of Zn nutritional status and represents the desirable daily intake that is expected to prevent deficiency in nearly 97.5% of individuals in a specific sex and life stage age group [20,51]. The EAR for Zn across various countries ranges from 5.5-12 mg/day (**Table 2.1**). The recommended tolerable upper intake level (UL) of Zn is set for 40 mg/day for adults, 34 mg/day for adolescents and 5 mg/day for infants (**Table 2.1**).

The European Food Safety Agency was the only agency that provided data that accounted for different levels of phytate intake. Increasing dietary phytate from 300 to 1200 mg/d doubles the dietary Zn requirements [65]. The inconsistencies and large heterogeneity between the currently available recommendations were noted and are discussed in great detail by Hambidge et al. (2011) [54], Lowe et al. (2013) [66] and by Gibson et al. (2016) [61].

* Factorial approach is based on the estimates of the amount of absorbed Zn required to offset all obligatory Zn losses plus additional requirements for absorbed Zn for growth, pregnancy or lactation.

Table 2.1 Estimated physiological requirements for absorbed Zn (mg/day), by age group and sex as defined by expert committees of the WHO, IOM, IZiNG and EFSA.

Age group/Life stage	WHO	IOM	IZiNCG	EFSA
Infants and children				
6-11 months	0.84	0.84	0.84	
1-3 years	0.83	0.74	0.53	1.07
4-6 years	0.97			1.39
4-8 years		1.20	0.83	
7-9 years	1.12			1.87
9-13 years		2.12	1.53	
Adolescents				
10-12, males	1.40			
12-15, males	1.82			2.63
14-18, males		3.37	2.52	
15-18, males	1.97			3.54
10-12, females	1.26			
12-15, females	1.55			2.66
14-18, females		3.02	1.98	
15-18, females	1.54			2.96
Adults				
19-50, males	1.40	3.84	2.68	
19-50, females	1.00	3.30	1.86	
Pregnancy				
14-18 years	0.10[a] 0.30[b]	0.16[a] 0.39[b]	+0.70	+0.40
19-50 years	0.70[c]	0.63[c]	+0.70	+0.40
Lactation				
14-18 years	1.40[a] 0.80[b]	+ 1.35	+1.00	+1.1
19-50 years	0.50[c]	+ 1.35	+1.00	+1.1

WHO: World Health Organization; IOM: Institute of Medicine, IZiNCG – International Zn Nutrition Consultative Group.

EFSA: European Food Safety Authority. Estimated additional average requirements for Zn needed during pregnancy and lactation (+).

[a] first trimester, [b] second trimester, [c] third trimester.

2.3 Dietary Zn Intake

2.3.1 Food Sources of Zn

Zinc (Zn) is found in a wide range of foods, however its bioavailability is variable [1,67]. Foods with a high protein content are rich in Zn, while foods and diets made of carbohydrates were found to be much lower in Zn content [68]. Richest sources of the Zn include oysters (25 to 39 mg per 100g), red meats (4.5 to 5.2 mg per 100g), liver (4 to 7 mg per 100g), nuts and seeds (6.5 to 7.8 mg per 100g) [69]. The highest content of Zn is found in animal source foods; 0.40 to 6.77 mg per 100 g. The grains have 0.30 to 2.54 mg per 100 g, dairy products between 0.36 to 0.49 mg per 100 g, vegetables from 0.12 to 0.60 mg per 100 g, and fruits have 0.02 to 0.26 mg per 100 g [69,70]. Moderate sources of Zn include: cheese, whole grain cereals, and legumes (**Table 2.2**). Zn in animal products is more easily absorbed when compared to Zn absorbed from plant foods. Cereal grains, legumes, and nuts are rich in phytate, a compound known to bind Zn in the intestine and this reduces its absorption [68,71]. The first recognized cases of Zn deficiency were linked with high phytate containing foods, unleavened bread from unrefined wheat flour and beans [72]. The intake of phytate around the world has been investigated to some degree and omnivorous adult intakes of phytates range from 395-1293 mg/day in the US and UK [73]. The molar ratio of phytate:Zn in the diet has been perceived as an indicator of Zn bioavailability, and ratios >15 have been related to suboptimal Zn status [68,71,74].

Currently there are several useful resources that provide data on Zn and phytate values of foods. The most complete source for low income countries is available from the World Food System International Mini-list [75,76]. Industrialized countries mainly use USDA database from the Nutritional Coordinating Center, University of Minnesota (USDA National database for standard reference) [77] as well as the publications by Reddy and Sathe (2002) [78] and Wessells and Brown (2012) [79].

Table 2.2 Food sources of Zn (high, moderate and low Zn content)

High	Moderate	Low
Shellfish: oysters, crab, lobster, shrimp	Legumes: chickpeas, lentils, beans	Fruits: avocado, blackberries, pomegranate, raspberries
Meat: red meat, ground beef, lamb, liver, poultry	Whole grain cereals: wheat, quinoa, rice, oat	Vegetables: mushrooms, potatoes, lentil sprouts
Seeds: hemp, squash, pumpkin, sesame seeds	Dairy products: milk, cheese, yoghurt	Green vegetables: spinach, broccoli, green beans, kale
Nuts: pine nuts, peanuts, cashews, almonds	Eggs	
	Cocoa: dark chocolate, cocoa powder	

2.3.2 Dietary Recommendations for Zn Intake

Table 2.3 A summary of the currently used dietary recommendations for Zn intake (mg/day) for various life stage and sex group as set by WHO, IOM, IZiNG and EFSA

Age group/life stage	WHO				IOM			IZiNCG			EFSA	
	LBA	MBA	HBA	UL	EAR	RDA	UL	A	B	UL	EAR	UL
Infants and children												
0-6 months	6.6	2.8	1.1				4					
7-12 months	8.4	4.1	2.5	13	2.5	3	5	3	4	6	2.4	
1-3 years	8.3	4.1	2.4	23	2.5	3	7	2	2	8	3.6	3
4-6 years	9.6	4.8	2.9	23							4.6	
4-8 years					4	5	12	3	4	14		4
7-9 years	11.2	5.6	3.3	28							6.2	
9-13 years					7	8	23	5	7	26		7
Adolescents												
10-12, males	17.1	8.6	5.1	34								
12-15, males	17.1	8.6	5.1	40							8.9	
14-18, males					8.5	11	34	8	11	44		22
15-18, males	17.1	8.6	5.1	48							11.8	
10-12, females	14.4	7.2	4.3	32								
12-15, females	14.4	7.2	4.3	36							8.9	
14-18, females					7.3	9	34	7	9	39		22
15-18, females	14.4	7.2	4.3	38							9.9	
Adults												
18-60, males	14.0	7.0	4.2	45								
19-50, males					9.4	11	40	10	15	40	7.5	25
>51, males	14.0	7.0	4.2	45	9.4	11	40	10	15	40		25
18-60, females	9.8	4.9	3.0	35								
19-50, females					6.8	8.0	40	6	7	40	6.2	25
>51, females	9.8	4.9	3.0	35	6.8	8.0	40	6	7	40		25
Pregnancy												
14-18 years	11.0[a]	5.5[a]	3.4[a]		10.5	13	34	+2	+3		+1.3	
19-50 years	14.0[b] 20.0[c]	70[b] 10.0[c]	4.2[b] 6.0[c]		9.5	11	40	+2	+3		+1.3	
Lactation												
14-18 years	19.0[a]	9.5[a]	5.8[a]		10.9	14	34	+1	+1		+2.4	
19-50 years	17.5[b] 14.4[c]	8.8[b] 7.2[c]	5.3[b] 4.3[c]		10.4	12	40	+1	+1		+2.4	

Table 2.3 Footnote

WHO: Worlds Health Organization; IOM: Institute of Medicine, IZiNCG – International Zn Nutrition Consultative Group. EFSA: European Food Safety Authority. A: IZiNCG P:Zn≤18; B: IZiNCG P:Zn >18. BA: Bioavailability LBA: Low bioavailability (15%); MBA: Moderate (30%) and HBA: High (50%). UL: Upper limit of intake (the highest intake likely to cause no risks of adverse health effects); EAR: Estimated Average Requirements (meets the needs of 50% of individuals in the life stage group); RDI/RNI/RDA: Recommended Dietary Intake/Reference Nutrient Intake/Recommended Dietary Allowance (meets the needs of nearly all individuals in the life stage group). There is insufficient data for upper level of Zn intake for children. Estimated additional average requirement for Zn needed during pregnancy and lactation (+) .[a] first trimester, [b] second trimester, [c] third trimester. Modified from WHO (1996) [56], IOM (2001) [57]; IZiNCG (2004) [58]; Lim et al. (2013) [59]; King et al. (2016) [60] and Gibson et al. (2016) [61].

2.3.3 Zn Intake in Developed and Developing Countries

The adequacy of dietary Zn intake is assessed by comparing the usual dietary Zn intakes with the Estimated Average Requirements (EAR). As the assessment of Zn status by biomarkers is still challenging, evaluation of inadequacy is typically based on the assessment of dietary intakes, prevalence of child stunting, and the accessibility of Zn from the food supply [80]. The US, Canada, New Zealand and Australian government agencies have evaluated population dietary Zn intakes and documented the main dietary sources [81,82,83,84].

In Europe, the European Micronutrient Recommendations Aligned Network of Excellence (EURRECA; www.eurereca.org) is responsible for tracking projected prevalence of inadequate Zn intakes and determining Zn requirements [85].

Results from the nutritional surveys conducted in the United States, Great Britain, Spain and Germany concluded that the recommended intake of Zn is not always achieved [86,87]. For example, 10% of 1-3 years old children from Poland had Zn intakes below the recommendations and 39% of girls age 4-10 from Denmark had Zn intakes below the EAR [87,88,89]. Similarly, the percentage of inadequate Zn intakes for adolescents in the UK was between 3%-48%, while the percentage of inadequacy for adults was a bit lower; between 10% to 21% [83,87,90]. 39% of Spanish man over 60 years of age had Zn intakes below the EAR [91,92].

People living in Ireland failed to meet estimated average requirements for Zn; 13% of the elderly, 11% of men and 29% of women [86]. Comparable findings were provided for the USA, where data from the NHANES indicated that 11%-17% of people had Zn intakes below the EAR [93].

Similarly, the Canadian Community Health Survey from 2004 demonstrated that 10%-35% of Canadians consume Zn in inadequate amounts and most vulnerable being men over 70 years of age where 41% were Zn deficient [81]. People living in the UK also had Zn intakes below the EAR and girls 11-18 years of age were at the highest risk of inadequate intakes [83]. The estimated prevalence of poor Zn intake among children in New Zealand ranged from 5.4% for males and 9.2% (5-6 years old) to 16.4% for girls' age 11-14 years [94].

An adult nutrition survey from New Zealand identified that 39% of males and 11% of female adults is in risk of dietary Zn deficiency [95]. In addition, 52% of men and 9% of women consumed less than the Australia/ New Zealand estimated average requirement for Zn, while 15% of men and 7% of women had low serum Zn levels [96]. Furthermore, low serum Zn was observed in 18% of men 50 years or older and 30% of men 70 years or older [96]. Similarly, 19% of premenopausal (age 18-50) Australian women were susceptible to inadequate Zn nutrition [97]. Finally, 7.8% of people living in China and around 10% of people from Central and Eastern Europe consume Zn in inadequate amounts [79].
The most current review of available micronutrient intake and status data in Europe [98], encompassing the majority of available research related to Zn intakes of people living in European countries, pointed out that data on Zn intakes in Europe are lacking for all life stages while for certain countries data on Zn intakes are either outdated or do not exist [98].

The nationally representative surveys that evaluated the adequacy of Zn intakes in low income countries are very limited due to the high cost and logistical challenges. The global risk of Zn deficiency decreased from 22 to 16%, between 1992 and 2011, but it still remains prevalent [99]. In 2011, 1.1 billion people were at risk of Zn deficiency [99]. Comparable findings were provided by Wessells and Brown in 2012 [79], when the national food balance sheet data taken from the Food and Agriculture Organization [100] of the United Nations were used to estimate a country and region specific risk of dietary Zn inadequacy in 188 countries. The global estimates on the prevalence of inadequate intakes were relatively stable over the 20-year period and there were no inter-regional variations in Zn intake among individual countries [79]. 17.3% of the world's population is at risk of inadequate Zn intakes [79], with more than 20% inadequacy seen in people living in South and South East Asia and the Pacific (22%), Sub-Saharan Africa (25%) and South Asia (close to 30%). The Zn deficiency risk for Africa in 2009 was estimated to be around 40% [101]. A more recent systematic review documented that 34% of women of reproductive age and 46%-76% of pregnant women in Kenya, Ethiopia, Nigeria and South Africa are Zn deficient [102].

The prevalence of inadequate Zn intakes is most common in low income countries, however it is also increasingly seen in developed country populations. In addition, there is no national data from a specific survey period that can show Zn intakes and its adequacy. No cross country comparison could have been appropriately made as many countries have no regular nutritional monitoring programs in place (this includes both developing and European countries).

Comparison of data on Zn intake between countries is a challenging task due to heterogeneity in methodologies and study purposes being used, various assessment methods of food intake and no representative nutritional surveys at the national level for all countries. Additional, up to date and higher quality studies are undoubtedly needed to address gaps in current knowledge. Regular follow ups are necessary to ensure that potential deficiencies of Zn do get acknowledged and addressed in a timely manner, particularly in countries where their existence is less expected. More targeted measurements of population Zn status including biochemical and dietary assessments are needed for countries recognized as being at the highest risk of inadequate Zn intakes (i.e. South Asia, Sub-Saharan Africa and Central America).
Supporting evidence and additional research on dietary Zn intake globally would not only help in obtaining a more accurate estimate of Zn inadequacy, but also, in adequately directing nutritional interventions aimed at controlling Zn deficiency.

2.4 Zn Bioavailability

2.4.1 Zn Bioavailability, Inhibitors and Enhancers

Bioavailability of Zn refers to the portion of dietary Zn intake that can be absorbed into the blood system and used for physiological functions within the body [34]. Main factors that define the bioavailability of Zn are the total Zn content of the diet, the individual's Zn status, and the availability of soluble Zn from the diet's food components [103]. If the individual's Zn status is reduced, Zn absorption mainly depends on Zn solubility in the intestinal lumen, which is determined by the chemical form of Zn and the occurrence of certain inhibitors and enhancers of Zn absorption [34]. Fractional Zn absorption is a fraction of Zn that could potentially be absorbed from a single meal [34,35]. It is inversely proportional to dietary Zn intake and is negatively associated with the phytate content of a meal [68,74]. In contrast, total Zn absorption is greater with Zn adequate diets compared with low Zn diets [35].
As demonstrated in a number of studies, feeding low Zn diets increases fractional Zn absorption in all age groups and homeostatic mechanisms up-regulate Zn absorption and retention [105,106,107]. Reduced fractional absorption of Zn at higher doses is due to saturation of the transport mechanisms of Zn and increased intestinal Zn excretion [35,103]. Adults on higher bioavailable diets are capable in regulating Zn absorption upwards or downwards to absorb 4-5mg of Zn per day [108,109]. With regard to the time of adjustment, the variation of Zn absorption from the low Zn diet took place within the four week period and the degree of adjustment was no greater after eight weeks [109].

In conclusion, the absorption efficacy of Zn is up-regulated in response to extended low Zn intakes and the major predictors of Zn absorption are daily Zn and phytate intakes [109]. Total Zn absorption will be the greatest from low phytate, Zn adequate meals [34,35].

2.4.2 Main Zn Inhibitor in Plants - Phytate

Phytate is the key dietary component known to limit Zn bioavailability and it does this by strongly binding Zn in the gastrointestinal tract [65,110,111]. Phytate is the magnesium, calcium, or potassium salt of phytic acid (myo-inositol hexakiphosphate, IP_6) and is present in seeds, cereal grains, nuts, and legumes [112].

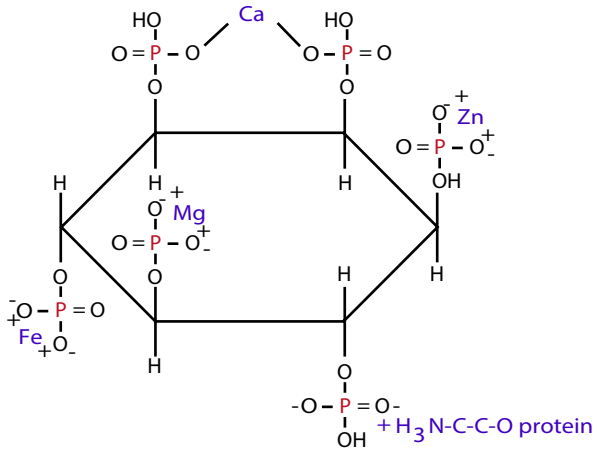

Figure 2.2 Arrangement of phytic acid; six phosphorous acid molecules attached to a phytic acid molecule

Modified from Coulibaly et al. (2011) [112].

In cereal grains (such as wheat) it is concentrated in the bran. The anti-nutritive effect of phytic acid is attributable to its molecular structure. At complete dissociation, the six phosphate groups of phytic acid carry twelve negative charges which, in weak acidic to neutral pH conditions, bind to various di and trivalent cations (Ca, Mg, Fe, Zn, Cu, Mn) into a stable complex (**Figure 2.2**).

The phytate:Zn molar ratio of a diet is used to assess the quantity of Zn available for absorption. In general, unrefined cereal grains have very high phytate:Zn molar ratios (ranged from 22-88). Diets with molar ratio >15 have poor Zn bioavailability and are linked to biochemical Zn insufficiency in human subjects [58,71,109,113]. The IZiNCG separates diets into high and low phytate:Zn molar ratios, with a cut-off of 18 [58].

The inhibitory effect of phytate on Zn absorption was originally demonstrated by Lonnerdal et al. (1988) [114] via a radioactive isotope study in infant rhesus monkeys and suckling rat pups. The results pointed out that the negative effect of phytate followed a dose-dependent response and that Zn absorption can be enhanced in humans by decreasing the phytate content of the diet. Similar findings were provided by others [115,116].

Dietary Zn intake that's needed to meet the Zn requirements of an adult doubles with every 1000 mg of phytate consumed [54,117]. With the phytate to Zn ratio of more than 15-20, any amount of Zn available for absorption is insufficient to up-regulate Zn absorption [109], so the goal for the phytate:Zn molar ratio should be less than 12 so that sufficient amounts of Zn are absorbed with unsupplemented diets [54,109]. Additionally, only the higher inositol phosphates (i.e., hexa- and penta-inositol phosphates) suppressed Zn absorption, whereas the lower phosphates had no negative effect [47,118].

Humans have a negligible capacity to adaptively increase Zn absorption from diets high in phytic acid [109,119,120]. In humans, unlike in rats, hydrolysis of the higher inositol phosphates does not happen in the gastrointestinal tract because of the absence of phytase enzymes [71]. Lower inositol phosphates are formed during certain food preparation and processing procedures such as soaking, germination and fermentation of whole grain cereals and legumes that stimulate enzymatic hydrolysis of phytic acid in whole grain cereals and legumes by increasing the activity of exogenous or endogenous phytase enzyme [71,121].
Similarly, non-enzymatic methods, i.e. milling, has also been shown to successfully lower the amount of phytic acid in plants [122].

2.4.3 Other Dietary Factors Known to Affect Zn Availability of Plant Foods

In addition to phytate, some other dietary ingredients can also reduce Zn absorption (i.e. polyphenols, fiber, oxalate, tannin, and lignin) [111,123]. Polyphenols, a class of antioxidant, reduce Zn bioavailability by forming complexes between the hydroxyl groups of the phenolic compounds and Zn molecules [124]. Generally, the degree of inhibition is inversely related to the condensed polyphenol content [125].

Calcium (Ca) is a dietary ingredient for which there is no definite evidence regarding the effect it has on Zn absorption and Zn bioavailability. Some believe that Ca may inhibit Zn absorption and additionally enhance the inhibition of Zn absorption by phytate [126], while others have shown no effect on Zn retention or balance. High levels of dietary Ca impair Zn absorption in animals [127,128] however, it is unclear if this also happens in humans. Current findings offer inconsistent results [111]. Increased Ca intake of post-menopausal women by 890 mg/day in the form of milk or Ca phosphate (total Ca intake; 1,360 mg/day) diminished Zn absorption and Zn balance in postmenopausal women [129]. However, increasing the Ca intake of adolescent girls by 1,000 mg/day (total Ca intake, 1,667 mg/day) did not disturb Zn absorption or balance [130]. Similarly, a study with ten healthy women (21-47 years old) demonstrated that high intake of dietary Ca (~1,800 mg/day) did not further impair Zn absorption of a high-phytate diet [131]. The effect of Ca is not significant and an enhancing effect is only observed in the diets with low phytate levels [109].

On the other hand, Sandström et al. (1989) [74] showed that the presence of Ca enhanced Zn absorption by the addition of dairy products to a high-phytate bread meal, but no change in absorption was evident in a low-phytate white bread meal [74].

There were also studies that provided evidence of a positive effect of Ca on Zn absorption showing that Zn absorption from soy formula was increased when Ca was added to it [132]. Authors explained this by hypothesizing that Ca is making complexes with phytate in the gut, hence making phytate unavailable to bind Zn. Miller et al. (2013) [133] based their mathematical model of Zn absorption on this hypothesis, stating that Ca has a positive effect on Zn absorption. The inconsistent findings of the Ca studies on Zn absorption clearly show that complex nutrient interactions exist (beyond that with phytate) and that further research is needed to clarify the effect of Ca on Zn availability and absorption.

The effect of dietary protein on Zn absorption is also ambiguous. Dietary protein and protein digestion products (i.e. casein phosphopeptides) have been shown to increase Zn absorption [74,103,134] inhibit absorption[135] and to have no effect [134,135]. The protein source, animal or plant, can also have an effect [103]. Likewise with Ca, inconsistencies in findings were explained by means of nutrient interactions where the phytate content is having a central role.

2.4.4 The Interaction Between Zn and Fe

Iron (Fe), is an essential micronutrient with important biological functions. Fe deficiency and Fe deficiency anemia remain a global problem, with approximately 25% of people worldwide being affected [136].

In some older nutrition textbooks, Fe deficiency anemia is recognized as a Zn deficiency symptom [137] and Zn deficiency was described as a cause of Fe deficiency anemia [138,139].

Zn is found in similar food sources to Fe and the same constituents disturb the absorption of both elements, so nutritional Zn and Fe insufficiencies often occur at the same time [140,141]. Nutritional deficiency of Fe in the developing world never arise alone and Zn deficiency always co-exists with Fe deficiency [142].

In recent years, an extensive progress in our understanding of Fe and Zn absorption and the interaction processes in humans has been made. A negative interaction among Zn and Fe for absorption ensues when elements are supplied simultaneously, in high doses, in an aqueous solution or in a simple food matrix [143]. However, the inhibitory effect is not observed in complex food matrices, except when Zn to Fe ratio is >1:25 [144].

The longstanding belief of a negative effect of Zn on Fe absorption and vice versa was forgotten as no antagonistic effect is constantly present, more so, when these nutrients are supplied in lower amounts (closer to 'physiological' concentrations) or with food [103,145,146]. Gradually, evidence has accumulated to show that plasma Zn is a good predictor of hemoglobin concentrations, independent of Fe status [71,147-153].
Additionally, a number of data sets have revealed a positive association between anemia and signs of the risk of Zn deficiency in children, pregnant women and adults [154-156]. Zn deficiency (low serum Zn concentrations) coexists with the presence of anemia, particularly in developing countries [149,157,158].
Moreover, Fe supplementation, on its own, was not always effective in treatment of anemia [159-161].
The positive influence of Zn in addition to Fe has been shown by Kolsteren et al. (1999) [162]; Alarcon et al. (2004) [163] ; Nishiyama et al. (1996) [164]; Nishiyama (1999) [165] and many others Shoham and Youdim (2002) [166]; Ramakrishnan et al. (2004) [167]; Hossain et al. (2011) [168]; Kaluza et al. (2014) [169]. Zn supplementation is known to improve hematological response among young children at risk of Zn deficiency [147,163,170] and to increase serum ferritin by approximately 50% [171,172]. Micronutrient interventions with simultaneous provision of both elements were more effective in the treatment of deficiencies [166,167]. Over the years, many studies have confirmed positive interactions between Zn and Fe during absorption [147,171-175]. The findings were initially summarized by Fisher-Walker et al. (2005) [176] who compiled findings from randomized placebo controlled trials of supplementation of Fe and Zn separately or in combination, showing no competitive interaction between Fe and Zn for absorption. A few years later Graham et al. (2012) [177] reviewed the medical literature on Zn and Fe deficiency, with a particular focus on the interaction of these elements during the absorption processes in the human gut. They proposed that a significant proportion of Fe deficiency anemia may be due to Zn deficiency [177]. New perspectives reveal that Zn in not inhibiting the Fe absorption process but in fact that adequate amounts of Zn are required for proper absorption of Fe and that Zn is having a major regulatory role in the Fe absorption path.
Certain justifications that could explain this link have been provided. It is believed that Zn affects hemoglobin via a Zn dependent enzyme system or that the link is based on erythropoietin stimulation [178-181]. DMT1 (divalent metal transporter 1, the main Fe import protein) has been ruled out as a site of Fe and Zn antagonism and what's more it has been proven that DMT1 is regulated by dietary/intestinal Zn concentrations [182-184]. The Fe export protein (FPN1, ferroportin 1) is also shown to be controlled by cellular Zn concentrations [183-185]. Finally, hepcidin, the major Fe absorption regulator located in the liver, is under the control by matriptase 2, a family of Zn dependent endopeptidases [186-188].

Despite all these achievements in explaining the Zn and Fe interaction, it is still unknown what is happening at the transporter level and how exactly Zn concentrations may affect the Fe absorption process. This PhD project aims to explain the biological mechanisms of this interaction that have not been elucidated so far. An improved understanding of the machinery that controls Zn and Fe absorption processes would help in a more appropriate treatment of both nutritional deficiencies; deficiency of Zn primarily, but also Fe deficiency and Fe deficiency anemia. Correcting Zn deficiency can possibly make more Fe available for absorption from a similar diet, which is important for planning nutrition intervention programs that would alleviate Zn deficiency and with it some of the anemia.

2.5 Zn Transporters in the Human Body

2.5.1 Zn Transporters in Human Enterocytes and Hepatocytes

Zn movement inside a cell is regulated by an activity of various Zn transporters. There are 24 Zn transporters in different parts of the human body and they are responsible for uptake, intracellular trafficking and efflux of Zn [9,189-192]. Ten Zn transporters are classified as ZnT family and they export Zn from the cytosol, while 14 members of the ZRT/IRT-like protein Zip family import Zn into the cytosol. These transporters control cellular Zn and its movement through the plasma membrane and between cytosol and different cellular segments and are described in great detail elsewhere [3,192,193].

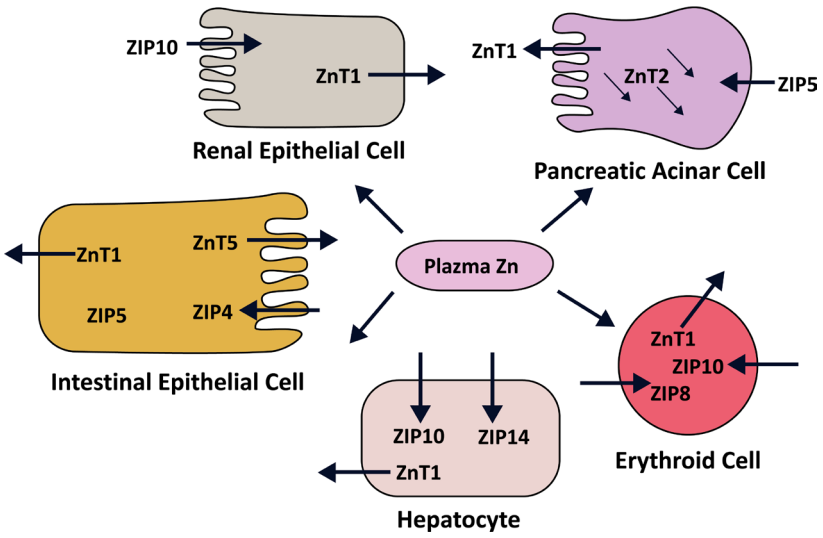

Figure 2.3 Zn transporters in the human body

Zn is present as a divalent cation and does not require redox reduction for the membrane transport process. Absorption occurs by means of a sophisticated process (control system) in which unique transport proteins are operative for each organ.

The activity of these transporters is regulated transcriptionally, translationally, and at the protein level via heterodimer formation, ubiquitination, phosphorylation, and proteolysis [3,7,192,193]. The expression and function of some of these proteins is stimulated by physiological stimuli (hormones and cytokines) and/or dietary conditions [195,196]. The activity of most of the Zn transporters is predominantly transcription controlled by the metal responsive element binding transcription factor 1 (MTF 1) [192,197,198].

2.5.2 Zn Transporters Regulated by Dietary Zn Concentrations

Out of the 24 Zn transporters identified to date, eight of them are shown to be regulated by dietary Zn intake. They include: ZnT1, ZnT2, ZnT4, ZnT5 and ZnT6 and three Zips: Zip3, Zip4 and Zip10 [9,195]. The transporters are mainly located within the small intestine, with only a few located in other tissues; blood cells: Zip3 and ZnT1 and Zip10 found in brain and liver (**Table 2.4**).

Table 2.4 Zn transporters regulated by dietary Zn intakes

Zinc transporter	Response during dietary Zn restriction	Tissue/cell
ZnT1	negative	Ubiquitous & blood cells
ZnT2	negative	Small intestine, liver, kidney
ZnT4	negative	Small intestine
ZnT5	negative	Small intestine
ZnT6	negative	Small intestine
Zip3	negative	Blood cells
Zip4	negative	Small intestine
Zip10	negative	Plasma membrane

Note: The structure and function of most important Zn transporters is described in detail within Chapter 3. In order to avoid unnecessary duplication the same information is not presented here. The role of Zip4, ZnT1, Zip14 & Zip8 in Zn, as well as in Fe, absorption processes was explained.

2.6 Assessment of Zn Status

2.6.1 Currently Used Biomarkers and their Limitations

The assessment of Zn status is perplexing as currently there is no single specific and reliable biomarker of Zn status. Zn status of individuals is estimated through assessment of dietary Zn intakes and by biochemical markers. Various categories of biomarkers have been examined over the years and they include tissue concentrations (plasma, hair, nail Zn content), mineral homeostasis and metabolism parameters (Zn excretion, measurements of the Zn pool sizes), body stores, response to increase in dietary intake. Out of 32 potential biomarkers from 46 publications, serum/plasma Zn concentrations, hair Zn concentration and urinary Zn excretion are the only three identified as potentially useful biomarkers of Zn status [199].
In 2016, the Biomarkers of Nutrition for Development (BOND), an international Zn Expert Panel [60] completed a thorough literature review of Zn biomarkers considering both functional and biochemical indicators of Zn status, and classified Zn related biomarkers into four categories: recommended, potential, emerging and not useful (**Table 2.5**).

Table 2.5 Summary of biomarkers of Zn status

Recommended	Potential	Emerging	Not useful
Dietary assessment	Hair Zn	Nail Zn	Zn dependent enzymes
Plasma/serum Zn	Urinary Zn	Zn dependent proteins	Erythrocyte and leukocyte zinc
Stunting	Neurobehavioral development	Oxidative stress and DNA integrity	
		Zn kinetics	
		Taste acuity	

The biomarkers were graded on the basis of their strength to be used for assessing Zn exposure, Zn status, Zn function & Zn effects [60].
Modified from King et al. (2016) [60] & Lowe (2016) [200].

2.6.2 Assessment Methods of Dietary Zn Intake

An inadequate dietary Zn intake is usually the main cause of Zn deficiency. The assessment of the dietary intake is accepted as the best scheme for estimating Zn exposure in individuals and populations and is the central element in evaluating the risk of Zn deficiency [60]. A comprehensive historical overview of the progress in the evaluation of dietary Zn intake as an indicator of Zn status was provided by Gibson in 2012 [201] .

The Dietary Zn intake of individuals can be assessed by several methods (**Table 2.6**). The details of methods presented here are described explicitly elsewhere [202,203].

Table 2.6 Assessment methods of dietary Zn intake for individuals

Method	Principle	Advantages	Disadvantages
Dietary record/Food dairy	Quantities of food consumed per day are recorded andweighed	Detailed information about the food intake, provides quantitative information about food consumed	Motivation bias, high burden on participants, expensive, not suitable for people who frequently eat outside the home
24-hour food intake recall	Food intake during the past 24 hours; subjective measure using open ended questionnaires	Provides detailed intake data, relatively small respondent burden, high literacy not required	Recall bias, requires skilled interviewers, depends strongly on the memory of participants, food intake may not be reported accurately, expensive, time consuming
Food frequency questionnaire (FFQ)	Questions on habitual consumption of certain foods (in the past several months or a year), data on frequency and size of food eaten	Easy and cheap to accomplish, cost effective, time saving, information of food intake over a longer period, no influence of eating behaviour, captures the intake of Zn rich foods that are irregularly consumed, useful in epidemiological studies	Closed ended questions, recalling and measurements errors, not appropriate for clinical setting, cognitive effort required, no specific quantification of amounts, depends on memory, no correlations between consumption of foods, dietary phytate may not be included in the food composition tables, portion sizes need to be cautiously quantified
Diet history	Frequency of food intake and food preparation, estimation of usual consumption, use both open and closed-ended questionnaires	No influence of eating behavior, information of food consumption over a longer period of time, information on the whole diet is obtained	High cost, time consuming, consumption often misreported and cannot be precisely quantified

Based on data provided by Shim et al. (2014) [204] & King et al. (2016) [60].

Once the daily food intake is known, the total Zn intake can be estimated by multiplying the amount of each food item that is consumed by its Zn content. The information on Zn content of certain foods is recorded in local food composition tables or databases. The values are based on the chemical analyses of food and beverages, or they are estimated from available data, including manufacturers' information [58,60,75]. The most commonly used and the most complete databases are the USDA food composition, FAO/INFOODS and EFSA food composition databases [63,75,75]. Nowadays, many countries have their own national food composition databases. Theoretically, local databases are advantageous as Zn content of foods can vary according to soil conditions, agronomic practices and local food processing techniques [58], however the country specific composition tables contain fewer food items and fewer replicate analyses per item [58,60]. The values for phytate content of local foods do not exist [60], and the information on Zn content of particular foods is very often taken from other more complete data-sources [58]. Even the most comprehensive and most widely used databases have their limitations; they do not contain information on the Zn content of all foods and the amount of Zn that is added as a fortificant may very among similar foods [58]. The information on dietary phytate, which helps in assessing Zn absorption, is rarely included in food composition databases [60]. Finally, internationally representative data may not always be useful for individuals, so they are not reliable enough for use in clinical studies where intakes are compared with biological measures [60].
It is important to highlight that the dietary Zn intake data provides only an approximation of Zn exposure or Zn insufficiency. The inadequacy cannot be identified with certainty as the actual Zn requirements of an individual are not always known. If dietary phytate can be assessed, the bioavailability of Zn can be defined. Nevertheless, the implications about the adequacy of an individual's Zn intake can be made by comparing the variance among the reported intake and the EAR, however certain conditions need to be met before the method can be used [205,206]. The day to day variation in dietary Zn intake needs to be addressed appropriately. Nowadays, certain software programs are available for making those corrections. They include the IMAPP, Intake, Modelling, Development and Planning Program and the National Cancer Institute method [61,207].

The assessment of the dietary Zn intake of populations can be determined from the food balance sheet data provided by FAO [80]. The balance sheets give an indication of the total amount of Zn accessible to populations within a certain country or region. Additional research is needed to evaluate the accurate proportion of a population affected by Zn deficiency or a population subgroups 'at risk' of Zn deficiency [60,208].

2.6.3 Methods for Evaluation of Intake of Bioavailable Zn

There are currently three algorithms that can be employed for assessing the intake of bioavailable Zn.
The initial algorithm, produced by Murphy et al. (1992) [209] was created on the semi-quantitative classification system of WHO [56] for diets in low income countries with a low content of animal protein, a moderate to low content of Ca and moderate to high content of phytate.
The second model was developed in 2004 by the IZiNCG group and used a regression model to calculate bioavailable dietary Zn [58,210]. Zn and the phytate:Zn molar ratios were involved in the final model and both were very important predictors of the percentage of Zn absorption (r^2=0.413, p<0.001) [51,201].
Hambidge et al. (2011) [54] calculated the effect of different levels of phytate on Zn absorption and intestinal excretion of endogenous Zn by using the staple isotope studies [46,54] and developed a new physiologically based mathematical model of Zn absorption based on the quantities of dietary Zn and phytate. The new trivariate model is the most commonly used nowadays and accounts for >80% of the discrepancy in the amount of Zn absorbed [54,60,133].

2.6.4 Blood Plasma or Serum Zn Concentrations

The concentration of Zn in blood plasma or serum is presently the best available biomarker for estimating Zn status in humans [60,66,200,211]. Previously, it was thought that plasma Zn is a more appropriate choice because of the lack of contamination of Zn from the erythrocytes, however when both samples were kept for identical periods of time before separation of the cells, the results between plasma and serum were no different [35,60,212]. Therefore, both plasma and serum Zn concentrations are seen as identically valid markers of Zn status.
The normal range of plasma Zn concentration in a healthy human population (PZC) is 15 µmol/L or 100 µg/dL.
The efficacy of plasma/serum Zn concentrations (referred to as plasma Zn hereafter) as a suitable biomarker of Zn status in humans is frequently evaluated. By combining the data from 46 publications, both the depletion and supplementation studies in various settings and population groups, an overall significant (p<0.001) response of PZC to dietary Zn intake has been found [61,199]. Every doubling in Zn intake leads to the 6% difference in PZCs [213]. However, there are studies that demonstrate no relationship amongst the dietary Zn intake and PZCs [30,214-217]. The discrepancies are explained by a good homeostatic mechanism that acts to keep PZC within the physiological range and prevent high plasma concentrations from being persistent over a prolonged period of time [60].
The PZCs oscillate as much as 20% during a 24-hour period and the highest levels are usually seen in the morning [218-221]. After a meal, there is an instant initial increase in Zn concentration after which it declines gradually for the next four hours and then increases until food is consumed again .

There are also discrepancies in PZCs when additional Zn was administered as a supplement or as a Zn fortified food; an increase in PZCs is observed after the provision of a supplement but not when the same quantity of Zn is given with food [222,224]. In addition, there is significant inter-individual variability in PZCs [60]. The reliability of PZCs as a marker of Zn status depends on several factors; health state of an individual, collection and storage of samples, the time of the day when the sample is collected, time of consumption of previous meal, time passed until centrifugation of sample. In addition, the PZCs are affected by infection, inflammation, stress and trauma, certain drugs, nutritional supplements and hormones [60,123,220,225-228].

During an acute infection and inflammation states the PZCs are reduced as a result of the redisposition of Zn from the plasma to the liver (cytokines released during acute phase response activate hepatic metalothionein synthesis and alter the hepatic uptake of Zn) [229]. Hemodilution, as seen during pregnancy, hormonal treatments and use of oral contraceptive also result in lower serum Zn levels [228,230]. Plasma Zn levels are significantly reduced in severe, acute Zn deficiency states; for example in acrodermatitis enterohepatica [231]. Low plasma Zn levels in the presence of hypoalbuminemia need to be interpreted cautiously, as albumin is the main carrier protein for circulating Zn [60,232]. Conditions causing an intrinsic or extrinsic hemolysis of blood cells can lead to extremely high plasma Zn levels since the concentration of intracellular Zn is significantly greater than in plasma [212].

As plasma Zn concentrations decline during acute and chronic infections and other conditions that cause systemic inflammation, it is suggested that biomarkers of the inflammatory response, such as C-reactive protein (CRP) and/or α 1-acid glycoprotein (AGP) are measured along with plasma Zn concentrations [19,60]. Subclinical inflammation is defined as ≥ 5 mg/L for CRP and/or 1 g/L for AGP [223]. The correction factors recommended to be used are the following: 1.08 for subjects in incubation stage (CRP>5 mg/L and/or AGP>1.0 g/L); 1.17 correction factor is used for those in early convalescence (CRP>5 mg/L and AGP>1.0 g/L) and 1.06 for those in late convalescence (CRP <5mg/L and AGP >1.0mg/L) [223]. Plasma Zn values should be adjusted with these correction factors, and certain statistical procedures are proposed for it, however further research is still needed to validate the sensitivity and specificity of this particular method [19].

The appropriate collection and storage of samples is crucial for proper determination of Zn concentration in a sample, as Zn can easily be added to samples by environmental exposure and unsuitable handling of samples [199]. The contamination can easily occur from the collection or storage vessel or by hemolysis of the sample when Zn is released from the red blood cells into the plasma [212]. The period between taking the sample and the separation of plasma from the red blood cells is also important [60]. Detailed guidelines of the suitable procedures for collection and processing of blood samples for assessing PZCs are presented by the International Zinc Nutrition Consultative Group [58,233].

In conclusion, the appropriate interpretation of plasma Zn concentration requires inclusion of various potential confounders [199]. However, despite obvious disadvantages the plasma/serum Zn is currently accepted as the most consistent biological marker of Zn status in humans.

2.6.5 Stunting

Stunting (height for age) is the best known biomarker of Zn status of children within a population. Stunting prevalence is calculated as the proportion of children under 5 years of age with height-for-age beneath the estimated range of a reference population (i.e., <2.0 standard deviations with respect to the reference median) [58]. An incidence of stunting larger than 20% of the population point to a public health concern, according to the WHO [58,234]. However, it must be acknowledged that Zn deficiency is not the only element affecting children's growth and that growth stunting could be the result of deficiency of one or several other nutrients. Similarly, the precise measurements of length or standing height involve the use of calibrated equipment and strict commitment to standard procedures which in developing countries may often be a problem. One person should complete all of the measurements to exclude between-examiner errors [60].

In brief, the assessment of growth and stunting can only be used to determine the risk of Zn deficiency in high-risk countries and mainly for children under the age of 5 years [34,58].

2.6.6 Hair Zn Concentrations, Urinary Zn Excretion, Neurobehavioral and Cognitive Function as Biomarkers of Zn Status

Zn is a structural component of the hair matrix that is formed in the follicle [200,235]. Hair Zn is a non-invasive technique that may give an indication of Zn status. The concentrations of Zn in hair are about three times higher than in plasma or serum (199 in hair and 72 ppm in plasma) [236]. Recently, studies have provided data to support the usefulness of hair Zn concentrations in predicting Zn status of individuals [237-239].
The hair Zn method has certain advantages, such as: low cost, practicability (samples can be collected, transported and stored at room temperature), higher concentrations (compared to concentrations of Zn in blood and urine) and less variability (not affected by a recent meal, inflammation, cosmetic hair treatments, hair color) [199,240-242]. However, hair Zn concentrations are affected by age, sex, rate of hair growth and season within a year [243-245]. To summarize, the hair Zn still lacks necessary evidence towards validity and reliability as a method of assessing Zn status [60,200]. Similarly, the reference values for interpreting hair Zn concentrations need to be established [60,246-248].

Urinary Zn excretion normally plays a minor role in Zn homeostasis, with 0.3-0.7 mg (15% of daily losses) of Zn being excreted daily in the urine [27,60,249]. The consumption of a low Zn diet reduces the excretion of Zn by 96% [27]. Lowe et al. (2013) [66] evaluated the response of urinary Zn excretion to alterations in dietary intake, by comparing the data extracted from five supplementation and four depletion studies. Primary analysis revealed a noteworthy effect of Zn intake on urinary Zn excretion without an important heterogeneity, so it was concluded that urinary Zn could be a useful marker of Zn status [66]. However, the urinary Zn excretion is increased in diabetes sufferers, during starvation, after strenuous physical exercise [43,60]. Certain metabolites, such as histidine and cysteine, also increase excretion of Zn via urine [43,250]. Urinary Zn excretion is reduced during pregnancy and lactation [251,252]. Finally, the need to obtain a 24 h urine sample additionally hinders the usefulness and simplicity of urinary Zn as a biomarker.

Zn acts as a neurotransmitter playing a role in learning and memory functions during the lifecycle [253]. Supportive evidence for the use of some aspects of cognitive functions (mainly memory associated characteristics) as functional markers of Zn status have been provided [254-257], nevertheless the efficacy of neurobehavioral functions in predicting Zn status in individuals still remains to be clarified.

In conclusion, considerable research is still needed before any of these biomarkers can be employed to assess the Zn status of humans (either individuals or populations).

2.7 New/Emerging Biomarkers of Zn Status in Individuals

2.7.1 Zn Dependent Proteins, Nail Zn Concentrations, Oxidative Stress, DNA Integrity, Erythrocyte and Leukocyte Zn Concentrations, Zn Kinetics and Taste Acuity

When a certain biomarker is theoretically linked to Zn intake or Zn status, but no sufficient testing is presented to confirm this relationship, this biomarker is referred to as emerging biomarker of Zn status [200]. Five emerging biomarkers have been identified by the BOND Zn Expert Panel (**Table 2.4**). A short description of each of these potential biomarkers follows.

Zn dependent proteins are responsible for the maintenance of cellular Zn homeostasis and thus it has been hypothesized that they respond to changes in dietary Zn intake. 3000 proteins are reliant on Zn as a cofactor [35,60].

Metallothionein (Zn binding proteins) and Zn transporters (ZnT1 and Zip1) were the most commonly measured. A few other proteins have also been proposed i.e. dematin, a cytoskeletal protein, and retinol binding protein but they have not been adequately tested [258,259]. The expression of metallothionein (MT) is seen as a potential biomarker as its function is dependent on MTF-1, metal transcription factor-1, regulated by cellular Zn concentrations [260]. Its expression is shown to be reduced in Zn deficiency and increased during Zn supplementation regardless of the baseline Zn status [261-265]. Inconsistent data exists on the responsiveness of Zn transporters to Zn concentrations. Generally, it is assumed that the expression of the cellular Zn efflux transporters (ZnT1 or ZnT2) is reduced while there is an increase in the expression of the influx transporters (Zips) with a reduction in cellular Zn concentrations [259,266,267]. However, there are studies that demonstrate the opposite effect; either decreased expression of Zips during Zn supplementation or no apparent effect on Zn transporter activity [109,268]. Hennigar et al. (2016) [265] provided a systematic review related to MT and Zn transporters expression in circulating human blood cells as biomarkers of Zn status. The general conclusion based on the sixteen identified studies is that changes in Zip transporters were not consistent across studies, while MT in leukocytes was the most consistent and receptive indicator out of all investigated [265]. Leukocytes MT was sensitive to changes in dietary Zn. In contrast, plasma and tissue Zn concentrations were unresponsive to variations in Zn intake due to the tight homeostatic regulation, which confirms that changes in the function of Zn transporters and Zn binding proteins, particularly these found in leukocytes (i.e. leukocyte metallothionein), may be a more appropriate measure of Zn status [265].
More recent data show that in the absence of pre-existing Zn deficiency, no changes in the expression of Zn transporters following supplementation is observed [269]. However, if Zn deficiency is present, there seems to be an increase in Zip1 and a decrease in Zip2 and Zip3 gene expression [266,267]. Briefly, the baseline dietary Zn intake was a significant predictor of the gene expression of Zn transporters. Further research is required to precisely determine the sensitivity of Zn proteins as markers of Zn status.

With respect to nail Zn concentrations as a biomarker, this has been shown to vary from between 80-200 μg/g. The usefulness of this biomarker has been limited by the lack of an appropriate measurement technique. Recently, a laser-Induced breakdown spectroscopy has been tested as a method for measuring nail Zn concentrations [270], but additional work is required to elucidate the efficacy of this potential biomarker.

As Zn is an essential constituent of proteins involved in defence against oxidative stress and DNA damage repair, it is therefore highly likely that Zn deficiency has undesirable effects on DNA integrity. In Zn deficient individuals, Zn supplementation reduces the cellular DNA damage by increasing the number of strand breaks [264,271,272].

The pro antioxidant nature of Zn has been shown in several studies, where Zn supplementation caused a reduction in both biomarkers of oxidative stress and inflammation [273,274]. Further work is necessary to delineate the ability of inflammation and oxidative stress to predict Zn status. Additional studies are also needed to clarify the influence of other nutrient deficiencies on DNA strand formation, and to confirm that the same effect exists in various settings and across different populations [271,272].

Blood cellular Zn concentration (both the erythrocyte and leukocyte Zn concentrations) has been investigated as a biomarker of Zn status, providing mixed results and unconvincing evidence for their use as indicators of Zn status [60,275].

Zn kinetics; the rate of movement of Zn in and out of plasma, has been explored as a biomarker of Zn status [35]. The plasma Zn turnover rate is normally 150 times/day, and it increases during Zn depletion [276,277,278]. The exchangeable body Zn pool (EZP) contains 150-200 mg Zn and has a turnover rate of 12.5 days [27,28]. The size of EZP fluctuates with dietary Zn intake (it reduces during Zn deficiency), which makes it a potentially good biomarker of Zn status [60,279-281]. However, EZP does not seem to be a valid indicator of modest short-term changes in Zn intake [282]. Additionally, the impact of age and sex on the EZP mass [27,60,280] demonstrates that the biomarker needs to be evaluated further.

Zn deficiency lead to increased absorption of Cu (hypercupremia) [283]. A Cu:Zn ratio above 1.5 is suggested as a biological marker of Zn deficiency, but it also requires additional testing [283]. Finally, gustin, a protein involved in taste perception, is a Zn dependent protein. Zn deficiency may impair taste acuity [284-287], but again further research is needed to validate it as a potential biomarker of Zn status.

The BOND group highlighted the need to identify and validate Zn biomarkers to assess Zn exposure, status and functional effects at both the individual and population levels [60]. A number of 'cross-cutting' issues were identified that affect biomarker discovery, development and implementation, especially in low and middle income countries [60].
The selection of a biomarker, regardless of use, is often constrained by the setting both in terms of the environment (i.e. sanitation, temperature) and technical capability (i.e. trained technicians, requisite equipment, sample collection procedures, storage needs and facilities) [60].

The costs involved for all aspects from specimen collection and specimen transport to laboratory analysis should also be considered as it can be a limiting factor. Finally, it should be acknowledged that there are often limitations to the ability to collect specimens due to social/cultural acceptability (i.e. a lack of community acceptance, resistance to venous blood collection) [60]. All these factors should be taken into account when a Zn biomarker is selected for use within a particular setting.

In summary, several new biomarkers of Zn status have been suggested over the last few years, some of them being tested to a certain degree, however additional research is necessary before any of these biomarkers can be employed as an accompanying biomarker of Zn status.

2.7.2 A Potentially New Biomarker of Zn Status in Humans (LA:DGLA Ratio)

Zn dependent enzymes have been evaluated as biomarkers of Zn status to a certain degree [60,199], with the alkaline phosphatase [153] being the most frequently studied and demonstrating no dependable variations with dietary Zn intake or PZCs [199,275]. For many of the Zn dependent enzymes, there was insufficient, up to date research available for evaluation of their potential, so they are classified as non-useful or non-recommended biomarkers of Zn status [60,200].

Battger et al. (1979) [288] were the first to propose an association between Zn and essential fatty acids (EFAs) signifying that Zn is an essential co-factor for the metabolism of fatty acids. Similarly, it was shown that desaturase enzymes need Zn as a cofactor for proper functioning [289]. Zn is necessary for at least two stages in essential fatty acid (EFA) metabolism; the transformation of linoleic acid to γ-linolenic acid (**Figure 2.4**) and the mobilization of dihomogamma linolenic acid (DGLA) to arachidonic acid [290]. Zn has an effect on delta 6 desaturase (Δ6 desaturase) itself, and affects linoleic acid absorption [291]. Deficiencies of Zn and essential fatty acids show remarkable similarities, which include growth retardation, dermal lesions, alopecia, delayed sexual maturation, decreased rate of wound healing supressed immune response and visual development and diminished gene expression, neurotransmission and cognition [36,288,291,293].

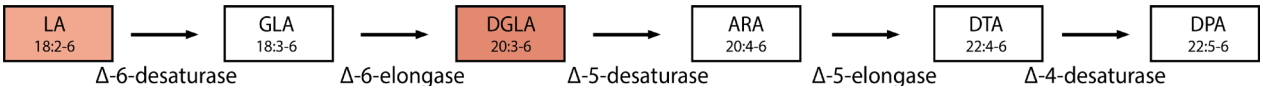

Figure 2.4 Desaturases involved in the biosynthesis of omega 6 polyunsaturated fatty acids

LA: linoleic acid; GLA: γ-linoleic acid; DGLA: dihomo-γ-linolenic acid; ARA: arachidonic acid; DTA: docosatetraenoic acid; DPA: docosapentaenoic acid. Δ6 desaturase is responsible for the formation of the carbon-carbon double bonds, and the function of an elongase is to lengthen fatty acid chains by the addition of two carbon units. LA (18:2-6) is desaturated by a Δ6 desaturase, introducing a Δ6 double bond into the substrate, giving γ-linolenic acid (GLA, 18:3-6). GLA is then elongated by a Δ6 elongase to dihomo-γ-linolenic acid (DGLA, 20:3-6). Modified from: Meesapyodsuk & Qiu (2012) [296].

Once it was recognized that Zn and essential fatty acid deficiencies give comparable symptoms, a close relationship between fatty acid metabolism and Zn status was suggested [288,290,291]. Numerous studies over the years demonstrated that Zn deficiency affects metabolism of essential fatty acids by impaired Δ6 desaturation activity [291-295]. Still, the activity of Δ6 desaturase and subsequent variation in the LA:DGLA ratio, as a biomarker of Zn status, was not investigated widely.

The concept of an essential role of Zn for Δ6 desaturase functioning was rediscovered in 2014, by Reed et al.[297] when the authors using the *Gallus gallus* (chicken) as a model demonstrated that the LA:DGLA ratio is able to differentiate Zn status between Zn adequate and Zn deficient subjects and that a change in the LA:DGLA ratio may be a sensitive biomarker of Zn status [297].
The initial findings for the development and application of the LA:DGLA ratio as a biomarker of Zn status looked promising. However, studies that test the usefulness of the newly proposed biomarker in humans are missing. If the LA:DGLA ratio is going to be used as a potential biomarker of Zn status in humans, its efficacy should be somehow validated within the human study population. Similarly, there is no up to date research data available to show how the biomarker operates when a typical food (diet consumed by people in Zn deficient populations) rather than a supplement is used, which indeed requires further research.

A sufficiently sensitive biomarker is needed to correctly evaluate the dietary Zn intake in regards to biochemical status, and correspondingly to accurately define Zn inadequacy as a public health problem. Finally, a discovery of a more suitable biomarker for assessing Zn status (a goal of this PhD project) would help in the development of targeted nutritional programs that could address Zn deficiency appropriately.

Note: With the intention of keeping this review succinct the material related to the chemical structure, regulation and function of the LA:DGLA ratio and Δ6 desaturase is not repeated here as it is comprehensively described within Chapter 6.

2.8. Overview of Zn Deficiency, Zn Biofortification and its Effectiveness

2.8.1 The Magnitude and Clinical Manifestations of Zn Deficiency

In humans, Zn deficiency was first recognised in early 1960s by Prasad and colleagues [298]. Initially, it was described in an adolescent male in Iran, and later on observed in Egypt [137,299]. A few years later, deficiency was documented among young children in the United States [300,301].

The historical aspect of Zn discovery as a vital element for humans and its pronounced impact on health and disease was nicely summarized by Prasad et al. (2013) [142]. Similarly, the circumstances leading to the discovery of human Zn insufficiency in the Middle East were presented by Sandstom et al. (2013) [302]. Although, it has long been disregarded as a 'global health problem', today Zn deficiency is acknowledged as one of the most severe problems of human malnutrition worldwide [6,303]. It is a public health challenge of global proportions [79,211]. As stated by the WHO, Zn deficiency ranks as the fifth most important health risk factors in developing countries and eleventh worldwide [6,304].

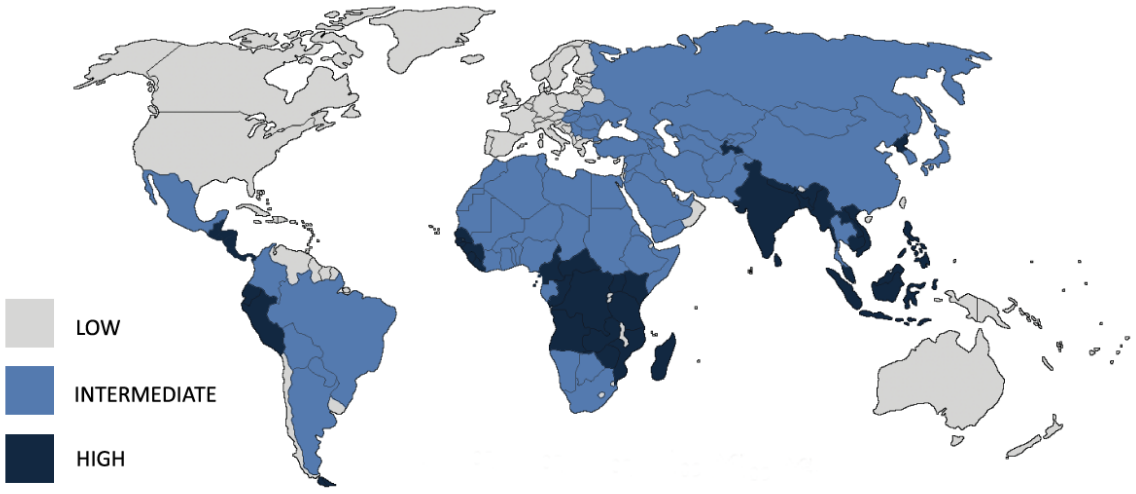

Figure 2.5 Prevalence of Zn deficiency in developing countries
Adjusted from Welch & Graham (1999) [308] and Wessells & Brown (2012) [208].

The magnitude of Zn deficiency is currently estimated based on: National Stunting Prevalence Data (documented in the WHO Global Database on Child Growth and Malnutrition and UNICEF's Annual Report on the State of the World's Children (SWOC)) [304,305]; on the information of Zn amounts presented in national food balance supplies derived from the Food and Agricultural Organization of the United Nations' food balance sheets (FBSs) [100], and finally the prevalence of Zn inadequacy is based on the evaluation of PZCs from national surveys. In view of all these analyses, it is estimated that Zn deficiency affects around 17%-20% of the global human population [79].

The populations at the highest risk of Zn deficiency are concentrated in South and South East Asia, Sub-Saharan Africa, Central America, and the Andean region (**Figure 2.5**) where the diets are mostly plant based and the intake of animal source foods is low. Additionally, in recent years, Zn deficiency becomes progressively more and more apparent in developed countries [306,307].

Table 2.7 Recommended cut-offs of plasma Zn concentrations for evaluating the risk of Zn deficiency by sex, age group and fasting status

Fasting status and time of day	Plasma Zn concentration, μg/dL		
	Children < 10 y	Females ≥ 10 y	Males ≥ 10 y
Morning, fasting		70	74
Morning, non-fasting	65	66	70
Afternoon	57	59	61

Modified from Hotz et al. (2004) [51]; Wieringa et al. (2015) [220] & Wessells et al. (2014) [317].

Zn deficiency generally exists due to one or more reasons: insufficient Zn intake, interference of other dietary factors with the absorption and bioavailability of dietary Zn, enlarged losses of Zn, reduced utilization, and increased requirements for Zn during physiological conditions, such as periods of rapid growth, pregnancy and lactation [37,309,310]. Currently used cut-offs of PZCs for assessing the risk of Zn deficiency are presented in **Table 2.7.**

Clinical manifestations of Zn insufficiency are nonspecific, differ extensively and depend on the severity of deficiency. The clinical features of severe Zn deficiency in humans are growth retardation, skin lesions, diarrhoea, dermatitis, alopecia, pneumonia, delayed sexual and bone maturation, impaired appetite, defects in the immune system, delayed wound healing, increased vulnerability to infections and the appearance of behavioural changes [218,311-314]. Zn deficiency has also been shown to be associated with sepsis [313,315] and may be a risk factor for developing asthma [316]. Impaired growth, child morbidity and mortality and preterm births are manifestations most commonly seen in populations with inadequate Zn intake in low income countries [60,310].

The variable estimates for Zn attributable mortality oscillate from 97,330 in the Global Burden of Disease Study 2010 [59], to 116,000 in the Lancet 2013 Maternal and Child Nutrition series [318], to 453,207 in a previous review [312]. An excessively high number of avertable childhood deaths is caused by Zn deficiency [60]. Poor Zn nutrition is associated with >50% of diarrhoea deaths [18], 10% of malaria and 7% of pneumonia deaths [312].

The consequences of marginal or mild Zn deficiency are less clear. The most vulnerable groups are prematurely born babies, infants and young children, particularly those 6-23 months of age. Mild Zn deficiency is frequently seen in healthy elderly subjects, contributing to impaired cell-mediated immune responses [319,320]. Moderate Zn deficiency can lead to hypogonadism, delayed puberty, reduced appetite, mental lethargy, hyperammonemia, dermatitis, cell mediated immune dysfunction, delayed wound healing and abnormal neurosensory changes [225,321].

Zn deficiency has been linked to certain health diseases in developed countries: cancer, diabetes, depression, multiple sclerosis, coronary heart disease [226,322-325]. Childhood obesity, insulin resistance, metabolic syndrome and atherosclerosis are also associated with Zn inadequacy [326-328]. Numerous clinical risk factors and pathologies related to Zn deficiency have been comprehensively described by Mocchegiani et al. (2000) [19] and Roohani et al. (2013) [34].

2.8.2. Biofortification: To Improve Staple Food Crops with Zn

Since there is no efficient reserve or body store for Zn, an appropriate consumption of dietary Zn is needed on a regular basis [303]. Staple diets in low income countries are primarily plant based. The use of animal protein foods that contain a higher level of Zn; for instance, red meat, fish and poultry, is often small for various reasons, including economic, cultural, or religious restrictions [201]. Consequently, the amount of bioavailable Zn from such diets is low and often the main source of Zn insufficiency [126]. Severe Zn deficiency is seen in people whose diets are mainly based on cereal grains produced on Zn-deficient soils, for instance in India, Pakistan, China, Iran and Turkey [51,329].

This relationship of poverty with micronutrient malnutrition advocates that it is not simple to accomplish adequate intake of Zn by dietary modifications (e.g. more fish, poultry and meat consumption), which would in theory provide an answer [330]. Furthermore, changes in dietary habits require primarily the availability of alternative foods at affordable prices, as well as individual and society acceptance. Alternatives to this approach are supplementation by oral provision, i.e. Zn salts in the form of tablets; or the fortification of staple food such as flours through the addition of Zn [331]. Supplementation with pharmaceutical Zn preparations can be efficient in alleviation of Zn deficiency on an individual basis, nevertheless this strategy is often shown as unsuccessful on a population level in developing countries, due to the absence of suitable infrastructure and education [332-334]. Similarly, food fortification can be implemented promptly at a national level without personal contact to and change of delivery habits by consumers, but its successful application into society involves existence of safe delivery systems, steady policies, suitable social infrastructures and constant financial support [33]. All these strategies have limited success in developing countries as they were often difficult to sustain or were too expensive [331,335].

Taking all these aspects into account, biofortification, i.e. the development of crop plants with greater levels of bioavailable Zn, is seen as the most manageable method for developing countries as it does not involve changes in customary diets and has the capacity to reach rural families with very restricted access to infrastructure [331,336,337]. Besides, it may provide a significant increase in plant growth and the extra benefit of considerable yield increases on Zn deprived soils [331,333,338].

Increased bioavailability of Zn in plant foods (i.e. wheat) can be accomplished by plant breeding or genetic engineering approaches that either increase the concentration of Zn, reduce the content of inhibitors (primarily phytate) or increase the expression of compounds that augment Zn absorption (i.e. amino acids) [339,340,341]. Zn biofortification through Zn application is generally suggested to increase grain Zn concentration [333,334,342,343] and Zn bioavailability [344]. This is termed, agronomic biofortification and this is achieved by application of minerals to the soil or by foliar application of fertilizers directly to the leaves of the plants. Foliar Zn application was shown to be effective in improving both Zn concentration and bioavailability of Zn in wheat grains without changing the phytic acid concentrations [335,343,345,346].
The HarvestPlus Fertilizer and HarvestZinc project (www.harvestzink.org) established that foliar application of Zn fertilizers to wheat can increase grain Zn concentration by 28%-68% [330,347,348]. Target Zn concentrations set by HarvestPlus program are 38 ppm of Zn in wheat [331]. The success of agronomic biofortification has been already reported for many wheat varieties [338,349,350].

The agronomic biofortification strategy, breeding nutrient rich staple food crops, is anticipated to be of special benefit to poor rural populations considerably affected by dietary Zn deficiency [351-354]. Economic analyses demonstrate that biofortification is the most concrete, cost effective and durable strategy for increasing dietary Zn intake of vulnerable populations [201,335,336,355]. Certain economic analyses propose that genetic approaches towards biofortification are more cost effective than dietary diversification, supplementation or food fortification programs [332,356-358]. More lately, the likely influence of Zn biofortification has been calculated as the saving of disability-adjusted life years [359]. The annual burden of Zn deficiency in India was shown to be 2.8 million lost DALYs and it was predicted that Zn biofortification of rice and wheat could cut this burden by 20%–51% [357].

In conclusion, improvement of Zn concentrations of crop plants via conventional breeding and genetic engineering procedures represents the core biofortification strategy. Zn biofortification of wheat grain by genetic and agronomic approaches is commonly suggested to resolve Zn deficiency problem in people dependent on wheat as a main food source [330,331]. Relevant actions have been organized in the past ten years, many of them being initiated by the HarvestPlus program.
Finally, biofortification strategies based on crop breeding and application of Zn fertilizers have a great potential to alleviate Zn malnutrition in humans. Additional research is certainly needed to assess various variables that will determine the ultimate success of biofortification of staple crops with Zn.

2.8.3. The Effectiveness of Zn Biofortified Wheat Grains on Zn Availability and Zn Absorption in Animals and Humans: Current Knowledge

As previously mentioned, methods to increase dietary diversification, mineral supplementation and food fortification have not always been effective in alleviating the problem of Zn deficiency in developing countries. The biofortification of crops by either plant breeding or by using mineral fertilizers has been suggested as a potential strategy that could produce a solution to the Zn deficiency problem [330,360]. It is crucial that biofortification strategies focus on the staple food that dominates people diets (i.e. wheat and rice which are staple food for almost a half of the world's population) [332,333,349].

In developing countries, a minimum of 60% of Zn in human diets is derived from grain and legumes [361]. Wheat is one of the three major cereal crops worldwide [332,349,362]. Wheat grains are major source of calorie and mineral intakes in many developing countries of the world [76,100,340]. Global wheat production exceeds 720 million tons per year, most of it being used as food for humans [76,100,362]. The concentration of Zn in wheat plants is low; 20-35 mg/kg of whole grain, due mainly to the low content of Zn of the soils where wheat is grown [334,343]. More than 40% wheat plants are grown on soils with low levels of Zn [329]. In addition, a significant amount of Zn in grain is lost during the wheat processing technique (i.e. removal of aleurone layer and embryo during milling) [363].

The concentration of Zn in refined wheat flour is less than 15 mg/kg [364]. However, the percentage of Zn retained in flour after milling is anticipated to be 60% [365]. Furthermore, besides being naturally low in Zn, wheat is rich in phytic acid which is known to limit Zn bioavailability to a great extent [308,346]. The Zn concentration in wheat grain needed to prevent Zn deficiency in humans is estimated to be 45 mg/kg [346,359]. A daily net absorption of approximately 3 mg of Zn from 300 mg of wheat flour is necessary for human health [58,352].

Theoretical studies indicate that biofortification strategy of staple crops would increase the delivery of Zn to human diets and improve nutritional status of vulnerable populations is developing countries [332]. The HarvestPlus group has recommended a target for added Zn in biofortified crops of 30% of the estimated average dietary requirements for humans [349,366]. Wheat varieties with enhanced Zn content have been produced, but before they can be introduced into the food supply, their efficiency in improving Zn status of consumers needs to be confirmed.

Over the years, both in vitro and in vivo methods (animal and humans studies) have been used to assess the efficacy of biofortified crops in improving nutritional status of consumers.
In in vitro studies (mainly using Caco-2 cells) are suitable for preliminary screening of biofortified wheat plants but these studies do not provide data that are necessarily applicable to humans. Similarly, a protein that can be used as an indicator of Zn uptake and is specific for Zn only is still missing [367] making this approach unsuitable for merely assessing Zn absorption. Human studies certainly provide the most valuable results, as they are capable of investigating host factors and physiological changes during digestion. However, they are time consuming and very expensive [368].
An alternative approach to in vitro and human studies are studies in animal models, that can provide a whole body assessment of absorption as dissection of individual tissue parts is possible [368-370]. In recent years, the chicken (*Gallus gallus*) model has often been used for evaluating the effectiveness of biofortified foods crops [297,371,372]. The *Gallus gallus* model has been shown as appropriate for Zn related studies [373-375]. Lately, a good correlation between the results obtained though this animal model and via human efficacy trials was confirmed, additionally proving the suitability of the model in examining mineral bioavailability [372].
The faster output, the ability to assess an extensive range of physiological and molecular parameters thoroughly, and the cost effectiveness are features that make the use of animal models appealing for testing dietary Zn bioavailability of staple crops.

The usefulness of biofortified wheat products to improve Zn nutritional status of subjects has rarely been tested. Welch et al. (2005) [351] were the first to demonstrate the beneficial effect of Zn biofortified wheat in improving Zn status of rats. The wheat genotypes with increased grain Zn concentrations provided increased amounts of bioavailable Zn to rats, supporting the idea that breeding for Zn enhanced wheat grain may contribute to decreasing Zn deficiency in target human populations [351].
In a 2009 human trial, Zn absorption from biofortified versus conventional wheat as 95% and 80% extraction flours was compared [352]. Adult women were given 300 g of the high or low extraction flours as tortillas for two consecutive days using either biofortified (41 mg Zn/g) or control (24 mg Zn/g) wheat. Zn intake from the biofortified wheat meals was 5.7 mg/day (72%) higher at 95% extraction ($p<0.001$) and 2.7 mg/day (68%) higher at 80% extraction compared with the corresponding control wheat ($p=0.007$). The absorption of Zn from the Zn bioforitfied wheat remained significantly higher than that from the control wheat.

Mean total Zn absorption from biofortified wheat was 2.1+0.7 mg/day and 2.0+0.4 mg/day for 95% and 80% extraction, respectively, 0.5 mg/day higher than for the control wheat ($p<0.05$) [352]. The higher absorption was maintained with moderate extraction of the grain, even though substantial quantities of Zn were lost with extraction (80%), which indicates that benefits of Zn biofortified wheat are not lost with a moderate degree of milling. Zn absorption is greater from biofortified wheat, than from typical wheat with lower Zn concentration, from the same quantities of each type of wheat flour consumed [352].
Carlson et al. (2012) [376] investigated the bioavailability of three wheat varieties using a pig as a model by collecting urine and faeces samples for seven days. The soluble Zn content of the three wheat samples was 9.9, 12.8 and 21.7 mg/kg. The Zn excretion in urine of pigs was very low (below 1 mg/day) for all treatments. The daily Zn intake differed between all dietary groups ($p<0.001$) with the highest intake in pigs fed the high Zn diet (57.5 mg/day) and the lowest intake in pigs fed the low Zn diet (7.3 mg/day). Consequently, the net Zn absorption in milligrams per day differed ($p<0.001$) between the dietary groups.

The measurement of Zn absorption from biofortified wheat varieties is a crucial first step in demonstrating the efficacy of these products in improving Zn status of consumers. Limited available evidence show that new biofortified varieties of wheat may be useful in improving Zn status of individuals. Nevertheless, it is still required to precisely determine the bioavailability of added Zn in wheat plants and to explore if the additional Zn is at least equally absorbable as the native Zn content, and if Zn enriched wheat varieties can be used to successfully improve Zn status of Zn deficient people in developing countries dependent on wheat as a basic food source. Finally, an appropriate assessment of Zn bioavailability and absorption from Zn biofortified wheat is fundamental in estimating the efficacy of wheat related breeding programs.

In conclusion, Zn deficiency is an important malnutrition public health problem worldwide with numerous negative impacts on human health, lifespan and productivity. Number of people affected by dietary Zn deficiency increased over recent years both in developed and developing countries due to the poor quality of people's diets. Biofortification is a strategy that can improve human nutrition on a global scale. Agronomic biofortification of staple plant foods is regarded as highly effective strategy for increasing dietary Zn intake. Zn biofortified crops are a feasible tool for addressing nutritional deficiencies, and understanding their efficiency in improving Zn status of consumers is fundamental and needs to be verified.

2.9 References

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You can trace every sickness, every disease, and every aliment to a mineral deficiency.

Dr. Linus Pauling

two-time Nobel Prize winner

Section 2 | The Zn and Fe Interaction

Preamble

The general idea for this PhD project emerged based on several well-known and well documented statements about Zn deficiency in relation to another major micronutrient inadequacy, Fe deficiency. As extensively described in the previous section:

- *Zn deficiency has been identified as a priority in public health on the World Health Organization website back in 2001. Nowadays, it is still a major nutritional problem estimated to affect approximately 17% of the total world's population.*
- *Iron (Fe) deficiency is recognized as the most common and widespread nutritional inadequacy, affecting approximately around 30% of people worldwide.*
- *Soil scientists and agronomists consistently reported that Zn-deficient soils are widespread, with about half of the major agriculturally productive soil types being Zn deficient. On the contrary, only 3% of the soils were Fe deficient.*
- *Fe deficiency persists despite the abundant presence of soils rich in Fe.*
- *Multiple micronutrient deficiencies were common and occur simultaneously.*
- *It was also noted that Fe supplementation/fortification on its own, was not always effective in the alleviation of Fe deficiency, so, it was assumed, that something other than Fe may be responsible for Fe deficiency and Fe deficiency anemia.*

Certain questions have logically appeared: Do we know the exact extent of Zn deficiency and potential consequences of inadequate Zn intakes? To what degree is Zn deficiency responsible for the development of the other major micronutrient deficiencies? Can this current magnitude of anemia and nutritional Fe deficiency be explained by insufficient dietary Zn intakes? Does Zn really have a role to play in the Fe absorption process? If so, what would be the mechanism of that interaction?

The scientific evidence collected over the last decade assisted in answering some of the queries. It was confirmed that micronutrient deficiencies occur concurrently and that Zn deficiency is very often accompanied by deficiencies of other micronutrients, i.e. vitamin A and Fe. Similarly, Zn deficiency was acknowledged as possibly responsible for insufficiencies of other major micronutrients, Fe in particular.

There were also some propositions made that a significant proportion of Fe deficiency anemia in humans may be due to an underlying Zn deficiency. However, the mechanism of the link between Zn and Fe absorption processes, that could explain the potential interaction, hasn't been elucidated. Consequently, that became an initial goal of this PhD research project, to enlighten the interaction between Zn and Fe and to propose mechanisms by which cellular Zn concentrations control Fe absorption processes.

An extensive review of the literature has been performed to identify all relevant information related to possible mechanism of this interaction. By combining and summarizing the available evidence it was demonstrated that Zn has an important role in the Fe absorption process. The structure, role and function of major Zn and Fe transport proteins have been described through this work. However, the major attainment of this effort was the proposition of the potential mechanism by which cellular Zn concentrations regulate Fe absorption processes, both at the systemic and local level. The entire mechanism is explained in **Chapter 3** *as a publication: Knez, M.; Graham, R.D.; Welch, R.M.; Stangoulis, J.C.R. New perspectives on the regulation of iron absorption via cellular zinc concentrations in humans, Critical Reviews in Food Science and Nutrition, 2015, 57 (10), 2128-2143, DOI: 10.1080/10408398.2015.1050483.*

The next step in the research process was an exploration of the relationship among Zn and Fe intake and status in humans, both in apparently healthy populations, as well as, in Fe and/or Zn deficient cohorts. Although, a number of studies have already demonstrated that the link exists, there was an aspiration to provide further, more current evidence, to this research field. The intention was to investigate the link between Fe and Zn intake and status in an apparently healthy population, but, in order to additionally increase the power of this work, that needed to be a population for which more recent data on the intake and status of these minerals remained limited.*

An opportunity for this arrived with a cross sectional study that was run in Serbia, Europe. Serbia was a suitable setting as no large proportions of people affected by Fe and Zn deficiencies are expected to be seen. If deficiencies existed, they were anticipated to be marginal. In this context, Serbia was representative of any other industrialized country (America, Australia or other European countries).

* Apparently Healthy refers to the absence of disease based on clinical signs and symptoms and function, normally assessed by routine laboratory methods and physical evaluation.

In addition, the study population also fulfilled our second criterion; the last time Zn and Fe status was measured in a Serbian population was in 1995, more than 20 years ago, so an update on the intake and status of these essential nutrients was undoubtedly needed. 754 healthy adults (25-65 years of age) living in Serbia were included in the study. The goal was to examine the relationship between dietary Zn and Fe intake and related biochemical parameters. Furthermore, the study allowed attainment of information on the influence of food choices and socio-economic factors on Fe and Zn dietary intake and status with an aim to identify groups at risk of dietary Zn and Fe deficiency and to suggest factors that may influence the status of these nutrients.
The findings of this work are presented in **Chapter 4,** *as a publication: Knez, M.; Nikolic, M.; Zekovic, M.; Stangoulis, J.; Gurinovic, M.; Glibetic, M. The influence of food consumption and socio-economic factors on the relationship between zinc and iron intake and status in a healthy population. Public Health Nutrition, 2017, 20 (14), 2486-2498. DOI:10.1017/S1368980017001240.*

In addition, there were efforts made to study the Zn and Fe interrelation in a Zn deficient human population. India was a perfect setting as there were clinical studies with Zn deficient people running at the time.
While, certain collaborations have been developed in order to obtain the samples for Zn and Fe analyses, the very strict rules on the handling and export of blood samples from India restricted import of samples to Australia and implementation of necessary analyses during the time of this research project.
Finally, additional efforts have been made to help in obtaining necessary financial support to allow further investigation of the role of Zn in intestinal Fe absorption, using an animal model (i.e. mice). Unfortunately, the resources were not accessible and the study hasn't been implemented during this PhD project.

Chapter 3

New Perspectives on the Regulation of Iron Absorption via Cellular Zinc Concentrations in Humans

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DOI: 10.1080/10408398.2015.1050483.

3.1 Abstract

Iron deficiency is the most prevalent nutritional deficiency, affecting more than 30% of the total world's population. It is a major public health problem in many countries around the world. Over the years various methods have been used with an effort to try and control Fe deficiency anemia. However, there has only been a marginal reduction in the global prevalence of anemia. Why is this so?

Fe and Zn are essential trace elements for humans. These metals influence the transport and absorption of one another across the enterocytes and hepatocytes, due to similar ionic properties. This paper describes the structure and roles of major Fe and Zn transport proteins, clarifies Fe and Zn interactions at these sites, and provides a model for the mechanism of these interactions both at the local and systemic level. This review provides evidence that much of the massive extent of Fe deficiency anemia in the world may be due to an underlying deficiency of Zn. It explains the reasons for predominance of cellular Zn status in determination of Fe and Zn interactions and for the first time thoroughly explains mechanisms by which Zn brings about these changes.

Keywords: Iron, zinc, Fe & Zn interaction, hepcidin, Fe transporters, Zn transporters

3.2 Introduction

Iron (Fe) and zinc (Zn) are essential trace elements for humans. Together, they play a major role in a wide variety of cellular functions, some of which are proliferation and differentiation, development and maintenance, oxidative metabolism, etc. Systemic Fe and Zn homeostasis is based on tightly coordinated processes and effective communication between the key sites of Fe and Zn uptake, utilization and storage. The enterocytes and the hepatocytes perform the most important roles in homeostasis. Considering the similarities in ionic properties between divalent ferrous Fe and divalent Zn, it is not unexpected that these trace metals influence the transport and absorption of one another across the enterocytes and hepatocytes.

For a long time, Fe and Zn absorption has been seen as competitive, where Zn had inhibitory effects on Fe absorption and vice versa [1]. However, while high doses of soluble forms of Fe and Zn are capable to produce this negative interaction, the probability of antagonistic interactions appears to be much lower when Zn and Fe intake are closer to 'physiological' concentrations and when they are provided with food [2-4]. The summary review of Fischer-Walker et al. (2005) [5] was one of the first reviews on this topic that provided considerable support for noncompetitive absorption between Fe and Zn. Over the years, more and more studies demonstrated evidence suggestive of positive interactions between Fe and Zn in absorption [6-10]. Additionally, a number of data sets have clearly showed a positive correlation between anemia and signs of the risk of Zn deficiency in adult males, children, and pregnant women [11-13]. Zn is shown to be a strong predictor of hemoglobin concentrations [6,14,15]. Moreover, Fe supplementation, by itself, was not always effective in treatment of anemia[16-19]. Positive effect of provision of Zn in addition to Fe has been proven by Kolsteren et al. (1999) [20], Nishiyama et al. (1996) [21], Nishiyama et al. (1999) [22], Alarcon et al. (2005) [23] and many others [15,24-26].

Although, there is more and more evidence accumulating showing the positive link between Fe and Zn and a strong positive influence of Zn on Fe absorption and Fe status, there is still a lack of information on the precise mechanisms of Zn involvement in the Fe absorption processes. Therefore, this paper presents a comprehensive overview on the structure and roles of major Fe and Zn transport proteins aimed at clarifying Fe and Zn interactions at these sites, and providing a model of potential mechanism of Zn impact on Fe absorption processes both at the local and systemic level.

3.3 Fe and Zn Transporters in the Human Body

3.3.1 Fe Transporters in Human Enterocytes and Hepatocytes

Fe is essential for every living organism. It is a key component of oxygen-carrying proteins, has a pivotal role in cellular metabolism and is essential to cell growth and differentiation. Intestinal Fe absorption involves a series of proteins including duodenal cytochrome b reductase (Dcytb), divalent metal transporter (DMT1), hephaestin (Heph), and ferroportin 1 (FPN1) (**Figure 3.1**).

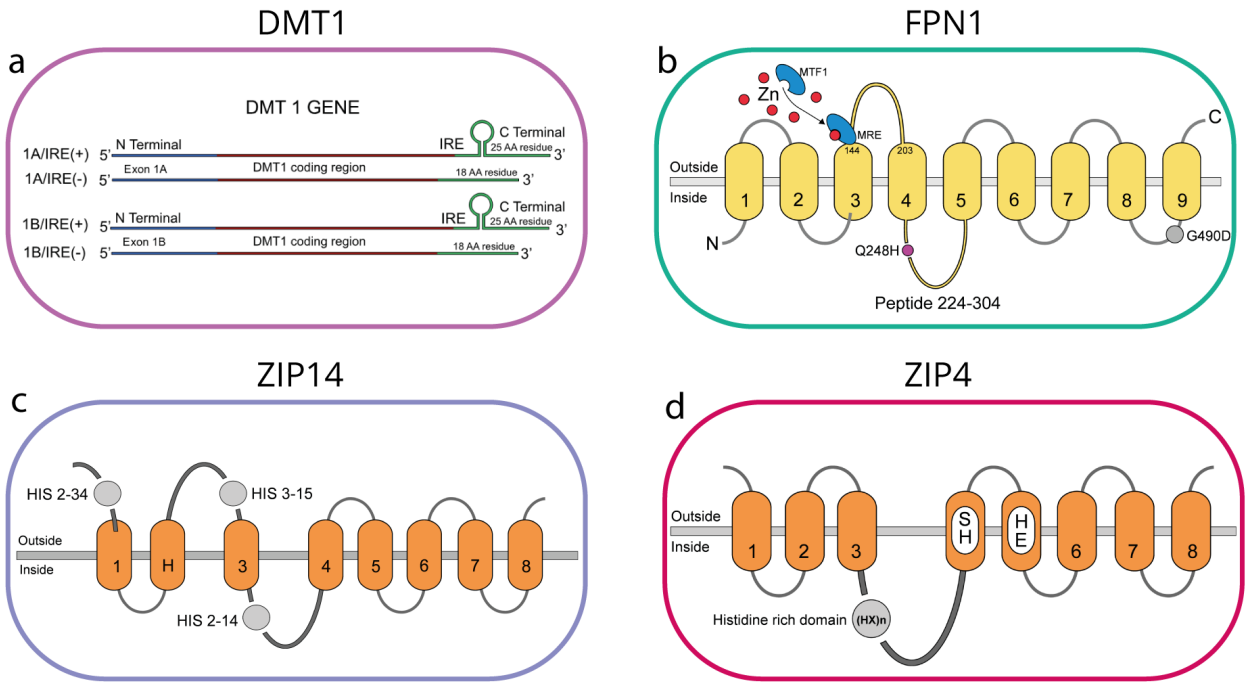

Figure 3.1 DMT1, FPN1, Zip4 and Zip14 gene

a. Four DMT1 isoforms that differ in their N- and C-termini arise from mRNA transcripts that vary both at their 5'-ends (exon 1A or 1B) and at their 3'- ends giving rise to mRNAs containing (+) or lacking (−) the 3'- IRE (iron-responsive element). The exon 1A/IRE (+) isoform is the predominant form in duodenal enterocytes. **b.** C terminal oriented towards extracellular medium. The extracellular loop 144-203 is a possible hepcidin bind The Zip4 gene has eight trans-membrane domains. **c.** Zip14 contains three histidine-rich repeats and six to eight trans-membrane domains. **d.** A histidine rich region within the large intracellular loop between putative transmembrane domains 3 and 4 plays a role in the response of Zip4 to Zn by regulating endocytosis and ubiquitination. Zn induces FPN1 transcription through the action of MTF1.

3.3.1.1 Divalent Metal Transporter 1 (DMT1) - Fe Import Protein

DMT1 (Divalent Metal Transporter 1), also known as Nramp2 (natural resistance-associated macrophage protein) or Dct1 (divalent cation transporter), is a proton-coupled metal-ion transporter that is commonly expressed in enterocytes. DMT1 is a 12-transmembrane domain protein that transports several divalent metals but operates mostly as a cotransporter of protons and ferrous Fe in the proximal duodenum.

DMT1 is encoded by the mammalian *Solute carrier family 11, member 2* gene (SLC11A2) which gives rise to 4 variant messenger RNA (mRNA) transcripts that differ in their tissue distribution and regulation 1A/+IRE, 1A/-IRE, 2/+IRE and 2/-IRE (Figure 1) [27]. All four isoforms of human DMT1 function as metal-ion transporters of equivalent efficiency [28]. They all transport Fe^{2+} at the same turnover rate and exhibit no differences in their functional properties, permeate ions or rate limiting steps. Similarly, N and C-terminal sequence variations among the DMT1 isoforms do not alter DMT1 functional properties [27]. Therefore, all of the hDMT1 isoforms are equally efficient in terms of the rate at which they transport Fe. It is interesting that although Zn^{2+} is a ligand for both the 1A and 1B isoforms of hDMT1, it is only poorly transported, if at all, relative to Fe^{2+} forms [27]. DMT1 forms the most stable substance binding with Fe^{2+}. The historic belief that Fe and Zn compete for absorption at the DMT1 has been proven false. A large number of studies over the years confirmed that Zn^{2+} is not transported by DMT1. More importantly, Zn has been reported to be a chief element in the regulation of DMT1 gene expression (**Table 3.1**).

One explanation for the way Zn affects DMT1 expression is that the promoter region of DMT1 contains several metal response elements suggesting that Zn exposure can positively regulate DMT1 mRNA level via metal transcription factor-1 activation [29] or perhaps through other Zn-dependent transcription factors, such as peroxisome proliferator activated receptor-g, nuclear factor-kB, or activator protein-1 [30].

Table 3.1 A summary of studies (published between 2000 and 2014) showing that Zn is not transported via DMT1 (Divalent Metal Transporter 1) and that intracellular Zn concentrations regulate the expression of DMT1 transporter.

Authors	Material used	Findings
Bishop et al. (2010)	Astrocytes	Zn^{2+} is not transported through DMT1
Deng et al. (2009)	Epithelial cells	Fe and Zn can enhance the uptake of each other
Espinoza et al. (2012)	Caco-2 cells	Zn is not transported by DMT1
Garrick et al. (2006)	HEK 293 cells	DMT1 has very low activity for Zn (almost no activity)
Iyengar et al. (2009)	Caco-2 cells	Zn does not inhibit transport of Fe, and is not transported via DMT1
Iyengar et al. (2012)	Caco-2 cells	DMT1 is not a site of Fe and Zn interaction, DMT1 expression increases with Zn supplementation
Kordas and Stoltzfus (2004)	Neural tissue	DMT1 is not a site of Zn absorption
Leong et al. (2003)	Rat pups	DMT1 changes in accordance to intestinal Zn concentrations
Lopez de Romana et al. (2003)	Caco-2 cells	Zn is not absorbed via DMT1
Mackenzie et al. (2007)	Xenopus laevis	DMT1 does not mediate Zn transport
Olivares et al. (2012)	A review of studies	No negative effect of Zn on Fe absorption
Rashed Abd (2011)	Caco-2 cells	Zn significantly increases DMT1 expression
Sacher et al. (2001)	Caco-2 cells	Km and Vmax of Fe and Zn uptake are very different, no competition for a single transporter
Sacher et al. (2004)	Xenopus laevis	DMT1 does not transport Zn
Tallkvist et al. (2000)	Caco-2 cells	DMT1 is not regulated by intestinal Fe concentrations
Tandy et al. (2000)	Caco-2 cells	Zn does not inhibit transport of Fe and is not transported by DMT1
Wang et al. (2005)	Epithelial cells	DMT1 mRNA increases with Zn exposure
Yamaji et al. (2001)	Caco-2 cells	Zn increases DMT1 expression and Fe^{2+} uptake

3.3.1.2 Ferroportin (FPN1) - Fe Export Protein

Ferroportin (FPN1), also known as solute carrier family 40 member 1 (SLC40A1) or iron-regulated transporter (IREG1) is a multipass transmembrane protein composed of 571 amino acid residue found on the basolateral membrane of enterocytes [31]. It is present in various forms from 9-12 transmembrane domains [32], with the N-terminus present inside the cells (**Figure 3.1**).

It is the only known mammalian Fe export protein that is responsible for the movement of Fe from the enterocytes into the circulation. Deletion of FPN1 in intestinal cells in mice results in a near complete block of intestinal Fe absorption and a consequent accumulation of Fe in intestinal enterocytes [33]. FPN1 transports Fe in the ferrous form, while plasma transferrin (Tf) only binds Fe in the ferric form, so ferroxidases (hephaestin) are needed to oxidize ferrous Fe to ferric Fe for transport by FPN1. Without the activity of a hephaestin, FPN1 is internalized and degraded [34].

The regulation of FPN1 expressionis still incompletely understood, but is believed to be mainly controlled at the posttranslational level, by hepcidin-mediated internalization and degradation. Following hepcidin binding to FPN1, FPN1 is internalized, phosphorylated and subsequently degraded by lysosomes [35-37]. When hepcidin concentration is low, the rate of FPN1 synthesis is greater than its degradation, resulting in higher amountsof FPN1 at the cell surface, thus increasing Fe export. In contrast, high hepcidin levels lead to FPN1 degradation and consequently to Fe retention [37]. However, FPN1 can also be regulated by other mechanisms [38], via translational regulation by the iron-regulatory element/iron-regulatory protein (IRE/IRP) system [39-41] and transcriptional regulation in the duodenal mucosa and macrophages [35,42].

Importantly, over the years, it has been shown that FPN1 levels are increased when cells are exposed to Zn [43-48]. Zn induces FPN1 transcription through the action of Metal Transcription Factor 1 (MTF1). In 2010, Troadec and colleagues [49] showed that Zn leads to MTF1 binding to the FPN1 promoter, while Fe does not. Recently, hypoxia inducible factor HIF2α has been shown to regulate FPN1 expression in intestinal cells [50]. HIF2α was responsible for adaptive increase of intestinal FPN1 during Fe deficiency in mice [50].

3.3.1.3 Dyodenal Cytochrome b (Dcytb) and Hephaestin (Hp)

Duodenal cytochrome b (Dcytb) is a plasma membrane protein localized predominantly on the duodenal brush-border membrane. It has ferric reductase activity and plays a physiological role in dietary Fe absorption. Dcytb is a member of the cytochrome b561 family, made of 286 amino acids with six trans-membrane domains. Dcytb mRNA and protein are believed to be rapidly induced in response to Fe deficiency and hypoxia, indicating a key role of this molecule in Fe metabolism [41].

Hephaestin (Hp) is a transmembrane bound ceruloplasmin homologue that functions as ferroxidase [35]. Hp ferroxidase activity is necessary for effective release of Fe following transport through the basolateral membrane by the Fe transporter FPN1.

There are findings which demonstrate that the duodenal ferroreductase Dcytb and ferroxidase Hp mRNA expression are not significantly altered by variations in Fe homeostasis and are not affected by Fe status [51-54]. Similarly, it has been demonstrated that Dcytb is most highly upregulated by hypoxia in the duodenum, through the activity of HIF2 [55,56]. It is still not completely clarified whether or not duodenal ferric reductase and oxidase mRNA expression have a pivotal role to play in Fe metabolism [57,58]. In addition, it has been demonstrated that hephaestin protein stability is sensitively regulated through polyubiquitination and proteosomal degradation, a process that is prompted by Zn exposure [59,60]. Increased hephaestin levels are demonstrated in Zn supplemented pups after weaning [45]. Although, there is still no conclusive evidence about the effect of Zn on Dcytb or hephaestin activity some initial findings show that Zn may have a role in determining stability and function of these proteins.

3.3.2 Zn Transporters in Human Enterocytes and Hepatocytes

Zinc (Zn) is an important trace element for many biological functions. It is present in over 300 enzymes as a catalytic metal and in at least 3000 proteins as a structural metal [61]. Zn is also an important regulator of enzyme activity; acting as an activator or inhibitor ion. These pleiotropic actions require very tight regulation. Zn homeostasis in humans is achieved through a balance between intestinal absorption and excretion involving adaptive mechanisms programmed by levels of dietary Zn [62,63].

There are 24 Zn transporters in various parts of the human body that handle uptake, efflux and intracellular trafficking [64]. Half of these proteins are expressed in the enterocytes or enterocyte-like cell lines [65].

There are two families of Zn transporters: ZnT and Zip members. The ZnT family of transporters is responsible for decreasing intracellular Zn levels by transporting Zn from the cytoplasm to the lumen of organelles or to the extracellular space [66]. The Zip family is responsible for increasing intracellular Zn levels by either transporting the metal from the extracellular space or the organellar lumen into the cytoplasm[67].

Intestinal concentrations of Zn are regulated by the activity of Zn transporters (mainly Zip4, Zip5, ZnT1 and ZnT5) and Zn binding proteins such as metallothioneins (MTs) (**Figure 3.2**) [67]. The expression and function of these proteins act in response to a variety of physiological stimuli and/or dietary conditions, while others appear to be constantly expressed [47]. The function of most of the Zn transporters is primarily transcription regulated by the metal responsive element binding transcription factor 1 (MTF1) [48,66].

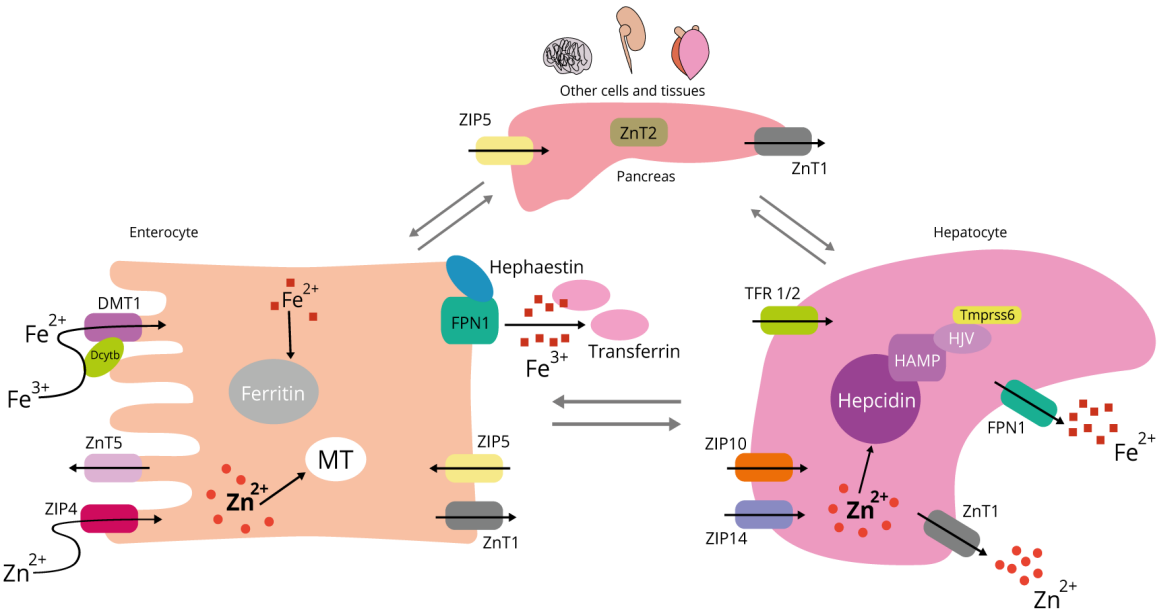

Figure 3.2 The mechanism of Fe and Zn absorption in human enterocytes and hepatocytes

Most Fe is moved across the enterocyte brush border membrane by the divalent metal-ion transporter 1 (DMT1), a process enhanced by the prior reduction of the Fe (ferric reductase activity) by duodenal cytochrome B (DcytB). Enterocyte Fe is exported to the blood via ferroportin 1 (FPN1) on the basolateral membrane. This transporter acts in partnership with the ferroxidase hephaestin that oxidizes exported ferrous Fe to facilitate its binding to plasma transferrin. Diferric transferrin binds to its specific receptor (TFR) and is endocytosed. Ferritin binds to its specific receptor and is endocytosed. At the high level of Fe in plasma, NTBI is reduced to Fe^{2+} by ferric reductase and is rapidly transported into the hepatocytes via Zip14 transporter. Zip4 is the most important transporter in the enterocytes responsible for the uptake of Zn into the cells, while ZnT1 is the main Zn export transporter. The activity of Zip4, Zip10, ZnT1 is regulated via dietary Zn intake.

3.3.2.1 Zip 4 - The Most Dominant Zn Import Protein in Human Enterocytes

The Zip 4 (SLC39A4) protein is the main Zip family transporter responsible for the uptake of dietary Zn into intestinal enterocytes [68]. It is localized at the apical plasma membrane of enterocytes. This transporter has eight transmembrane domains [68]; most loops between TM domains are very short; a longer loop region is frequently found between TM domains three and four (**Figure 3.1**). This longer region often contains a histidine-rich domain with the sequence (HX)n where n ranges from 3 to 5. The function of this domains not clear [69]. Transmembrane domains four and five are particularly amphipathic and contain conserved histidine residues frequently with adjacent polar or charged amino acids. Given their sequence conservation and amphipathic nature, TM four and five are predicted to line a cavity in the transporter through which the substrate passes and as such these regions are essential for function [69].

Zip4 localization and expression are thought to be the primary regulators of intestinal Zn absorption in humans [70]. This transporter is the only Zn transporter that adequately compensates for the Zn deficiency in acrodermatitis enteropathica that result from hereditary defects in Zip4 [71]. Zip4 is expressed in the stomach, small intestine, colon and most likely in the cecum [65,72,73].

Expression of Zip4 appears to be regulated by both transcriptional and post transcriptional mechanisms in response to Zn availability (**Table 3.2**). The abundance of Zip4 mRNA, cellular localization and turnover of this protein are regulated by Zn availability in the intestine. The transcription factor Kr uppel-like factor 4 (KLF4) is an important component of the mechanism responsible for the transcriptional upregulation of Zip4 [65,74]. Important to note is that transcription factors are also regulating the expression of proteins involved in Fe homeostasis [56].

3.3.2.2 ZnT1 - The Main Zn Export Protein

ZnT1 (SLC30A1) is the first Zn transporter discovered. It is the main transporter controlling cellular Zn efflux. The transporter is most highly expressed in tissues involved in Zn acquisition, recycling, or transfer, such as the small intestine [75]. ZnT1 is increasingly abundant along basolateral membranes of enterocytes where it participates in Zn transfer into the circulation [76]. ZnT1 expression can be influenced differentially by the dietary Zn supply (**Table 3.2**). In addition, it was also shown that ZnT1 regulation is under the control of the transcription factor MTF-1, which is Zn-responsive [66,77].

3.3.2.3 Zip14 - The Main Zn Import Protein in Human Hepatocytes

Zip14 (SLC39A14) is a part of LZT subfamily members, distinguished from other Zip transporters by their consensus sequence HEXPHEXGD in TMDV [78]. Amino acid sequence analysis of human Zip14 revealed a slightly altered motif, EEXPHEXGD in TMD V. Multiple studies and tissue array data show that Zip14 is mainly expressed in the liver, though also found in the pancreas and heart [79].

In addition to Zn, the Zip14 was identified as a protein capable of transporting Fe [78,80]. DMT1 transports Fe optimally at low pH 5.2-5.5 [27] while Zip14 exhibits maximal Fe transport at pH 7.5 [80], making it suitable for Fe uptake from the plasma, such as from non-transferrin-bound Fe (NTBI) during Fe overload. In addition, it has been demonstrated that Zip14 is found at the apical and basolateral membrane of enterocytes [65,78].

3.3.2.4 The Important Role of Zip14 in Inflammation

Inflammation is initiated by proinflammatory cytokines that have powerful effects on nutrient metabolism and function. During the acute phase response the liver prioritizes nutrient flows toward production of acute phase proteins [81,82]. Trace elements are among those nutrients that exhibit atypical metabolic profiles during inflammation and infectious episodes [61].

Using a global screening approach for the ZnT and Zip transporter genes it was shown that Zip14 was the gene most profoundly upregulated by these proinflammatory conditions [81]. Northern analysis showed that Zip14 upregulation was specific for the liver [83]. Abundant Zip14 mRNA was also found in duodenum and jejunum [78].

Induction of Zip14 leads to enhanced expression of Zip14 in the hepatic plasma membrane and is associated with increased Zn transport into hepatocytes via interleukin 6 (IL-6) and other mediators (**Figure 3.3**). Under these conditions, serum Zn levels drop and liver levels rise in a likely effort to withhold this essential metal nutrient from the invading pathogen [84]. This suggests that induction of Zip14by IL-6 is responsible for the serum hypozincemia associated with infection. The binding sites of metallothioneins (MT), particularly those that comprise the N-terminal (β-cluster) metal-binding domain of the protein [85] are the ultimate recipients of Zn ions lost from the plasma pool during stress and inflammation.

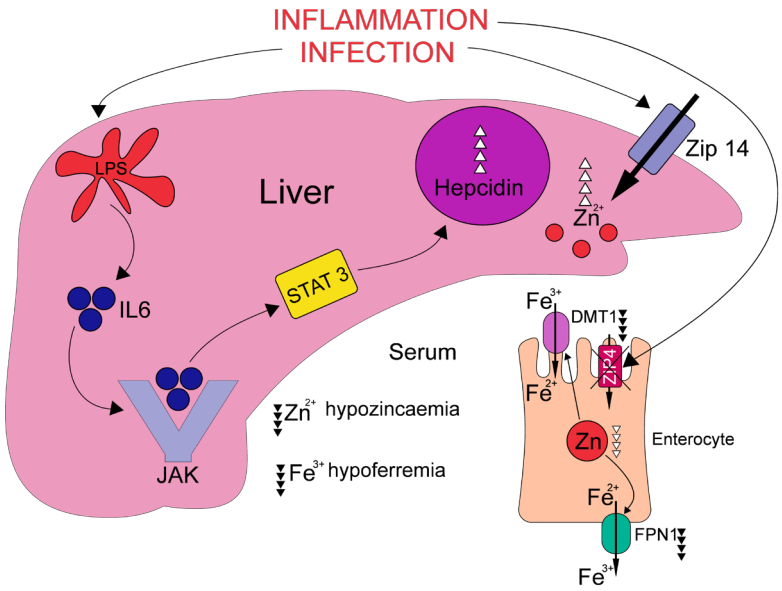

Figure 3.3 The effect of inflammation on Fe and Zn absorption in the intestine and liver

Inflammation → increased Zip14 expression → increased levels of Zn in the liver and decreased in the serum (hypozincaemia) and hypoferrimia. Zip4 reduced to nearly undetectable levels so there is no Zn uptake in the enterocytes. Infection →interleukin (IL1b)→IL6→ JAK/ STAT3→ increased hepcidin. Hepcidin can also be upregulated by LPS. Low concentrations of Zn in enterocytes + high hepcidin→ low expression of FPN1 and DMT1→ reduced absorption of Fe→ anemia of inflammation. During the inflammation pH in the duodenum increases which decreases Fe solubility.

During infections, multiple cytokines may contribute to hepcidin regulation [83]. The inflammation produces increases in IL-6 signal, which transduction requires STAT3 that is activated by Jak-kinase 1 (JAK) to upregulate Type II acute-phase genes [83]. In addition, hepcidin is upregulated by lipopolysaccharide (LPS) [86]. LPS stimulated cytokines such as tumor necrosis factor (TNFα), interleukins IL-1α, as well as IL-1β, which, sequentially, stimulate IL-6 production [87].

Hypoferremia is also associated with inflammation and infection. Mechanisms that result in reduced serum Fe in response to both acute and chronic stimuli focus on the regulatory peptide hepcidin.

Hepcidin controls Fe levels via regulation of the FPN1 through a mechanism that regulates degradation of the transporter. However, there may well be an additional mechanism responsible for low Fe levels. A decreased concentration of Zn in enterocytes caused by reduced expression of Zip4 and consequentially low expression of DMT1 and FPN1 transporters could also contribute to reduced plasma Fe concentrations (see discussion below).

3.3.2.5 Zip8 (SLC39A8)

ZIP8 was first identified in 2002 in a screen of monocyte cDNAs induced by infection and inflammation [88]. This protein is mainly expressed in the lung, kidney, testis, liver, brain and small intestine [66]. Among the 14 mammalian Zip family members, Zip8 is most closely related to Zip14 [89]. Mouse Zip14 and Zip8 are similar in length (489 *versus* 462 amino acids), ~50% of their amino acids are identical, and they each contain a long extracellular N-terminal region with multiple potential glycosylation sites. Notably, Zip14 and Zip8 are 90% identical [90]. Wang et al. in 2012 [90] demonstrated that Zip8 can transport Fe at physiologic pH. Zip8 is expressed at the cell surface, and mediates the uptake of Fe from ferric citrate, the predominant form of non-transferrin bound iron (NTBI) in the plasma of individuals with Fe overload.

The data from the H4IIE hepatoma cells are showing that Fe loading increases plasma membrane levels of Zip8 which suggests that similarly to Zip14, Zip8 can also be recruited to the cell surface to enhance the uptake of NTBI. The upregulation of the Zn/Fe importer Zip8 in response to Fe loading may partially account for hepatic Zn accumulation that occurs during Fe overload [90]. Notably, the Fe transport activity of Zip8 decreases with decreasing pH. At pH 6.5, the pH by which~50% of the Fe dissociates from transferrin-transferrin receptor complex in endosomes, Zip8 shows the highest transport activity, while at pH 5.5 Zip8 shows no Fe transport activity [90].

3.3.2.6 The Key Role of Zip8 in Fe Transport Across the Placenta

It is still unknown how Fe is transported across the placenta to the developing fetus [91]. What is known is that DMT1 and Zip14 are not required for materno-fetal Fe transfer because DMT1 and Zip14 knock-out mice are born alive with adequate amounts of Fe [54]. These observations leave Zip8 as the only remaining known Fe transport protein that could function in the placenta. The uptake of NTBI is increased during the Fe loading of hepatocytes [92-93]. The upregulation of cell-surface Zip8 by Fe loading suggests that Zip8 may contribute to NTBI uptake [94].

Recently, it was shown that Zip8 hypomorphic mice (which carries a neomycin resistance gene in intron 3 of Zip8) exhibit severe anemia and embryos and neonates and do not survive more than 48 h after birth [95]. The anemia appears to result from Fe deficiency as indicated by low serum Fe, total Fe binding capacity, and low hepatic Fe. The Fe-deficient phenotype of the hypomorphic mice strongly suggests that Zip8 plays a direct physiologic role in Fe metabolism. What is interesting is that Zip8 mRNA levels do not change according to Fe status in rat liver and are not affected by Fe loading, whereas Zip8 protein levels increase, which suggest a post transcriptional regulation of this transporter [96].

3.3.3 Zn and Fe Transporters - Cellular Location and Tissue-Specific Expression

Zn and Fe transporters are expressed all over the intestinal tract. DMT1 and FPN1 transporters are most highly expressed in the proximal duodenum, but they are also found in the distal parts and colon, although with a much lower expression rates (**Table 3.2**). Zn transporters are produced in the epithelium lining of the entire gastrointestinal tract, so it is likely that some Zn absorption occurs in the entire intestinal tract [65]. Zip14 is mainly expressed in the liver but it is also found in the duodenum and jejunum [66].

Similarly, DMT1 and FPN1 are expressed in the liver cells. As previously discussed, besides being regulated by other mechanisms (hepcidin, hypoxia, inflammation, hormones) major Fe and Zn import and export transporters are all responsive to cellular Zn concentrations.

3.3.4 The Role of Pancreas in Zn Homeostasis

The pancreas is well-known as a site of high Zn turnover, with pancreatic acinar cells having much higher Zn turnover rates than islet tissue [77]. Pancreatic release of Zn by acinar cells is through the secretory process at the apical membrane and involves transporters ZnT2 and ZnT1, respectively [74]. Under standard dietary conditions, 1-2 mg/day Zn enters the digestive tract via zymogen granules secreted from pancreatic acinar cells [97,98]. Enterocytes and acinar cells constitutively express Zip5 at the basolateral membrane, which serve as a monitor of Zn status and play a role in Zn import into the cell.

Table 3.2 Fe and Zn transporters: cellular location, tissue-specific expression and the mechanism of regulation

Transporter name	Cellular location	Tissue expression	The mechanism of regulation	Zn dependent regulation	Expression along intestinal axis
DMT1	Apical membranes	Small intestine, kidney, liver	Cellular Zn, Hepcidin, Hypoxia, Inflammation	Increased expression and activity by Zn treatment	Expressed all over the intestinal tract; and liver, most strongly in the proximal duodenum
FPN1	Plasma membrane	Ubiquitously expressed	Hepcidin, Zn, Fe, Hypoxia, Inflammation	Increased expression and activity by Zn treatment	Expressed all over the intestinal tract and liver most strongly in the proximal intestine, lower expression in distal parts and colon
Zip4	Apical membranes	Small intestine, stomach, colon, kidney, brain	Dietary Zn, Transcriptional and post-transcriptional regulation	Up-regulated by Zn deficiency	Abundantly expressed throughout the small intestine
Zip14	Plasma membranes	Ubiquitously expressed, liver cells, heart, kidney	IL-6 and IL-1, nitric oxide	-	Expressed in small intestine (found in duodenum and jejunum)
ZnT1	Basolateral membranes	Ubiquitously expressed, small intestine	Dietary Zn supply	Down-regulated by Zn deficiency	Most abundant in the proximal small intestine, cecum
ZnT2	Vesicles, secretory granules	Small intestine, liver, pancreatic acinar cells, kidney	Dietary Zn and hormones	Down-regulated by Zn deficiency	Expressed in small intestine

Data modified from: Litchten and Cousins (2009) [100] & Wang and Pantopoulos (2011) [105].

Dietary Zn restriction significantly decreased the Zn concentration; over 50% in both pancreatic cell cytoplasm and in zymogen granules, and was correlated with decreased expression of ZnT1 and ZnT2 [99]. These two transporters function closely in acinar cell Zn secretion and produce an important component of the entero-pancreatic Zn circulation.

Upregulation of Zip4 in enterocytes, and concurrent downregulation of ZnT1 and ZnT2 in pancreatic acinar cells, when dietary Zn intake is low, are the key factors that may balance the intestinal intake with endogenous loss via pancreatic secretions [72].

The pancreas has the potential to act as a key component of Zn homeostasis in humans, which may also mean that the pancreatic Zn is also a major determinant of Fe absorption, however this requires further investigation, as the molecular mechanisms, pathways, and the transporters for pancreatic Zn secretion and their role in Fe absorption are still not entirely clear.

3.4 The Mechanism of Local and Systemic Regulation of Fe Absorption Under Physiological and Pathological Conditions

3.4.1 The Effect of Fe/Zn Depletion/Supplementation on the Expression of Fe Transporters and their Storage Proteins

The most recently published study that looked at Fe and Zn interaction at the transporter level during Fe and/or Zn depletion/supplementation conditions was completed by Iyengar et al. in 2012 [48]. The authors used Caco-2 cells, an established model of absorptive enterocytes, to investigate Fe/Zn interaction and they reported the following:

Apical DMT1 expression increased upon Zn supplementation while Fe supplementation did not have any significant effect on the localization of DMT1. Depletion of Fe and Zn leads to movement of DMT1 away from the apical membrane and accumulation towards the center of the cell. Expression of FPN1 increased upon supplementation with Fe or Zn and upon depletion of Zn. These findings suggest that Zn supplemented cells not only take up more Fe but also transfer the excess Fe out of the cell. Total cellular IRP1 protein expression did not change upon supplementation or depletion of Fe and/or Zn [48]. The RNA bound form decreased upon Fe supplementation while notably increased upon Zn supplementation.

An interesting finding from this study is that Zn supplementation increased both the RNA bound form of IRP1 and IRP2 expression, resulting in increased DMT1, which suggests that the observed changes in DMT1 expression upon Zn supplementation may be under the control of the IRP/IRE system [48].

Over the years, the evidence for increased expression of DMT1 during Zn supplementation was also provided by Yamaji et al. (2001) [43], Leong et al. (2003) [100], Kelleher and Lonnerdal (2006) [45] and many others (**Table 3.1**). Comparable results were reported for the activity of basolateral transporter, the expression of FPN1 increased in Zn treated cells [43-47]. However, inconsistencies exist regarding the mechanism that controls the activity of Fe transporters in the presence of Zn. Iyengar et al. (2012) [48], show some evidence for the involvement of the IRE/IRP system in the control of DMT1. Others believe that the effect is post translational and that the IRE/IRP system is unlikely to play a role in the regulation [53,101]. Transcriptional regulation of both Fe transporters has also been demonstrated [35,42,43,51]. It is tempting to postulate that the activity of various mechanisms involved in the regulation of Fe absorption is coordinated by a common component. We believe that cellular Zn concentrations, in addition to intracellular Fe levels, may be the key element that controls and modulate the function of the entire Fe absorption machinery (see later sections).

3.4.2 The Effect of Fe/Zn Depletion/Supplementation on the Expression of Zn Transporters

Zn absorption is influenced by dietary Zn intake, not Zn status [62,63]. Expression of Zip4 appears to be regulated by both transcriptional and posttranscriptional mechanisms in response to Zn availability [102,103].
In addition, Iyengar et al. (2012) [48] reported that Zip14 transcript level decreased with Zn supplementation, with no change in Zip1 transcript levels. Supplementation with Fe and Zn significantly increased ZnT1 expression; the increase with Zn supplementation was higher when compared with Fe supplementation.
No significant changes in ZnT1 localization (cell height at maximum amplitude) were observed except for diffuse punctuate fluorescence upon Zn supplementation. Similarly, Zn supplementation resulted in a marked increase in discrete punctate ZnT4 fluorescence [48].

3.4.3 The Transporter Models of Fe and Zn Uptake

Diverse models have been proposed for Fe and Zn absorption. The uptake of Fe is dependent on a two component model while a three compartment model is suggested for Zn uptake [46]. There are two transporters capable of transporting Fe, one of which is the well-known DMT1 transporter.
Based on the Ki (the inhibition constant) value, which indicates high affinity for Zn, the second Fe transporter is a Zn influx transporter (most likely it is Zip14 that is able to transport NTBI along with Zn) [46]. In the presence of concomitant Zn, the second Fe transporter is less capable to transport Fe, thus halving Fe uptake. Upon Zn pretreatment the second transporter becomes non functional.

The Zn absorption model contains three compartments. Component I of this model represents paracellular uptake that is diffusion limited, component 2 characterizes Zn influx (most likely Zip4 transporter) and component 3 is an efflux transporter (probably ZnT1).

The gastrointestinal tract is the major site for regulation of Zn homeostasis. Unlike Fe, Zn absorption is influenced by dietary Zn intake, not Zn status [61]. Fractional Zn absorption is inversely related to dietary Zn intake, whereas intestinal absorptive capacity is high at low levels of Zn intake.

During periods of higher levels of Zn intake, there is a decrease in absorptive efficiency but an increase in the total amount of Zn absorbed. At intakes below 9 mg/day, Zn absorption occurs primarily by a saturable process involving Zip4, ZnT1, and other transporters. There is no evidence that past Zn intakes, or status, influence Zn absorption. Instead, current Zn intake is the chief determinant of Zn absorption [62].

However, an upper limit exists where increasing Zn intake >20mg in solution does not result in greater Zn absorption [70]. The Fe absorption is also saturable with a limited number of carriers mediating uptake; it is two fold greater at pH 5.5 than at pH 7.4 [104].

3.4.4 The Mechanism of Local Regulation of Intestinal Fe Absorption

Besides systemic regulation (via hepcidin) Fe absorption is also regulated at the local level, in intestinal enterocytes. The Fe responsive element (IRE)/iron regulatory protein (IRP) system is the major player in this regulation, which affects the post-transcriptional regulation of proteins involved in Fe metabolism [105].

The mRNAs encoding TFR1 and two isoforms of DMT1 have IREs in their 3'UTRs which are believed to be stabilized under Fe deficient conditions when an IRP (IRP1 or IRP2) binds to them [106]. This stabilization increases the half-life of the mRNA and consequently increases the amount of protein translated. Ferritin and one isoform of FPN1 have IREs in their 5'UTR. In this location, stabilization of the stem loop by IRP binding blocks translation (**Figure 3.4**).

The major form of FPN1 in intestinal cells, however, lacks the 5'IRE and thus FPN1 protein levels can be maintained even if Fe levels in the cell are low [38]. Interestingly, the FPN1 transcript IRE that is present in the 5'UTR when activated via IRP binding should lead to decreased FPN1 expression. However, an increase is noted, implying that FPN1 may not be under the control of the IRP/IRE system [38], or that some other factor is also controlling the expression of the FPN1 transporter. Similarly, two of four isoforms of DMT1 and Dcytb mRNA have no IRE, and therefore their regulation has also never been adequately explained by the IRE/IRP system. Clearly, local regulation of Fe absorption does not rely solely on intestinal Fe concentrations, but an additional mechanism is involved.

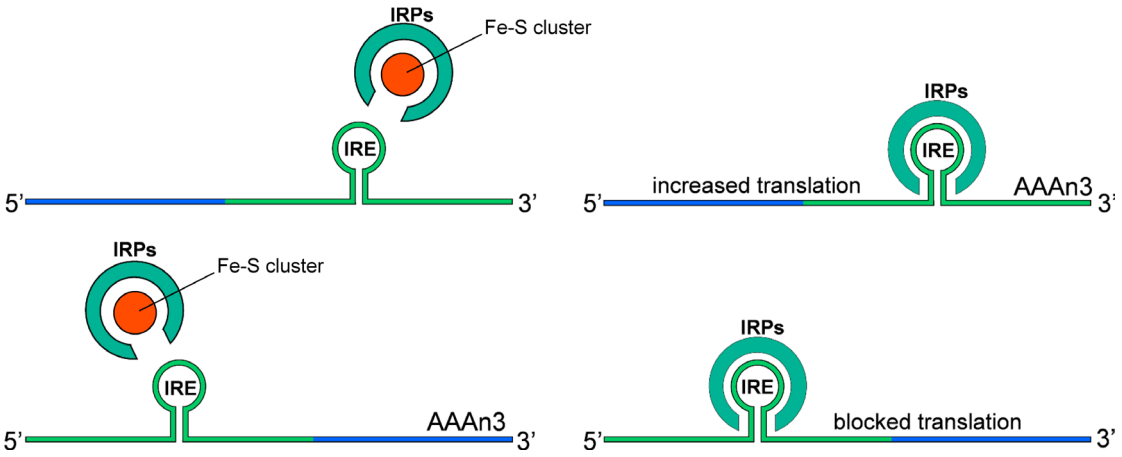

Figure 3.4 The iron responsive element (IRE)/iron regulatory protein (IRP) system

Cytosolic aconitase with a 4Fe-4S-cluster senses the cellular Fe levels and can function as a Fe-regulatory protein (IRP). The Fe-sulfur cluster cannot assemble under Fe starved conditions, in which case the cytosolic enzyme undergoes a dramatic conformational change; aconitase assumes a 3Fe–4S configuration and loses the enzyme activity. This reorganized form of c-aconitase is called Fe regulatory protein (IRP). IRP bind to IRE in 3'UTRs and so improves stabilization that in turn increases translation of the transporter protein. In contrast, binding of IRPs to IRE in 5'UTRs blocks translation. Fe deficiency per se is not sufficient to promote loss of the cluster. Zn can replace 4Fe-4S clusters in proteins; Zn has an intrinsic preference for the fourth site over Fe.

A molecular basis by which elements other than Fe can selectively modulate Fe metabolism in cells and tissues in the presence or absence of changes in Fe availability is based on the regulation of IRP function through changes in their phosphorylation state [107]. IRPs bind to Fe responsive elements in the untranslated regions of mRNA and regulate translation of key proteins involved in Fe metabolism, ferritin, and transferrin receptor. The formation or loss of the Fe-S cluster is a mean by which changes in cellular Fe status could be transmitted into alterations in RNA binding capacity of IRP1 [108]. An enzyme, aconitase, has a central role in determination of the labile Fe pool and overall regulation of the intracellular Fe homeostasis. In order for the Fe-S cluster of IRP to serve as a biosensor, its assembly and/or disassembly needs to be adequately regulated.

However, the Fe-S clusters do not disassemble spontaneously. In other words, Fe deficiency per se is not sufficient to promote loss of the cluster [109,110]. Consequently, we postulate that cellular Zn concentration, in addition to Fe, can affect the stability of the Fe-S cluster and determine if the cluster is going to be removed or not. Further support to this idea comes from the studies that demonstrated an inhibiting effect of Zn on aconitase, showing low aconitase activity during Zn supplementation periods [48,105,111].

Similarly, there is some evidence that Zn can replace/alter 4Fe-4S clusters in proteins; Zn has an intrinsic preference for the fourth site over Fe [109]. In addition, newly identified IRP mRNAs fall into the category of "metal ion binding" proteins that are shown to interact selectively and non-covalently with Zn ions [68].

Recently, Iyengar et al. (2012) [48] showed that Zn supplementation increased the RNA-bound forms of both IRP1 and IRP2 expression, resulting in increased DMT1 activity [48]. Similarly, the expression of FPN1 was also increased upon supplementation with Zn [43,44,45,48]. Furthermore, it appears that Zn is able to block the Fe storage capacity of ferritin [48,112,113]. Besides, it has been shown that the induction of the DMT1 IRE isoform by Fe depletion was completely dependent on HIF2, suggesting molecular crosstalk between HIF2 and IRP signaling, where IRP1 and/or IRP2 are critical for basal gene expression of DMT1 IRE isoform [56] and HIF2 is required for transcriptional response (**Figure 3.5** for an association between Zn and HIF2). These results noticeably raise the probability that fine-tuned coordination of Fe and Zn metabolism may also take place via the IRE/IRP system, but this requires further investigation.

When low concentrations of Zn are present in the cells, Zn is not able to bind to ferritin and does not block its storage capacity, which in turn, contributes to increased accumulation of Fe in the enterocytes. This implies that the availability of Zn in the cells plays a crucial role in Fe absorption and transfer to circulation. Although, the Fe may be supplied in sufficient amounts it cannot be absorbed (Zn deficiency leads to DMT1 delocalization) and transported out of the enterocyte (as ferritin storage capacity increases) without an adequate supply of Zn.

However, the increased expression of FPN1 and ZnT1 has also been noted during Zn depletion [48]. Briefly, this initial increase in FPN1 and ZnT1 expression in response to Zn deficiency, in our opinion, exists in order to improve the transfer of Fe and Zn to systemic circulation, which helps in minimizing the difference between Fe and Zn supply and demand, and alleviates negative consequences of prolonged Fe and Zn depletion. Similarly, Zn plays a protective role against Fe induced oxidative damage, so decreased concentration of Zn in the cells indicate that the levels of free Fe in the cells should be reduced in order to avoid oxidant induced damage, which additionally explains the increased storage capacity of ferritin, as well as increased FPN1 expression, during the periods of cellular Zn depletion.

3.5 The Regulation of Fe Absorption During Pathological Conditions (Inflammation, Hypoxia, IRIDA and Fe Overload Conditions)

3.5.1 Hypoxia

Fe is essential for supplying cells and tissues with oxygen. Hypoxia (oxygen deprivation) creates a strong stimulus to Fe absorption, regulating the function of Fe transporters by hypoxia inducible factors (HIF1 and HIF2). HIFs are a part of a transcription factor complex that binds to promoters containing HIF responsive elements (HREs) and induces transcription. Fe metabolism genes that contain HREs include Dcytb, DMT1, and FPN1. HIF2 levels, and thus the transcription of target genes, rise when oxygen levels decrease. Increased expression of HIF2 in the intestine in genetically modified mice leads to increased DMT1 and Dcytb expression and increased Fe absorption, whereas intestinal knockout of HIF2 leads to low levels of DMT1, Dcytb and FPN1and systemic Fe deficiency despite low levels of hepcidin [55]. Dcytb and DMT1 are the genes most highly upregulated by hypoxia in duodenum and both transporters are regulated by HIF2 [56].
HIFs (hypoxia-inducible factors) have been shown basically to control the cellular response to hypoxia. Recently, it was shown that NF-κB (nuclear factor κB) is a modulator of HIF2 expression in the presence of normal oxygen pressure [114,115]. NF-κB indirectly controls HIF2 αthrough its control of HIF1β [116].

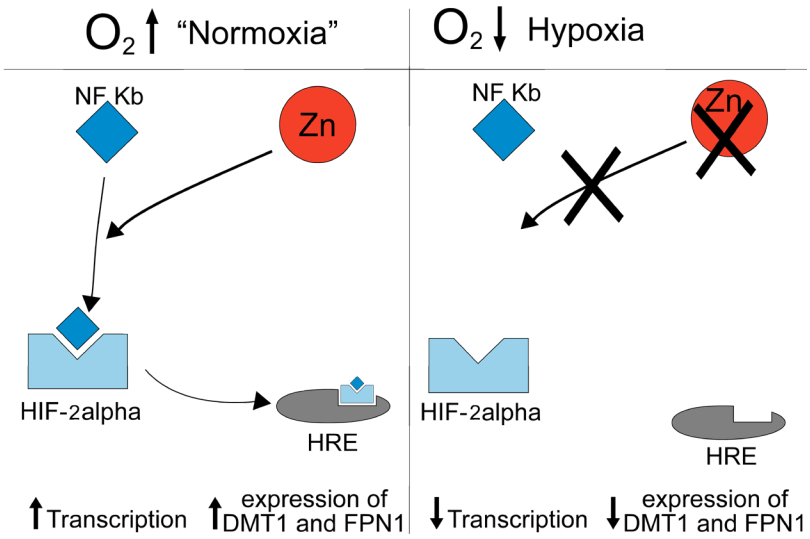

Figure 3.5 Proposed model for the role of Zn in the cell response to hypoxia

Nuclear factor Kb stabilises HIF1β, HIF2α binds to HREs and induces transcription, which increases DMT1 expression, Dcytb and FPN1 expression and consequentially increases the levels of Fe and oxygen in the cells. Nuclear factor binding activity declines when Zn concentrations in the cells are decreased.

A number of studies over the years confirmed that Zn plays an important role in the activation of NF-kappaB [117,118]. Under low Zn conditions, NF-kappaB nuclear binding activity is lower [119,120,121].
The initial reduction in NF-kappaB binding most likely reflects an early response to Zn deficiency-induced oxidative stress [119].
At times of prolonged Zn deprivation, oxidative stress (ROS-reactive oxygen species) continues to activate NF-kappaB which increases the production of growth factors, antiapoptotic molecules and inflammatory cytokines. In these situations, the provision of adequate concentrations of Zn to the cells is crucial, as Zn has been shown to decrease ROS by several mechanisms. One mechanism by which Zn reduces inflammatory cytokine production involves the Zn-induced upregulation of a Zn-finger protein, A20, which inhibits NF-kB activation via TRAF pathway [122]. Thus, Zn functions as an antioxidant and as an important anti-inflammatory agent.

3.5.2 IRIDA

Iron refractory iron deficiency anemia (IRIDA) is an inherited Fe deficiency disorder manifested by a profound anemia that is unable to respond effectively to oral Fe therapy. The gene mutated in most such cases is transmembrane protease serine 6 (Tmprss6), a hepatocyte plasma membrane protease that degrades HJV and effectively acts to repress hepcidin production. When Tmprss6 is mutated, hepcidin levels are relatively high and body Fe intake declines.

3.5.3 Fe Overload Conditions

Fe overload conditions (e.g. thalassemia, hereditary hemochromatosis) are characterized by increased Fe absorption despite the presence of adequate or increased body Fe stores. They are mainly caused by mutations in the genes encoding, HFE, TFR2, JH, and they share the common feature of reduced or absent hepcidin expression [123]. Under normal circumstances and during Fe deficiency, the liver acquires Fe via receptor-mediated endocytosis of transferrin, the circulating Fe transport protein [124].
During conditions of Fe overload, the Fe-carrying capacity of transferrin is exceeded, giving rise to non-transferrin bound Fe (NTBI). As previously discussed, Zip14 mediates the uptake of NTBI into hepatocytes [78]. The role of Zip14 in HFE-mediated Fe overload was examined in HepG2 cells [125].
The expression of HFE in HepG2 cells resulted in a lower abundance of Zip14, possibly by a post-transcriptional mechanism. Interestingly, Fe uptake was unaffected by HFE expression after Zip14 knockdown, implying that HFE has a direct effect on Zip14-mediated Fe transport.

3.6 The Mechanism of Systemic Fe Regulation: The Important Role of Hepcidin (Major Fe Regulatory Protein in Hepatocytes)

Hepcidin is a small peptide, composed of 25 amino acids, produced mainly by hepatocytes in the liver. It is a major regulator of Fe homeostasis that acts at two sites, tissue macrophages and the small intestine.

While there is general agreement on the essential role of hepcidin in Fe absorption, there is still no conclusive evidence about the exact site of hepcidin action in enterocytes. Hepcidin has been shown to induce a significant reduction in intestinal Fe transport and DMT1 protein levels, but no change in FPN1 levels [53,126,127]. On the other hand, there are studies that demonstrated a link between decreased hepcidin and elevated intestinal FPN1 expression [53,128,129].

Looking at the structural properties of this molecule, the first five N-terminal amino acids are essential for regulation of Fe metabolism [130,131]. The N-terminal region of hepcidin is essential for binding to FPN1 because sequential truncation of the N-terminal residues results in a progressive loss in activity of the peptide. However, the N-terminal region alone is not sufficient to induce FPN1 internalization [36]. In addition, it has been shown that Cys326 of FPN1 is required for the interaction between hepcidin and FPN1 [132,133].

The regulation of hepcidin production is complex. Animal models and human diseases that lead to inappropriate Fe levels have helped in the clarification of hepcidin regulatory mechanisms, although some of these mechanisms are still incompletely understood [34]. It is now clear that there are several pathways involved in the regulation of this hormone, including inflammation, infection, erythropoietic demand, hypoxia and body Fe status [134]. Hepcidin synthesis by the liver is increased by inflammation and Fe stores and is decreased by hypoxia and anemia.

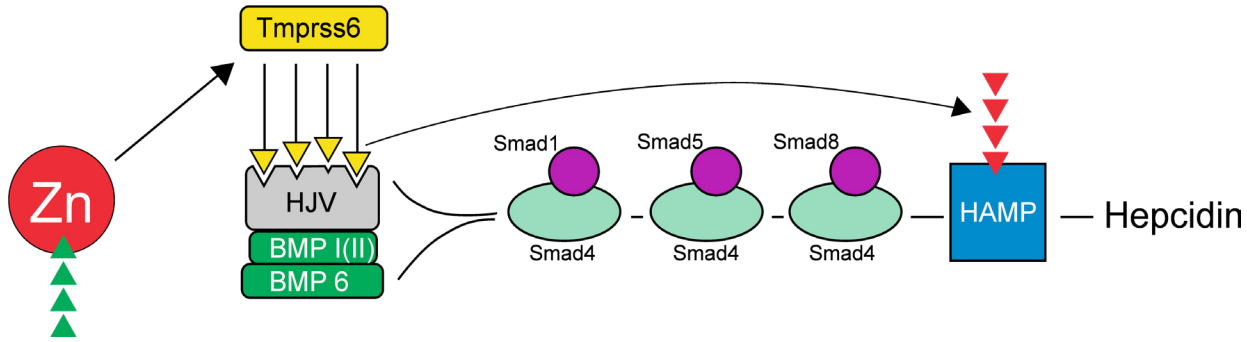

Figure 3.6 Proposed mechanism for the role of Zn in hepcidin regulation

BMP6 binds to a BMP receptor I/II complex on hepatocytes, leading to phosphorylation of receptor-regulated SMADs 1, 5, and 8. Phosphorylation allows these proteins to interact with Smad 4, and the resulting heteromeric complexes stimulate transcription of the gene encoding hepcidin antimicrobial peptide (HAMP). Tmprss6s are Zn dependent endopeptidases, so adequate cellular Zn levels contribute to natural function of Tmprss6 (degradation of HJV) that reduces hepcidin production and consequently contribute to increased Fe absorption.

Among the multiple mechanisms that regulate hepcidin expression the BMP6/SMAD pathway takes the central role [34,135,136]. BMP6 is a transforming growth factor beta family cytokine produced and secreted by hepatocytes in proportion to their Fe load. The BMP6 receptor complex includes a co-receptor called hemojuvelin (HJV) that is critical for this pathway to function [34,134]. Individuals with a congenital defect preventing HJV synthesis have very low hepcidin levels and develop the severe Fe loading disease juvenile hemochromatosis. HJV is normally found bound to the plasma membrane of cells, but a soluble form can be produced by the action of the protease furin [136]. Soluble HJV can compete with membrane-bound HJV for BMP binding and thus is able to ameliorate BMP/SMAD signaling [106].

Tmprss6, a cell surface serine proteases that degrades HJV and is able to reduce BMP signaling [135]. Tmprss6 is an essential component of a pathway that detects Fe deficiency and blocks *HAMP* transcription, permitting enhanced dietary Fe absorption. Tmprss6 is a Zn dependent endopeptidase. Therefore, inadequate Zn levels lead to improper function of Tmprss6 [137,138]. In this situation, Tmprss6 cannot bind HJV which accordingly increases hepcidin production and lowers the absorption of Fe (**Figure 3.6**).

3.6.1 The Role of Intracellular Zn Concentrations on Hepcidin Expression and Regulation of Intestinal FPN1 Transporter

The inhibitory role of hepcidin is believed to be initiated by a direct binding of this peptide toFPN1 transporters [36,133]. However, there is more and more evidence showing the absence of the hepcidin effect on FPN1 in the intestine [38,39,50]. The explanation could be the presence of a second FPN1 isoform that is unable to interact with hepcidin.

A new FPN1B transcript lacking the IRE sequence has been described recently in enterocytes [106]; however it is still unknown how the protein expression of this isoform is controlled. The FPN1B form represents 25% of total FPN1 mRNA in duodenum [38]. The translation of FPN1B is insensitive to Fe but the protein can export Fe under conditions of Fe deficiency [38]. Similarly, in vitro studies using either hepatoma cell lines or primary hepatocytes have failed to demonstrate increased synthesis of hepcidin in response to Fe loading [34,36]. Moreover, analysis of hepcidin mRNA and protein levels in hepatoma cells suggests that its expression is regulated by divalent metal ions, with Zn inducing maximal effects on hepcidin levels [139]. MTF1, a divalent metal ion sensitive transcription factor, regulates hepcidin transcription by binding to its cognate response elements within the hepcidin promoter. Out of four elements which activity was tested (Fe, Zn, Cu, Co) only Zn was shown to induce metal sensitivity fully [139].

In addition, hepcidin belongs to the family of metallothioneins (MTs), proteins regulated by intracellular Zn ion levels through MTF1/MREs interactions [140]. MTs are cysteine-rich, metal-binding proteins involved in Zn homeostasis and are also involved in protection against oxidative stress. MTF1 has been shown as important in the Zn-mediated induction of FPN1 mRNA (**Figure 3.1**). Troadec et al. in 2010 [49] demonstrated that MTF1 binds to the FPN1 promoter in the presence of Zn and initiates its transcription. It has also been shown that although some other metals (Cd, Co) can induce metallothionein expression by MTF1, only Zn can activate the DNA binding activity of this transcription factor by its reversible interactions with specific Zn fingers [49]. FPN1 is the main hepcidin receptor [33,136,141]. Acute hepcidin exposure has little effect on FPN1 while chronically high levels of hepcidin lead to loss of intestinal FPN1 [129,142,143]. The alterations in Fe transport activity in enterocytes seems to result from the changes in DMT1 protein levels [53,123,126,127]. How hepcidin affects DMT1 levels is not clear, but it is believed that the process is guided by ubiquitin-dependent proteasome degradation of DMT1 [123]. The inconsistent evidence about the exact site of hepcidin action in the intestine could be explained by the fact that changes at the apical side of the enterocyte most likely contribute to the changes at the basolateral side and vice versa; and possibly indicate the presence of an additional mechanism controlling the entire process. The presented findings demonstrate that cellular Zn concentrations modulate the expression and function of the transporters.

3.7 The Mechanism of Fe Absorption Under Physiologically Favorable Conditions (Adequate Supply of Both Minerals Zn and Fe, without Inflammation or Infection). The Significance of Suitable Cellular Zn Concentrations.

Zn adequacy: Zip4 and ZnT1 respond positively to adequate dietary Zn intake and transport Zn inside the enterocytes (Zip4) and outside of it, to the plasma (ZnT1) (**Figure 3.7**). The satisfactory levels of Zn in the cells, in addition to Fe, stimulate the expression of both DMT1 and FPN1 transporters. Consequently, there is a transfer of Fe across the enterocytes (Fe is not stored as ferritin), Fe goes out to the plasma. Due to the presence of adequate levels of Zn in the hepatocytes, Tmprss6 is functioning properly, hepcidin production is decreased (HJV, BMP pathway) and FPN1 and DMT1 expression increases, so the cells are able to absorb Fe further. In order for the cells to respond appropriately to Fe deficiency the presence of adequate intracellular concentrations of Zn is vital.

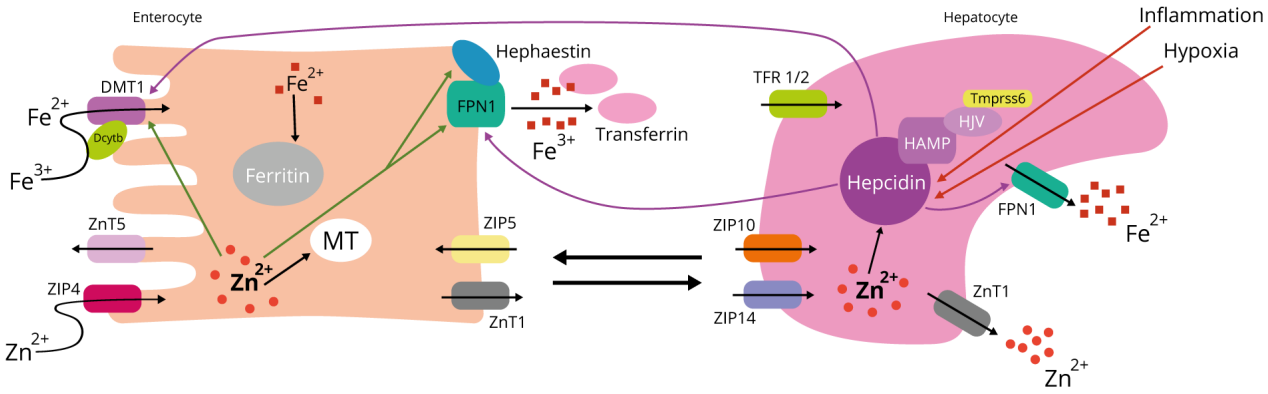

Figure 3.7 Proposed model for the role of Zn in systemic regulation of Fe absorption

In order for the cells to respond appropriately to Fe deficiency the presence of adequate intracellular concentrations of Zn is vital. Cellular Zn concentrations regulate the expression and activity of both intestinal Fe transporters: DMT1 and FPN1. In addition, the activity of Zip transporters (Zip4, ZnT1, Zip10) including the Zip14 transporter is also regulated by Zn. Cellular Zn concentrations are playing a role in hepcidin expression.

Zn deficiency: Dietary Zn intake is the main determinant of Zn absorption (**Figure 3.7**). During the states of prolonged marginal intake of Zn, homeostatic adjustments are not sufficient to replace Zn losses and a negative Zn balance occurs. Even if Fe is supplied in sufficient amounts it cannot be absorbed without an adequate supply of Zn. Low intracellular Zn concentrations cause DMT1 delocalization, reduce FPN1 expression and there is no transport of Fe into the enterocytes. Additionally, in an effort to adjust to low dietary Zn intake, the system will start to withdraw Zn from the tissues (plasma, pancreas and liver are among the most affected organs). Low concentrations of Zn in the liver will affect the activity of Tmprss6 (HJV will not be degraded) which will cause an increased hepcidin synthesis and consequently, through limited expression and activity of DMT1 and FPN1 transporters lead to reduced Fe uptake.

3.8 Concluding Remarks

In recent years, substantial progress in the understanding of Fe and Zn absorption and interaction processes in humans has been made. The mode of Fe absorption is complex and it is challenging to distinguish between the different players involved in the process. There are still missing pieces of information to fully explain certain inconsistencies.

This paper describes the main Fe and Zn transporters found in major sites of Fe and Zn uptake and storage, the enterocytes and the hepatocytes. It also explains Fe and Zn interactions at the transporter level.

Finally, this review, for the first time, proposes the entire mechanism of Zn involvement in the Fe absorption processes (both at the local and systemic level) and further clarifies some of the discrepancies related to Fe and Zn absorption and interactions.

DMT1 is not the site of negative interactions between Fe and Zn. Zip14 is a second Fe transporter that can transport both NTBI Fe and Zn. Cellular Zn dictates the course of events that determine the expression of proteins involved in cellular Fe metabolism. Zn controls the expression of both DMT1 and FPN1, as well as the expression of Zip4, Zip14 and ZnT1 transporters. Anemia develops due to impaired mobilization of Fe from diet and stores rather than inadequate dietary intake, which demonstrates the crucial role of Zn on Fe transporters. In addition, the major protein of systemic Fe regulation, hepcidin, is coordinated by intracellular Zn ion levels through MTF1/MREs interactions. Tmprss6, an essential component of a pathway that detects Fe deficiency, is a Zn dependent endopeptidase.

In conclusion, this review clarifies the role of cellular Zn status in determination of Fe/Zn interactions and illuminates mechanisms by which Zn could possibly control the Fe absorption process. The challenge is certainly to further utilize this new information, to provide a more comprehensive understanding of the link between Zn and Fe absorption pathways using both in vitro and in vivo models. Finally, research into the mechanism by which Zn status influences Fe absorption in humans is, and will remain, an important and interesting area of investigation for many years to come.

3.9 References

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Chapter 4

The Influence of Food Consumption and Socio-Economic Factors on the Relationship Between Zn and Fe Intake and Status in a Healthy Population

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DOI: 10.1017/S1368980017001240.

4.1 Abstract

Objective: To examine Zn and Fe nutritional status of a healthy population by means of anthropometric, dietary, and biochemical measurements and to investigate the relationship of usual Zn and Fe dietary intakes to Zn and Fe status. In addition, to examine the impact of food choices and socio-economic factors on Fe and Zn dietary intakes and status with an aim to identify groups at risk of dietary deficiency and suggest factors that may influence the status of these nutrients. **Design:** Food consumption was assessed by 24 h recall questionnaires. Twenty biochemical parameters were measured, of which Hb, haematocrit, red blood cells count and plasma concentrations of Fe and Zn were directly related to Fe and Zn nutrition. The prevalence of study participants with inadequate micronutrient intakes was calculated using the Estimated Average Requirement cut-point method. **Setting:** Serbia, Europe. **Subjects:** Apparently healthy adults (25-65 years of age). **Results:** Mean daily Zn and Fe intakes were 9.1 mg and 11.6 mg for males and 7.3 mg and 9.5 mg for females, respectively. Five percent of the study population had inadequate dietary Fe intake and 15-25% had inadequate Zn intake. Lower hemoglobin concentrations were measured in women with lower Zn intakes. No differences in Fe and Zn intakes and status among various socio-economic groups were observed, except for Fe intake between the low-income and affluent groups. **Conclusion:** Regular follow-ups are needed to ensure that potential deficiencies of Zn and Fe do get recognized and addressed in a timely manner.

Keywords: Zn, Fe, nutritional status, minerals, adults, healthy population, socio-economic factors, dietary intake

4.2 Introduction

Zinc (Zn) and iron (Fe) are essential micronutrients for human health and are involved in many complex enzyme systems that function in various biological processes [1-3]. Deficiencies of these two nutrients remain a global problem, with the worldwide prevalence of Fe deficiency estimated to be around 30% [4] while approximately 20% of the world's population is affected by Zn deficiency [5]. Although severe Zn deficiency is uncommon in developed countries, marginal deficiency is likely to be much more prevalent [6] and is associated with certain functional outcomes; for instance, delayed wound healing, disturbances of taste and smell acuity, decreased work capacity and diminished immunological response [6-8].

The burden of Fe and Zn deficiencies is most common in low income countries; however inadequate intake of these micronutrients is also seen in resource-rich areas of the world where overeating becomes a public health concern. Recent results from the nutritional surveys conducted in the USA, Great Britain and Germany indicate that the recommended intakes of Fe and Zn are not always achieved [9,10]. The population-based data from Europe suggested substantial variability in micronutrient intakes among healthy adults [9,10]. Data from the National Health and Nutrition Examination Survey 2001-2002 showed that 12% of the USA population had Zn intakes below the Estimated Average Requirements [11]. Similarly, 10-35% of Canadians from most age and sex groups consumed Zn in inadequate amounts [12]. Between 11% and 21% of the population from Greece, the UK and Finland had Fe intakes below the EAR [13-15]. Similarly, a high percentage (39%) of the population in the USA was also found to be at risk of inadequate Fe intake [16]. Women aged 14-50 years are at particular risk of low Fe intake, as confirmed recently by results obtained from six European countries [10]. The prevalence of inadequate intakes of Fe and Zn is not consistent between studies performed in different developed countries. For Serbia in particular, the relevant data for adults are either old or scarce, therefore requesting additional investigation.

Zn is found in similar food sources to Fe, so insufficient dietary Fe and Zn intakes usually occur simultaneously [17,18]. Considering these diet-based similarities, there is a possibility that Fe and Zn status are also positively correlated. Zn metabolism interacts with the metabolism of Fe [19-21]. Similarly, it was noted that Zn and Fe nutriture are affected by many of the same dietary factors (i.e. Ca, phytate and polyphenols) [22,23]. The simultaneous occurrence of Zn and Fe deficiencies in humans has been known since the discovery of Zn deficiency. Zn deficiency associated with Fe-deficiency anemia was first recorded by Prasad et al. [24,25].

A number of studies over the years have demonstrated that Zn intake is correlated with hemoglobin (Hb) concentrations [26,27]. However, it is not established if suboptimal Zn intake or slightly lower Zn status infer low Fe intake per se or low Fe status. Therefore, our objective was to identify relationships between Zn and Fe intakes and status in an apparently healthy population, where only marginal Fe and Zn deficiencies may occur. The influence of socio-economic and demographic factors on Zn and Fe nutriture have been investigated to some degree [28,29]. However, information on the influence of educational level, marital and socio-economic position on mineral status in healthy population is scarce, especially for Zn intake.

This cross-sectional study reports on the current nutritional intake of Zn and Fe in apparently healthy adults (25-65 years of age) living in Serbia and examines the relationship between dietary Zn and Fe intakes and related biochemical parameters. In addition, the study investigates the impact of food choices and socio-economic factors on Fe and Zn dietary intakes and status with an aim to identify groups at risk of dietary deficiency and to suggest factors that may influence the status of these nutrients.

4.3 Materials and Methods

4.3.1 Study Participants

The study participants (n= 754) were apparently healthy 25-65 year old male and female volunteers recruited within an 18-month period from June 2013 to January 2015, through two different projects: National project III 41030 and the Bacchus project (EU FP7, project no. 312090). The study participants were recruited through primary health-care facilities (community pharmacies and health centres) where flyers about participation in the study were distributed. Eligible subjects included apparently healthy people living in Serbia (as indicated by brief questionnaires, measure of blood pressure and blood glucose). Subjects without any clinical signs of an acute condition or chronic disease and without the need for medical treatment were included in the study. Pregnant and lactating women were excluded. All subjects went through the informed consent process, both verbal and written. The study protocols were approved by the Clinical Hospital Centre Zemun, Belgrade, Serbia, Ethics Committee (number 2125, 2013) and by SAC HREC Adelaide, South Australia (EC00188; 96.15).

The protocols and procedures were in agreement with the ethical guidelines on biomedical research on human subjects of The Code of Ethics of the World Medical Association's Declaration of Helsinki (1964) and its further amendments.

4.3.2 Assessment of Dietary Zn and Fe Intake

Dietary intake data were collected for three non-consecutive days, two working days and one weekend day using the 24 h recall technique. All the interviews were done face to face and led by a trained professional according to standardized protocol. The estimated time of data collection was 15-30 min. During the interview, a food atlas with colored photographs of 125 items (foods and composite dishes), illustrating a range of portion sizes in increasing order, was used in order to enhance the accuracy of portion size estimation [30]. In addition, to enhance interpretation of dietary estimates, general questions related to education, previous medical conditions, detailed food supplements, medication use, and lifestyle habits regarding smoking, physical activity/exercise, alcohol as well as coffee and tea consumption were also incorporated in the questionnaire. All questionnaires were checked for errors/omissions together with study participants.

The Zn and Fe content of foods were determined using the Serbian food composition database, harmonized with EuroFIR food platform and Balkan food platform [30,31]. DIETS ASSESS & PLAN, a nutritional tool validated in different national and regional surveys and international projects, and evaluated in the European Food Safety Authority project, was used to obtain comprehensive dietary intake assessments [32]. Dietary data were entered in duplicate to ensure accuracy of data entry and to identify potential inconsistencies. Both the dietary intake assessment software used and food composition data management system have integrated quality control mechanisms including mandatory fields, standard templates and error warnings [30,31]. The dietary intakes from administered questionnaires were calculated by multiplying the frequency of consumption of each food item by composition of that food using adequate portion sizes. In addition to Zn and Fe dietary intakes, data were obtained for energy, macronutrients and main food groups.

Zn and Fe content of participants' diets were verified using the Estimated Average Requirement, as defined in the Dietary Reference Intakes (DRIs) provided by the Institute of Medicine [33]. To check for under-reporting, the ratio of reported energy intake (EI) to predicted basal metabolic rate (BMR) was used, where BMR was estimated according to Schofield equations [34] taking into account age, sex and body weight.

4.3.3 Collection of Blood Samples, Socio-Economic Data and Anthropometric Measurements

Blood samples were collected from a random sub-sample of 177 participants (23% of the study population) between 08.00 and 09.00 hours after an overnight fast (>10 h fasting). Whole-blood samples were collected from participants in a seated position into trace-mineral- free tubes by venepuncture from an antecubital vein using butterfly needles (Sarstedt, Inc.). All samples were centrifuged (1000g for 15 min). The serum and plasma samples were removed and 1 ml aliquots were stored at -80°C until further analysis.

Data on the socio-economic and demographic characteristics of participants were collected via questionnaires. The risk of poverty is defined as 'the share of people with an equalized disposable income (after social transfer) below the at-risk-of-poverty threshold, which is set at 60% of the national median equalized disposable income' [35,36].

Anthropometric variables, height and weight, were measured to the nearest 0.1 cm and 0.1 kg, respectively. Body weight and body composition (muscle mass and fat tissue mass) of participants were measured using a TANITA UM072 balance (TANITA Health Equipment H.K. Ltd). BMI was computed as the ratio of weight to height squared (kg/m^2). BMI was used to assess the prevalence of overweight (25.0-29.9 kg/m^2) and obesity (≥30.0 kg/m^2) according to WHO criteria [37].

4.3.4 Biochemical Analysis

The biochemical parameters were measured using a Cobas e411 clinical chemistry analyzer (Roche Diagnostics, Basel, Switzerland) and using Roche Diagnostics Kits according to the manufacturer's instructions. Plasma Fe, Hb, hematocrit and erythrocyte count were used as markers of Fe status and plasma Zn was used for Zn status. Individuals were considered to be at risk of Fe deficiency anemia if they had below-normal values for Hb (<120 g/L).

4.3.5 Determination of Plasma Zn & Fe Concentrations

The assessment of Fe and Zn mineral status was performed for eighty-two participants (approximately 10% of the study population). The analysis of plasma Zn and Fe was conducted at The Institute of Public Health, Pozarevac, Serbia, using flame atomic absorption spectrometry (AAS) on a Varian SpectrAA-10 instrument with instrument parameters: wavelength 213.9 nm, slit width 1.0 nm and air-acetylene flame according to the method described by Jian Xin (1990) [38].

The concentrations of Zn and Fe were measured after dilution 1:10 with high purity Milli-Q water (>18 MΩ resistivity). To verify the accuracy of the method for Zn, control serum ClinChek-Control (Recipe, Chemical + Instruments GmbH; catalogue number 8880) with Zn concentration of 889 ± 178 µg/L (Level I) and 1738 ± 261 µg/L (Level II) was analysed. Likewise, for Fe, control serum ClinChek-Control (Recipe, Chemical and Instruments GmbH, Germany, catalogue number 8882) with Fe content of 612 ± 122 µg/L (Level I) and 1538 ± 154 µg/L (Level II) was used. Method performances were monitored by analysis of the same control serum within each series. The mean results for Zn concentration were 914 ± 23 µg/L (Level I) and 1801 ± 35 µg/L (Level II) which is in accordance with the certified values. In order to avoid Zn contamination, all tubes and utensils were either soaked in HNO_3 (25%, w/w) for 16 h, or were known from previous studies to be Zn-free. The average results obtained for Fe content were 598 ± 24 µg/L (Level I) and 1529 ± 55 µg/L (Level II) which was in agreement with the certified values.

4.3.6 Statistical Analysis

The distribution of data was tested using the Shapiro-Wilk's test for normality. The non-parametric statistical methods were applied for data that were not normally distributed.

The association of Zn and Fe intakes and status with biochemical parameters were established by Spearman rank correlation. The effect of demographic and socio-economic factors on Fe and Zn intakes and status was analyzed using Kruskal-Wallis test for factors that were divided in more than two categories and Wilcoxon test for independent samples (Wilcoxon rank-sum test), for factors with two categories. Data are presented as means and standard deviations and results are considered significant at $p<0.05$. All analyses were performed using R software package [39].

4.4 Results

4.4.1 General Characteristics of Study Participants

4.4.1.1 Macronutrients, Fe and Zn Intakes of Study Participants

The study sample included 754 participants, 128 males (17%) and 626 (83%) females. **Table 4.1** shows mean intakes of macronutrients, Fe and Zn together with their percentile distributions to the total energy (TE) intake.

Table 4.1 Macronutrient, Fe & Zn intakes of apparently healthy Serbian adults, June 2013-January 2015

	Males (25-65 years, n = 128)		Females (25-65 years, n = 626)	
Macronutrients	Mean (SD)	% TE	Mean (SD)	% TE
Energy (kcal/d)	2135.9 (730.1)		1705.3 (430.7)	
Proteins (g/d)	83.4 (26.0)	16.5 (4.2)	64.3 (17.7)	15.7 (3.5)
Total carbohydrates (g/d)	207.7 (99.0)	39.1 (10.1)	183.5 (55.4)	44.5 (7.5)
Total fats (g/d)	94.8 (36.6)	40.9 (8.4)	69.6 (24.3)	37.4 (7.1)
Minerals	Mean (SD)		Mean (SD)	
Zinc (mg/d)	9.1 (4.6)		7.3 (3.8)	
			(< 50 years, n = 501)	(>= 50 years, n = 125)
Iron (mg/d)	11.6 (5.4)		9.4 (4)	10.0 (5.7)

TE: Total energy intake; mg/d: milligram per day; g/d: gram per day.

Energy, protein and mineral crude intakes were significantly higher in men than in women (p=0.001). The under-reporting of food consumption was estimated to occur in 34% (260/754) of our study participants. The mean dietary Zn intake was 9.1 mg/day for males and 7.3 mg/day for females, while mean dietary Fe intake was 11.6 mg/day for males and 9.7 mg/day for females. Zn intake correlated with energy (r=0.45), protein (r=0.64), carbohydrate (r=0.32) and Fe (r=0.57) intakes (all p<0.001).

Energy, protein, carbohydrates, Fe and Zn intakes were adequate according to the Dietary References Intakes by age and sex of the adult population [40]. However, the percentage of energy coming from fats was higher than the recommended amount, 40.9% and 37.4% of energy for males and females, respectively, compared with the recommended 20-35% [40].

4.4.1.2 Percentage of Population with Fe and Zn Intakes Below Dietary Recommendations

The percentage of the study population with low Zn intake was greater than the percentage with low Fe intake. After taking under-reporting into account, we estimated that 5% of the study population was at risk of inadequate Fe and 15-25% of inadequate Zn intakes. Females were at much higher risk of inadequate Fe, while males were at higher risk of inadequate Zn intakes. Females below 50 years of age are at the highest risk of inadequate Fe intakes. There were significant differences in the level of inadequacy observed using dietary recommendations proposed by different expert groups (**Tables 4.2 and 4.3**).

Table 4.2 Percentage of apparently healthy Serbian adults with Fe intakes bellow the recommendations, June 2013-January 2015

	Males (25-65 years, n = 128)		Females (< 50 years, n = 501)		Females (>= 50 years, n = 125)	
	Fe RNI (mg/d)	% below RNI	Fe RNI (mg/d)	% below RNI	Fe RNI (mg/d)	% below RNI
FAO/WHO[53]	9.1	39.8	19.6	97.8	7.5	32.8
IOM[33]	8	23.4	18	96.6	8	36.7
IOM (EAR)[33]	6	7.03	8.1	38.3	5	11.8
NNR[52]	9	35.9	15	94.6	9	50

EAR: Estimated Average Requirement - how much of a nutrient meets the needs of 50% of the healthy subjects of a specific population's group; RNI: Recommended Nutrient Intake - the daily intake that meets the nutrient requirements of almost all (97.5%) apparently healthy individuals in an age and sex-specific population group; WHO: World Health Organization; IOM: Institute of Medicine; NNR: Nordic Nutrition Recommendation; mg/d: milligram per day.

Table 4.3 Percentage of apparently healthy Serbian adults with Zn intakes bellow recommendations, June 2013-January 2015

	Males (25-65 years, n = 128)		Females (25-65 years, n = 626)	
	Zn RNI (mg/d)	% below RNI	Zn RNI (mg/d)	% below RNI
FAO/WHO[53]	4.2	10.2	3	3.7
IOM[33]	11	73.4	8	69.4
IOM (EAR)[33]	9.4	60.2	6.8	50.6
NNR[52]	9.0	58.6	7	53.8
IZiNCG[92]	10	66.4	6	38.9

EAR: Estimated Average Requirement - how much of a nutrient meets the needs of 50% of the healthy subjects of a specific population's group; RNI: Recommended Nutrient Intake - the daily intake that meets the nutrient requirements of almost all (97.5%) apparently healthy individuals in an age and sex-specific population group. WHO: World Health Organization; IOM: Institute of Medicine; NNR: Nordic Nutrition Recommendation; IZiNCG: International Zinc Nutrition Consultative Group; mg/d: milligram per day.

4.4.1.3 Anthropometric and Socio-Economic Characteristics of Study Participants. Biochemical Data

Main anthropometric and demographic characteristics of study participants are presented in **Tables 4.4 and 4.5.**

Anthropometric parameters measured in this study fall within the reference ranges for healthy adult population. The average BMI of our study population was 26.8 kg/m^2 for males and 24.0 kg/m^2 for females. 30% of males and 70% of female participants in our study (**Table 4.4**) had BMI values in the normal range (n = 476), while about 30% of participants were overweight (n=277) and 10% were obese. An assessment of the marital status and education showed that 50% of participants were married and 34% were tertiary educated (**Table 4.4**). Of the 754 participants included in the study, biochemical data were obtained for 23% of our study population (**Tables 4.5 and 4.6**). Different biochemical and hematological parameters were assessed (twenty different indicators) with only parameters related to Fe status being presented (**Table 4.5**).

Table 4.4 Anthropometric measurements of apparently healthy Serbian adults, June 2013-January 2015

	Males (n = 128)	Females (n = 626)
Mean weight, kg (SD)	88.9 (14.0)	68.0 (11.8)
Mean height, cm (SD)	181.8 (0.1)	168.4 (0.1)
Mean BMI, kg/m2 (SD)	26.8 (3.5)	24.0 (4)
Waist circumference, cm (SD)	95.4 (16.9)	78.5 (14.3)
Nutritional status (by BMI)	%	%
Underweight (< 18.5)	0.8	2.1
Normal range (18,5-24.9)	30.5	69.2
Overweight (>= 25)	68.8	28.8
Pre-obese (25-29.9)	50.8	20.0
Obese class 1 (30-34.9)	18.0	6.5
Obese class 2 (35-39.9)	0.0	1.8
Obese class 3 (>= 40)	0.0	0.5
Obesity comorbidity risk (by WC)		
Level 1 (female - WC > 80 cm; male - WC > 94 cm)	57.0	32.9
Level 2 (female - WC > 88 cm; male - WC > 102 cm)	32.0	19.0

WC: Waist Circumference

Table 4.5 Effect of age, gender, educational level, obesity, marital status, SES status and Fe and Zn intake adequacy on mean daily crude Fe & Zn dietary intakes in apparently healthy Serbian adults, June 2013 - January 2015

		Dietary intake (mg/day)	
	N (%)	Fe (mg/d)	Zn (mg/d)
Gender			
Male	128 (17)	11.6 ± 5.4***	9.1 ± 4.6***
Female	626 (83)	9.5 ± 4.4	7.3 ± 3.8
Age			
20-29	231 (31)	9.5 ± 3.5	7.5 ± 2.9
30-39	197 (26)	10.0 ± 5.2	7.4 ± 3.3
40-49	154 (20)	9.7 ± 4.4	7.8 ± 4.5
50-59	118 (16)	10.4 ± 5.5	8.2 ± 5.9
60-69	54 (7)	10.2 ± 5.3	6.9 ± 3.5
Obesity			
Obese (BMI > 30)	79 (10)	10.4 ± 4.7*	8.2 ± 4.3
Non-obese (BMI < 30)	675 (90)	9.8 ± 3.9	7.6 ± 3.9
Iron intake adequacy			
Adequate	538 (72)	11.3 ± 4.7***	8.4 ± 4.3***
Inadequate	215 (28)	6.3 ± 1.4	5.5 ± 1.9
Zinc intake adequacy			
Adequate	360 (48)	11.5 ± 5.2***	10.1 ± 4.3***
Inadequate	393 (52)	8.3 ± 3.4	5.3 ± 1.5
SES status			
Low income	247 (33)	10.5 ± 5.5*	7.5 ± 3.4
Affluent	507 (67)	9.5 ± 4.1	7.7 ± 4.2
Education			
Primary	58 (8)	10.5 ± 5.4	7.2 ± 3.4
Secondary	443 (59)	9.7 ± 4.7	7.3 ± 3.0
Tertiary	253 (34)	9.9 ± 4.2	8.2 ± 5.3
Marital status			
Married	383 (51)	9.9 ± 4.7	7.7 ± 4.7
Single	203 (27)	9.7 ± 4.1	7.5 ± 2.9
Other	168 (22)	10.1 ± 6.2	7.3 ± 3.3

Kruskal Wallis (for > 2 factors) and Wilcoxon (for 2 factors categorization) test *** p<0.001; * p<0.05 - between factor comparison.

mg/d: milligram per day.

Table 4.6 Effect of age, gender, educational level, obesity, marital status, SES status and Fe and Zn intake adequacy on biochemical parameters and plasma concentrations of Fe and Zn in apparently healthy Serbian adults, June 2013-January 2015

		Biochemical parameters			Plasma concentrations	
	N (%)	HCT (L/L)	Hb (g/L)	RBC (10^{12}/L)	Fe (mg/dL)	Zn (mg/dL)
Gender						
Male	31 (38)	0.4 ± 0.04***	150.6 ± 10.9***	5.1 ± 0.4***	1.3 ± 0.4*	1.1 ± 0.2*
Female	51 (62)	0.4 ± 0.03	127.1 ± 11.4	4.5 ± 0.4	1.3 ± 0.9	1.0 ± 0.2
Age						
20-29	3 (4)	0.4 ± 0.03	131.3 ± 9.1	4.3 ± 0.5	1.6 ± 0.6	1.0 ± 0.1
30-39	28 (34)	0.4 ± 0.10	141.6 ± 18.1	4.9 ± 0.6	1.1 ± 0.5	1.1 ± 0.2
40-49	38 (46)	0.4 ± 0.03	133.7 ± 13.1	4.7 ± 0.4	1.3 ± 0.7	1.1 ± 0.2
50-59	13 (16)	0.4 ± 0.05	131.8 ± 18.2	4.5 ± 0.6	1.6 ± 1.1	1.0 ± 0.1
Obesity						
Obese (BMI > 30)	26 (32)	0.4 ± 0.04	138.5 ± 17.8	4.8 ± 0.6	1.3 ± 0.4	1.1 ± 0.2
Non-obese (BMI < 30)	56 (68)	0.4 ± 0.05	134.9 ± 15.1	4.7 ± 0.5	1.2 ± 0.8	1.1 ± 0.2
Iron intake adequacy						
Adequate	70 (85)	0.4 ± 0.04*	137.5 ± 16.6*	4.8 ± 0.5	1.3 ± 0.6	1.1 ± 0.2
Inadequate	12 (15)	0.4 ± 0.03	127.4 ± 7.3	4.5 ± 0.4	1.3 ± 1.3	1.1 ± 0.2
Zinc intake adequacy						
Adequate	52 (63)	0.4 ± 0.05*	137.1 ± 16.8*	4.8 ± 0.5	1.3 ± 0.6	1.0 ± 0.2
Inadequate	30 (37)	0.4 ± 0.04	134.2 ± 14.7	4.6 ± 0.5	1.3 ± 0.9	1.1 ± 0.2
SES status						
Low income	0	-	-	-	-	-
Affluent	82 (100)	0.4 ± 0.04	136.0 ± 16.0	4.7 ± 0.5	1.3 ± 0.7	1.1 ± 0.2
Education						
Primary	1 (1)	0.4	125	4.5	0.6	1.1
Secondary	17 (21)	0.4 ± 0.04	132.6 ± 13.7	4.7 ± 0.4	1.3 ± 0.6	1.1 ± 0.2
Tertiary	64 (78)	0.4 ± 0.05	137.1 ± 16.6	4.7 ± 0.5	1.3 ± 0.7	1.1 ± 0.2
Marital status						
Married	55 (67)	0.4 ± 0.04	134.6 ± 16.5	4.7 ± 0.5	1.3 ± 0.8	1.1 ± 0.2
Single	18 (22)	0.4 ± 0.05	138.7 ± 16.9	4.8 ± 0.5	1.3 ± 0.5	1.0 ± 0.2
Other	9 (11)	0.4 ± 0.03	139.6 ± 9.9	4.9 ± 0.4	1.0 ± 0.3	1.1 ± 0.2

Between - factor comparison (using the Kruskal-Wallis test for factors with more than two categories and the Wilcoxon test for factors with two categories): *p<0.05, *** p<0.001. HCT: Hematocrit; Hb: Hemoglobin; RBC: Red blood cells; Fe: Plasma Fe and Zn concentration determined by flame atomic spectometry analysis.

4.4.2 Dietary Intake of Zn and Fe

4.4.2.1 Different Food Groups in Relation to Fe and Zn Intake

Grains (mainly wheat, rye and rice) were the most important source of dietary Fe. The proportion of total dietary Fe coming from grains was about 30% for both genders. Other important sources of Fe were meats (17%) and vegetables (mainly beans and legumes, 16-21%, **Table 4.7**). Similarly, red meats were the most essential contributor to dietary Zn intake in this study group (30% of total Zn intake), followed by grains and grain products and vegetables, with a contribution of 17% and 12%, respectively. Good sources of Zn were also milk and milk products, where more than 20% of the Zn intake was coming from products such as cheese and yoghurt (**Table 4.7**). The intakes of both Fe and Zn were correlated with the intakes of certain food groups.

Table 4.7 Contribution (%) of the main food groups to the total Zn and Fe intakes of apparently healthy Serbian adults, June 2013-January 2015

	Iron			Zinc		
	Males	Females	Total	Males	Females	Total
Meat and meat products	17.9	16.7	17.2	35.2	29.2	30.6
Milk and milk products	1.9	2.4	2.3	20.0	22.6	22.2
Grains and grain products	30.1	32.8	32.6	13.9	17.3	16.6
Vegetable and vegetable products	14.9	11.4	12.3	11.2	7.7	8.3
Legumes	6.1	4.8	5.1	3.6	2.9	3.1
Egg and egg products	5.1	3.5	3.8	4.9	3.9	4.1
Nuts, seeds, kernel products	8.4	9.6	9.4	3.1	3.5	3.3
Beverage (non-milk)	4.2	4.9	4.8	2.0	3.2	3.0
Seafood and related products	2.4	2.6	2.6	1.6	3.1	2.7
Fruit and fruit products	3.4	4.6	4.4	1.7	2.4	2.2
Sugar and sugar products	3.3	3.9	2.6	1.9	2.1	2.1
Miscellaneous food products	1.9	2.6	2.5	1.0	2.0	1.8
Fats and oils	0.3	0.3	0.3	0.0	0.1	0.1

Table 4.8 Top ten foods contributing to Fe and Zn intake (both genders)

Food name	Average amount (g)	Fe (mg/100g)	Fe (mg/d)	Food name	Average amount (g)	Zn (mg/100g)	Zn (mg/d)
Bread white	60.6	1.5	0.91	**Cheese, low fat**	2.8	12.3	0.35
Herbal tea- hibiscus	5.5	8.6	0.47	**Yoghurt 2.8% mf**	61.0	0.5	0.32
Beans	5.0	5.5	0.28	**Roasted meat**	0.8	30.3	0.24
Egg, whole	13.3	2.0	0.27	**Egg, whole**	13.3	1.4	0.19
Pork, liver	0.6	29	0.18	**Pork steak, fried**	3.9	4.6	0.18
Potatoes	25.3	0.7	0.18	**Potatoes**	25.3	0.6	0.15
Potato peeled	24.1	0.7	0.17	**Yoghurt 1.2% mf**	16.8	0.9	0.15
Apple, raw	54.5	0.3	0.16	**Beef**	3.0	5.0	0.15
Phyllo	10.9	1.4	0.16	**Pork, leg, raw**	6.0	2.4	0.15
Coffee beans	4.1	3.9	0.16	**Beans**	5.0	2.7	0.14

The most noteworthy correlations among various food groups were those for grain products (r=0.43 for Fe and r=0.28 for Zn, both p<0.001), vegetables (r=0.33 for Fe and r=0.22 for Zn, both P<0.001) and meat (r=0.25 for Fe and r=0.41 for Zn, both p<0.001). The intakes of Fe and Zn were correlated with the energy intake (r=0.56 and r=0.45, respectively, both p<0.001).

4.4.2.2 Contribution of Grains and Pulses to the Total Fe and Zn Intake

Phytate in the diet comes mainly from cereal grains, grain products and legumes. White bread and wheat flour are two of the most consumed cereal foods in the Serbian diet and beans and peas are the most commonly used legumes by our study participants (**Tables 4.9 and 4.10** for the top ten foods from the cereal and legumes food groups contributing to Zn and Fe intakes).

The negative effect of phytic acid on Zn absorption follows a dose-dependent response, and the molar ratio of phytate to Zn of a diet can be used to predict the proportion of absorbable dietary Zn [41].

Table 4.9 Grains and grain products: top ten foods contributing to Fe & Zn intake

Food name	Average amount (g)	Fe (mg/100g)	Fe (mg/d)	Food name	Average amount (g)	Zn (mg/100g)	Zn (mg/d)
Bread white	60.61	1.5	0.90	Wheat flour	11.5	0.7	0.08
Wheat flour	11.54	0.9	0.10	Phyllo	10.9	0.6	0.06
Phyllo	10.91	1.5	0.16	Macaroni, spaghetti boiled	10.7	0.8	0.08
Puff pastry	10.41	0.8	0.08	Puff pastry	10.4	0.4	0.04
White wheat roll	10.32	0.9	0.09	White wheat roll	10.3	0.7	0.07
Bread, wholemeal	6.62	2.1	0.13	Bread, wholemeal	6.6	1.5	0.10
Plazma biscuit	4.36	3.3	0.14	Rice, polished	4.7	1.7	0.08
Musli, cereal	4.34	3.3	0.14	Musli, cereal	4.3	2.5	0.11
Noodles, enriched	2.70	4.0	0.10	Noodles, enriched	2.7	1.9	0.05
Lady finger	2.13	3.6	0.07	Pop corn	1.8	2.5	0.04

Table 4.10 Legumes: Top ten foods contributing to Fe & Zn intake

Food name	Average amount (g)	Fe (mg/100g)	Fe (mg/d)	Zn (mg/100g)	Zn (mg/d)
Beans	5.02	5.5	0.27	2.8	0.14
String beans, green, fried	4.87	1.0	0.04	0.3	0.02
Peas, green, frozen	4.07	1.4	0.05	0.9	0.04
Peas, green	2.89	1.8	0.05	0.8	0.02
String beans, raw	2.39	1.0	0.02	0.6	0.01
String beans, canned	0.29	0.5	0.01	0.2	0.00
Lentils, brown, dried	0.23	11.1	0.03	3.9	0.00
Soybean	0.04	15.7	0.01	1.0	0.00
Chick pea, dry	0.01	4.94	0.01	3.5	0.00

Table 4.11 Bioavailability assessment

	Males	Females
Total energy, kcal	2131.0	1716.5
Energy by protein from fish and meat, kcal (% TE)	149.3 (7% TE)	99.6 (5.8% TE)
Energy by grains & nuts & pulses, kcal (% TE)	698.6 (32.8% TE)	634.4 (36.9% TE)
Fe bioavailability	15%	15%
Zn bioavailability	High	High

TE: Total energy; Kcal: Kilocalories. The method by Jati et al. (2014) [42] was applied.

Due to inadequate dietary data on the phytate content of foods in Serbian databases, we were not able to calculate phyate:Zn molar ratios. However, we employed the method by Jati et al. [42] to determine the bioavailability (**Table 4.11**) and confirmed that there is a high bioavailability of both minerals in participants' diets.

4.4.3 Correlation Between Zn and Fe Intake and Status and Fe Biochemical Parameters

Mean plasma Zn and Fe status of our study population appeared adequate in spite of the lower dietary intake of these minerals (**Table 4.5**).

Intakes of Fe and Zn were strongly correlated (r=0.57, p=0.001), while the correlations between biochemical parameters and Fe and Zn dietary intakes were weak. The plasma Zn concentration did not reflect Zn intake (p=0.81). Women below 50 years of age are the most vulnerable group for development of Fe deficiencies due to increased requirements (menstrual bleeding) an increased need for Fe, and also very often poor intake and poor bioabsorption of Fe. Out of 142 woman for whom biochemical data were available, around 23% had lower Hb concentrations (<120 g/L). There was a statistically significant difference in the intake of Zn between the groups of women with different Hb concentrations (p=0.01, **Table 4.12**) with no variation in any other biochemical or anthropometrical indicators measured.

Table 4.12 Dissimilarities in various parameters among apparently healthy Serbian women with low and high Hb concentration, June 2013-January 2015

	Females (n=142)	Low Hb (n=33)	High Hb (n=108)	p value
Age (years)	23.3 (0.8)	30.2 (10.5)	23.0 (0.8)	
Weight (kg)	60.3 (5.5)	67.2 (10.3)	61.0 (5.0)	
Height (cm)	169.9 (4.4)	169.5 (6.0)	170.7 (4.1)	
BMI (kg/m^2)	20.9 (1.4)	23.4 (3.8)	20.9 (1.3)	
Waist circumference (cm)	72.0 (4.4)	76.4 (9.8)	72.8 (4.4)	
Hemoglobin (g/L)	130.4 (10.7)	112.4 (7.8)	135.0 (8.1)	
Hematocrit (L/L)	0.4 (0.0)	0.4 (0.0)	0.4 (0.0)	
Erytrocyte count (x 10^{12}/L)	4.4 (0.3)	4.2 (0.3)	4.5 (0.3)	
Fe (µmol/L)	15.8 (4.8)	12.5 (6.1)	16.5 (4.5)	
Diet Fe (mg)	9.6 (5.1)	10.2 (4.1)	9.5 (4.5)	0.04
Diet Zn (mg)	7.1 (2.2)	9.0 (5.5)	7.3 (2.2)	0.01

BMI: Body mass index; Low Hb: Hemoglobin values below 120 g/L. Values presented are means ± SD (standard deviations). Dietary Fe and Zn data wer obtained from 24 h recalls. Only significant p values are given.

4.4.4 The Effect of Lifestyle Factors on Fe and Zn Intake and Status

Plasma Zn levels ranged from 0.72 to 1.67 mg/dL in males and from 0.78 to 1.64 mg/dL in females. Mean plasma concentrations of Fe and Zn were different between males and females ($p<0.05$ and $p=0.02$, respectively). Likewise, the Fe and Zn dietary intakes were gender-dependent. Generally, mean dietary intake levels of Fe and Zn were significantly higher in males compared with females, being approximately 20% higher in males. Similarly, Hb concentration and plasma concentrations of Fe and Zn were higher in males than in females (**Table 4.6**). We did not observe any differences in the Zn and Fe intake or status data within different age groups ($p=0.16$).

Fe intake was related to BMI ($p<0.05$), while Zn intake was not different between obese and non-obese individuals ($p=0.07$). No correlation was seen between BMI and Fe and Zn status ($p=0.44$ and $p=0.25$, respectively). We estimated the degree of association between biochemical parameters (plasma Fe, Hb, red blood cells count and plasma Zn), energy and protein intake and lifestyle factors, and did not detect any significant associations.

No association was found between dietary Fe and Zn intake with education level or marital status (**Table 4.5**). Similarly, components of socio-economic status (SES) measured in this study showed no correlation with any of the biochemistry-related parameters, including Fe and Zn status. However, there was a statistically significant difference in Fe intake between the low-income and affluent participants ($p=0.03$), while no such difference was observed for Zn intake ($p=0.08$). Differences were also observed in the intake of Fe between the affluent and low-income female groups ($p=0.01$; **Table 4.5**). Finally, there was a statistically significant difference in Hb concentrations between the groups of women with Zn adequate and Zn inadequate diets.

4.5 Discussion

The results of this study demonstrate that inadequate intake of Zn was present in this healthy population cohort. There was no strong relation between dietary Zn intake and Fe biochemical parameters. However, there was a statistically significant difference in Zn intake among groups of women with dissimilar Hb concentrations. Zn and Fe dietary intakes were strongly correlated. Grains and meat were identified as major sources of dietary Fe intake and meat and dairy products as main foods consumed contributing to Zn intake. Generally, no differences were seen for Fe and Zn intakes and status among various SES groups, except for Fe intake between those on low income and affluent. The plasma Zn and Fe concentrations were within the reference ranges despite the insufficient dietary intakes of these minerals. However, a prolonged inadequate intake of Zn and Fe may contribute to the development/manifestation of Zn and/or Fe deficiencies, so regular monitoring of mineral intake and status in this population cohort is necessary.

The mean values for Fe and Zn intake of our study population were similar to the values of these elements reported for the adult population in other developed countries: Hungary, Croatia, Eastern and Central European countries, Australia, Canada [29,43-45]. A recent review of available micronutrient intake and status data in Europe [29] showed that Fe and Zn intake data for a number of countries are very limited. More current measurements of adult Fe and Zn intake and status data are rare [46]. In this sense, results presented in the current study can be considered a valuable resource.

The intakes of Fe and Zn were different between males and females, and there was a strong correlation between Zn and Fe intake, which is in agreement with previous reports [47-49]. There was also a positive correlation between mineral intakes and energy and this relationship has been highlighted by others [28,50]. The EAR cut-point method was used to assess the adequacy of Fe and Zn intakes. The EAR cut-point method is identified as an appropriate dietary reference intake to use when assessing the adequacy of group intakes and has been adopted for assessing nutrient inadequacy [51]. In addition, we also compared the intake data with other available dietary recommendations [52,53] and showed that the risk of inadequate intakes was very different. The fact that the method employed can affect the estimation of nutrient intake inadequacy [53,54] has been confirmed again. The Nordic and FAO/WHO recommendations overestimate the true prevalence of inadequate intake compared with the EAR cut-point method. Still, it must be noted that while the intakes below the recommendation are not synonymous with deficiency, they certainly indicate increased risk of deficiency [54].

The estimation and adjustment of bias (such as under- or over-reporting of food intake) is a relatively unexplored field. An evaluation of the effect of misreporting on energy and nutrient intake assessment indicated that low energy reporters had lower mean intakes for several micronutrients (from 25% to 36%) compared with non-under-reporters [55]. Likely under-reporting of food consumption was estimated to occur in 34% (260/754) of the study participants. Of our study population, 28% had low Fe intake and 52% had Zn intake below current dietary recommendations [33]. Taking under-reporting into account, it is estimated that 5% of the study population had inadequate Fe and 15-25% had unsatisfactory Zn intake. Sixty-five percent of our study population were women within the age range of 20-50 years. Women in this age group are known as the most vulnerable group for development of Fe and Zn deficiencies because they are more likely to have excessive menstrual bleeding, infrequently consume sources of bioavailable Zn and Fe, rarely consume rich sources of enhancers and often consume rich sources of inhibitors of Zn and Fe absorption [56]. We found that women with dietary Fe intakes below recommendations (EAR of 8.1 mg/d) were at greater risk of inadequate dietary Zn intakes: 38% of women in the present study had inadequate dietary Fe intake and 76% of these with low dietary Fe intakes show concurrent insufficient intake of Zn.

In many developed countries, micronutrient deficiencies are not related to the quantity of food consumed, but rather to the quality of the diet [57]. Inadequate dietary intakes of Zn and Fe that failed to meet the high physiological demands of adolescent girls in Australia and New Zealand were shown to be related to food choices [3].

Relevant to these findings is the observation in fifty-two non-pregnant premenopausal women from Seattle, Washington, USA, whose diets provided similar amounts of Fe, where the consumption of red meat five times per week was more efficacious for body Fe stores than the consumption of lacto-ovo vegetarian foods, or the flesh of chicken and fish [58]. In further support of this are the findings of the National Food Survey of the British population [59] showing that intake and red meat consumption have declined during the past three decades, leading to low Fe stores of females aged 11-64 years.

Being overweight is a worldwide problem that affects developed and developing countries similarly[37,40]. Approximately 30% of our study population was overweight and 10% was obese. Other European countries have shown a similar percentage of overweight and obesity [37]. However, even with excess dietary intake of energy and macronutrients, it is still uncertain if people are taking the recommended intakes of micronutrients. For instance, a number of national epidemiological surveys conducted in several developed countries have reported the co-occurrence of obesity with inadequate intakes of certain vitamins and minerals, particularly Ca, Fe, Zn, thiamine, riboflavine, vitamins B6 and D and folate [15,60,61].
In the present study we found higher intake of Fe in the obese population than in non-obese individuals. A possible explanation for the higher intakes of Fe in obese people is that their energy intakes were higher because of the greater consumption of fatty (energy-dense) foods, (i.e. meats) and refined grain products (white bread is a staple food). The correlations of energy intake, total grain products and meat with Fe intake support this explanation. However, not only inappropriate intakes, but also impaired bioavailability and utilization of micronutrients may be involved in the inadequate micronutrient status in obesity.
The low-level inflammation that accompanies obesity is likely to decrease Fe absorption [62]. This needs to be taken into account when evaluating the adequacy of Fe intake in a population.

Zn and Fe nutrition are often associated. Red meat and grain products are the most important common dietary source of Fe and Zn. Our findings confirm observations that food sources of Zn are also important food sources of Fe, as well as supports the theory that usual low intakes of either mineral increases the likelihood of low intakes of the other [3,17,18]. Furthermore, other factors influence the daily intakes of Zn and Fe: their concentration in food, the amount of food consumed, and the consumption of dietary components that delay Fe and Zn absorption [23]. Zn and Fe are most bioavailable from many of the same foods and their absorption is repressed by many of the same dietary substances [56]. Although we were not able to estimate the exact phytate:Zn molar ratio of the diets, by employing the method of Jati et al. [42] we showed that there was a high bioavailability of both nutrients in the diets of people we studied.

Food composition database needs to be updated because, as in many other databases, our data did not contain information on the content of phytate. Food preparation and processing methods can be used to reduce the phytate content of foods based on cereals and legumes. These methods are based on the enzymatic hydrolysis of phytic acid to lower inositol phosphates that are induced by germination and fermentation[26]. Soaking, followed by empting out of the soaking water, can also be used to reduce the phytate content of cereal and legume flours by passive diffusion of the water-soluble sodium, potassium and magnesium phytates [63]. Household-level information was obtained for people where cereals and legumes contributed a large proportion to total Fe and Zn intakes. Almost all of our participants (97%) employed preparation techniques that are known to reduce phytate content of foods and increase Zn bioavailability (i.e. soaking of beans, fermentation of bread dough).

Overall, the plasma concentrations of Fe and Zn in the present study were within the expected normal ranges for adult population and similar to reported levels of these elements in healthy individuals elsewhere [64,65]. Similarly, the mean values of Fe-related biochemical parameters were within the expected ranges for a healthy population of subjects [64,66,67]. Mean plasma levels of both elements were significantly different between males and females. Obesity, age, education and marital status did not contribute to the differences in plasma levels of the studied minerals. These findings are in agreement with the results of Sian et al. (1996) [68], Baily et al. (1997) [69] and Sanchez et al. (2009) [28].

Our observation of a strong association between usual Fe and Zn intakes was not accompanied by a similar relation between Fe and Zn status. The lack of association may be due to several factors.
First, the inter-individual variation in biological availability of dietary Fe and Zn is determined by the degree of intestinal absorption that depends on the composition of meals; the absorption from two different meals with similar Fe/Zn content may vary considerably due to the interaction between dietary Fe/Zn and absorption enhancers and inhibitors [70,71]. It could also be that good homeostatic regulation of Zn contributes to these dissimilarities [44,72]. Second, the calculation of reported dietary intake is performed using food composition tables (calculated and chemically measured content of the diet may be different). Finally, the reported intake of dietary Zn and Fe may deviate to some degree from the true intake due to diet reporting error. In the present study we attempted to correct for some of these discrepancies by taking under-reporting into account; however, we need to acknowledge that a certain level of error may still be present.

The correlation of Zn intake and Zn status with Fe biochemical parameters in healthy populations has not been investigated widely. Only a few studies have been initiated and provide inconsistent results [28,73,74]. Various studies in Fe- and Zn- deficient populations have shown a positive link between Zn intakes and Fe status indicators [6,27]. In our healthy population cohort we did not find any significant correlations, most likely due to having a small number of people with inadequate Fe status. Still, it was evident that Hb concentration was statistically different between women with appropriate and insufficient Zn intakes.
Further work may be needed to examine the role of dietary Zn on Fe status indicators more fully. Measurements of ferritin, transferrin and total Fe-binding capacity may provide more detailed analysis of this relationship as low Hb, which was used in this study, is not the best indicator of Fe status and it is mainly apparent during the late stages of Fe deficiency. In addition, as a number of studies over the years have demonstrated the positive link between Zn intake and Fe status and the role of Zn in Fe absorption process [21,27,73,75], further work in this area should aim to elucidate the specific point in Fe deficiency progression when inadequate Zn intake is beginning to have its role.

As shown by many, SES, conventionally measured by education, occupation or income, can contribute to inequalities in health [76,77]. Those with limited incomes or lower educational levels are more likely to have poor quality diets [81]. Energy-dense and nutrient-poor diets are cheap and usually consumed by those with limited budgets [77]. A limited number of studies has shown inconsistent results on the variation in Fe and Zn intake between SES groups. Higher (2%-5%) Zn levels were found in low-SES groups [28,79], while others found no differences among the groups [80]. Similarly, higher values of plasma Fe have been reported in high-SES groups [81,82], while again there are studies that show no differences in Fe intake or status among various SES groups [83].
In the current study we looked at the association between marital status, educational level and occupation with Fe and Zn intakes and status, and found no differences in intake or status between SES groups.
The only difference was seen in the intake of Fe between the affluent and those on poor income, which is most likely due to the higher consumption of grain products and pulses (staple foods) by people in the low-income group.

The findings of the current study should be interpreted in view of its strengths and limitations.
The strength of our study is that we investigated a homogenous population with similar demographic and genetic background. In this way, we were able to look at the influence of nutrition on Fe and Zn intake and status and their relationship in the healthy adult population.

Another strong point of our work is the collection of dietary data. We administered three 24 h recall questionnaires, on three non-consecutive days, which is the gold standard method for assessing the adequacy of Zn and Fe intakes [84].

We identify a few limitations of the present study. Plasma (serum) zinc is a good indicator of Zn deficiency; however, plasma Zn levels do not reflect marginal status and may not reflect dietary intakes [85]. Plasma Zn is insensitive to Zn intakes within this range [71,86], and the Institute of Medicine ruled out plasma Zn as a useful status indicator for evaluating human Zn requirements for Canada and the USA [33]. It is also accepted that plasma Zn concentration is a valid indicator of whole-body Zn status in the absence of confounding factors, such as infection or stress [87]. Nevertheless, with all its limitations, it is the best biomarker currently available. Studies have pointed out that the ratio of blood fatty acids (linoleic acid:dihomo-γ-linolenic acid) may be a more appropriate biomarker of Zn status that is able to differentiate between various Zn deficiency/adequacy states [88,89]; however, additional research is needed to fully determine the efficacy of this biomarker. Another weakness of this study was the inability to calculate the phytate:Zn molar ratios due to the lack of available data on phytate content of foods in our database. However, we believe that this was overcome by estimating the bioavailability of Fe and Zn using the method by Jati et al. (2014) [42].

In countries where fresh vegetables, fruits and proteins are freely available and well embodied in the average diet, as is the case in many European countries, nutritional deficiencies of Fe and Zn are expected to be rare. However, easy access to food does not ensure healthy food choices required to achieve adequate nutrition [63]. Similarly, lifestyle changes occurring over the last few decades in many developed countries are characterized by increased consumption of low-cost, but energy-dense foods and by reduced physical activity levels. These lifestyle changes are the major factors implicated in the aetiology of the current obesity epidemic [90]. It is well known that an unbalanced diet can contribute to micronutrient malnutrition [91,92]. Dietary decisions made by individuals may lead to Fe and Zn deficiencies. Finally, with the present global trend of people in developed countries to decrease meat intake and increase intake of grains, there is a tendency that more individuals may end up having inadequate intakes of these important minerals.

In conclusion, considering that more than 20% of our study population had inadequate dietary intake of Zn and bearing in mind the health consequences of prolonged inappropriate Zn intake on both Zn and Fe nutritional status, more regular follow-ups are needed to monitor the intake and status of these nutrients and to make sure that potential deficiencies do get recognized and addressed in a timely manner.

Acknowledgments: The authors gratefully thank all volunteers who participated in the study for their cooperation, time and motivation. **Financial support:** This work was supported by a grant from Ministry of Education and Science of the Republic of Serbia (Grant III 41030) and EU FP7 project BACCHUS (Grant agreement no. 312090) and an Australian Postgraduate Award Scholarship, provided to M.K. The financing organizations had no role in the design, analysis or writing of this article.

Ethics of human subject participation: All subjects went through the informed consent process, both verbal and written. Verbal consent was witnessed and formally recorded. The study protocols were approved by the Clinical Hospital Centre Zemun, Belgrade, Serbia, Ethics Committee Approval, No: 2125, 2013; and by SAC HREC EC00188 (96.15), Adelaide, South Australia. The protocols and procedures were in agreement with the ethical guidelines on biomedical research on human subjects of The Code of Ethics of the World Medical Association's Declaration of Helsinki (1964) and its further amendments.

Authorship: MK: designed and conducted part of the research, analyzed data, wrote the manuscript and had primary responsibility for the final content. JS: designed part of the research and revised the final version of the paper. MN: responsible for statistical analysis. MZ: helped with dietary data intake and analysis. MGu: proofread the paper and provided constructive advice. MGl: provided essential materials and revised the final draft the paper. All authors have read the manuscript and approved the final version of the paper.

Conflict of interest: The authors declare no conflict of interest.

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Let food be thy medicine and medicine be thy food

Hippocrates

Section 3 | Potentially New Biomarker of Zn Status

Preamble

The examination of the link between Zn and Fe dietary intake and status brought some important insights:

- *There was a strong indication from the studies (Chapter 3) that Zn may have a role to play in the Fe absorption process. The potential mechanism of the Zn and Fe interaction was further illuminated.*
- *During the course of the human trial it was noticed that while Fe status was straightforward to measure, the measurement of Zn concentrations was a bit more challenging as no single sufficiently sensitive biomarker of Zn status exists up to date.*
- *The plasma Zn, currently referred to as the most reliable biomarker of Zn status in humans, has its own well known limitations.*

At that stage of the project, it became evident that if one wants to explore the magnitude of Zn deficiency in detail, and the consequences of inadequate Zn intakes in various populations, a more accurate biomarker of Zn status is needed.
As highlighted in the literature review (Chapter 2), several biomarkers have been classified as 'emerging' biomarkers of Zn status. These include: nail Zn concentration, Zn-dependent proteins, oxidative stress, DNA integrity, and taste acuity. Most of these biomarkers have somehow already been, or were planned to be, tested in humans. At that time, the linoleic acid:dihomo-γ-linolenic acid (LA:DGLA) ratio was the only indicator that was studied solely through an animal model, and still not nominated as 'emerging', but rather, as a 'non useful' biomarker of Zn status. The initial animal study performed in Gallus gallus demonstrated that the LA:DGLA ratio was able to differentiate Zn status between Zn adequate and Zn deficient subjects, and that the ratio can be employed as an effective tool to detect an early stage of Zn deficiency. It was reasonable to hypothesize that the same relationship observed in animals may exist in humans. Could the LA:DGLA ratio be used as a sufficiently sensitive and effective biomarker of Zn status in humans?

This question turned into the next goal of the PhD project, to examine the efficacy of the LA:DGLA ratio in predicting Zn status in humans. As it was sound to implement the initial testing of the biomarker within a healthy population setting, where the effect of inflammatory and infections conditions is assumed to be insignificant, and since the number of marginally Zn deficient people has already been identified (Chapter 4), the same population cohort was used to examine the LA:DGLA ratio.

The outcomes of this study are explained in detail in the following **Chapter 5** *through the publication Knez, M.; Stangoulis, J.C.R.; Zec, M.; Debeljak-Martacic, J.; Pavlovic, Z.; Gurinovic, M.; Glibetic, M. An initial evaluation of newly proposed biomarker of zinc status in humans - linoleic acid: Dihomo-γ-linolenic acid (LA:DGLA) ratio. Clinical nutrition ESPEN, 2016, 15, 85-92. DOI: 10.1016/j.clnesp.2016.06.013.*
Additionally, the efficacy of the LA:DGLA ratio to predict Zn status of Zn deficient subjects consuming wheat based diets, a diet more representative of a diet of the target Zn-deficient populations, was evaluated in vivo by using Gallus gallus as a model. As initially hypothesized the analysis confirmed that LA:DGLA ratio responds to dietary Zn manipulations and that LA:DGLA ratio can be used as an additional biomarker of Zn status (**Chapter 7**).

Finally, after the two studies have been successfully accomplished and provided comparable outcomes there was a need to describe the LA:DGLA biomarker in more detail, to explain its molecular composition, structure and role. Furthermore, to make an up to date summary of major findings related to the efficacy of this biomarker in predicating Zn status, and to provide straightforward directions for further work in this research area. **Chapter 6**, *presented as a review paper: Knez, M.; Stangoulis, J.C.R.; Glibetic, M.; Tako, E. The Linoleic Acid: Dihomo-γ-Linolenic Acid Ratio (LA:DGLA)-An Emerging Biomarker of Zn Status. Nutrients, 2017, 9 (8), 825. DOI: 10.3390/nu9080825.*

Note: Chimhashu et al. recently provided additional evidence that Zn status may play an important role in FA desaturation and/or elongation, and that the LA:DGLA can be used as a biomarker of Zn status.
Chimhashu, T.; Malan, L.; Baumgartner, J.; Van Jaarsveld, P.; Galetti, V.; Moretti, D.;. ...Zimmermann, M. Sensitivity of fatty acid desaturation and elongation to plasma zinc concentration: A randomised controlled trial in Beninese children. *Br. J. Nutr.* **2018**, *119* (6), 610-619. doi: 10.1017/S000711451700366X.

Chapter 5

An Initial Evaluation of Newly Proposed Biomarker of Zn Status in Humans - Linoleic Acid:Dihomo-ɣ-linolenic Acid (LA:DGLA) Ratio

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DOI: 10.1016/j.clnesp.2016.06.013.

5.1 Abstract

Background: Zn is an essential micronutrient for humans with important physiological functions. A sensitive and specific biomarker for assessing Zn status is still needed. **Objective:** The major aim of this study was to examine if the changes in the content of plasma phospholipid LA, DGLA and LA:DGLA ratio can be used to efficiently predict the dietary Zn intake and plasma Zn status of humans. **Methods:** The study was performed on healthy human volunteers, 25-55 years of age. The dietary Zn intake was assessed using 24 h recall questionnaires. Plasma phospholipid fatty acid analysis was done by gas chromatography, and plasma analysis of minerals by atomic absorption spectrometry. Biochemical, anthropometrical and hematological parameters were assessed. **Results:** No significant relationship was found between the dietary and plasma Zn status (r=0.07; p=0.6). There was a statistically significant correlation between DGLA and plasma Zn (r=0.39, p=0.00). No relationship was observed between the linoleic acid and plasma Zn, while there was a significant negative correlation between LA:DGLA ratio and plasma Zn status (r=-0.35, p=0.01). Similarly, there were statistically significant difference in DGLA status (p=0.01) and LA:DGLA ratio (p=0.04) between the Zn formed groups. **Conclusions:** This study is an initial step in evaluating LA:DGLA ratio as a biomarker of Zn status in humans. The results are encouraging as they show that concentration of DGLA is decreased and LA:DGLA ratio increased in people with lower dietary Zn intake. However, additional studies are needed to fully examine the sensitivity of this biomarker.

Keywords: Zinc, fatty acid composition, LA:DGLA, healthy population, dietary Zn intake, plasma Zn status, Zn biomarker

5.2 Introduction

Zinc (Zn) is an essential nutrient for human health with many important biological functions. It plays a significant role in growth and development, cell mediated immunity, protein synthesis, skin and bone metabolism, enzyme function, gene expression, and hormonal excretion [1-3]. Deficiency of Zn may severely affect the homeostasis of a biological system and insufficient Zn intake has profound consequences at all points of the human lifecycle, from the point of conception through to old age [4]. Zn deficiency is very common, with an estimated 2 billion people worldwide being affected by dietary Zn deficiency [5]. Zn inadequacy is identified as a major contributor to the burden of disease in developing countries [6,7].

Nonetheless, a suboptimal Zn status is not easily determined due to the lack of clinical signs and reliable biochemical indicators of Zn status. It is generally accepted that there is currently no specific, reliable biomarker of Zn status [4]. Out of 32 potential biomarkers from 46 publications in humans, serum/plasma Zn concentrations, hair Zn concentration and urinary Zn excretion are the only three biomarkers identified as potentially useful [4]. However, there are still considerable reservations in terms of reliability of these biomarkers due to the effect of multiple confounders such as infection, inflammatory conditions and the time of last meal [8]. Similarly, the serum/plasma Zn biomarker is perceived as an unresponsive index of Zn nutritional status due to effective homeostatic regulation which responds to alterations in Zn intake, up-regulating absorption and conserving losses via GI tract and kidneys, when intakes fall [4]. Thus, it clear that there is a need for the development /discovery of a new biological marker of Zn status.

Recent studies by Reed et al. [9] showed a significant negative correlation between dietary Zn deficiency and the ratio of erythrocyte phospholipid linoleic acid: dihomo-γ-linolenic acid (LA:DGLA) in vivo using the chicken model, *Gallus gallus*. The authors clearly demonstrated that LA:DGLA is able to differentiate Zn status between Zn adequate and Zn deficient subjects, showing that LA:DGLA ratio can be used as an effective tool to detect an early stage of Zn deficiency before the onset of symptoms [9]. It seems reasonable to postulate that the same relationship may exist in humans and that the LA:DGLA ratio may well prove to be a novel, effective, noninvasive, sensitive and reliable biomarker of Zn status in humans.

The major aim of this study was to assess the correlations of plasma phospholipid content of linoleic (LA, 18:2n-6), dihomo-γ-linolenic acid (DGLA, 20:3n-6) and LA:DGLA ratio, with plasma Zn status and dietary Zn intake in human subjects. This study presents an initial, and at the same time, an essential step, in the process of validating if the newly proposed biomarker of Zn status, LA:DGLA ratio, can be used as a valid, sufficiently sensitive and reliable biomarker of Zn status in human subjects.

In addition to this, in this study we examine the correlations of plasma Zn status and dietary Zn intake with the content of ten additional fatty acids in plasma phospholipids. Furthermore, this study looks at the correlations between Zn and other trace elements, comments on the dietary Zn intake of the study population and explains the correlations between the plasma Zn status, dietary Zn intake and plasma LA:DGLA ratio with numerous anthropometrical, biochemical and hematological measures.

5.3 Materials and Methods

5.3.1 Study Participants

The study participants (n=54) were apparently healthy 28-55 years old males and females volunteers. Eligible subjects included non-smoking volunteers, without any clinical signs of an acute condition or chronic disease, and without the need for medical treatment. Strict inclusion/exclusion criteria has been used (please refer to supplementary material for more information). All subjects went through the informed consent process, both verbal and written. The study protocol was approved by the Clinical Hospital Centre Zemun, Belgrade, Serbia, Ethics Committee Approval, No: 2125, 2013; and by SAC HREC EC00188 (96.15), Adelaide, South Australia. The protocols and procedures of the study were in agreement with the ethical guidelines on biomedical research on human subjects of The Code of Ethics of the World Medical Association's Declaration of Helsinki (1964) and its further amendments.

5.3.2 Collection of Blood Samples and Anthropometric Measurements

Blood samples were collected between 8 and 9 a.m. after an overnight fast (>10hr fasting). Whole-blood samples were collected from participants in seated position at in a trace mineral free tube by venipuncture from an antecubital vein using butterfly needles (Sarstedt, Inc.). All samples were centrifuged (1000 X g for 15 mm). The serum and plasma samples were removed and 1 ml aliquots were stored at –80°C until further analysis.

Anthropometric variables height and weight were measured to the nearest 0.1 cm and 0.1 kg, respectively. The weight and percent of fat mass in the body composition of participants were measured using a TANITA UM072 balance (TANITA Health Equipment H.K. LTD). The body mass index (BMI) was computed as the ratio of weight (kg) to height squared (m^2). BMI was used to assess the prevalence of overweight (25-29.9 kg/m^2) and obesity (≥30 kg/m^2) according to WHO criteria [10].

5.3.3 The Assessment of Dietary Zn Intake

Three 24 h recall questionnaires (interactive, validated) run on three non-consecutive days (two working days and one weekend day) were used for assessing dietary Zn intake of participants. The photographs of different foods and composite dishes were used during the interview to help improve the portion size estimations [11]. The Zn content of foods was determined using the Serbian food composition data base (FCDB) [12,13]. DIETS ASSESS & PLAN, a nutritional tool validated in different national and regional surveys and international projects, evaluated in the EFSA project [14] was used for obtaining comprehensive dietary intake assessments. The dietary intakes from administered questionnaires were calculated by multiplying the frequency of consumption of each food item consumed by composition of that food, using adequate portion sizes. In addition to Zn, dietary intake data were obtained for energy, macronutrients, Fe and certain fatty acids. Total Zn intakes were adjusted for total energy intake using the residual method [15,16]. Zn levels in the participants' diets were verified using the Estimated Average Requirement, as defined in the Dietary Reference Intakes (DRIs) provided by the Institute of Medicine [17].

5.3.4 Biochemical Analysis

The biochemical parameters were measured using a Cobas e411 clinical chemistry analyser (Roche Diagnostics, Basel, Switzerland) and using Roche Diagnostics Kits according to the manufacturer's instructions.

5.3.5 Plasma Phospholipid Fatty Acid Analysis

Fatty acid concentrations were determined by gas-liquid chromatography (GC). Total lipids were extracted from the plasma according to a method described by Milutinovic et al. (2012) [18].
In short, the phospholipid fraction was isolated from the extracted lipids by one-dimensional neutral lipid solvent using the system of petroleum ether, diethyl ether and glacial acetic acid (87:12:1, by volume) and separated on Silica Gel Chromatography plates (C. Merck, Darmstadt, Germany). The phospholipid fraction was scraped into glass tubes and the phospholipid fatty acid methyl esters were prepared by transmethylation with sodium hydroxide in methanol (heated at 85°C for 1 h) and after that sulfuric acid in methanol (heated 85°C for 2 h). After 30 min, samples of esters were centrifuged and the upper phase samples were put into tubes and evaporated with technical grade nitrogen. Fatty acid methyl ester derivatives were separated by gas chromatography (GC) using a Shimadzu (Kyoto, Japan) GC 2014 equipped with a flame ionization detector and a Chronus GC-CN100 column (60m x 0.32mmID, film thickness 0.2 μm, SMI-Labhut, Churcham, Gloucester, UK).

Adequate separation was obtained over a 50 min period with an initial temperature of 140°C held for 5 min. The temperature was increased to 220°C at a rate of 3°C/min and held on final temperature for 20 min. Individual peaks were identified by comparison with known standard mixtures (PUFA-2 and/or 37 FAMEs mix, Supelco, Bellefonte, PA), and each peak was quantified by calculating the area under the peak. Finally, the content of individual fatty acids was expressed as a percent of total fatty acids identified.

5.3.6 Determination of Plasma Zn Concentrations

The analysis of plasma Zn was conducted at The Institute of Public Health, Pozarevac, Serbia, using flame atomic absorption spectrometry (AAS) on a Varian SpectrAA-10 instrument with instrument parameters: wavelength 213.9 nm, slit width 1.0 nm and air-acetylene flame according to the method described by Jian Xin, 1990 [19]. The concentration of Zn was measured after dilution 1:10 with MilliQ water. To verify the accuracy of the method, the control serum ClinChek-Control (Recipe, Chemical + Instruments Gmbh, Germany) with a Zn content of 889 ± 178 μg/L (Level I) and 1738 ± 261 μg/L (Level II) was analysed. Method performances were monitored by the analysis of the same control serum within the each series. The average obtained results for Zn content were 914 ± 23 μg/L (Level I) and 1801 ± 35μg/L (Level II) which is in accordance with the certified values. In order to avoid Zn contamination, all tubes and utensils were either soaked in HNO3 (25%, w/w) for 16 h, or were known from previous studies to be Zn-free.

5.3.7 Statistical Analysis

Data analysis was done using the statistical package SPSS 22 for Windows. All models were checked as to their appropriateness to the data. Normality of the distributions was assessed by a Shapiro-Wilk test. Spearman's rank-correlation coefficients (r) adjusted for sex, age, BMI, weight, height and energy intake were calculated to determine correlations between fatty acid composition, plasma Zn status and dietary Zn intake. Between-group differences in variables were compared with unpaired t-tests or by Mann-Whitney U tests. Where variables were not normally distributed, logarithmic transformation was undertaken to normalize the distribution. Data are presented as means ± standard errors. p values <0.05 were considered significant.

5.4 Results

5.4.1 General Characteristics of Study Participants

The average age of participants (n=54) was 40.4 ± 7 years, with average height of 173 cm ± 7.8 and weight of 83 ± 2.4 kg. The average BMI of this group was 27.7 ± 4.7 kg/m^2, there were no underweight individuals, 50% of participants were in the healthy weight range and ~25% were obese, according to WHO criteria [10].

5.4.2 Plasma Zn is Not Correlated with Plasma Concentrations of Other Trace Elements

Plasma Zn concentrations of our study participants ranged from 0.72 mg/L to 1.37 mg/L The overall mean plasma Zn concentration was 1.04 ± 0.16 mg/L (15.9 µmol/L).

All subjects had adequate plasma Zn concentrations (reference range 0.7-1.6 mg/L) and no deficiencies were observed (no plasma Zn values <0.7 mg/L). Generally, the trace element concentrations in this study (**Table 5.1**) were within the expected normal ranges for healthy humans and similar to reported levels of trace element concentrations in healthy individuals elsewhere [20-25].

Table 5.1 Plasma concentrations of Zn, Fe, Cu, Mg and Ca and their correlations

	Zinc (Zn)	Iron (Fe)	Copper (Cu)	Magnesium (Mg)	Calcium (Ca)
Concentration (mg/L)	1.04 ± 0.22	1.25 ± 0.11	0.94 ± 0.28	21.28 ± 0.26	101.7 ± 2.92
Correlation with Zn (r)	–	0.14 (0.33)	0.92 (0.51)	0.78 (0.58)	0.03 (0.85)

The values presented are means ± standard errors (SE); n=54, two replicates; r=correlation coefficient with p values in brackets. $p<0.05$ is considered statistically significant.

Overall, trace element concentrations were not gender dependent, there were no differences in the concentration of measured nutrients between the male and female participants. No statistically significant difference in plasma Zn concentrations was seen between the genders (p=0.14) which is consistent with results of several other studies [26,27]. The only statistically significant difference was observed for Cu, with females having higher mean levels of Cu compared to males (1.0 ± 0.03 and 0.78 ± 0.02 correspondingly; $p<0.00$).

The observed difference in Cu levels is most likely due to the higher estrogen levels in females. The average Zn:Cu ratio of subjects in this study was 0.91 ± 0.22 which demonstrates the absence of Zn deficiency and inflammatory conditions in our study population, since the increment of this ratio above 1.5 reflects an inflammatory response or a decreased nutritional Zn status [28].

There were no correlations between the plasma Zn and other elements (**Table 5.1**). Similarly, the LA:DGLA ratio was correlated with plasma Zn (r=-0.35, p=0.01), without any association with Fe, Ca, Cu and Mg, r= -0.12, p=0.39; r= 0.16, p=0.26; r= -0.21, p=0.12; r= 0.78, p=0.95, respectively.

5.4.3 Correlations of Plasma Zn with Anthropometric, Hematological and Biochemical Measures

Similarly to previous findings [26,29,30] in this study we have not found a relationship between the Zn status and age (**Table 5.2,** p=0.87). Alike, no relationship (p=0.2) was found between the plasma Zn and BMI (**Table 5.2**). However, there was statistically significant link between the weight (r=0.27; p=0.04), waist circumference (r=0.35, p=0.01) and free-fat mass (r=0.29; p=0.03) with plasma Zn (**Table 5.2**), as was also shown by others [31-33].

Comparable to plasma Zn, the LA:DGLA ratio was correlated with weight related parameters. The observed correlations were stronger, waist circumference (r=-0.43; p=0.01), hip circumference (r=-0.36, p=0.01), weight (r=-0.35, p-0.01) and BMI (r=0.36, p=0.01). There were also a significant correlation between the plasma Zn and glucose (r=0.28, p=0.04).

In summary, except for weak correlations between Zn and glucose and Zn and weight related parameters, we found no correlation between the plasma Zn status and any other biochemical, anthropometrical or hematological indicator measured (**Table 5.2**).

Table 5.2 Correlations of plasma Zn with anthropometrical, biochemical and hematological indicators

Indicator	Age	Height (cm)	Weight (kg)	BMI (kg/m^2)	Hip Circ. (cm)	Waist Circ. (cm)
Value	40.41 ± 0.95	173.15 ± 1.19	83.62 ± 2.42	27.68 ± 0.64	108.79 ± 1.53	91.83 ± 1.73
Correlation with Zn	0.23 (0.87)	0.17 (0.21)	0.27 (0.04)*	0.21 (0.13)	0.18 (0.19)	0.35 (0.01)*
Indicator	**FF Mass (kg)**	**HCT (L/L)**	**Glu (mmol/L)**	**Se (mm)**	**Cho (mmol/L)**	**TAG (mmol/L)**
Value	52.26 ± 1.47	0.41 ± 0.01	4.93 ± 0.07	7.85 ± 0.91	5.29 ± 0.14	1.02 ± 0.05
Correlation with Zn	0.29 (0.03)*	0.02 (0.87)	0.28 (0.04)*	0.02 (0.86)	- 0.54 (0.69)	0.26 (0.05)
Indicator	**HDL (mmol/L)**	**LDL (mmol/L)**	**Cre (µmol/L)**	**Urea (mmol/L)**	**BILT (µmol/L)**	**BILD (µmol/L)**
Value	1.74 ± 0.06	3.57 ± 0.13	6.96 ± 1.73	3.43 ± 0.13	9.39 ± 0.52	2.92 ± 0.14
Correlation with Zn	- 0.11 (0.45)	- 0.03 (0.81)	0.19 (0.16)	0.68 (0.63)	0.20 (0.15)	0.19 (0.16)
Indicator	**UA (µmol/L)**	**ALT (U/L)**	**AST (U/L)**	**Gamma GT (U/L)**	**LDH (U/L)**	**Hgb (g/L)**
Value	263.16 ± 10.07	26.25 ± 2.19	22.49 ± 1.20	15.43 ± 1.18	153.19 ± 4.64	135.50 ± 1.93
Correlation with Zn	0.24 (0.08)	0.15 (0.27)	0.19 (0.16)	0.22 (0.11)	0.26 (0.06)	- 0.02 (0.98)
Indicator	**WBC x 10^9/L**	**RBC x 10^{12}/L**	**PLT x 10^9/L**	**Lym (%)**	**Mon (%)**	**Gra (%)**
Value	5.78 ± 0.18	4.69 ± 0.06	253.05 ± 7.26	34.26 ± 0.80	6.40 ± 0.31	59.34 ±0.84
Correlation with Zn	0.19 (0.16)	0.12 (0.39)	0.11 (0.45)	- 0.17 (0.21)	0.06 (0.65)	0.15 (0.27)

BMI: Body mass index; Circ.: Circumference; FF: Free fat; HCT: Hematocrit; Glu: Glucose; Se: Sedimentation; Cho: Cholesterol; TAG: Triglyceride; HDL: High density lipoprotein; LDL: Low density lipoprotein; Cre: Creatinine; BILT: Bilirubin total; BILD: Bilirubin direct; UA: Urine analysis; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Gamma GT: Gamma-glutamyl transpeptidase; LDH: Lactate dehydrogenase; Hgb: Hemoglobin; WBC: White blood cells; RBC: Red blood cells; HCT: Hematocrit; PLT: Platelet count; Lym: Lymphocytes; Mon: Monocytes; Gra: Granulocytes. Values presented are means ± SE (standard errors). r - Correlation coefficient with p values in brackets. *p<0.05 is considered statistically significant.

5.4.4 Dietary Zn intake. Plasma Zn is not Correlated with Dietary Zn Intake

The average intake of Zn obtained from the 24 h dietary recalls was 9.98 ± 0.86 mg/day (mean ± SE). Around 30% of participants consumed Zn at levels below the EAR (6.8 mg/day for women and 9.4 mg/day for men) [17] and none of the study participants reported taking Zn supplements. The meat, nuts and grain products were the main sources of the dietary Zn intake of our study population. Additionally, good sources of dietary Zn were cheese products, yeast, sesame and poppy seeds, beans and cocoa powder.

Intakes of energy and protein in this study population were 1927 ± 112 kcal/day and 75 g/day of protein. The ranked data show significant correlations between the meat consumption (r=0.49; p=0.00) and energy (r=0.39, p=0.003) with Zn intake. Among the Fe biochemical indices, hemoglobin and RBC were correlated with dietary Zn intake (r=0.36, p=0.01; r=0.35, p=0.01 respectively). Similarly to other studies [34-37] this study shows no significant relationship between the dietary and plasma Zn status (r=0.07; p=0.6).

5.4.5 Correlations of Plasma Zn with Fatty Acids. LA:DGLA is Correlated with Plasma Zn Status in Healthy Subjects

The mean plasma fatty acid values of our study population are similar to the average serum/plasma fatty acid composition described by others [38-41].

Dietary Zn intake was mainly not correlated with various fatty acids measured in this study, the exception is the 22:5n-3, the end product of metabolic pathway of alpha linolenic acid. Plasma Zn status was not related to the status of SFA or MUFA and most of the PUFA. However, as presented in **Table 5.3**, there is statistically significant correlation between the DGLA and plasma Zn (r=0.39, p=0.00). No relation was observed between the linoleic acid and plasma Zn status while there was a significant negative correlation between LA:DGLA ratio and plasma Zn status (r=-0.35, p=0.01). Out of all fatty acids measured in this study DGLA was the only one that shows significant association with plasma Zn status. Multiple regression analysis (controlled for confounders) revealed that dietary and plasma Zn can predict changes in the LA:DGLA ratio (R^2=0.23, F=2.39, p=0.04).

Table 5.3 Correlations of major plasma phospholipid fatty acids content with plasma Zn status and dietary Zn intake

Fatty acids	Common name	Content (%)	Correlation with plasma Zn status	Correlation with dietary Zn intake
SFA	Palmitic acid (16:0)	30.13 ± 0.26	- 0.81 (0.56)	- 0.04 (0.77)
	Stearic acid (18:0)	16.69 ± 0.18	- 0.12 (0.43)	- 0.23 (0.09)
MUFA	Palmitoleic acid (16:1n-7)	0.58 ± 0.03	0.12 (0.42)	0.68 (0.63)
	Cis-vaccenic acid (18:1n-7)	2.51 ± 0.07	- 2.38 (0.08)	0.12 (0.40)
	Oleic acid (18:1n-9)	7.83 ± 0.14	0.23 (0.97)	0.06 (0.66)
PUFA	Linoleic acid (LA; 18:2n-6)	24.07 ± 0.37	0.01 (0.93)	0.08 (0.55)
	Dihomo-gamma-linolenic acid (DGLA, 20:3n-6)	2.88 ± 0.09	0.39 (0.00)*	0.04 (0.75)
	Arachidonic acid (AA, 20:4n-6	11.17 ± 0.27	- 0.05 (0.69)	0.06 (0.67)
	Adrenic acid (22:4n-6)	0.43 ± 0.02	0.06 (0.67)	0.05 (0.69)
	Docosapentaenoic acid (22:5n-3)	0.56 ± 0.03	- 0.15 (0.26)	0.30 (0.03)*
	Docosahexaenoic acid (22:6n-3)	2.82 ± 0.12	0.01 (0.98)	0.05 (0.73)
	Eicosapentaenoic acid (20:5n-3)	0.32 ± 0.02	- 0.98 (0.48)	0.17 (0.20)
	n-6:n-3	11.39 ± 0.49	0.01 (0.98)	- 0.11 (0.45)
	LA:DGLA	8.87 ± 0.33	- 0.35 (0.01)*#	- 0.01 (0.94)

FA: Fatty acid; SFA: Saturated fatty acid; MUFA: Mono-unsaturated fatty acid; PUFA: Poly-unsaturated fatty acid. Values presented are means ± SE (standard errors) of the % of total fatty acids. r- Correlation coefficient with p values in brackets. #Correlations are calculated controlling for age, sex, BMI, energy intake and dietary Zn intake. *p<0.05 is considered statistically significant

5.4.6 LA:DGLA Ratio Responds to the Changes in Dietary Zn Intake While Plasma Zn Does Not

In order to test the proposition that LA:DGLA is responsive to dietary Zn manipulations we divided samples into two groups with statistically significant differences in dietary Zn intake **(Table 5.4)**.
We examined the extent the differences in the plasma Zn status, dietary intake of fatty acids and alterations in the concentrations of LA and DGLA (individually), as well as LA:DGLA ratio follow the pattern of statistically significant differences in the dietary Zn intake among the groups.

Table 5.4 Differences in the dietary content and plasma concentrations of LA, DGLA and Zn between the two Zn groups

	Group 1 (n=27)	Group 2 (n=27)	Significance (2-tailed)
Dietary Zn (mg)	7.01 ± 0.52	12.78 ± 1.54	0.01*
Dietary LA (g)	17.54 ± 3.54	19.75 ± 2.78	0.65
Dietary PUFA (g)	12.61 ± 2.86	12.15 ± 2.17	0.75
Plasma Zn (mg/L)	1.02 ± 0.03	1.07 ± 0.03	0.28
LA (%)	24.01 ± 0.49	24.15 ± 0.54	0.53
DGLA (%)	2.61 ± 0.12	3.14 ± 0.14	0.01*
LA:DGLA	9.53 ± 0.43	8.21 ± 0.47	0.04*

Dietary Zn: Zn content of the diets based on three 24 h recall questionnaires n=54; 27 in each of the group. LA: Linoleic acid; DGLA: Dihomo-gamma-linolenic acid; PUFA: Poly-unsaturated fatty acid. Values presented are means ± SE (standard errors). *$p<0.05$ is considered statistically significant.

Except for the statistically significant difference in intake of meat ($p=0.02$) there were no statistically important dissimilarities in the consumption of any other food item that contributes to Zn intake (i.e. seafood, grain, vegetables, fruits, nuts). Similarly, no statistically significant variance was seen in dietary Fe intake ($p=0.85$). Furthermore, there were no statistically significant differences between the Zn groups for any of the trace elements measured. Finally, no dissimilarities were observed for any of the biochemical parameters between the groups.

For anthropometrical measures there were variations in % fat (p=0.03) and fat free mass (p=0.01) between participants in two groups, but no differences were seen for BMI (p=0.71). Hemoglobin (p=0.03) and red blood cells (p=0.01) were the only two hematological parameters with statistically significant differences. No dissimilarities were seen in the dietary intake of LA or PUFA between the groups. Plasma Zn did not reflect changes in the dietary Zn intake, so no statistically significant variances were seen in plasma Zn concentrations among the groups.

On the contrary, there was statistically significant difference in the DGLA status (p=0.01) and the LA:DGLA ratio (p=0.04) between the groups (**Table 5.4**). The effect size, Cohen's d for the LA:DGLA ratio was 0.58, which demonstrates the medium size effect. Considering other fatty acids, generally there were no variations observed between the Zn groups. The exceptions are the 22:4 n-6 (adrenic acid) and 18:1n-9 (oleic acid) with statistically significant differences 0.39 ± 0.02 and 0.47 ± 0.03, mean ± SE, p=0.014 and 7.5 ± 0.16 and 8.12 ± 0.21, p=0.03, respectively.

5.5 Discussion

This study is an initial step in the evaluation of the LA:DGLA ratio as a biomarker of Zn status in humans. Our findings demonstrate that while plasma Zn concentrations remain stable the LA:DGLA ratio responds to dietary Zn intake; the concentration of DGLA is decreased and the LA:DGLA ratio is increased in people with lower dietary Zn intake.

It is generally accepted that plasma Zn concentration is a valid indicator of whole-body Zn status in the absence of confounding factors, such as infection or stress [4,8]. However, as shown by many [34,35,37,42] plasma Zn level is not reflecting the nutritional state and dietary Zn intake of an individual faithfully. This clearly means that we need a new biomarker that responds more effectively to alterations in dietary Zn intake.

The aim of this study was to test if the changes in recently proposed biomarker of Zn status (LA:DGLA ratio) can successfully predict dietary Zn intake and Zn status of an individual.

In this study plasma Zn, rather than serum was measured, in order to avoid contamination of Zn from the erythrocytes. Blood was collected in accordance with the standard protocol suggested by the International Zinc Nutrition Consultative Group [43]. Fatty acids in plasma/serum phospholipids stored at -80°C for 7 to 12 years showed minimal degradation over time [44].

In order to avoid variations in Zn concentrations caused by the time of the day when the blood was taken and the time since last meal, in this study the blood was taken in the morning (between 8 and 9 am) and after an overnight fast (>10hr).
The trace element concentrations in this study were within the anticipated normal ranges for healthy humans reported elsewhere [20-25]. There were no correlation of plasma Zn with any other trace element measured in this study, which is consistent with previous findings [45,46].

Strict inclusion/exclusion criteria was used in order to exclude participants with infections, inflammatory conditions and allergies. In addition, we looked at the Cu:Zn to show the overall health status of participants. The ratio of copper to Zn (Cu:Zn) is believed to be clinically more important than the individual concentrations of either of these trace metals. Cu:Zn ratio is often used to show the general health state of an individual, as it is easily affected by inflammatory parameters [28]. Numerous studies have shown that the serum Cu:Zn ratio is a sensitive indicator for the identification of various diseases [22,47,48]. It has also been proposed that the ratio of these metals can be used as reference information for diagnosing Zn deficiency [42,49]. The optimal plasma or serum Cu:Zn ratio is 0.70-1.00 [28]. The increment of this ratio above 1.5 reflects an inflammatory response or a decreased nutritional Zn status [28]. The average Cu:Zn ratio of subjects in this study was 0.91 (reference range 0.7-1) which indicates the absence of Zn deficiency and inflammatory conditions in our study population.

All values obtained for various biochemical and hematological parameters measured in this study fall within the reference ranges for healthy population subjects [50-52]. The correlation of plasma Zn with various biochemical, anthropometrical and hematological parameters in healthy population subjects has not been investigated widely. The researchers were mainly interested in following the changes of these parameters and Zn status as a consequence of certain diseases (liver disease, diabetes, cardiovascular disease, various tumors). In general, no correlations were seen between the Zn status and biochemical, anthropometrical or hematological indicators measured in this study. The exemption is the weak correlation between the plasma Zn and glucose and Zn and weight related parameters. Zn is known to have an insulin like effect and is required for the synthesis and release of insulin from pancreatic β cells [53,54]. Zn ions have also been shown to suppress protein tyrosine phosphatases associated with the insulin signaling cascade; Zn stimulates glycemic control by modulating the insulin signalling patway [55], which explains the observed correlation.

Our results are in agreement with previous findings, no relationship was found between the lipid profile (TAG, CHO, HDL, LDL) and plasma Zn concentrations [32,33].

The assessment of dietary Zn intake confirmed that Zn is obtained from a wide range of foods, the richest sources include red meats and liver, nuts, seeds and grains. The red meat and grain products were the main sources of the dietary Zn intake of our study population. Moderate sources included whole grain cereals, and legumes, with lower quantities being taken from other vegetables, fruits, and refined cereals.

Zn in animal products is more readily absorbed than from plant foods. Cereal grains, legumes, and nuts are rich in phytate, which binds Zn in the intestine and reduces its absorption [6]. The molar ratio of phytate: Zn in the diet has been proposed as a predictor of Zn bioavailability, and ratios >15 have been associated with suboptimal Zn status [6,56]. One of the limitations of the present study is the lack of information on the intake of phytate. However, in an omnivorous population, the impact of phytate is likely to be less significant than expected in vegetarians and those whose diets are mainly based on plant foods [57]. Similarly, according to recently developed calculator for inadequate micronutrient intake, Zn bioavailability from the diets consumed by our study population belongs to the group of 'high Zn bioavailability', where ≤50% of total energy intake is accounted for from rice, other grains, other starchy staples, pulses and nuts (38% in our population) and >5% (15% in our study population) of total energy intake is accounted for by protein from fish, eggs, dairy and meat [57].

Dietary Zn intake has not been correlated with plasma Zn status which has also previously been demonstrated by others [34,35,37]. Due to the effective homeostatic regulation, plasma Zn does not reflect realistically dietary Zn intake and nutritional state of an individual. Unchanged plasma/serum Zn concentrations were observed with the intakes as low as 2.8 mg/kg to as much as 40 mg/kg, showing the limitation of plasma Zn status to reliably present the dietary Zn intake [16,58]. In addition, it is not unusual that the plasma Zn level falls at the lower end of the normal range even in the presence of Zn deficiency [42].

In this study we have participants with Zn intakes below the estimated average requirements for Zn [17] (around 30%) and still their plasma Zn levels remain stable (within the reference ranges). This finding, once again proves that plasma Zn is able to show relatively large variations in Zn status, but is not sensitive enough to reveal the early changes in Zn status or the changes in and between the deficient states. Mild to moderate Zn deficiency is not usually presented with specific organ pathologies [59], and basing the determination of Zn status solely on plasma Zn concentrations, early Zn deficiency states easily remain undiagnosed. The better biomarker of Zn status is undoubtedly needed.

The role of Zn in fatty acid metabolism has been demonstrated in several ways [60,61]. Zn modulates cyclooxygenase activity [62] and it is a co-enzyme for delta desaturase [63,64]. As desaturase enzymes require Zn and have a relatively low binding constant their activity is quite sensitive to early stage Zn deficiency. Zn deficiency leads to inconsistencies in the ratio of desaturase substrates and products, in this case linoleic acid (LA) and dihomo-γ-linolenic acid (DGLA) respectively [63]. The delta 6-catalyzed step required for conversion of LA to DGLA is usually the highest flux pathway, so an elevation in the LA:DGLA ratio may be a sensitive marker for Zn deficiency [9].

This study is an initial step in examining the LA:DGLA ratio as a biomarker of Zn status in humans. In addition to this, we looked at the correlation of plasma Zn and dietary Zn intake with other polyunsaturated, as well as some, saturated and monounsaturated fatty acids. Out of the twelve fatty acids examined plasma Zn was correlated with only one polyunsaturated acid, DGLA. Comparison of the differences in fatty acid content among the groups with different dietary Zn intake demonstrate that the concentration of oleic acid (18:1n-9; delta 9 desaturase product) and adrenic acid (22:4n-6; delta 5 desaturase) was lower in the group of people with lower Zn intake. Similar findings were provided by others [60,65,66]. The lower concentrations of oleic and adrenic acid in the group with lower dietary Zn intake indicate that Zn may have a role to play in desaturase activity.

We found no statistically important dissimilarities in the consumption of any other food item that contributes to Zn intake (i.e. seafood, grain, vegetables, fruits, nuts) among the investigated groups. The changes in fatty acid composition that are caused by food restrictions are different from the changes caused by Zn deficiency [67,68]. For example Kudo et al. (1990) [67], demonstrated that during Zn deficiency oleic acid is reduced but increased during food restrictions. Analogous findings were provided by Cunnane et al. (2005) [68]. The authors state that the inhibition of the desaturases by Zn deficiency is so strong that it causes a more rapid decline in tissue arachidonic acid and docosahexaenoic acid than does the direct dietary deficiency of all the omega 6 or omega 3 polyunsaturated fatty acids.

Our results suggest that the activities of delta 6 and delta 9 desaturase are reduced when lower intake of dietary Zn is present, which confirms the sensitivity of desaturases to Zn intake. Desaturase enzymes are coupled to the NAD (P) H-cytochrome b5 electron transferrin chain and lower dietary Zn intake most likely affects the electron transferring chain and subsequently changes the activities of desaturases [68].

In addition to Zn, Fe has also been shown to inhibit delta 9 desaturase activity [68]. Fe is a structural component of the desaturase enzymes, which are required to add double bonds to long chain fatty acids. Besides the changes in the concentrations of oleic acid between the Zn groups, there were also statistically significant differences in hemoglobin and RBC status. The new findings propose that dietary Zn intake is having a role to play in Fe deficiency [69,70], so the observed changes in the production of oleic acid may mean that Zn is also indirectly controlling the activity of Fe ions.
In further support to this argument, it is interesting to note that while there was no relationship between the dietary Zn intake and plasma Zn status, dietary Zn intake correlated with Fe indices, hemoglobin and red blood cell count. Zn is shown to be a strong predictor of hemoglobin concentrations [59,71]. Additionally, a number of data sets over the years, have clearly shown a positive correlation between anemia and signs of the risk of Zn deficiency in adult males, children, and pregnant women [72,73]. The negative interaction between Fe and Zn for absorption has been forgotten. There is more and more evidence accumulating showing the positive link between Fe and Zn and a strong positive influence of Zn on Fe absorption and Fe status.
The information on the precise mechanisms of Zn involvement in the Fe absorption processes is accumulating [69,70,74].

There is a possibility that other confounding variables may not have been controlled for in our analysis and this suggests that our estimate of the effect of dietary Zn on the changes in the LA:DGLA may be subject to some residual confounding. Nevertheless, given that the most important dietary (energy, fat and protein intake) and non-dietary confounders (sex, age, BMI) have been controlled for this outstanding confounding is likely to be very small.

In summary, our study results show that the LA:DGLA ratio changes in accordance to dietary Zn intake. Similar findings were provided by Reed et al. (2014) [9] who were first to propose that the LA:DGLA can potentially be used as a new biomarker of Zn status. With their chicken model the authors illustrate that the LA:DGLA is sensitive to changes in the dietary Zn intake, and that the biomarker can be used to assess the outcomes of changing levels of dietary Zn rapidly [9]. While plasma Zn concentrations of our study population remained unchanged (most likely due to the good homeostatic regulation) there was a statistically significant difference in DGLA production and the LA:DGLA ratio between the groups of subjects with statistically different dietary Zn intake. The effect size of this difference shows that the observed variation is of moderate effect size.

Finally, the percentages of adrenic acid (22:4n-6), the main end product of linoleic acid, and oleic acid (18:1n-9), the end product of stearic acid, were clearly different between the corresponding dietary Zn groups, which supports the idea that dietary Zn deficiency can affect the chain elongation/desaturation pathway of essential fatty acids [9,63].

This study is an initial step in evaluating the LA:DGLA ratio as a biomarker of Zn status in humans. Indeed, further studies and dietary intervention trials are needed to entirely describe the effectiveness of this biomarker in relation to Zn status and Zn bioavailability over time. The initial results are encouraging as they show that the LA:DGLA ratio changes in accordance to dietary Zn intake in humans.
However, additional studies are needed to examine the sensitivity of this biomarker in different setting: in larger study populations, in Zn deficient populations, as well as in the treatment groups with various levels of Zn deficiency. Additional work is needed to clarify any potential limitations of this biomarker, i.e. the effect of inflammatory conditions and infection states on this biomarker. The usefulness of the LA:DGLA ratio in reflecting the Zn status of an individual should further be examined by looking at the changes of this biomarker during different time frames (long vs. short low/high Zn intake). The kinetics of desaturase enzymes in humans should also be examined. Similarly, the changes in the LA:DGLA ratio may be investigated in relation to the alterations of Zn depended proteins and genes in various tissues (i.e. Zip4, ZnT1).

5.6 Conclusion

This study investigated the changes in the newly proposed biomarker of Zn status, the LA:DGLA ratio and the correlation of the ratio with plasma Zn status and dietary Zn intake in healthy human subjects.
In addition, the correlations of Zn related indices with fatty acids and various biochemical, anthropometrical and hematological parameters were investigated. This initial study confirms that the LA:DGLA ratio responds to dietary Zn manipulations. The study provided new information on the link between plasma Zn, fatty acid status and dietary Zn intake. In conclusion, additional dietary intervention trials are needed to investigate the efficacy of newly proposed biomarker of Zn status fully.

Acknowledgments: This work was supported by a grant from Ministry of Education and Science of the Republic of Serbia (Grant III 41030); EU FP7 project BACCHUS (Grant agreement no. 312090). The authors would like to thank Filip Stojanović for immense technical assistance.Financial support provided by an Australian Postgraduate Award is also acknowledged.

Author Contributions: MK: designed and conducted part of the research, analyzed data and wrote the manuscript and had primary responsibility for the final content. JS: designed research and corrected the paper. MZ & JM: conducted fatty acid analysis. ZP: performed mineral analysis. MG: proofread the paper and provided constructive advice. MG: provided essential materials, checked the paper. All authors have proofread the manuscript and approved the final version of the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 6

The Linoleic Acid:Dihomo-ɣ-Linolenic Acid Ratio (LA:DGLA) - An Emerging Biomarker Of Zn Status

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DOI: 10.3390/nu9080825.

6.1 Abstract

Zinc (Zn) deficiency is a common aliment predicted to affect 17% of the world's population. Zn is a vital micronutrient used for over 300 enzymatic reactions and multiple biochemical and structural processes in the body. Although whole blood, plasma, and urine Zn decrease in severe Zn deficiency, accurate assessment of Zn status, especially in mild to moderate deficiency, is difficult as studies with these biomarkers are often contradictory and inconsistent. Hence, as suggested by the World Health Organization, sensitive and specific biological markers of Zn status are still needed. In this review, we provide evidence to demonstrate that the LA:DGLA ratio (linoleic acid:dihomo-γ-linolenic acid ratio) may be a useful additional indicator for assessing Zn status more precisely. However, this biomarker needs to be tested further in order to determine its full potential.

Keywords: biomarker, Zn, LA:DGLA, Zn status, fatty acid

6.2 Introduction

Zinc (Zn) deficiency was first described in humans in the early 1960s, in Middle Eastern, male adolescent dwarfs consuming plant-based diets [1]. Subsequently, Zn deficiency has been identified in many other regions of the world, and it became evident that dietary deficiency of Zn in humans is a widespread phenomenon. Today, Zn deficiency affects around 17% of the world's population [2]. During the last 50 years, tremendous advances have been made both in our basic and clinical understanding of Zn metabolism [3]. Major progress has been made in understanding the importance of Zn as a structural and catalytic factor in a wide range of biological reactions, in uncovering the cellular Zn absorption and excretion mechanisms, and in clarifying the activities of major Zn transporters (15 Zip and 10 ZnT transporters) [4,5]. A significant research effort has tried to identify a physiological biomarker that predicts Zn status truthfully, especially in mild to moderate Zn deficiency. However, to this day, we are still without an entirely accurate biomarker of Zn status. In 2009, Lowe and colleagues evaluated 32 biomarkers in their review and identified only three as potentially useful: serum/plasma Zn concentrations, hair Zn concentration, and urinary Zn excretion [6]. Similarly, the BOND (Biomarkers of Nutrition for Development) Zn Expert Panel recommended the following Zn biomarkers for use: dietary intakes, plasma/serum Zn concentrations, and stunting [7]. Last year, an update on Zn biomarkers was provided, recognizing a few as emerging biomarkers that require further investigation: Zn-dependent proteins, taste acuity, oxidative stress, and DNA integrity [8].

This review is a summary of research related to the LA:DGLA ratio (linoleic acid:dihomo-γ-linolenic acid ratio) as a novel biomarker of Zn status. We describe the chemical structure and function of the Δ6 desaturase enzyme, outline the current knowledge related to the role of Zn in desaturase activity and fatty acid metabolism, and provide recent data that demonstrates the usefulness of the LA:DGLA ratio to be used as a potentially new biomarker of Zn status. Lastly, we allude to further research needed on this topic.

6.3 The Limitations of the Currently Used Biomarkers. "Emerging" Biomarkers

All of the currently accepted and commonly used biomarkers of Zn status have certain limitations. Serum/plasma Zn, hair Zn concentration, and urinary Zn levels tend to fall in severe Zn depletion [9]. However, while plasma Zn concentration responds to altered intake over a short period of time, low plasma Zn concentrations do not remain constant for an extended period due to the homeostatic mechanisms that act to maintain plasma Zn concentration within the physiologic range; by maintaining losses via the GI (gastrointestinal) tract and kidneys [6]. Similarly, dietary Zn intake very often is not correlated with plasma Zn status, and does not realistically reflect the nutritional state of an individual [7,10-12]. For example, unchanged plasma/serum Zn concentrations were observed with intakes as low as 2.8 mg/kg to as high as 40 mg/kg, showing the limitation of plasma Zn status to reliably present the dietary Zn intake [13,14]. Serum Zn levels tend to rise and then fall after a meal [15].

While urinary Zn decreases under severe Zn deficiency [16], the precise evaluation of Zn status in mild to moderate Zn deficiency is challenging, as studies with this biomarker give inconsistent and conflicting results [6]. Urinary Zn has been shown to be a poor indicator of early stage Zn deficiency [14,16].

Recently, a few studies provided some evidence to support the effectiveness of hair Zn concentrations in predicting the Zn status of individuals [17–19]. The hair Zn biomarker has some advantages, such as low cost and viability; however, hair Zn still lacks sufficient evidence towards validity as a method to assess Zn status [8,20].

Finally, the reliability of all currently used biomarkers under infection and inflammatory conditions is intricate [6.7]. Plasma Zn concentration can fall as a result of factors not related to Zn status or dietary Zn intake, i.e. infections, inflammation, trauma, and stress [7]. An indicator that truthfully represents Zn status under various physiological conditions in humans is still missing [7]. The role of Zn in various processes and pathways in the body is multifaceted, and this may indicate that one single biomarker may never be sufficiently sensitive and that we need to use a spectrum of Zn biomarkers to be able to precisely differentiate between various Zn deficiency states.

Emerging biomarkers are defined as 'biomarkers for which there is some theoretical basis of a relationship to Zn intake or status, but the testing is insufficient to confirm the relation' [8]. Currently, nail Zn concentration, Zn-dependent proteins, oxidative stress, DNA integrity, and taste acuity are placed in the group of emerging biomarkers [8].

However, for many of the newly identified biomarkers, insufficient evidence is available to demonstrate their true potential and further studies are needed to confirm which ones can be used as biomarkers of Zn status. In this review, we provide the existing evidence to show that the LA:DGLA ratio is likewise an emerging biomarker of Zn status that needs to be assessed further.

6.4 Delta 6 Desaturase: Structure, Regulation and Function

Delta 6 desaturase (Δ6 desaturase, D6D or Δ-6-desaturase) is a membrane-bound desaturase enzyme required for the synthesis of polyunsaturated fatty acids (PUFA). The enzyme is molecularly identical across all living organisms. Δ6 desaturase is widely expressed in human tissues, in the liver, the membrane of red blood cells, lung, and heart, with the highest levels being present in the brain [21,22].

Linoleic acid (LA) is an essential omega 6 (*n*=6) fatty acid that cannot be synthetized in the human body and must be obtained from the diet to ensure the appropriate development of various cells throughout the body [23]. It is the most abundant PUFA in human tissues [24]. LA is a metabolic precursor of dihomo-γ-linolenic acid (DGLA) (**Figure 6.1**). Δ6 desaturases are rate-limiting enzymes in the synthesis of PUFA, responsible for conversion of LA to DGLA, and the enzyme catalyzes the addition of a double bond at the sixth carbon-carbon bond position from the carboxylic acid end in fatty acids [22,25].

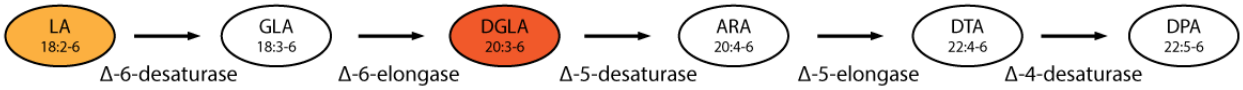

Figure 6.1 Desaturases involved in the biosynthesis of omega 6 polyunsaturated fatty acids.

LA: linoleic acid; GLA: γ-linoleic acid; DGLA: dihomo-γ-linolenic acid; ARA: arachidonic acid; DTA: docosatetraenoic acid; DPA: docosapentaenoic acid. Δ6 desaturase is responsible for the formation of the carbon-carbon double bonds, and the function of an elongase is to lengthen fatty acid chains by the addition of two carbon units. LA (18:2-6) is desaturated by a Δ6 desaturase, introducing a D6 double bond into the substrate, giving γ-linolenic acid (GLA, 18:3-6). GLA is then elongated by a Δ6 elongase to dihomo-γ-linolenic acid (DGLA, 20:3-6). Modified from: Meesapyodsuk & Qiu, 2012 [25].

The first Δ6 desaturase gene was cloned in 1993 from a cyanobacterium, *Synechocystis* [26]. Subsequently, desaturases have been identified and characterized from a wide range of species, and in 1997 the first eukaryotic Δ6 desaturase gene was cloned [27,28].

Mammalian Δ6 desaturase encoded gene is a protein made of a cytochrome b5-like domain, attached to the N-terminus of the main desaturation domain, and a histidine motif, located on a desaturation domain at the C-terminus [29]. The histidine sequence is made of three highly conserved histidine-rich motifs, i.e., HX3-4H, HX2-3HH, and H/QX2-3HH (**Figure 6.2**).

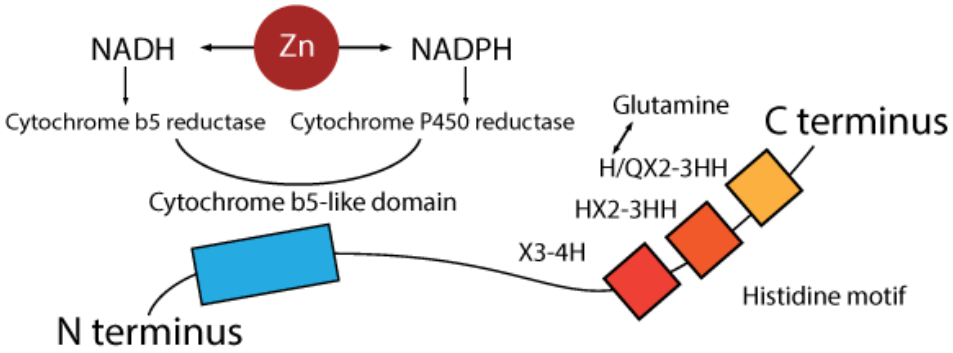

Figure 6.2 Schematic presentation of the structure of a Δ6 desaturase enzyme.

A cytochrome b5-like domain is attached to the N-terminus and a histidine motif is located at the C-terminus. The histidine sequence is made of three histidine-rich motifs. The first histidine of the third motif is often replaced by glutamine. NADPH reductase has a Zn-dependent activity. NADH-nicotinamide adenine dinucleotide hydride; NADPH - nicotinamide adenine dinucleotide phosphate-oxidase.

The first histidine of the third motif is commonly substituted by glutamine [27]. The conversion of glutamine back to histidine results in the loss of activity, suggesting that the process might be very important not only for the structural configuration of desaturases, but also for their activity [30]. The gene coding for Δ6 production is located on human chromosome 11 (11q12.2-13.1), and is made of 12 exons and 11 introns [31].

To date, there has been limited information available on how the expression of the Δ6 desaturase is regulated. Some scientists believe that the regulation is achieved by the feedback control of the transcriptional regulation of fatty acid desaturase genes, mediated through signaling pathways activated by sensors embedded in cellular membranes, in response to environmental factors [32]. There is also some evidence showing that Δ6 desaturase enzymatic activity may be determined by tissue-specific mechanisms that involve both pre- and post-translational events [25]. NADPH reductase is important in the action of the Δ6 desaturase and is a Zn-dependent enzyme [33–35]. Typically, the Zn atom is bound to three or four ligands, which are composed of amino acids residues with histidine being the most frequent, followed by glutamic acid, aspartic acid, and cysteine [36]. Finally, the mutation of the cytochrome b5 domain is critical for the activity of Δ6 desaturases [37,38].

6.5 The Role of Zn in the Regulation of Δ6 Desaturase Activity and Fatty Acid Metabolism

Zn is an essential component of many enzymes and constitutes a part of their prostetic group [39].
It is present in DNA and RNA polymerases, dehydrogenases, and desaturases, regulating their functions via its catalytic, structural, or regulatory role. Over the years it has been noted that Zn is an important co-factor for the metabolism of fatty acids [40]. Zn is also necessary for at least two stages in essential fatty acid (EFA) metabolism; the conversion of linoleic acid to γ-linolenic acid, and the mobilization of dihomo-γ-linolenic acid (DGLA) to arachidonic acid [41]. Zn has an effect on Δ6 desaturase itself [41], and affects linoleic acid absorption [42]. Once it was identified that Zn and essential fatty acid deficiencies give similar symptoms, a close association between fatty acid metabolism and Zn status was proposed [41,43,44].

Over the years, a number of studies have demonstrated an effect of Zn deficiency on the metabolism of essential fatty acids by impaired Δ6 desaturation activity [34,42,44–46]. On the other hand, there are a few studies completed in early 1990 that showed no role of Zn in fatty acid desaturation [47–51].
The effect of Zn deficiency on Δ5 and Δ6 activity was initially investigated by a number of groups in the early 1980s [42,45,52]. The findings were consistent, proving the reduced activity of Δ6 desaturase during Zn deficiency. Ayala and Branner (1983) [42] used male weaning Wistar rats and examined the influence of Zn on desaturating enzymes of liver and testes microsomes and their impact on fatty acid and lipid alterations of the tissues. The rats were fed Zn-adequate (55 ppm of Zn) or Zn-deficient diets (1.2 ppm of Zn) for 60 days. The progress of the effect of Zn deficiency was notable; Zn deficiency induced a decrease of essential fatty acids of the linoleic family in plasma after only 18 days, which indicates that Zn deficiency causes a rapid change in desaturase activity [42]. The activities of both desaturases were affected by Zn deficiency, but to a different degree. The same level of Zn deficiency caused a 45% reduction in Δ6 activity, while Δ5 was almost completely attenuated [42].
Similar findings were provided by Cunnane and Wahle (1981) [53] when it was shown that Zn modulates linoleic acid metabolism in rat mammary glands, modifying the Δ6 desaturation of microsomes. Specifically, 38 Sprague-Dawley rats were fed either a purified Zn-supplemented or a Zn-deficient diet for six weeks. The effects of Zn deficiency on the fatty acid composition of plasma lipids and microsomes of liver, intestine, and testes were studied. Among the polyunsaturated fatty acids, DGLA was significantly reduced by the Zn-deficient diet.

Interestingly, the activity of Δ6 activity in liver microsomes was decreased by 25%, while the Δ5 desaturation was reduced by 53% in Zn-depleted rats. In addition, hypertriglyceridemia was observed in the serum of Zn-deficient rats. This study demonstrated that Zn supplementation returned serum triglycerides to normal levels, which shows a strong physiological interaction between Zn and EFAs (essential fatty acids) and confirms that Zn deficiency is responsible for the defects in desaturation [54].

Ten years later, studies completed by Eder and Kirchgessner (1994 & 1995) [44,55,56] provided somewhat contradictory results, demonstrating that Zn deficiency does not affect Δ5 and Δ6 desaturation. The experiments were conducted using various types of dietary fats, including coconut, sunflower, or linseed oil. The proposition was that the activities of Δ5 and Δ6 desaturase depend on the type of dietary fat consumed. Diets rich in fats with high levels of polyunsaturated fatty acids suppress activities of desaturases, while fat free diets significantly raise the activities of these desaturases.

Later in 1995, the authors suggested that one reason for the contradiction between the findings might be the experimental design used in the studies, where the effects of Zn deficiency on desaturase activity was misperceived by a low food intake. After that, the role of Zn in desaturase activity was examined by a serious of experiments with Zn-deficient rats using a force-feeding technique that ensures identical food intake [44]. Δ5 and Δ6 desaturation was investigated in the presence of Zn deficiency in force-fed rats by previously raising the levels of enzymes by feeding a fat-free diet [44]. Zn-deficient rats fed a diet consisting of 5% safflower oil had lower levels of total PUFA than the corresponding rats fed a Zn-adequate diet. The authors clearly demonstrated the role of Zn in Δ5 and Δ6 desaturation in subjects with adequate food and energy intake. Similarly, in subjects with a low-fat intake (fat-free diets), the effect of Zn deficiency on Δ6 desaturation activity was even more pronounced, with a significantly lower activity of the enzyme being observed [44].

In 1999, Waldhauser and colleagues [57] looked at the ratio between *n*-3 PUFA and *n*-6 PUFA in Zn-deficient animals. Four groups of rats were fed Zn-deficient (0.5 mg Zn/kg) or Zn-adequate (45 mg Zn/kg) diets with either olive oil or linseed oil as the source of fat. To ensure an adequate food intake, the rats were force-fed by gastric tube over a period of 13 days. The study confirmed that Zn deficiency influences the metabolic balance between *n*-3 and *n*-6 PUFA, whereas saturated and MUFA (monounsaturated fatty acids) seem to remain unaffected by Zn deficiency. In the rats that were fed linseed oil, Zn deficiency caused a marked increase in the ratio between *n*-3 and *n*-6 polyunsaturated fatty acids in liver phospholipids, particularly in phosphatidylcholine.

In contrast, in the rats that were fed olive oil, Zn deficiency had only slight effects on the fatty acid composition of the liver phospholipids. Therefore, this study confirms the previous results demonstrating that the effects of Zn deficiency on lipid metabolism may be influenced by the type of dietary fat. However, it must be noted that only hepatic Δ6 desaturase enzymatic activity may be reliant upon the composition of dietary fat [58]. This is not applicable to all other tissues. While, the consumption of an essential fatty acid-deficient diet is paralleled by a similar increase in the hepatic abundance of Δ6 desaturase mRNA and the increase in hepatic Δ6 desaturase activity [21], Δ6 desaturase activity was very low in non-hepatic tissues [58,59].

It seems that the potential role of dietary fat on desaturase activity, under relevant conditions, is only related to hepatic tissue. Similarly, in situations when EFA deficiency is of dietary origin, there is an increased attempt to synthetize more linoleic acid, so Δ6 desaturase activity is increased. However, when EFA deficiency is metabolic (as in Zn deficiency) Δ6 activity is inhibited [60]. Finally, increased Δ6 desaturase activity will not necessarily lead to the elevated metabolizing of linoleic acid and its conversion to DGLA. Below, we summarize the findings that confirm the interaction between Zn deficiency and the metabolism of linoleic acid via desaturase enzymes:

- Zn may have a role in the absorption of linoleic acid. Lower levels of Zn produce lower levels of linoleic acid [60].
- Zn has a role in relation to the NADH-NADPH cycle (**Figure 6.2**) [35].
- Cytochrome P-450 activity is significantly reduced under Zn deficiency [34].
- Zn deficiency reduces the availability of linoleic acid metabolites γ-linolenic and arachidonic acid [52,61].
- Zn deficiency decreases the mobilization of DGLA from tissue stores [62].
- Zn is needed in the formation of GLA and in the mobilization of DGLA [41].
- EFA supplementation worsens the effect of Zn deficiency [43,52,63].
- Zn deficiency decreases the esterification of essential fatty acids into phospholipids [46].
- During Zn deficiency, linoleic acid accumulates in tissues when EFA supplements are administered [63].
- Zn deficiency results in a higher concentration of linoleic and a lower concentration of arachidonic acid in tissue phospholipids [34].
- Zn-deficient subjects have an increased β-oxidation of linoleic acid, resulting in decreased amounts of linoleic acid available to be metabolized into arachidonic acid [64].
- The most important EFA functions are carried out by molecules downstream of GLA [41].
- In animals exposed to diets deficient in essential fatty acids, the characteristic symptoms develop much more rapidly if the diets are also deficient in Zn [43,65].

- The inhibition of the desaturases by Zn deficiency is so strong that it causes a more rapid decline in tissue arachidonic acid and docosahexaenoic acid than does the direct dietary deficiency of all the omega 6 or omega 3 polyunsaturated fatty acids [64].
- Enzymes involved in prostaglandin synthesis are also Zn-dependent, and defects in prostaglandin synthesis are observed under Zn deficiency [52].

In summary, Zn has both a direct role in the modulation of desaturase activities involved in the fatty acid metabolism and an indirect effect on fatty acids by influencing their absorption, oxidation, and incorporation [34]. Zn deficiency causes inconsistencies in the ratio of desaturase substrates and products, and linoleic acid (LA) and dihomo-γ-linolenic acid (DGLA), respectively (**Figure 6.1**). The most important EFA functions are carried out by molecules downstream of GLA. The Δ6-catalyzed step required for the transformation of LA to DGLA is generally the highest flux pathway, so an elevation in the LA:DGLA ratio could be a sensitive marker for Zn deficiency.

6.6 The LA:DGLA Ratio as a Biomarker of Zn Status, Current Evidence

In 2014, the concept of the essential role of Zn for Δ6 desaturase activity was reinvented. For the first time, Reed at al. [66] tested and implemented a previously unexplored biomarker of Zn status related to erythrocyte Δ6 desaturation, the LA:DGLA ratio. By using the chicken (*Gallus gallus*) as a model, the authors evaluated the sensitivity of the erythrocyte LA:DGLA ratio to changes in supplemental Zn intake. A significant negative correlation was found between dietary Zn deficiency and the LA:DGLA ratio.

The *Gallus gallus* has a similar membrane fatty acid composition to mammals [67] and is highly sensitive to dietary Zn manipulations [66,68–70], which makes it a potentially ideal animal model for exploring changes in the production of essential fatty acids in relation to Zn nutrition. In the original study, birds were fed either a Zn-adequate control diet (42.3 μg Zn/g) or a Zn-deficient diet (2.5 μg Zn/g). Diets had identical FA (fatty acids) content/profile. The body weight, feed consumption, Zn intake, and serum Zn concentrations of the birds were measured weekly, showing higher values of all parameters in the Zn control versus the Zn-deficient diet group of birds ($p<0.05$). There was a relative increase in gene expression of the cytokines: tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and interleukin-6 (IL-6) in the control group.

Other assessed parameters included metal transporters (i.e., ZnT1, ZnT5, ZnT7, Zip6, Zip9); transcription factor: nuclear factor kappa B (NF-κB); brush border enzymes: aminopeptidase, sucrose-isomaltase, Na+K+ATPase, sodium glucose transport protein 1 (SGLT-1), and binding metallothionein-4 protein (MT4). These parameters were found to not be significantly different between the groups. However, the expression of hepatic Δ6 desaturase was significantly higher in the control group ($p<0.001$). Accordingly, the LA:DGLA ratio was noticeably elevated in the low Zn compared to the control Zn group (22.6±0.5% and 18.5±0.5% w/w, respectively, $p < 0.001$). This study demonstrated that the erythrocyte LA:DGLA is able to differentiate Zn status between Zn-adequate and Zn-deficient subjects. Furthermore, variations in the LA:DGLA ratio were noticeable within seven days, signifying that this biomarker can show changes in the dietary Zn status quickly and that it may be able to detect early stages of Zn deficiency that usually, due to the lack of obvious signs and symptoms, pass unrecognized.

This proposed biomarker has been evaluated further in humans [71]. A study was completed on healthy human volunteers, 25–55 years of age. The content of plasma phospholipid LA, DGLA, and changes in the LA:DGLA ratio were compared to the dietary Zn intake and plasma Zn status in human subjects. Participants were separated into two groups based on dietary Zn intakes, assessed using three 24 h recall questionnaires provided on three non-consecutive days. There were no statistically significant differences in the dietary intake of LA and PUFA among the groups of participants. Plasma phospholipid fatty acid analysis was conducted by gas chromatography, and plasma analysis of minerals was conducted by atomic absorption spectrometry.

In addition, the study assessed correlations of plasma Zn and the LA:DGLA ratio with various biochemical, anthropometrical, and hematological parameters. It was shown that while the plasma Zn concentrations of participants remained unchanged (most likely due to the good homeostatic regulation), there was a statistically significant difference in DGLA production and the LA:DGLA ratio between the groups ($p<0.05$). The concentration of plasma DGLA was decreased and the LA:DGLA ratio was increased in people with lower dietary Zn intakes. Besides, docosatetraenoic acid (22:4n-6; D5D) was also lower in the group of people with lower dietary Zn intake.

Finally, the efficacy of the LA:DGLA ratio to predict the Zn status of subjects consuming a wheat-based diet, a diet more representative of a diet of the target Zn-deficient populations, was recently evaluated in vivo by using the *Gallus gallus* model [72]. Two groups of birds (n=15) were fed two different diets, a "high-Zn" diet (46.5 ppm Zn) and a "low-Zn" diet (32.8 ppm Zn), for six weeks. Dietary Zn intake, body weight, serum Zn, and the erythrocyte fatty acid profile were assessed. Serum Zn concentrations were greater in the high-Zn group ($p<0.05$). Similarly, the concentration of Zn in tissues (feather and nail) was higher in the high-Zn group of birds as opposed to the birds fed a low-Zn diet ($p<0.05$). Duodenal mRNA expression of various Zn transporters (i.e., Zip4, Zip6, Zip9, ZnT1, ZnT5 and ZnT7) demonstrated a higher mean value in the tissues collected from the birds fed a low-Zn diet ($n=15$, $p<0.05$). The measurements of hepatic Δ6 desaturase expression showed significant differences between the groups, with a higher mean value in birds fed high-Zn diets. The LA:DGLA ratio was higher in the low-Zn group of birds at all time points measured (weeks 2, 4, and 6). Once more, the LA:DGLA ratio responded to changes in dietary Zn intake. Even though both groups of birds were fed Zn-deficient diets, with only 14 ppm differential in dietary Zn content, still the LA:DGLA ratio differentiated clearly between the groups, which demonstrates the sensitivity of the biomarker to change in accordance with dietary Zn intake.

6.7 Conclusions and Recommendations for Further Research

The evidence provided in this review demonstrate the potential of the LA:DGLA ratio to be used as an additional biomarker of Zn status in humans. To date, research shows that the LA:DGLA ratio corresponds to dietary Zn manipulations, both in animals and humans. This biomarker should be tested and evaluated further to illuminate its full potential. This review provides some evidence to justify the requirements for further research.

Well-controlled human dietary intervention trials are needed to examine the sensitivity of this biomarker in larger healthy cohorts, as well as in Zn-deficient populations. Hence, additional research is needed to elucidate any potential limitations of this biomarker, i.e. the effect of inflammatory conditions and infection states on this biomarker. Similarly, the enzymatic activity of hepatic desaturases should be compared to the expression and activity of Δ6 desaturases in non-hepatic tissues in order to determine the exact role of dietary fats in Δ6 desaturase activity. Additional studies are needed to clarify the potential impact of other nutrient deficiencies on the LA:DGLA ratio, in particular the effect of Fe and Cu deficiencies.
The effectiveness of the LA:DGLA ratio, in relation to Zn status and Zn bioavailability over time, requires further investigation. The kinetics of other desaturase enzymes in relation to Zn intake should also be tested (i.e. Δ5 desaturase). Finally, the modifications of Zn-dependent proteins and genes at the main sites of Zn absorption, namely, in the small intestine, in relation to Zn intake and the LA:DGLA ratio need to be tested and evaluated further.

Acknowledgments: Australian Postgraduate Award Scholarship was provided to the principle author. The financing organization had no role in the design, analysis or writing of this article.

Author Contributions: MK: conducted the literature search, synthetized information, and wrote the manuscript and had primary responsibility for the final content. JS & MG: revised the final version of the paper. ET: proofread the paper and provided constructive advice. All authors have proofread the manuscript and approved the final version of the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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When the diet is wrong medicine is of no use,
when the diet is correct medicine is of no need.

Ancient Ayervedic Proverb

Section 4 | Zinc Biofortification

Preamble

The research project, up to this point, provided important and convincing findings:

- *In the apparently healthy Serbian cohort tested there is a number of people affected by marginal Zn deficiency.*
- *Zn deficiency appears to contribute towards Fe deficiency and Zn is having a role in the Fe absorption process. The potential mechanism of Fe & Zn interaction was described in detail.*
- *The LA:DGLA ratio was identified as a new 'emerging' biomarker of Zn status and the evidence is provided to demonstrate its effectiveness in both animals and humans.*

The ultimate goal of all research efforts related to Zn deficiency as a health problem is to try to reduce the incidence of the deficiency by providing suitable solutions. In many developing countries, Zn deficiency is attributable to a lack of diversity in the diet, with the low consumption of animal products, and often an increased intake of cereal based foods. For example in North Africa and the Eastern Mediterranean nearly 50% of the daily food energy is derived from wheat. However, most of the cereals that make the basis of diets for the majority of people are Zn deficient. Similarly, data obtained through the Serbian study demonstrated that bread (mainly made of wheat) is the staple food and that more than 30% of daily energy is coming from cereals/wheat. Likewise, more than 30% of daily dietary intake of Zn and Fe is coming from cereals, with the white bread and wheat flour being the most used foods in this category. Hence, in both developed and developing countries, wheat is seen as a potential vehicle for the delivery of Zn to people.

As highlighted in the literature review (Chapter 2), Zn biofortification has already been proposed to complement existing efforts for the alleviation of Zn deficiency. However, when the literature was looked at in some detail, it become evident that the quantitative data on the efficacy of the Zn biofortified wheat in improving Zn status is still very limited. A more precise measurements of Zn bioavailability from Zn biofortified products is required. This was the last major aim of this PhD project, to examine the usefulness of Zn biofortified wheat in improving Zn status of consumers.

With an employment of a very experienced farmer from Horsham, Victoria, sufficient quantities of Zn biofortified wheat material was produced and the product was tested through an animal (Gallus gallus) model. The increased amount of Zn in the biofortified wheat resulted in a higher relative bioavailability of Zn and an increased uptake of Zn by the intestinal cells. This demonstrated the potential of Zn biofortified wheat varieties to improve Zn status of consumers. The comprehensive explanation of the study protocol and major findings are presented in **Chapter 7** *as a publication: Knez, M.; Tako, E.; Glahn, R.P.; Kolba, N.; de Courcy-Ireland, E.; Stangoulis, J.C.R. Linoleic acid:Dihomo-γ-linolenic acid ratio predicts the efficacy of Zn biofortified wheat in chicken (Gallus gallus). Journal of Agricultural and Food Chemistry, 2018, 66, 1394-1400, DOI: 10.1021/acs.jafc.7b04905.*

Chapter 7

Linoleic Acid:Dihomo-ɣ-Linolenic Acid Ratio Predicts the Efficacy of Zn Biofortified Wheat in Chicken (Gallus gallus)

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DOI: 10.1021/acs.jafc.7b04905.

7.1 Abstract

The amount of Zn absorbed from Zn biofortified wheat material has been determined using an in vivo model of Zn absorption. Erythrocyte linoleic: dihomo-γ-linolenic acid (LA:DGLA) ratio was used as a biomarker of Zn status. Two groups of chickens (n=15) were fed different diets: a high Zn (46.5 µg Zn/g) and a low Zn wheat based diet (32.8 µg Zn/g). Dietary Zn intakes, body weight, serum Zn, and the erythrocyte fatty acid profile were measured, and tissues were taken for gene expression analysis. Serum Zn concentrations were greater in the high Zn group ($p<0.05$). Duodenal mRNA expression of various Zn transporters demonstrated expression upregulation in the birds fed a low Zn diet ($n=15$, $p<0.05$). The LA:DGLA ratio was higher in the birds fed the low Zn diet ($p<0.05$). The higher amount of Zn in the biofortified wheat resulted in a greater Zn uptake.

Keywords: zinc, Zn biofortification, wheat, Zn transporters, LA:DGLA, Zn biomarker

7.2 Introduction

Zinc (Zn) deficiency is a worldwide health problem that is projected to affect 17% of the total world's population [1]. Zn inadequacy has been related to poor growth, reduced immune function, increased susceptibility to and severity of infection, neurobehavioral abnormalities, and adverse outcomes of pregnancy [2-5]. Zn deficiency is a major cause of early childhood morbidity and mortality [6].
In many developing countries, Zn deficiency is attributable to the low consumption of animal products, and an increased intake of cereals that contain substantial amounts of phytate; a compound known to inhibit Zn absorption [7]. Wheat is one of those cereals with a high phytate content, yet it is a major food staple for almost a half of the worlds' population [8]. In many developing countries (i.e. North Africa and the eastern Mediterranean) nearly 50% of the daily food energy originate from wheat [2]. However, after significant extraction, the remaining white flour of modern wheat cultivars is inherently poor in Zn [9].

The wide-ranging occurrence of Zn deficiency in developing countries and a large number of people dependent on wheat as a major food source, encouraged the development of biofortified wheat varieties with enhanced Zn concentration. Bio-fortification, the delivery of Zn via staple food crops, has been proposed to facilitate current efforts for the alleviation of Zn deficiency [10]. The trait for high Zn concentration in grain can be backcrossed into local varieties that are suited to a particular regional conditions [11-13]. While these conventional plant breeding initiatives are particularly beneficial to poor rural populations that are affected by dietary Zn deficiency [11,13,14], quantitative data on efficacy of the biofortified product is still limited. Welch et al. (2005) [13] and Rosado et al. (2009) [14] provided limited evidence that newly developed Zn biofortified varieties of wheat may be useful in improving the Zn status of consumers. Nevertheless, we still require a more precise measurement of Zn bioavailability.
Additionally, is the extra Zn in biofortified wheat seed at least similarly absorbable as Zn in seed that hasn't been biofortified, and can the Zn-enriched wheat varieties be used to successfully improve the Zn status of Zn deficient people in developing countries?

In recent years, the chicken (*Gallus gallus*) model has been used for assessing the efficacy of biofortified foods crops [15-17]. Recently, a strong correlation between the results obtained via this animal model and through human efficacy trials was confirmed, additionally proving the suitability of the model in examining mineral bioavailability [16].

The *Gallus gallus* model has also been shown to be highly sensitive for Zn related studies [18-20].
The cost effectiveness, faster output and the ability to assess a wide range of physiological and molecular parameters in great detail are characteristics that make the use of the model appealing for testing dietary Zn bioavailability of wheat crops. The LA:DGLA ratio, a potentially new biomarker of Zn status, has been previously tested, demonstrating the efficacy of the biomarker in predicting Zn status in both animals and humans, as the LA:DGLA ratio responded to the changes in supplemental Zn intake within G*allus gallus* [15] and closely tracked dietary Zn intakes within humans [21].

The major aim of this study was to determine the amount of Zn absorbed from Zn biofortified wheat material using an in vivo (*Gallus gallus*) model of Zn absorption. In addition, this study assesses the usefulness of the LA:DGLA ratio to predict the Zn status of Zn deficient subjects consuming wheat based diets

7.3 Materials and Methods

7.3.1 Development of Zn Biofortified Wheat

The commercial wheat variety, Correll (*Triticum aestivum*) was grown by Jordan Farms at Murtoa in the West Wimmera Shire of Victoria, Australia. Seeds were sown in the last week of May 2015 and harvested late November to early December.
The high Zn grain was obtained by a foliar application of 1.5 L/ha $ZnSO_4$ during the mid-vegetative growth stage (mid-August), followed by 2 L/ha $ZnSO_4$ two weeks after flowering (mid-October). The lower Zn grain was grown in the same paddock and was treated with one foliar application of $ZnSO_4$ at the rate of 2 L/ha one week after flowering and this was a common practice in the region to mitigate any effects of Zn deficiency in the soil. Wheat samples were prepared under trace element-free conditions to eliminate contaminant sources of Zn. The samples were dried at 80°C in a conventional oven, and subsequently, they were milled using a trace-element-free mill. The wheat flour was transported to Cornell University, Ithaca, New York in sealed containers for testing in the *Gallus gallus* model. Zn, Fe, phytate, calcium, fatty acid and protein concentrations were measured in the original grain and in experimental wheat-based diets (**Table 7.1**).

Table 7.1 Zn, Fe, phytate, Ca content and fatty acid concentration of wheat flours

	High Zn flour	Low Zn flour
Zn concentration[a] (µg/g)	47.2	33.6
Fe concentration (µg/g)	58	53
Phytate[b] (mg/g)	11 .1	9.9
Calcium (mg/kg)	340	330
Fatty acids		
Total saturated	338.8	330.3
Total transaturated	0.4	0.3
Total monounsaturated	286.1	274.4
Total omega 3	55.2	55.8
Total omega 7	19.0	19.1
Total omega 9	265.8	254.1
Total omega 6	975.8	981.0
LA (18:2n-6)	973.1	978.4
DGLA (20:3n-6)	0.6	0.5

[a,b]The methods employed for mineral and phytate analyses and determination of fatty acids are described in the Materials and Methods section. LA - Linoleic Acid; DGLA - Dihomo-γ-linolenic Acid. Fatty acids mg/100g.

7.3.2 Assessment of Micronutrient and Protein Content of Wheat Flour

Micronutrient content was measured by inductively coupled-mass spectrometry (ICPMS 7500cx, Agilent Technologies, USA) following closed-tube digestion of 0.2 g of flour with nitric acid and hydrogen peroxide. Digest solutions were further diluted 10 times with the addition of a mixture of internal standards.

The Agilent 7500cx is equipped with a CETAC ASX560 autosampler and the ASXpress Plus Rapid Sample Introduction System, a Glass Expansion OpalMist nebuliser (0.4 mL/min) and nickel cones. It was run in the helium (He) collision mode using two He tune methods to allow full analysis of all elements. NIST reference material was analysed concurrently for quality control. Nitrogen analysis was done using a Vario EL Cube (Elementar, Germany) using a modified Dumas combustion as outlined in Muñoz-Huerta et al. [22] and Watson and Galliher [23]. 19±2 mg of sample was weighed into a 4 x 4 x 11 mm tin boat and sealed tightly.

Samples were analyzed using the default settings of the instrumentation, the method used for analysis had a 120 second oxygen dose. Sulfanilamide (Elementar, 15.00-0062) was used to adjust the calibration and a NIST reference material (Durum Wheat, 8436) was run at the start of each batch.

7.3.3 Phytate Content of Wheat Flour

Phytate content, in the form of myo-inositol hexaphosphate (IP-6) was measured by Dionex liquid chromatography (Dionex Corporation, Sunnyvale, CA, USA) using a method developed by Lehrfeld, 1989; Lehrfeld, 1994; Dionex, 1990 [24-26]. A Dionex liquid chromatograph system (AS50 autosampler), equipped with conductivity detector model ED50, and gradient pump GS50 was used along with an IonPac AG11 guard column and IonPac AS11 column (4 × 250 mm) to quantify phytate. PeakNet 6.40 software was used to process chromatographic data. The mobile phase was 200 mmol/L NaOH (carbonate-free) and deionized water, using a flow rate of 1 mL/min. Phytate was extracted from 250 mg of dry, lyophilized diet sample, in 10 mL of a 1.25% H_2SO_4 solution; the extraction process was carried out for 2 hours, after which the samples were centrifuged at 3660 g for 10 minutes. Subsamples were diluted 1:10 with deionized water, and 10 µL was inserted and analyzed (n=3).

7.3.4 Animals, Diets and Study Design

Thirty Cornish cross fertile broiler eggs were taken from a commercial hatchery (Moyer's chicks, Quakertown, PA, USA). The eggs were incubated in optimal conditions at the Cornell University Animal Science poultry farm incubator. The procedure was described in detail elsewhere [15-17]. Upon hatching (hatchability rate was 94%), chicks were assigned to two treatment groups based on gender and body weight to make an equal dissemination between groups (n=15): 1. 'High Zn': 75% Zn wheat diet (46.5 µg Zn/g); 2. 'Low Zn'= 75% wheat diet (32.8 µg Zn/g).

The NRC recommendations and requirements for poultry [27] were consulted to formulate the wheat based diets that meet the nutrient supplies for the broiler, excluding Zn (**Table 7.2**). Chicks were housed in a total-confinement building (1 chick per 0.5 m^2 metal cage) and birds were under indoor controlled temperatures and were provided with 16 h of light. Each cage was equipped with an automatic nipple drinker and manual self-feeder. All birds were given ad libitum access to purified water and food. Feed intakes were measured daily (as from day one), and body weight was measured weekly. Zn intakes were calculated from feed intakes and Zn concentration in the diets. At the end of the experiment (day 42), birds were euthanized by carbon dioxide exposure.

The digestive tracts (colon and small intestine) and liver were quickly removed from the carcass and separated into various sections for tissue analysis (~1-2 cm; ~2-3 g was taken from small intestine and liver, respectively). The samples were immediately frozen in liquid nitrogen, and then stored in a -80°C freezer until analysis. All animal protocols were approved by the Cornell University Institutional Animal Care and Use Committee (no. 2007-0129).

Table 7.2 Composition of the experimental diets

	High-Zn (biofortified)	Low-Zn (standard)
Ingredients	g/kg (by formulation)	
High-Zn Wheat	750	–
Low-Zn Wheat	–	750
Skim milk, dry	100	100
DL- Methionine	2.5	2.5
Corn starch	46.75	46.75
Corn oil	30	30
Choline chloride	0.75	0.75
Vitamin/mineral premix[a] (no Zn)	70	70
Total (g)	1000	1000
Selected component	mean ± SEM, n = 5 (by analysis)	
Dietary Zn concentration[b] (µg/g)	46.5 ± 0.99[c]	32.8 ± 0.17[d]

[a] Vitamin and mineral premix provided/kg diet (330002 Chick vitamin mixture; 235001 Salt mix for chick diet; Dyets Inc. Bethlehem, PA).
[b] Zn concentrations in the diets were determined by an inductively-coupled argon-plasma/atomic emission spectrophotometer (ICAP 61E Thermal Jarrell Ash Trace Analyzer, Jarrell Ash Co. Franklin, MA) following wet ashing. c,d Within a row, means without a common letter are significantly different ($p<0.05$).

7.3.5 Blood Collection and Serum, Nail and Feather Zn and Fe Content Measurements

On a weekly basis, approximately 100 µL of blood was collected from the wing vein (n=15) using a micro-hematocrit heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA, USA). Samples were taken in the morning after an 8 h overnight fast. Nail and feather samples (1-2g) were gathered on the last day of the experiment. Serum, nail and feather Zn and Fe concentrations were measured by an inductively-coupled argon-plasma/atomic emission spectrophotometer (ICAP 61E Thermal Jarrell Ash Trace Analyzer, Jarrell Ash Co., Franklin, MA, USA) following wet ashing.

7.3.6 Fatty Acids Analysis of Erythrocytes and Experimental Wheat Based Diets

Blood samples were centrifuged at ~2000 g for 10-15 minutes at room temperature to fractionate whole blood. Total lipids were extracted from red blood cells and experimental diets according to a modified Bligh and Dyer method.

Fatty acid methyl esters (FAMEs) were prepared using 14% boron trifluoride in methanol (Sigma Chemical, St. Louis, MO, USA). Butylated hydroxytoluene was added to the methanol as an antioxidant. Heptadecanoic acid (Sigma Chemical, St Louis, MO, USA) in chloroform was used as an internal standard. FAME analyses were performed using a Hewlett Packard 5890 Gas Chromatograph (GC) (GMI Inc., Ramsey, MN, USA) with a flame ionization detector (FID) (GMI Inc., Ramsey, MN, USA). A BPX-70 column (25 m × 0.22 mm × 0.25 µm, SGE, Austin, TX, USA) was used for the analysis with H_2 as the carrier gas. FAME identities were determined by a chemical ionization (CI) mass spectrometry, using a Varian Star 3400 GC (Varian Inc., Walnut Creek, CA, USA) coupled to a Varian Saturn 2000 Ion Trap MS (Varian Inc., Walnut Creek, CA, USA). FAME identities were based on GC retention time of each substance and its CI mass spectra. An equal weight of FAME mixture (68A; Nuchek Prep, Elysian, MN, USA) was used to calculate response factors. Fatty acid levels were expressed as weight % of total FA (% w/w). Fatty acid analysis of the wheat based diets was performed at the Waite Lipid Analysis Services according to their standard fatty acid analysis protocol.

7.3.7 Isolation of Total RNA

Total RNA was extracted as previously described by Tako et al. (2014) [28]. The extraction was completed using 30 mg of duodenal (proximal duodenum, n=15) and liver tissues (n=15) using Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. All steps were carried out under RNase free conditions. RNA was quantified by absorbance at 260-280 nm. Integrity of the 28S and 18S rRNA was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. RNA was stored at -80°C until used.

7.3.8 The Gene Expression Analysis

The procedure was carried out as described previously [29,30]. To create the cDNA, a 20 µL reverse transcriptase (RT) reaction was completed in a BioRad C1000 touch thermocycler using the Promega-Improm-II Reverse Transcriptase Kit (Catalog #A1250). The first step consisted of 1 µg of total RNA template, 10 µM of random hexamer primers, and 2 mM of oligo-dT primers. The RT protocol was to anneal primers to RNA at 94˚C for 5 minutes, copy the first strand for 60 minutes at 42˚C (optimum temperature for the enzyme), then heat inactivate at 70˚C for 15 minutes and hold at 4˚C until ready to analyze by Nanodrop. The concentration of cDNA obtained was determined by measuring the absorbance at 260 nm and 280 nm using an extinction coefficient of 33 (for single stranded DNA). Genomic DNA contamination was assessed by a real-time RT-PCR assay for the reference genes samples.

7.3.9 Primer Design

The Zn and Fe primers used in the real-time PCR were designed based on 19 gene sequences from Genbank database, using Real-Time Primer Design Tool software (IDT DNA, Coralvilla, IA). The sequences and the description of the primers used in this work are summarized in **Table 7.3**. The amplicon length was limited to 90 to 150 bp. The length of the primers was from 17- to 25- mer and the GC content was between 41% and 55%. The specificity of the primers was tested by performing a BLAST search against the genomic NCBI database. The primers set ActB and 18S rRNA were designed as reference genes. Results obtained from the qPCR system were used to normalize those obtained from the specific systems as described below.

Table 7.3 DNA sequences of the primers used in this study

Analyte	Organ	Forward Primer (5′-3′) (Nucleotide Position)	Reverse Primer (5′-3′)	Base Pairs Length	GI Identifier
ZnT1	Int	GGTAACAGAGCTGCCTTAACT	GGTAACAGAGCTGCCTTAACT	105	54109718
ZnT5	Int	TGGTTGGTATCTGTGCCTTTAG	GGCTGTGTCCATGGTAAGATT	99	56555150
ZnT7	Int	GGAAGATGTCAGGATGGTTCA	CGAAGGACAAATTGAGGCAAAG	87	56555152
ZIP6	Int	GCTACTGGGTAATGGTGAAGAA	GCTGTGCCAGAACTGTAGAA	99	66735072
ZIP9	Int	CTAAGCAAGAGCAGCAAAGAAG	CATGAACTGTGGCAACGTAAAG	100	237874618
Zip4	Int	TCTCCTTAGCAGACAATTGAG	GTGACAAACAAGTAGGCGAAAC	95	107050877
NF-κB	Liv	CACAGCTGGAGGGAAGTAAAT	TTGAGTAAGGAAGTGAGGTTGAG	100	2130627
SI	Int	CCAGCAATGCCAGCATATTG	CGGTTTCTCCTTACCACTTCTT	95	2246388
Na+ K+ ATPase	Int	CCTTGGAGGTTTCTTCACCTATT	GGTCATCCCACTGAAGTCTAATC	92	14330321
SGLT-1	Int	GCATCCTTACTCTGTGGTACTG	TATCCGCACATCACACATCC	106	8346783
Δ6 desaturase	Liv	GGCGAAAGTCAGCCTATTGA	AGGTGGGAAGATGAGGAAGA	93	261865208
DMT-1	Int	TTGATTCAGAGCCTCCCATTAG	GCGAGGAGTAGGCTTGTATTT	101	206597489
Ferroportin	Int	CTCAGCAATCACTGGCATCA	ACTGGGCAACTCCAGAAATAAG	98	61098365
DcytB	Int	CATGTGCATTCTCTTCCAAAGTC	CTCCTTGGTGACCGCATTAT	103	20380692
18S rRNA	Liv, Int	GCAAGACGAACTAAAGCGAAAG	TCGGAACTACGACGGTATCT	100	7262899
ActinB	Liv, Int	CCAAAGCCAACAGAGAGAAGA	ATCACCAGAGTCCATCACAATAC	137	NM 205518

7.3.10 Real-time qPCR Design

cDNA (2 µg) was used for each 10 µL reaction together with 2X Applied Biosystems qPCR Master Mix (Kit # 4367659, Applied Bio-systems). Specific primers (forward and reverse) and cDNA or water (for no template control) were added to each PCR reaction. For each gene, the optimal $MgCl_2$ concentration produced the amplification plot with the lowest Cp, the highest fluorescence intensity and the steepest amplification slope. Master mix (8µL) was pipetted into the 384-well plate and 2 µL cDNA was added as PCR template. Each run contained 7 standard curve points in duplicate. A no template control of nuclease-free water was included to exclude DNA contamination in the PCR mix. The double stranded DNA was amplified in the ABI Prism 7900HT Real-Time System using the following PCR conditions: 50˚C for 2 minutes, initial denaturing at 95˚C for 2 minutes, 40 cycles of denaturing at 95˚C for 15 seconds, various annealing temperatures according to IDT for 15 seconds and elongating at 72˚C for one minute. The data on the expression levels of the genes were obtained as Cp values based on the 'second derivative maximum' (=automated method) as computed by the software. For each of the 19 genes, the reactions were run in duplicates. All assays were quantified by including a standard curve in the real-time qPCR analysis. The next four points of the standard curve were prepared by a 1:10 dilution. Each point of the standard curve was included in duplicate. A graph of Cp vs. log 10 concentrations was produced by the software and the efficiencies were calculated as 10 (1/slope). The specificity of the amplified real-time RT-PCR products was verified by melting curve analysis (60-95°C) after 40 cycles, which should result in a number of different specific products, each with a specific melting temperature. In addition, an agarose gel electrophoresis followed by ethidium bromide staining was performed and the identities of the PCR products were positively confirmed by sequencing using an ABI PrismTM Genetic Analyzer 3100 (Applied Biosystems, Foster City, CA, USA).
Real-time RT-PCR efficiency (E) values for the five genes were as follows: NK-kB, 1.33; SGLT-1, 1.32; LepR, 1.205; ActinB, 1.09; DMT-1, 1.11; Ferroportin, 1.27; ZnT5, 1.43; Na+K+/ATPase, 1.33; ZnT7, 1.39; 18s rRNA, 1.28; Zip4, 1.11; Δ6-desaturase, 1.34; Zip9, 1.37; DcytB, 1.36; ZnT1, 1.31; Zip6, 1.33; SI, 1.43 (**Table 7.3**).

7.3.11 Liver Fe and Ferritin Analysis

Liver samples were treated as described by Mete et al. [31] and Passaniti and Roth [32.]. Briefly, 1 g of sample was diluted into 1 mL of 50 mM Hepes buffer, pH 7.4, and homogenized on ice for 2 minutes at 5000 g. One microliter of each homogenate was subjected to heat treatment for 10 min at 75°C to aid isolation of ferritin since other proteins are not stable at that temperature [31,32]. After a heat treatment the samples were immediately cooled down on ice for 30 minutes.

Afterwards, samples were centrifuged at 13000 g for 30 min at 4°C until a clear supernatant was obtained and the pellet containing most of the insoluble denaturated proteins was discarded. All tests were conducted in duplicates for each of the animals.

Electrophoresis, staining and measurement of gels native polyacrylamide gel electrophoresis was conducted using a 6% separating gel and a 5% stacking gel. Samples were run at a constant voltage of 100 V.

After electrophoresis, the gels were treated with either of the two stains: Coomasie blue G-250 stain, specific for proteins, or potassium ferricyanide (K3Fe(CN)6) stain, specific for Fe. The corresponding band found in the protein and Fe stained gel was considered to be ferritin [31,32]. The gels were scanned with a Bio-Rad densitometer. Measurements of the bands were conducted using the Quantity-One 1-D analysis program (Bio-Rad, Hercules, CA). The local background was subtracted from each sample.

Horse spleen ferritin (Sigma Aldrich Co., St. Louis, MO) was used as a standard for calibrating ferritin protein and Fe concentrations of the samples. Dilutions of the horse spleen ferritin were made and treated similarly to the liver supernatant samples in order to create a reference line for both protein and Fe-stained gels.

Fe levels were determined using the same calibration since approximately 20% of the weight of horse spleen ferritin is Fe [32]. Saturation levels of ferritin with Fe were calculated as the percentage of the Fe present in the protein to the maximum amount of Fe atoms (4500 Fe atoms/ferritin molecule) ferritin can incorporate [31].

7.3.12 Statistical Analysis

Results were analyzed by ANOVA using SAS software (SAS Institute Inc. Cary, NC). Dissimilarities among treatments were computed by Tukey's test. $p<0.05$ was considered statistically significant. Data are presented as means ± SEM.

7.4 Results

7.4.1 Composition of the Wheat Based Diets

Composition of the wheat based diets is presented in **Table 7.2**. High and low Zn wheat made 75% of the total diet. The concentrations of Zn in the high and the low Zn based diets were 46.5 ± 0.99 and 32.8 ± 0.17 mg/kg, respectively. Except for the variation in Zn concentration there were no statistically significant variances in the fatty acid content or Fe concentrations between the low and high Zn diets (**Table 7.1**). The phytate concentration of the high Zn flour was higher; 11.1 compared to 9.9 mg/g in the low Zn wheat.

7.4.2 General Information (Body Weight, Feed Consumption and Dietary Zn Intake)

There were no statistically significant differences in feed consumption and body weights between groups of birds fed different diets. However, Zn intakes were consistently lower in the low Zn group versus high Zn group ($p<0.05$, **Table 7.4**).

Table 7.4 Body weight, feed consumption and Zn intake of chickens fed a low and a high Zn diet from day 0 to day 42

	Day 0	Day 14	Day 28	Day 42
Body weight (g)				
Low Zn group	41.1 ± 0.7^{a}	125.4 ± 4.8^{a}	301.5 ± 11.4^{a}	598.6 ± 22.8^{a}
High Zn group	41.4 ± 0.8^{a}	122.5 ± 3.9^{a}	298.7 ± 12.8^{a}	585.8 ± 29.7^{a}
Feed consumption (kg/day)				
Low Zn group		0.41 ± 0.2^{a}	1.13 ± 0.5^{a}	2.12 ± 0.8^{a}
High Zn group		0.40 ± 0.3^{a}	1.12 ± 0.6^{a}	2.11 ± 0.7^{a}
Zinc intake (g)				
Low Zn group		13.15 ± 0.6^{b}	36.21 ± 4.8^{b}	69.18 ± 7.5^{b}
High Zn group		19.58 ± 0.8^{a}	49.85 ± 5.1^{a}	100.14 ± 9.8^{a}

[1]Values are mean daily feed intakes for the 14 days preceding the day designated in the column heading. Values are cumulative weekly from day 0. [a,b] Within a column and for each parameter, means without a common letter are significantly different ($n=15$, $p<0.05$). Values are means ± SEM.

7.4.3 Differences in Zn Status Among the Groups (Various Biomarkers)

Serum Zn concentrations were lower in the low Zn group with statistically significant differences between the groups noted at each time point ($p<0.05$). Similarly, the concentration of Zn in both tissues (feather and nail) was lower in the low Zn group of birds versus the birds fed high Zn diet (day 42, $n=15$, $p<0.05$, **Table 7.5**). There were statistically significant differences in the LA:DGLA ratio among the groups, with the higher ratio measured in the group of birds fed the low Zn diet. The LA:DGLA ratio was increasing as the study progressed from day 0 to day 42 and was dissimilar among the treatment groups at each time point ($p<0.05$, **Figure 7.1**).

Table 7.5 Differences in Zn status among the groups as assessed by various Zn biomarkers (serum, nail, and feather Zn concentration)

	Day 0	Day 14	Day 28	Day 42
Serum Zn (µg/g)				
Low Zn group	1.07 ± 0.15^{a}	0.48 ± 0.05^{a}	0.35 ± 0.03^{a}	0.43 ± 0.04^{a}
High Zn group	1.07 ± 0.15^{a}	0.66 ± 0.08^{b}	0.69 ± 0.07^{b}	0.55 ± 0.04^{b}
Feather Zn (µg/g)				
Low Zn group				89.6 ± 7.7^{a}
High Zn group				117.9 ± 10.2^{b}
Nail Zn (µg/g)				
Low Zn group				67.9 ± 3.9^{a}
High Zn group				85.8 ± 3.9^{b}

^{a,b} Within a column and for each parameter, means without a common letter are significantly different (n=15, p<0.05). Day 0-day 42. Values are means ± SEM.

7.4.4 Changes in the Expression of Zn and Fe Transporters Among the Groups

Duodenal mRNA expression of numerous Zn transporters (**Figure 7.2**) confirmed a higher mean arbitrary unit (AU) value in the tissues taken from the birds fed the low Zn diet (n=15, p<0.05). Similarly, the expression of iron (Fe) related transporters (DMT1, FPN1 and DcytB) was statistically different among the groups with the higher expression measured in the low Zn group (n=15, p<0.05).

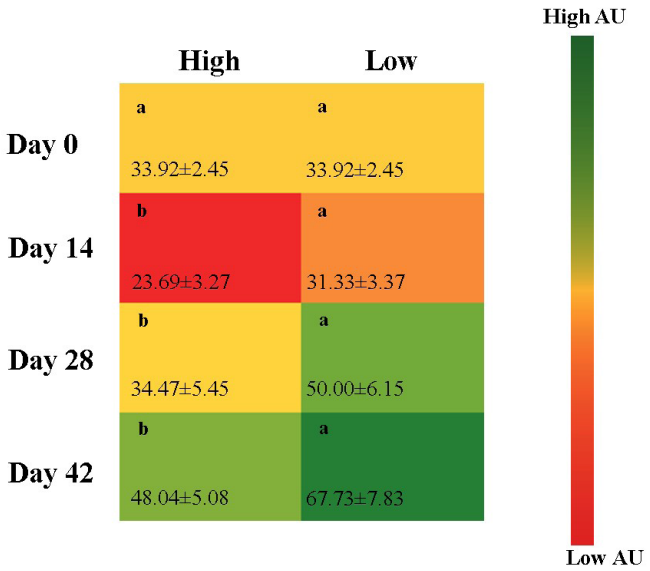

Figure 7.1 Changes in the LA:DGLA ratio among the groups from day 0 to day 42

Further, higher expression of functional genes (SGLT-1, SI and ATPase) was measured in the birds fed with the low Zn wheat based diet. Finally, the measurements of hepatic Δ6 desaturase revealed significant variations among the groups, with a lower mean value for birds in the low Zn group (**Figure 7.2**).

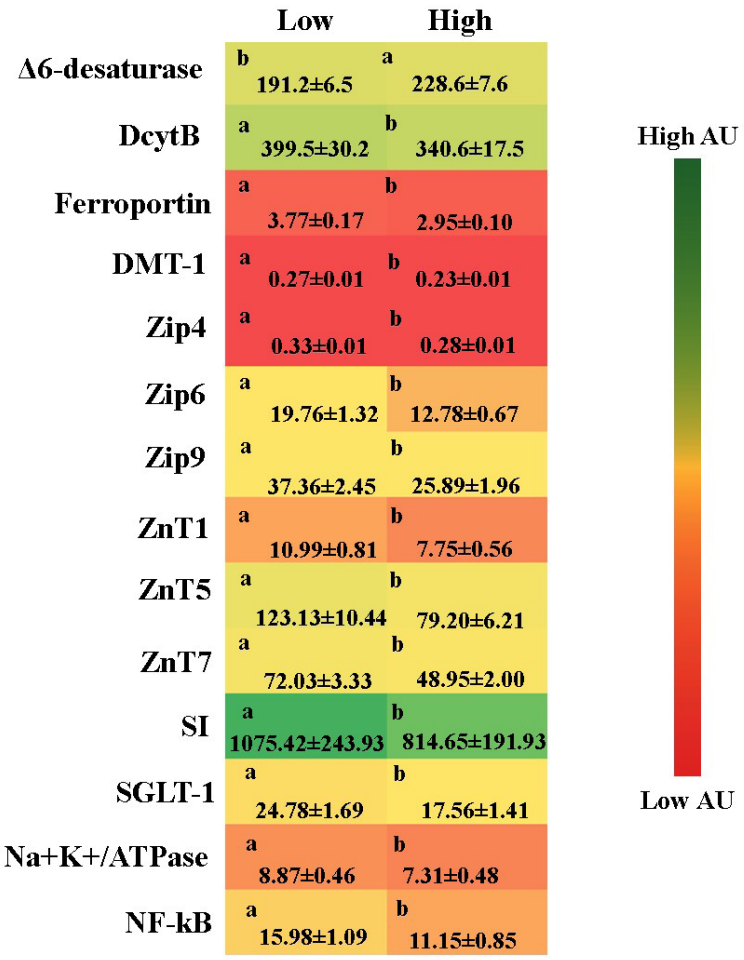

Figure 7.2 Gene expression of Zn and Fe transporters in duodenum and liver

Chicken mRNA expression of hepatic Δ6 desaturase and NF-κB1 and duodenal transporters: cytochrome B (DcytB), ferroportin, divalent metal transporter 1 (DMT1), solute carrier family 39 member 4 (Zip4), solute carrier family 39 member 6 (Zip6), solute carrier family 39 member 9 (Zip9), Zn transporter 1 (ZnT1), Zn transporter 5 (ZnT5) and Zn transporter 7 (ZnT7), sucrose-isomaltase (SI), sodium/glucose cotransporter (SGLT1), ATPase Na+/K+ transporter (NaKATPase), nuclear factor kappa B subunit 1 (NF-kB) in birds given 'high Zn wheat' diet and 'low Zn wheat'. Changes in mRNA expression are shown relative to expression to 18s rRNA in arbitrary units (AU). Values are means ± SEM. n=15, $p<0.05$.

7.4.5 Liver Zn, Fe and Ferritin Concentrations

No significant dissimilarities in the liver Zn, Fe and ferritin concentrations were noticed between the treatment groups (**Table 7.6**).

Table 7.6 Concentrations of Zn, Fe and ferritin in the liver of birds on low and high Zn diets

	Zn concentration (µg/g)	Fe concentration (µg/g)	Liver Ferritin (µg/g wet weight)
Low Zn group	16.72 ± 0.82[a]	144.59 ± 10.95[a]	254.5 ± 12.6[a]
High Zn group	16.76 ± 0.82[a]	145.31 ± 11.53[a]	272.5 ± 15.3[a]

[a] Within a column and for each parameter, means without a common letter are significantly different (n=15, $p<0.05$).
Values are means ± SEM.

7.5 Discussion

Nutritional deficiency of Zn in humans is prevalent throughout the world, particularly in areas where cereal grains are the primary staple in local diets [6,33]. Zn-biofortified crops are a means of addressing nutritional deficiencies and knowledge of their efficacy in improving Zn status of subjects needs to be demonstrated.

This study shows that the additional Zn present in the biofortified wheat is readily available for absorption; the higher amount of Zn in biofortified wheat contributed to a larger uptake of Zn by the intestinal enterocytes. A 14 µg Zn/g differential in dietary Zn intake was sufficient to discriminate between the Zn statuses of the birds used in the study. Higher serum, feather and nail Zn concentrations were measured in the group of birds consuming a high-Zn wheat based diet. The absorption of Zn was greater from the Zn- biofortified wheat dietary treatment than from the wheat with lower Zn concentration, when the same amounts of each type of wheat flour were consumed.

Our results are consistent with previous studies, providing evidence that valuable increases in Zn absorption can be accomplished by a consumption of a Zn-biofortified wheat product [13,14]. Welch et al. (2005) [13] were the first to demonstrate the beneficial effect of Zn-biofortified wheat in improving the Zn status of rats. The wheat genotypes with enriched grain Zn concentrations had increased quantities of bioavailable Zn, supporting the idea that breeding for Zn enhanced wheat grain may lead to reducing Zn deficiency problem in target populations [13].

Similar findings were provided by Rosado et al. (2009) [14] who confirmed greater net absorption of Zn in women eating Zn-biofortified wheat. In that study, adult women were given 300 g of the high or low extraction flours as tortillas for two successive days using either biofortified (41 mg Zn/g) or control (24 mg Zn/g) wheat. The absorption of Zn from the Zn-biofortified wheat was significantly higher than that from the control wheat.

In addition, it was established that the amount of Zn absorbed from cereal products with high phytate content is greater from those fortified with Zn than when they are not fortified [14]. Phytate is a major inhibitory compound present in plant foods and is known to inhibit dietary Zn bioavailability and absorption [34,35]. In this study, slightly higher levels of phytate were measured in the biofortified wheat variety (11 vs. 9 mg/g in the low Zn wheat grain); however, this increase in phytate did not change Zn absorption negatively as the phytate to Zn molar ratio was not elevated.

Serum Zn responded to dietary Zn intake, as birds given the low Zn diet had a consistently lower level of Zn in serum. There were also significant differences in feather and nail Zn concentrations among the groups. These results are compatible with previous studies in humans that indicated serum, hair and nail Zn concentration are contemplative of dietary Zn intake [36-38]. However, over the years, it has been shown that the sensitivity of these biomarkers is very often affected by factors not related to Zn intake/status, i.e. infection, inflammation, stress, sample handling [38,39]. Hence, the development of a sensitive Zn biomarker is still a high priority, as proposed by the World Health Organization [1,40].

The current study demonstrated the effectiveness of the LA:DGLA ratio to predict Zn status of subjects consuming a wheat based diet - a diet more representative of a diet in target Zn-deficient populations. The erythrocyte LA:DGLA ratio reacted to dietary Zn manipulations rapidly (within 2 weeks). A difference of 14 µg Zn/g in Zn concentration among the wheat based diets was enough to show that the production of the DGLA was reduced and accordingly the LA:DGLA ratio was increased in subjects consuming wheat diets with lower Zn concentrations.

The Δ6 desaturase expression was increased in the group of birds fed the high Zn wheat based diet. Zn is an essential and vital cofactor for a proper activity of the Δ6 desaturase enzyme [41,42]; therefore, the higher concentrations of Zn lead to the higher expression of this enzyme. In contrast, Zn deficiency inhibits functioning and expression of the hepatic Δ6 desaturase [41].

The LA:DGLA ratio was statistically different among the groups of birds, with a higher LA:DGLA ratio measured in the subjects that were fed the low Zn wheat based diet, at each time point (weeks 2, 4 and 6). The ratio increased in both groups as the study progressed (from day 0 to day 42), which is due to the fact that both groups of birds were getting progressively more Zn deficient. It is important to note that with only 14 μg Zn/g distinction in dietary Zn concentration, the LA:DGLA ratio still distinguished clearly between the groups, which demonstrates the sensitivity of the biomarker to change according to the dietary Zn intake. Besides, variations in the LA:DGLA ratio were evident within 7 days, indicating that this biomarker can display changes in the dietary Zn status reasonably quickly and it may be able to identify early stages of Zn deficiency that usually, due to the lack of obvious signs and symptoms, pass unrecognized.

The results are in agreement with our former studies [15,21] demonstrating that LA:DGLA is able to distinct Zn status between Zn adequate and Zn deficient subjects. When birds were fed either Zn adequate control diet (42.3 μg Zn/g) or a Zn deficient diet (2.5 μg Zn/g), the hepatic Δ6 desaturase expression was considerably higher in the control group [15]. Consequently, the LA:DGLA ratio was markedly reduced in the group of birds fed a high Zn diet.

The dietary Zn intake and plasma Zn status were similarly compared to the content of plasma phospholipid LA, DGLA and variations in the LA:DGLA ratio in healthy human subjects [21]. It was demonstrated that while the plasma Zn concentrations of participants stayed unchanged (presumably due to the good homeostatic regulation), there was a statistically significant discrepancy in DGLA production and the LA:DGLA ratio between the study participants. The concentration of DGLA declined and the LA:DGLA ratio increased in people with lower dietary Zn intakes. The LA:DGLA biomarker needs additional testing in order to determine its full potential, but a number of studies up to date confirm that the LA:DGLA ratio may be a valuable additional indicator for assessing Zn status more precisely [15,21].

In this study, the expression of various Zn transporters were also responsive to dietary Zn manipulations, with higher expression of all investigated transporters found in birds fed the lower Zn diet. Comparable findings have been observed by others; a rapid upregulation of Zip4 expression in the small intestine was seen during Zn deficient conditions [43-45]. Zip4 is the most important import protein, so rapid Zip4 accumulation demonstrates the molecular basis of systemic Zn homeostatic regulation [44,46].

Similarly, the expression of other Zn import proteins, Zip6 and Zip9, was also significantly greater in subjects on lower Zn diets. The increased expression of the major Zn export protein, ZnT1, has been previously noted during Zn deficiency [47,48]. As was previously suggested, this initial increase in the expression most likely occurred in order to improve the transfer of Zn into systemic circulation in order to minimize the difference between the Zn demand and supply and to alleviate the undesirable consequences of extended Zn depletion [47,49]. The same applies for Fe uptake, which explains the increased activity of the ferroportin in the group of birds fed lower Zn diets. In addition, increased ferroportin expression may be explained by the point that Zn has a protecting role against Fe induced oxidative damage, so reduced amounts of Zn in the cells signify that the level of Fe in the cells should also be reduced [49].

ZnT5 and ZnT7 are transporters ubiquitously expressed in the small intestine and are believed to perform partly overlapping functions in intestinal Zn homeostasis [20]. The expression patterns of ZnT5 and ZnT7 suggests a role in dietary Zn absorption, and in this study increased expression was measured in birds fed the low Zn diet. Both increased and reduced expression of ZnT5 and ZnT7 has been found in response to Zn [45,50].

Expression of DcytB reductase and ferroportin transporter was different among the groups, which confirms that cellular concentrations of Zn may also affect the process of Fe absorption by modulating the activities of transporters responsible for the uptake of Fe into the cells. This proposition has already been confirmed by a number of studies [47,49,51,52].

In conclusion, this study demonstrated that the increased amount of Zn in the biofortified wheat produced a higher dietary bioavailability of Zn and therefore enhanced uptake of Zn by the intestinal enterocytes.

This shows the potential of Zn biofortified wheat varieties in improving the Zn status of consumers.

The LA:DGLA ratio responded to dietary Zn manipulations and the consumption of the Zn-biofortified wheat lowered the LA:DGLA ratio. Hence, these observations suggest that the LA:DGLA ratio can be utilized as an additional physiological indicator of Zn status.

Funding: Financial support provided by an Australian Postgraduate Award is acknowledged.

Acknowledgments: The authors would like to thank Jamie and Craig Jordan of Jordan Farms, Murtoa, Victoria, Australia for producing the wheat used in this chicken study. The authors would like to thank Dr.J. Tom Brenna (Division of Nutritional Science, Cornell University, Ithaca, NY) for the red blood cell fatty acid analysis.

Conflict of Interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Every time you eat or drink you are either feeding disease or fighting it.

Heather Morgan

Section 5 | Conclusion and Research Implications

Chapter 8

Conclusion and Research Implications

8.1 Research Implications

The Main Research Findings Defined in this Thesis are:

- Zn appears to play a role in Fe absorption process and the mechanism by which Zn may control Fe absorption at the local and systemic level has been further elucidated.
- An apparently healthy population living in Serbia have inadequate dietary intake of Zn that may lead to a suboptimal Zn status.
- The LA:DGLA ratio changes in accordance to dietary Zn intake in a Serbian human cohort.
- The LA:DGLA ratio varies with dietary Zn modifications in animals and the ratio is a candidate physiological biomarker of Zn status.
- The consumption of Zn biofortified wheat improves the Zn status of consumers and Zn biofortification is therefore considered an acceptable solution for alleviating Zn deficiency.

8.2 General Discussion with Recommendations for Further Research

More than 50 years has passed since Zn deficiency was first discovered in humans [1] and research efforts are still trying to better understand its effects, and potential solutions to ameliorate the condition.
This PhD project contributed to studies on Zn deficiency as a worldwide health problem and this was achieved via the following:

- Examining the extent of Zn deficiency through its impact on the development of another major micronutrient inadequacy, Fe deficiency and Fe deficiency anemia;
- Studying the mechanism of the Zn/Fe interaction and absorption processes;
- Reviewing the link between Zn and Fe intake and status data in an apparently healthy human population;
- Exploring a new biomarker of Zn status; the LA:DGLA ratio and examining its efficacy and applicability to measure Zn status;
- Investigating the effectiveness of Zn biofortification as a potential strategy for alleviating the consequences of Zn deficiency.

Within this chapter, each of these major aspects/findings will be discussed, highlighting the key research points, explaining certain limitations of the work presented in this thesis and proposing recommendations for further research. The major contributions to knowledge attained through this PhD work are once again summarized in the final section of this chapter.

8.2.1 Zn Deficiency as an Underlying Cause for Development of Fe Deficiency: The Proposed Mechanism of the Fe and Zn Interaction

Over many years, evidence has been accumulating on a relationship between dietary Zn intake and Fe absorption. To summarize this interaction, it was shown that deficiencies of Fe and Zn occur simultaneously [2-4], that there is a positive correlation between anemia and signs of the risk of Zn deficiency in various populations [5-6], that Zn deficiency is likely to contribute to Fe deficiency and that Zn is a significant predictor of hemoglobin concentrations independent of Fe status [4,7-12], that the provision of Fe is not always effective in the treatment of Fe deficiency and Fe deficiency anemia and delivery of Zn in addition to Fe is beneficial and improves hematological indices and Fe status [13-21]. While, the evidence indicates a synergistic relationship between Zn and Fe, the mechanism of the interaction was not so clear. Knowledge of the physiology that controls Zn and Fe absorption is crucial for introducing appropriate treatments and planning nutrition intervention strategies for alleviating the problem of inadequacy of both micronutrients simultaneously.

The information provided in **Chapter 3** elucidates the prospective mechanism of the Zn and Fe interaction, in a more comprehensive manner, and provides a model of the potential mechanism of Zn impact on the Fe absorption process, both at the local and systemic level. The structure and the function of major Zn and Fe transporters found in the intestine and the liver are described (**Chapter 3**). It was demonstrated that cellular Zn directs a sequence of events that determines the expression of proteins involved in cellular Fe metabolism. Zn controls the expression of both major Fe transporters, DMT1 and FPN1 (**Chapter 3, Table 3.1**). The role of Zn in the regulation of Fe absorption under a number of physiological and pathological conditions is explained. The involvement of Zn in the Fe regulatory protein system is presented and an entire mechanism of Zn involvement in the control of hepcidin action; the major protein of systemic Fe regulation, is clarified (**Chapter 3, Figure 3.6**). Finally, a comprehensive model for the role of Zn in the overall regulation of Fe absorption was presented (**Chapter 3, Figure 3.7**).

The positive link between dietary Zn intake and Fe status indicators has been additionally confirmed through both the animal and the human studies.

In the human trial, statistically significant alterations in hemoglobin and RBC status between the groups of people with different dietary Zn intakes was observed **(Chapter 5**). While there was no relationship between the dietary Zn intake and plasma Zn status, dietary Zn intake correlated with Fe indices, hemoglobin and red blood cell count.

In the chicken study, the expression of various Fe transporters were responsive to dietary Zn manipulations, with the higher expression of investigated transporters found in birds fed the lower Zn diet (**Chapter 7, Figure 7.2**). Zn has a protective role against Fe induced oxidative damage, so reduced amounts of Zn in the cells signify that the level of Fe in the cells should also be reduced [22,23]. Expression of DcytB reductase and ferroportin transporter (FPN1) was different among the groups (**Chapter 7, Figure 7.2**) which supports the hypothesis that cellular concentrations of Zn affect the process of Fe absorption by modulating the activities of transporters responsible for the uptake of Fe into the cells.

The entire mechanism of potential Zn involvement in the Fe absorption process (both at the local and systemic level) has been illuminated. The challenge is certainly to further use this new information in such a way as to provide a more complete understanding of the link between Zn and Fe absorption pathways, using both in vitro and in vivo models.

For example, Caco-2 cells are the human-derived cell line with various morphological and functional characteristics of absorptive small intestinal cells, so they can be employed for mechanistic studies on the Fe and Zn interaction and for investigating Zn related dietary factors that influence Fe absorption [23,24].

Caco-2 cells contain all relevant transport proteins for Fe (DMT1, Dcytb, FPN1) [25], and ferritin formation is a sensitive indicator of the Fe uptake within these cells [26,27]. However, the effect of hepcidin (HAMP) on Fe status cannot be examined with this model, as the regulation of Fe absorption by serum levels of hepcidin relies on hepcidin expression in the liver [25].

There is mixed evidence regarding the correlation between Fe uptake in Caco-2 cells and absorption ratios in humans; some demonstrated negative and others a strong positive correlation [28-35]. It seems that the Caco-2 cell model predicts the direction of response, but the magnitude varies from human studies, so further development of the model may be needed. However, with all its limitations, the Caco-2 cells are a good starting point for understanding the processes occurring in intestinal cells in vivo and for suggesting future experiments that can be performed in whole organisms, either animals or humans.

The Zn and Fe interaction could also be tested in animals before any human trial was initiated. This would allow the whole body assessment of Fe and Zn absorption machinery as dissection of individual tissue parts would be possible [36]. An animal model study has the capacity to assess an extensive range of physiological and molecular parameters more thoroughly and provides fast output. Such a research would help in studying the Fe and Zn absorption processes in Zn deficient subjects, and simultaneously examine the mechanism of the Zn and Fe interaction (the machinery by which Zn deficiency causes Fe deficiency anemia) in greater detail.

Dietary Zn and Fe deficiencies are the leading causes of major global health problems and early childhood deaths [37,38] so suggested studies will offer the evidence to more appropriately direct the future efforts towards resolving the problem of both Zn and Fe insufficiencies. Finally, evidence of improvements in Fe metabolism due to the presence of adequate Zn concentrations in the animal model would justify undertaking human trials, a necessary step for changing the present targets for improving human Zn and Fe nutrition of the most affected groups, the resource-poor subsistence farming populations in developing countries.

8.2.2 An Update on Zn and Fe Intake and Status Data in an Apparently Healthy Population

A recent review of available data on micronutrient intake emphasized that the information about Zn and Fe intake and status data for a number of countries is limited, outdated or do not exist at all [39-41]. Therefore, more current measurements of Zn intake and status data for a number of developed countries is needed. Similarly, the correlation of Zn intake and Zn status with Fe biochemical parameters in healthy population subjects has not been widely investigated. A few studies provided inconsistent results [12,42-44] so additional research in this area is required.

The data presented in **Chapter 4** provides an up to date estimate of the nutritional status and interrelation of Zn and Fe intake and status in an apparently healthy adult population living in Serbia, Europe. In this study, the Zn and Fe nutritional status of adults was examined by means of anthropometric, dietary, and biochemical measurements and the relationship between Zn and Fe dietary intake to Zn and Fe status was evaluated.

Zn and Fe dietary intakes were strongly correlated, while there was no statistically significant relationship between the intakes and plasma concentrations of these elements (**Chapter 4, Tables 4.5 & 4.6**).

Generally, no differences were seen for Zn and Fe intake and status among various socioeconomic groups (**Chapter 4, Tables 4.5 & 4.6**). No strong correlations were found between dietary Zn intake and Fe biochemical parameters, however there was a statistically significant difference in Zn intakes among groups of women with dissimilar hemoglobin concentrations (**Chapter 4; Table 4.12**).
The study demonstrated that lower than recommended dietary intake of Zn exists in otherwise healthy people. Five percent of the study population had inadequate Fe and around 20% had unsatisfactory Zn intakes (**Chapter 4; Tables 4.2 & 4.3**). Women with dietary Fe intakes below recommendations were at greater risk of inadequate dietary Zn intakes. 38% of women had inadequate dietary Fe intakes and 76% of these with low dietary Fe intakes show concurrent insufficient intake of Zn.

A prolonged inadequate intake of Zn and Fe may contribute to the development/manifestation of Zn and Fe deficiencies, so regular monitoring of mineral intake and status for various population cohorts is necessary to make sure that deficiencies do get recognized and addressed in a timely manner. In many developed countries, micronutrient deficiencies are not related to the quantity of food consumed, but rather to the quality of the diet [45,46]. Inadequate dietary intakes of Zn and Fe that failed to meet the high physiological demands were seen in different countries for various age groups (i.e. adolescent girls in Australia and New Zealand; non-pregnant premenopausal women from Seattle, Washington, USA) [45,47-49]. Dietary decisions made by individuals may lead to Fe and Zn deficiencies. Similarly, lifestyle changes occurring over the last few decades in many developed countries are characterized by increased consumption of low-cost, but energy-dense foods and by reduced physical activity levels [50-53]. Finally, with the present global trend of people in developed countries eating less meat and increasing intake of grains [54,55] there is a tendency that more individuals may end up having inadequate intakes of many important nutrients. Therefore, regular monitoring of Zn and Fe intake and status data for various age groups is very important.

Further work is needed to examine the role of dietary Zn on Fe status indicators in humans more fully. Measurements of ferritin, transferrin and total Fe binding capacity may provide more detailed analysis of this relationship.

Being overweight is a worldwide problem that affects developed and developing countries [53,56], however even with excess dietary intake of energy and macronutrients, it is still uncertain if people are taking the recommended intakes of micronutrients. A number of national epidemiological surveys conducted in several developed countries reported the co-occurrence of obesity with inadequate intakes for certain vitamins and minerals, particularly calcium, Fe, Zn, vitamins B1, B2, B6, D and folate [57-59]. Not only inappropriate intakes, but also impaired bioavailability and utilization of micronutrients may be involved in the inadequate micronutrient status in obesity. The low level inflammation that accompanies obesity is likely to decrease Fe and Zn absorption [60]. This needs to be taken into account when the adequacy of Zn and Fe intake in a population is evaluated.

Furthermore, other factors influence the daily intake of Zn and Fe; their concentration in food, the amount of food consumed, and consumption of dietary components that delay Fe and Zn absorption, i.e. polyphenols and phytate [61-64]. Food composition databases need to be updated, as many of them do not contain information on the content of phytate.

To sum up, additional up to date and higher quality studies are needed to address gaps in current knowledge. Regular follow ups are necessary to ensure that potential deficiencies of Zn do get acknowledged and addressed in a timely manner, particularly in countries where their existence is less expected. Supporting evidence and additional research on dietary Zn intake globally would not only help in obtaining a more accurate estimate of Zn inadequacy, but also, in adequately directing nutrition interventions aimed at controlling Zn deficiency. Finally, further work is needed to look at the role of Zn nutrition in the Fe absorption process in more detail.

8.2.3 The LA:DGLA Ratio - A Novel Biomarker of Zn Status

Ten years ago, the World Health Organization announced the identification of a sensitive biomarker of Zn status as a high priority [65]. Despite various research efforts accomplished over the years, an indicator that could present Zn status truthfully, under numerous physiological conditions, is still missing. A more accurate biomarker is needed to appropriately assess the dietary Zn intake in relation to biochemical status, and correspondingly to correctly define Zn inadequacy as a public health problem.

The results presented within this thesis support the hypotheses that the LA:DGLA ratio corresponds to dietary Zn manipulations, both in animals and humans, and that the ratio can be used as an additional physiological biomarker of Zn status (**Chapters 5 & 7**).

The content of plasma phospholipid LA, DGLA and changes in the LA:DGLA ratio were compared to the dietary Zn intake and plasma Zn status in healthy human subjects (**Chapter 5)**. It was shown that while plasma Zn concentration of participants remained unchanged, there was a statistically significant difference in DGLA production and the LA:DGLA ratio between the groups of subjects with statistically different dietary Zn intakes (**Chapter 5, Table 5.4**).
In addition, when the correlation of plasma Zn and dietary Zn intake was evaluated in relation to other polyunsaturated, and some saturated and monounsaturated fatty acids, out of the twelve fatty acids examined plasma Zn was associated with only one polyunsaturated acid, DGLA (**Chapter 5, Table 5.3**).
The activity of delta 6 desaturase was reduced when lower intake of dietary Zn was present, which confirms the sensitivity of delta 6 desaturases to Zn intake. The concentration of DGLA declined and the LA:DGLA ratio increased in people with lower dietary Zn intakes (**Chapter 5, Table 5.4**).

The ability of the LA:DGLA ratio to discriminate the status between Zn adequate and Zn deficient subjects was additionally shown in animals (**Chapter 7**). The LA:DGLA ratio was statistically different among the groups of birds, with a higher LA:DGLA ratio measured in the subjects fed the low Zn wheat based diet, at each time point (**Chapter 7, Figure 7.1**). When the birds were fed either a Zn adequate control diet (46.5 μg Zn/g) or a Zn deficient diet (32.8 μg Zn/g), the hepatic delta 6 desaturase expression was significantly higher in the control group (**Chapter 7, Figure 7.2**). With only a 14 μg Zn/g differential in dietary Zn concentration, the LA:DGLA ratio still differentiated clearly between the groups, which demonstrates the sensitivity of the biomarker to change in accordance to the dietary Zn intake.

A further advantage of the LA:DGLA ratio as a biomarker of Zn status is its ability to rapidly assess the outcomes of changing levels of dietary Zn. The erythrocyte LA:DGLA ratio responded to dietary Zn manipulations quickly (within two weeks) (**Chapter 7, Table 7.4**), signifying that this biomarker can show changes in the dietary Zn status quite quickly and it may be able to detect early stages of Zn deficiency that usually, due to the lack of obvious signs and symptoms, pass unrecognized.

The new findings are very encouraging as they demonstrate that the LA:DGLA ratio changes in accordance to dietary Zn intake in both animals and humans and that it has the potential to be used as a new physiological indicator of Zn status. Indeed, further studies and dietary intervention trials are needed to entirely describe the effectiveness of this biomarker in relation to Zn status and Zn bioavailability over time.

Examining the sensitivity of this biomarker in different settings is critical. For example, in larger study groups, in Zn deficient populations, as well as in treatment groups with various levels of Zn deficiency. Well controlled human dietary intervention trials are needed to elucidate any potential limitations of this biomarker (i.e. the effect of inflammatory conditions and infection states on this biomarker).
The effectiveness of the LA:DGLA ratio in relation to Zn status and Zn bioavailability over time (long vs. short low/high Zn intake) requires further investigation. Additional studies are needed to clarify the potential impact of other nutrient deficiencies on the LA:DGLA ratio, in particular the effect of Fe and Cu deficiency. The kinetics of desaturase enzymes in humans should also be examined.
Similarly, the enzymatic activity of hepatic desaturases should be compared to the expression and activity of delta 6 desaturases in non-hepatic tissues in order to determine the exact role of dietary fats in delta 6 desaturase activity. The kinetics of other desaturase enzymes in relation to Zn intake should also be tested (i.e. delta 5 desaturase). Finally, the modifications of Zn dependent proteins and genes, at the main sites of Zn absorption (i.e. the small intestine) in relation to Zn intake and the LA:DGLA ratio, needs to be tested and evaluated further.

8.2.4 The Efficacy of Zn Biofortified Wheat in Improving Zn Status of Consumers

The introduction of Zn biofortified wheat crops is suggested as an approach for addressing nutritional Zn deficiency in people dependent on wheat as a basic food source [66,67]. As such, knowledge of its efficacy in improving Zn status of consumers needs to be confirmed. Additionally, an appropriate assessment of Zn bioavailability and absorption from Zn biofortified wheat is essential in estimating the effectiveness of wheat related breeding programs and health impacts of biofortified wheat products.

The work presented in this thesis (**Chapter 7**) provides evidence that valuable increases in Zn absorption can be achieved with the intake of Zn biofortified wheat products. Using *Gallus gallus* (chicken) as a model, it was confirmed that the additional Zn present in biofortified wheat is readily available for absorption. The wheat genotypes with increased grain Zn concentrations contained increased amounts of bioavailable Zn (**Chapter 7, Table 7.1**), supporting the idea that breeding for Zn enhanced wheat grain may contribute to decreasing Zn deficiency in target populations. The greater amount of Zn in the biofortified wheat resulted in a higher dietary bioavailability of Zn, increased the uptake of Zn by the intestinal cells and improved Zn status of birds. Higher serum, feather and nail Zn concentrations were measured in the group of birds consuming a high Zn wheat based diet (**Chapter 7, Table 7.5**).

The absorption of Zn was greater from the Zn biofortified wheat dietary treatment than from the wheat with lower Zn concentration, when the same quantities of each type of wheat flour were consumed. A 14 μg Zn/g differential in dietary Zn intake was sufficient to discriminate between the Zn status of the birds used in the study (**Chapter 7, Figures 7.1 & 7.2**).

The nutritional benefits of Zn biofortified wheat are shown, however, people do not eat wheat flour raw and processing and baking are common practices worldwide. There is increasing evidence that the food processing, including milling, fermentation and heat treatment during baking can change the nutritional content and nutraceutical properties of cereal based products [68-72]. The effect of bread making processes on the concentration and availability of Zn and phytate in biofortified wheat breads made of different wheat varieties should be evaluated and compared as variable responses with different wheat cultivars may be observed.

Currently available evidence propose that the levels of Zn do not change, while the concentration of phytate declines during fermentation and baking procedures [70,71,73-75], which in theory would mean that the bioavailability and absorption of Zn from baked products should be additionally augmented due to the lower phytate:Zn ratio. Zn absorption is considerably increased when the phytate:Zn molar ratio is below 15 [62,76]. Therefore, the magnitude of this increase and its consequences on availability of Zn and its absorption from processed Zn biofortified wheat products of various wheat varieties needs to be explored further.

In addition, it is still not entirely known how rheological properties of flour get affected by Zn biofortification procedures. It should be examined if Zn biofortified wheat varieties are having similar rheological behaviour to traditional wheat flour and in which way the biofortification affects functional properties of this newly formed wheat material in terms of its stability, viscosity, elastic properties, formulation of the flour in the presence of Zn enhancers, consistency modification when adding fertilisers, water absorption properties and enzymatic activity.

The effect of foliar agronomic biofortification of wheat plants with Zn on the concentration of health promoting compounds (i.e. phytate and polyphenols) and other minerals (i.e. Fe, Cu) has had limited testing and shown contrasting results. The estimated bioavailability of Fe, Mn, and Cu was unaffected by Zn biofortification [77] or an antagonism between Zn biofortification and content of other important minerals in wheat grains is shown [78], so further research in this area is required to clarify these discrepancies.

The efficacy of Zn biofortified wheat products in improving the Zn status of consumers should be tested for various biofortified wheat varieties available across the globe as each wheat variety may not necessarily produce an identical effect. Moreover, great variability in the number of traits and properties between the wheat varieties has been noticed [71,79-83]. Subsequently, different wheat cultivars need to be examined to reach biofortification targets aimed at enhancement of Zn content and its maximal possible bioavailability in grains.

A large number of wheat samples may possibly be tested simultaneously using the high throughput Caco-2 cells model. Duodenal enterocyte modulate their nutrient absorption in response to dietary intake, so the model can predict availability, uptake of Zn into the enterocytes and sometimes even the absorption [25]. The cell model is less work expensive, quicker and allows a number of wheat varieties to be compared simultaneously [84,85]. In addition, a well-developed cell model could diminish the need for isotopic labelling of the foods to measure Zn uptake [84,85]. However, there is mixed evidence of the efficacy of cell monolayers in predicting Zn absorption from food. When the formation of metallothionein, a cytoplasmatic protein that stores Zn, was tested as a proxy for Zn absorption, it has been shown that the measurements of the cellular Zn and MT concentrations are less reliable as their expression is often affected not only by Zn but also by other dietary components (i.e. phytate, casein, other metals) [84-86]. On the other hand, Caco-2 cells were successfully used for assessing Zn uptake into the cells [84,87-91]. The Caco-2 cell technique provided comparable result to certain in vivo methods and is suggested as a technique for preliminary screening of a large variety of Zn biofortified cereal lines [36,84,92]. Nevertheless, a protein that can be used as an indicator of Zn uptake and is specific for Zn only is still missing [85,93] and the conditions for the in vitro digestion should be measured and selected cautiously, making this approach imperfect, so further improvement and optimization of Caco-2 cells as a model for estimating Zn absorption from food is recommended.

Cultural acceptance of Zn biofortified wheat based foods should be examined before biofortified wheat varieties are disseminated and introduced on a wide-scale. It is important to determine if potential consumers like the biofortified wheat foods as much as their conventional counterparts. Sensory perspective and sociocultural adoption of new products to both rural and urban populations should be explored, either when wheat is prepared following traditional methods or when used as an ingredient in non-traditional food products. The willingness of producers and consumers to accept new wheat varieties will determine whether Zn biofortification of wheat crops can be successfully implemented.

Zn biofortified wheat varieties are developed primarily for resource poor populations dependent on wheat as a basic food source. They are meant to reach malnourished rural populations with a restricted access to supplements and commercially fortified foods [94]. While these populations would certainly benefit the most from the consumption of Zn biofortified wheat, the use of the newly developed product could also be helpful to people in industrialized countries, where wheaten bread is a staple food. As it has been demonstrated in this thesis, a marginal Zn deficiency is present in apparently healthy people living in Serbia, Europe (20% of the study population was with inadequate dietary intake of Zn). Similarly, the assessment of Zn availability in soils and wheat grains revealed that while only 13% of the soil samples in Serbia were Zn deficient, the levels of this micronutrient in wheat grain were relatively low (median values of 21 mg/kg for Zn) and even lower in the white flour [95]. The concentration of Zn in bread wheat grains in Serbia is modest and in the lower range of concentrations reported globally, 15-40 mg/kg [95,96]. Despite the evidence, the public attention on this issue in Serbia is still missing [95] and actions should be taken to address the problem in a timely manner.

The Serbian population is dependent on cereal based diets with cereals providing approximately 40% of the daily per capita energy intake (http://faostat3.fao.org/download/FB/FBS/E). Wheat is the staple food and the second most important crop in relation to cultivated area and total production [95]. The typical annual per capita consumption of wheat bread made of white flour is about 106 kg [97], which is three times more than the typical intake in the EU. The usual daily per capita intake of wheat bread in Serbia is high (over 300 g), while the dietary intake of animal products is four times lower than in the EU [95]. For such a diet type, recommendations regarding public health are to fortify white wheat flour by adding 4-9 times more Zn than currently present in Serbia [95,98]. Zn biofortification could be a suitable solution to the problem of inadequate Zn intakes in the Serbian and other developed country populations where wheaten bread is a staple. A short term intervention with foliar application of Zn fertilizers and/or a long-term breeding program to augment the Zn concentration of major bread wheat cultivars should be considered to alleviate the problem of Zn inadequacy in these settings.

Recently, the gut microbial environment has been recognized as an important organ in the absorption and utilization of Zn from the diet [99,100]. The gut microbial environment is fundamental to Zn homeostasis and it is undesirably affected by suboptimal Zn status [99-101]. As the intake of Zn biofortified staple food crops is projected to grow substantially due to increasing implementation of population-wide biofortification strategies [66,102,103], characterization of potential alterations in the gut microbiota following consumption of a Zn biofortified wheat based diet needs to be investigated.

The supplemental Zn alters the composition of the gut microbiome [101]. Zn deficiency deleteriously changes the composition of the gut microbiota through global reductions in taxonomic richness and diversity, decreases in beneficial short chain fatty acids (SCFAs), and changes in expression of bacterial micronutrient pathways [101]. Additionally, the protective effects of therapeutic Zn supplementation, including modifying intestinal permeability (via proliferation of the absorptive mucosa), reducing villous apoptosis, influencing the immune response, reducing pathogenic infections and subsequent diarrheal episodes, are documented [101,104-107]. Further studies should evaluate how the consumption of Zn biofortified wheat based diets modify the gut microbiota and whether the biofortified Zn diet influence the metabolic and functional capacity of the host microbiome *. With the current intention of increasing the consumption of Zn biofortified diets worldwide, exploring the role of the gut microbiota in Zn biofortification remains important in order to further improve the nutritional outcomes provided by biofortification.
Finally, the efficacy of the biofortified staple food crops in improving Zn status of humans should be directly measured. Thus, human studies are desirable to confirm positive impact of Zn biofortified wheat on Zn status and support the further release of Zn biofortified crops.

To conclude, the biofortification strategies based on wheat breeding and application of Zn fertilizers has the potential to alleviate Zn malnutrition, both in developing and industrialized countries. Additional research is certainly needed to assess various variables (i.e. health impacts of biofortified products, product acceptance by farmers, by consumers, decision makers, recognition of the product by regulatory agencies, integration into development policies and private sector entities) that will determine the ultimate success of biofortification of wheat crops with Zn.

*** Note:** Please see the paper presented in Appendix 2.
Publication: Reed, S.; Knez, M.; Uzan, A.; Stangoulis, J.C.R.; Glahn, R.P.; Koren, O.; Tako, E. Alterations in the gut (*Gallus gallus*) microbiota following the consumption of zinc biofortified wheat (*Triticum aestivum*)-based diet. *J Agric Food Chem.* **2018**, *66* (25), 6291-6299.

8.3. Contributions to Knowledge

A Summary of the Main Contributions to Knowledge Presented Within this Thesis:

- An improved understanding of the role of Zn in the Fe absorption process;
- The proposition of mechanisms by which dietary Zn controls Fe absorption processes, both at the local and systemic level;
- New information of the link between Zn and Fe intake and status data in an apparently healthy population living in Serbia, Europe;
- Confirmation that the LA:DGLA ratio can be considered as an additional biomarker of Zn status, both in animals and humans;
- Evidence that the intake of Zn biofortified wheat improves Zn status of chickens.

8.4 References

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Appendix 1

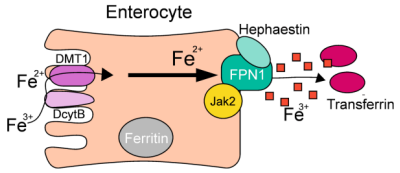
Conference Posters

NEW PERSPECTIVES ON THE REGULATION OF IRON ABSORPTION VIA CELLULAR ZINC CONCENTRATIONS IN HUMANS

Marija Knez, Robin D. Graham, Ross M. Welch
and James C.R. Stangoulis

BIOAVAILABILITY 2014

The mechanism of Fe absorption in human enterocytes

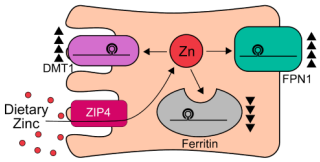


Iron is moved across the enterocyte brush border membrane by DMT1, a process enhanced by the prior reduction of Fe (ferric reductase activity) by duodenal cytochrome B (DcytB). Enterocyte Fe is exported to the blood via FPN1 which acts in partnership with the ferroxidase hephaestin that oxidizes exported ferrous Fe and facilitate its binding to plasma TF.

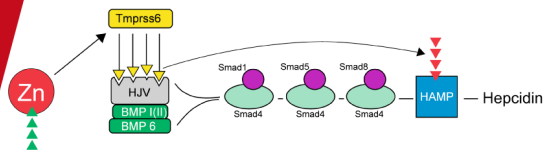
- Fe and Zn influence the transport and absorption of one another across the enterocytes and hepatocytes
- No conclusive evidence about the mechanisms that regulate these interactions
- Fe and Zn do not compete for absorption at the DMT1; Zn2+ is not transported by DMT1
- Zn is an important element in the regulation of DMT1 gene expression
- FPN1 levels are increased when cells are exposed to Zn

Proposed model for the role of Zn in regulation of intestinal Fe absorption

The availability of Zn in the cells plays a crucial role in Fe absorption and transfer to circulation. Although, Fe is supplied in sufficient amounts it cannot be absorbed (Zn deficiency leads to DMT1 delocalization) and transported out of the enterocyte (as ferritin storage capacity increases) without the adequate supply of Zn. With sufficient Zn supply the expression of both transporters is increased. The storage capacity of ferritin is reduced, and more Fe is transported into the plasma.



The expected influence of Zn on hepcidin regulation



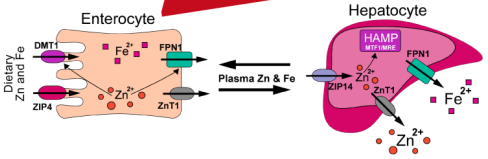
- Hepcidin – small peptide, a major regulator of Fe homeostasis
- BMP6/SMAD pathway takes the central role
- Critical for this pathway to function is a co-receptor - hemojuvelin (HJV)
- Tmprss6, a cell surface serine protease that degrades HJV, is able to reduce BMP signaling
- Tmprss6 is Zn dependent endopeptidase; inadequate Zn levels lead to improper function of Tmprss6

BMP6 binds to a BMP receptor I/II complex, leading to phosphorylation of receptor-regulated SMADs 1, 5, and 8. Phosphorylation allows these proteins to interact with Smad 4, and the resulting heteromeric complexes stimulate transcription of the gene encoding hepcidin antimicrobial peptide (HAMP). Tmprss6s are Zn dependent endopeptidases, so adequate cellular Zn levels contribute to natural function of Tmprss6 (degradation of HJV) that reduces hepcidin production and consequently contribute to increased Fe absorption.

The anticipated mechanism of Zn involvement in Fe homeostasis

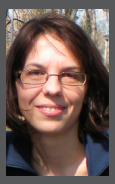
Dietary Zn intake is the main determinant of Zn absorption. Adequate Zn, increased expression of DMT1 and FPN1, Fe is not stored as ferritin, transfer of Fe across the enterocytes .

Zip 14 transports Zn to the liver. Adequate levels of Zn in the hepatocytes, Tmprss6 is functioning properly, hepcidin production decreased (HJV, BMP pathway) and FPN1 and DMT1 expression increases, cells absorb Fe further. In order for the cells to respond appropriately to Fe deficiency the presence of adequate intracellular concentrations of Zn is vital.



The anticipated mechanism of Fe absorption under physiologically favourable conditions (adequate supply of both minerals Zn and Fe, without inflammation or infection).

An initial step in the evaluation of newly proposed biomarker of Zn status, linoleic acid: dihomo-γ-linolenic acid, in humans.



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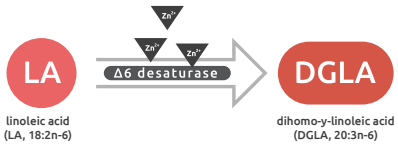
Marija Knez, James C.R. Stangoulis, Elad Tako, Manja Zec, Jasmina Debeljak-Martacic, Zoran Pavlovic, Mirjana Gurinovic, Maria Glibetic

Introduction:

Zinc is an essential micronutrient for humans and hasimportant physiological functions. To date, a sensitive and specific biomarker for assessing Zn status is still needed.



Objective:

The aim of this study was to examine if changes in the content of plasma phospholipid LA, DGLA and LA: DGLA ratio can be used to efficiently predict the dietary Zn intake and plasma Zn status of humans.

Zinc modulates cyclooxy-genase activity and it is a co-enzyme for delta desaturase. As desaturase enzymes require zinc and have a relatively low binding constant their activity is quite sensitive to early stage zinc deficiency. Zn deficiency leads to inconsistencies in the ratio of desaturase substrates and products, in this case linoleic acid (LA) and dihomo-γ-linolenic acid (DGLA) respectively (Bettger et al. 1979; Eder & Kirchgessner 1996). The delta 6-catalyzed step required for conversion of LA to DGLA is usually the highest flux pathway, so an elevation in the LA: DGLA ratio may be a sensitive marker for Zn deficiency (Reed et al. 2014).

Methods:

The study was performed on apparently healthy human volunteers (n=54). Dietary Zn intake was assessed using three 24h recall questionnaires, conducted on non-consecutive days. Plasma phospholipid fatty acid analysis was done by gas chromatography, and plasma analysis of minerals by atomic absorption spectrometry. Biochemical, anthropometrical and haematological parameters were assessed.

Results:

The average age of participants was 40 ± 7 years, with average height of 173 cm ± 7 and weight of 83 ± 2.4 kg. All subjects had adequate plasma Zn concentrations (reference range 0.7-1.6mg/l) and no deficiencies were observed (no plasma Zn values <0.7 mg/l). Mean intake of zinc obtained from the 24h dietary recalls was 12.75 ± 2.18 mg/day (mean ± SE). Around 30 % of participants consumed Zn at levels below the EAR (6.8 mg/day for women and 9.4 mg/day for men).

Differences in the dietary content and plasma concentrations of LA, DGLA and Zn between Zn groups.

	Group 1 (n=27)	Group 2 (n=27)	Significance (2-tailed)
Dietary Zn (mg)	7.01 ± 0.52	12.78 ± 1.54	0.001*
Dietary LA (g)	17.54 ± 3.54	19.75 ± 2.78	0.650
Dietary PUFA (g)	12.61 ± 2.86	2.15 ± 2.17	0.755
Plasma Zn (mg/L)	1.02 ± 0.03	1.07 ± 0.03	0.283
LA (%)	24.01 ± 0.49	24.15 ± 0.54	0.528
DGLA (%)	2.61 ± 0.12	3.14 ± 0.14	0.004*
LA/DGLA	9.53 ± 0.43	8.21 ± 0.47	0.040*

The meat, nuts and grain products were the main source of the dietary Zn intake. Within the study population, Zn bioavailability from the diets consumed belong to the group of 'high Zn bioavailability'. Out of the twelve fatty acids examined, plasma Zn was correlated with only one polyunsaturated acid, DGLA. No significant relationship was found between the dietary and plasma zinc status (r=0.07; p=0.6). There was a statistically significant correlation between DGLA and plasma Zn (r=0.39, p=0.00). No relationship was observed between the linoleic acid and plasma Zn, while there was a significant negative correlation between LA: DGLA ratio and plasma Zn status (r=-0.35, p=0.01). Similarly, there were statistically significant differences in DGLA status (p=0.004) and LA: DGLA ratio (p=0.042) between the two Zn groups.

Except for the statistically significant difference in intake of meat (p=0.015), there were no statistically important dissimilarities in the consumption of any other food items that contributes to Zn intake (i.e. seafood, grain, vegetables, fruits, nuts). No statistically significant variation was seen in dietary Fe intake (p=0.85). There were no statistically significant differences between the zinc groups for any of the trace elements measured. No dissimilarities were observed for any of the biochemical parameters between the groups. No dissimilarities were seen in the dietary intake of LA or PUFA between the groups. Plasma Zn did not reflect changes in the dietary Zn intake, so no statistically significant variances were seen in plasma Zn concentrations among the groups.

Conclusions:

This study is an initial step in evaluating the LA: DGLA ratio as a biomarker of Zn status in humans. The results are encouraging as they show that the concentration of DGLA declines and the LA:DGLA ratio increasesin people with lower dietary Zn intake. However, additional studies are needed to fully examine the sensitivity of this biomarker.

Further

To examine the sensitivity of this biomarker in different settings: in larger study populations, in Zn deficient populations, as well as in the treatment groups with various levels of zinc deficiency.

To clarify any potential limitations of this biomarker, i.e. the effect of inflammatory conditions and infection states on this biomarker. The usefulness of LA: DGLA ratio in reflecting the Zn status of an individual should further be examined by looking at the changes of this biomarker during different time frames (long vs. short low/high Zn intake).

The kinetics of the desaturase enzymes should also be examined in humans. Similarly, the changes in LA: DGLA ratio may be investigated in relation to the alterations of Zn depended proteins and genes in various tissues (i.e. ZnT1, Zip4).

Acknowledgments:

This work was supported by a grant from Ministry of Education and Science of the Republic of Serbia (Grant III 41030) and EU FP7 project BACCHUS (Grant agreement no. 312090) and by an Australian Postgraduate Award Scholarship, provided to the principle author.

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The linoleic acid: dihomo-γ-linolenic acid ratio predicts the efficacy of Zn biofortified wheat in *Gallus gallus*

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INTRODUCTION:

Zn deficiency is a common micronutrient deficiency in developing country populations, many of which eat wheat as their primary staple[1]. Zn biofortified wheat is one recently developed approach for alleviation of Zn deficiency, however, its efficacy needs to be confirmed[2]. The lack of a sensitive and specific Zn biomarker limits our ability to assess the impact of biofortified products.

The quantity of Zn absorbed from Zn biofortified wheat material using an *in-Vivo* (*Gallus gallus*) model of Zn absorption was determined through measurement of a range of physiological and molecular parameters. The efficacy of the LA:DGLA ratio to predict Zn status of Zn deficient subjects consuming wheat based diets was also assessed.

Table 1. Zn, Fe, phytate, Ca content and fatty acid concentration of wheat flours

Components	High Zn flour	Low Zn flour
Zn concentration (ppm)	47.2 ± 0.47	33.6 ± 0.43
Fe concentration (ppm)	58	53
Phytate (mg/g)	11 .1 ± 1.5	9.9 ± 0.4
Calcium (mg/kg)	340	330
Fatty acids		
Total saturated	338.8	330.3
Total transaturated	0.4	0.3
Total monounsaturated	286.1	274.4
Total omega 3	55.2	55.8
Total omega 7	19.0	19.1
Total omega 9	265.8	254.1
Total omega 6	975.8	981.0
LA (18:2n -6)	973.1	978.4
DGLA (20:3n -6)	0.6	0.5

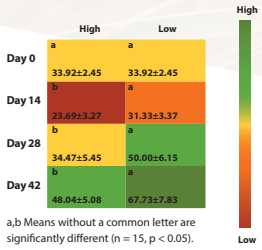
LA - Linoleic acid; DGLA - Dihomo -γ-linolenic acid. Fatty acids – mg/100g.

METHODS:

The commercial wheat variety, Correll (*Triticum aestivum*) was grown by Jordan Farms at Murtoa in the West Wimmera Shire of Victoria, Australia. Biofortified grain was obtained by a foliar application of 1.5 L ha^{-1} $ZnSO_4$ during the mid-vegetative growth stage (mid-August), followed by 2 L ha^{-1} $ZnSO_4$ two weeks after flowering (mid-October).

Except for the variation in seed Zn concentration, there were no statistically significant differences in the fatty acid content or Fe concentrations between the low and high Zn diets (Table 1).

Two groups of chickens (n = 15) were randomly separated upon hatching and fed two different diets: a high Zn diet (46.5 ppm Zn) and a low Zn diet (32.8 ppm Zn). Dietary Zn intakes, body weight, serum Zn, and the erythrocyte fatty acid profile were measured weekly. At the end of the study (day 42) tissues were collected for gene expression analysis.



a,b Means without a common letter are significantly different (n = 15, p < 0.05).

Figure 1. Changes in the LA: DGLA ratio among the groups from day 0 to day 42

References:

[1] Wessells, K.R., Brown, K.H. (2012) Estimating the global prevalence of zinc deficiency: Results based on zinc availability in national food supplies and the prevalence of stunting. PLoS ONE 7:11:e50568.

[2] Shahzad, Z., Rouached, H., and Rakha, A. (2014) Combating Mineral Malnutrition through Iron and Zinc Biofortification of Cereals. Comprehensive Reviews in Food Science and Food Safety 14:329-46.

Manuscript:

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RESULTS:

Serum Zn concentrations were greater in the high Zn group versus the low Zn group at each time point ($p<0.05$). The concentration of Zn in feather and nail was higher in the high Zn group versus the low Zn group (day 42, $p < 0.05$).

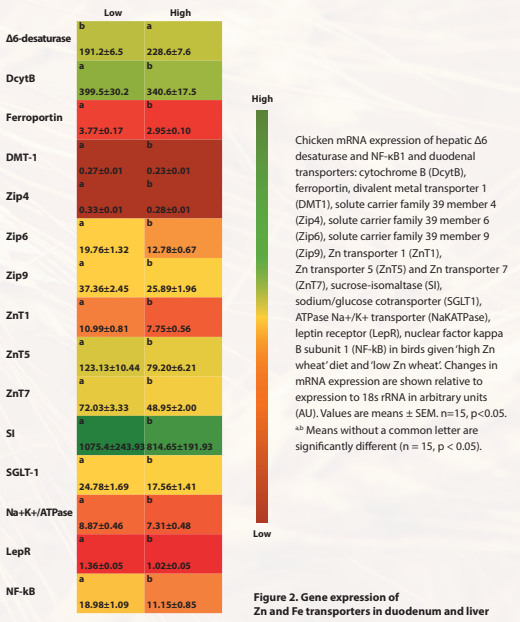
Duodenal mRNA expression of various Zn transporters demonstrated expression upregulation in the birds fed a low Zn diet (n=15, $p<0.05$).

The Δ6 desaturase expression was higher in the high Zn group and the LA: DGLA ratio was higher in the birds fed the low Zn diet ($p<0.05$). The LA: DGLA ratio was increasing as the study progressed from day 0 to day 42 and was dissimilar among the treatment groups at each time point (Figure 1).

Table 2 . Differences in Zn status among the groups as assessed by various Zn biomarkers (serum, nail, and feather Zn concentration)

	Day 0	Day 14	Day 28	Day 42
Serum Zn (μg/g)				
Low Zn group	1.07 ± 0.15 [a]	0.48 ± 0.05 [a]	0.35 ± 0.03 [a]	0.43 ± 0.04 [a]
High Zn group	1.07 ± 0.15 [a]	0.66 ± 0.08 [b]	0.69 ± 0.07 [b]	0.55 ± 0.04 [b]
Feather Zn (μg/g)				
Low Zn group				89.6 ± 7.7 [a]
High Zn group				117.9 ± 10.2 [b]
Nail Zn (μg/g)				
Low Zn group				67.9 ± 3.9 [a]
High Zn group				85.8 ± 3.9 [b]

[ab] Within a column and for each parameter, means without a common letter are significantly different ($n = 15$, $p < 0.05$). Day 0 -day 42. Values are means ± SEM.



Chicken mRNA expression of hepatic Δ6 desaturase and NF-κB1 and duodenal transporters: cytochrome B (DcytB), ferroportin, divalent metal transporter 1 (DMT1), solute carrier family 39 member 4 (Zip4), solute carrier family 39 member 6 (Zip6), solute carrier family 39 member 9 (Zip9), Zn transporter 1 (ZnT1), Zn transporter 5 (ZnT5) and Zn transporter 7 (ZnT7), sucrose-isomaltase (SI), sodium/glucose cotransporter (SGLT1), ATPase Na+/K+ transporter (NaKATPase), leptin receptor (LepR), nuclear factor kappa B subunit 1 (NF-kB) in birds given 'high Zn wheat' diet and 'low Zn wheat'. Changes in mRNA expression are shown relative to expression to 18s rRNA in arbitrary units (AU). Values are means ± SEM. n=15, $p<0.05$. [ab] Means without a common letter are significantly different ($n = 15$, $p < 0.05$).

Figure 2. Gene expression of Zn and Fe transporters in duodenum and liver

CONCLUSION:

The increased amount of Zn in the biofortified wheat resulted in a higher relative bioavailability of Zn and an increased uptake of Zn by the cells. This demonstrates the potential of Zn biofortified wheat varieties in improving the Zn status of consumers.

The LA: DGLA ratio responded to dietary Zn manipulations and the consumption of the Zn biofortified wheat lowered the LA: DGLA ratio. The LA:DGLA ratio can be used as an additional biomarker of Zn status.

Acknowledgment:

The authors acknowledge Jamie and Craig Jordan of Jordan Farms, Murtoa, Victoria, Australia for producing the wheat used in this chicken study. The authors would also like to thank Dr.J.Tom Brenna (Division of Nutritional Science, Cornell University, Ithaca, NY) for the red blood cell fatty acid analysis. Financial support provided by an Australian Postgraduate Award is also acknowledged.

The influence of food consumption and socio-economic factors on the relationship between Zn and Fe intake and status in a healthy Serbian population.

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James C.R. Stangoulis, Mirjana Gurinović, Maria Glibetić

INTRODUCTION

In countries where people have good access to a diverse food source severe zinc (Zn) and iron (Fe) deficiencies are rare. However, ready access to food does not ensure adequate nutrition and marginal Zn and Fe deficiencies still occur.
This study reports on Fe and Zn intake and investigates the link between dietary intakes and associated biochemical parameters in a healthy Serbian adult population; 25-65 years old. In addition, this study identifies patterns of Fe and Zn intake, recognizes main dietary sources, describes biochemical status of Fe and Zn and assesses the effect of some socio-economic factors on Fe and Zn intake and plasma status of these minerals.

RESULTS

Table 1. Macronutrients, Fe & Zn intake of participants

	Males (25 - 65 years, n = 128)		Females (25 - 65 years, n = 626)	
MACRONUTRIENTS	Mean (SD)	% TE	Mean (SD)	% TE
Energy (kcal/day)	2135.9 (730.1)		1705.3 (430.7)	
Proteins (g/day)	83.4 (26.0)	16.5 (4.2)	64.3 (17.7)	15.7 (3.5)
Total carbohydrates (g/day)	207.7 (99.0)	39.1 (10.1)	183.5 (55.4)	44.5 (7.5)
Total fats (g/day)	94.8 (36.6)	40.9 (8.4)	69.6 (24.3)	37.4 (7.1)
MINERALS				
Zinc (mg/day)	9.1 (4.6)		7.3 (3.8)	
			Females (< 50 years, n = 501)	Females (>= 50 years, n = 125)
Iron (mg/day)	11.6 (5.4)		9.4 (4)	10.0 (5.7)

TE- total energy intake

Energy, protein and mineral crude intakes were significantly higher in men than in women (p=0.001). Zn intake correlated with energy (r=0.45), protein (r=0.64), carbohydrate (r=0.32) and Fe (r=0.57) intakes, p<0.001.
Energy, protein, carbohydrates, Fe, and Zn intakes were adequate according to the Dietary References Intakes by age and gender of the adult population [40]. However, the percentage of energy coming from fats was higher than the recommended amount (40 % and 37.4 % of energy for males and females, respectively) compared with the recommended 20-35 % (Table 1).

Table 2. Percentage of the participants with Fe intakes bellow the recommendations

	Males (25 - 65 years, n = 128)		Females (< 50 years, n = 501)		Females (>= 50 years, n = 125)	
	RNI - Iron (mg/day)	% below RNI	RNI	% below RNI	RNI	% below RNI
WHO/FAO	9.1	39.8	19.6	97.8	7.5	32.8
IOM*	8	23.4	18	96.6	8	36.7
IOM (EAR)	6	7.03	8.1	38.3	5	11.8
NNR	9	35.9	15	94.6	9	50

EAR (Estimated Average Requirement) - how much of a nutrient meets the needs of 50 % of the healthy subjects of a specific population's group;
RNI (Recommended Nutrient Intake) - the daily intake that meets the nutrient requirements of almost all (97.5 %) apparently healthy individuals in an age and sex-specific population group.
**IOM, Institute of Medicine FoNB: Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. (Press NA ed. Washington, DC, USA2011*

Five percent of the study population had inadequate Fe and 15-25 % had inadequate Zn intakes. Females were at much higher risk of inadequate Fe, while males were at higher risk of inadequate Zn intakes.
Females below 50 years of age are at the highest risk of inadequate Fe intakes. There were significant differences in the level of inadequacy observed using dietary recommendations proposed by different expert groups (Tables 2 & 3).

Biochemical parameters measured in this study fall within the reference ranges for healthy adult population. The average BMI of our study population was 26 kg/m2 for males and 24 kg/m2 for females. 30 % of males and 70 % of female participants in our study had BMI values in the normal range (n = 476), while ~ 30 % of participants were overweight (n=277), with 10 % of participants being obese. An assessment of the marital status and education showed that 50 % of participant were married and 34 % were tertiary educated.

Table 3. Percentage of participants with Zn intakes bellow recommendations

	Males (25 - 65 years, n = 128)		Females (25 - 65 years, n = 626)	
	RNI - Zinc (mg/day)	% below RNI	RNI	% below RNI
WHO/FAO	4.2	10.2	3	3.7
IOM *	11	73.4	8	69.4
IOM (EAR)	9.4	60.2	6.8	50.6
NNR	9	58.6	7	53.8
IZINCG	10	66.4	6	38.9

EAR (Estimated Average Requirement) - how much of a nutrient meets the needs of 50 % of the healthy subjects of a specific population's group;
RNI (Recommended Nutrient Intake) - the daily intake that meets the nutrient requirements of almost all (97.5 %) apparently healthy individuals in an age and sex-specific population group.
**IOM, Institute of Medicine FoNB: Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. (Press NA ed. Washington, DC, USA2011*

METHODS

Food consumption was assessed by 24 h recall and food frequency questionnaires. Twenty biochemical parameters were measured, of which haemoglobin, haematocrit, red blood cells and plasma concentrations of Fe and Zn were directly related to Fe and Zn nutrition. The prevalence of study participants with inadequate micronutrient intakes was calculated using the estimated average requirement, cut-point method.

Table 4. Contribution of the main food groups to the total Fe & Zn intake (%)

	Iron (Fe)			Zinc (Zn)		
	Males	Females	Total	Males	Females	Total
Meat and meat products	17.9	16.7	17.2	35.2	29.2	30.6
Milk and milk products	1.9	2.4	2.3	20.0	22.6	22.2
Grains and grain products	30.1	32.8	32.6	13.9	17.3	16.6
Vegetable and vegetable products	14.9	11.4	12.3	11.2	7.7	8.3
Legumes	6.1	4.8	5.1	3.6	2.9	3.1
Egg and egg products	5.1	3.5	3.8	4.9	3.9	4.1
Nuts, seeds, kernel products	8.4	9.6	9.4	3.1	3.5	3.3
Beverage (non -milk)	4.2	4.9	4.8	2.0	3.2	3.0
Seafood and related products	2.4	2.6	2.6	1.6	3.1	2.7
Fruit and fruit products	3.4	4.6	4.4	1.7	2.4	2.2
Sugar and sugar products	3.3	3.9	2.6	1.9	2.1	2.1
Miscellaneous food products	1.9	2.6	2.5	1.0	2.0	1.8
Fats and oils	0.3	0.3	0.3	0.0	0.1	0.1

Table 5. Dissimilarities in various parameters among women with low and high Hb

	Females (n=142)	Low Hb (n=33)	High Hb (n=108)	p value
Age	23.3 (0.8)	30.2 (10.5)	23.0 (0.8)	
Weight (kg)	60.3 (5.5)	67.2 (10.3)	61.0 (5.0)	
Height (cm)	169.9 (4.4)	169.5 (6.0)	170.7 (4.1)	
BMI (kg/m^2)	20.9 (1.4)	23.4 (3.8)	20.9 (1.3)	
Waist circumference (cm)	72.0 (4.4)	76.4 (9.8)	72.8 (4.4)	
Hemoglobin (g/L)	130.4 (10.7)	112.4 (7.8)	135.0 (8.1)	
Hematocrit (L/L)	0.4 (0.0)	0.4 (0.0)	0.4 (0.0)	
Red blood cells (x 10^{12}/L)	4.4 (0.3)	4.2 (0.3)	4.5 (0.3)	
Fe (µmol/L)	15.8 (4.8)	12.5 (6.1)	16.5 (4.5)	
Diet Fe (mg)	9.6 (5.1)	10.2 (4.1)	9.5 (4.5)	**0.036**
Diet Zn (mg)	7.1 (2.2)	9.0 (5.5)	7.3 (2.2)	**0.010**

BMI: Body mass index, Low Hb – Hemoglobin values below 120 g/L. Values presented are means ± SD (standard deviations). Diet Fe and Zn data – 24 h recalls. p<0.05 is considered statistically significant.

Women were more at risk of inadequate Fe intakes, while Zn inadequacy was more prominent in males. Women below fifty years of age are the most vulnerable group for development of Fe deficiencies due to increased requirements (menstrual bleeding) an increased need for Fe, and also very often poor intake and poor bio-absorption of Fe.

There is no association between dietary Fe and Zn intake with education level or marital status.
Similarly, SES components measured in this study show no correlation with any of the biochemistry related parameters, including Fe and Zn status. Finally, no differences in the Fe and Zn intake and status among various SES groups were observed, except for Fe intake between low income and affluent groups (p=0.034), while no such difference was observed for Zn intake (p=0.084).

Plasma Zn and Fe concentrations were within the references ranges for healthy population adults. No correlations were seen between the Fe and Zn intake data and plasma status of these elements. However, there was a statistically significant difference in hemoglobin concentrations between those on Zn adequate and Zn inadequate diets (Table 5).

CONCLUSION

Considering that more than twenty percent of our study population may be at risk of Zn deficiency and bearing in mind the health consequences of prolonged inadequate Zn intakes on both Zn and Fe status, more regular follow ups are needed to determine the intake and status of these nutrients and to make sure that deficiencies do get recognised and addressed in a timely manner. Finally, further work is needed to look at the role of Zn nutrition in the Fe absorption process in more detail.

ACKNOWLEDGMENTS

This work was supported by a grant from Ministry of Education and Science of the Republic of Serbia (Grant III 41030) and EU FP7 project BACCHUS (Grant agreement no. 312090) and by an Australian Postgraduate Award Scholarship, provided to the principle author.

Appendix 2

Microbiota Manuscript

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. XXXX, XXX, XXX−XXX

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Alterations in the Gut (*Gallus gallus*) Microbiota Following the Consumption of Zinc Biofortified Wheat (*Triticum aestivum*)-Based Diet

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Supporting Information

ABSTRACT: The structure and function of cecal microbiota following the consumption of a zinc (Zn) biofortified wheat diet was evaluated in a well-studied animal model of human nutrition (*Gallus gallus*) during a six-week efficacy trial. Using 16S rRNA gene sequencing, a significant increase in β- but not α-microbial diversity was observed in the animals receiving the Zn biofortified wheat diet, relative to the control. No significant taxonomic differences were found between the two groups. Linear discriminant analysis revealed a group of metagenomic biomarkers that delineated the Zn replete versus Zn deficient phenotypes, such that enrichment of lactic acid bacteria and concomitant increases in Zn-dependent bacterial metabolic pathways were observed in the Zn biofortified group, and expansion of mucin-degraders and specific bacterial groups able to participate in maintaining host Zn homeostasis were observed in the control group. Additionally, the *Ruminococcus* genus appeared to be a key player in delineating the Zn replete microbiota from the control group, as it strongly predicts host Zn adequacy. Our data demonstrate that the gut microbiome associated with Zn biofortified wheat ingestion is unique and may influence host Zn status. Microbiota analysis in biofortification trials represents a crucial area for study as Zn biofortified diets are increasingly delivered on a population-wide scale.

KEYWORDS: *zinc deficiency, zinc biofortification, wheat biofortification, gut microbiome, microbiota, Gallus gallus*

INTRODUCTION

Zinc (Zn) is a vital nutrient for nearly all organisms, and, in a healthy adult, is second only to iron (Fe) as the most abundant micronutrient.[1,2] Clinically, even mild deficiencies can decrease cellular differentiation, delay immune system maturation, and profoundly impact growth and development.[3,4] Dietary Zn deficiency is affecting approximately 17% of the global population.[5] Indeed, it is the most common micronutrient deficiency in populations living primarily on cereal based diets, such as wheat, where insufficient dietary Zn intake, low consumption of animal products, and poor Zn bioavailability remain major contributors to this condition.[6,7] In many of these populations, such as those in Northern Africa and Eastern Mediterranean regions, up to 50% of the dietary caloric intake is derived from wheat, although present-day refining and extraction techniques lead to wheat that is inherently poor in bioavailable Zn.[8] To decrease the prevalence Zn deficiency, biofortification has been proposed to complement current efforts in improving Zn status.[9] Biofortification, the delivery of Zn via staple food crops, uses both conventional plant breeding and genetic modification approaches to increase concentrations (and bioavailability) of minerals in staple food crops and has become a relevant and effective approach to potentially alleviate micronutrient deficiencies in numerous vulnerable populations.[10] Indeed, our group recently published evidence of a significant improvement in Zn status provided by a novel Zn biofortified wheat-based diet, the same diet used in the current study.[11]

The intestinal microbial environment is crucial for Zn metabolism[12,13] and is in turn influenced by inferior Zn status. Recent work has demonstrated that severe Zn deficiency (dietary Zn concentrations of ∼2.5 μg/g Zn) deleteriously affects the composition of the intestinal microbial populations such as through global reductions in taxonomic richness and diversity, decreases in beneficial short chain fatty acids (SCFAs), and changes in expression of bacterial micronutrient pathways.[14] Collectively, these microbial perturbations may serve as possible effectors of the Zn deficient phenotype by limiting Zn solubility and precluding optimal host Zn availability. Additionally, others have demonstrated the defensive effects of therapeutic Zn supplementation including modifying intestinal functionality (via proliferation of the digestive and absorptive mucosal layer),[15,16] reducing villus apoptosis,[17] affecting immune system response,[18] and decreasing pathogenic infections and consecutive diarrheal incidents.[15]

Received: March 22, 2018
Revised: June 5, 2018
Accepted: June 5, 2018
Published: June 6, 2018

DOI: 10.1021/acs.jafc.8b01481
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Although supplemental Zn alters the composition of the intestinal microbiome, studies have yet to evaluate how Zn biofortified diets modify the gut microbiota. As the consumption of Zn biofortified staple food crop based diets is elevated due to the increasing application of crop biofortification strategies, understanding the risk−benefit ratio from the perspective of the intestinal microbiota remains significant if we are to further advance the nutritional outcomes of strategic biofortification efforts.[19] Hence, by utilizing a well-studied animal model of human nutrition,[14,20] this six-week feeding trial investigated the compositional and functional alterations of the intestinal microbiota in broiler chickens fed a Zn biofortified wheat-based diet (BZn) versus a pair-fed Zn control wheat-based diet (CZn).

We hypothesized that the increased dietary Zn content in the biofortified wheat-based diets would positively alter the gut microbiome in the BZn group and that the relative deficit of Zn in the CZn group would cause an expansion of pathogenic microbiota. A spectrum of Zn status biomarkers was measured on a weekly basis to screen the level of Zn deficiency, and gene expression of a variety of Zn-dependent proteins was measured from relevant tissues at the conclusion of the study. Duodenal samples were taken for identification of villus morphological changes at the conclusion of the study. 16S rRNA gene sequencing was utilized to analyze the microbial population's variations in the intestinal cecal contents.

MATERIALS AND METHODS

Animals, Diets, and Study Design. Hatchlings (n = 30) were randomly distributed into two treatment groups based on body weight and gender (aimed to ensure equal distribution between groups): (1) biofortified Zn (BZn), Zn biofortified wheat (75% Zn wheat based diet, 46.5 ± 0.99 μg Zn/g, n = 15); and (2) control Zn (CZn), standard wheat (75% wheat based diet, 32.8 ± 0.17 μg Zn/g, n = 15). Experimental diets are shown in Supplementary Table 1. At the conclusion of the study, birds were euthanized, and the digestive tracts (colon and small intestine) and liver were quickly removed and stored as was previously described.[21] All animal protocols were approved by the Cornell University Institutional Animal Care and Use committee.

Determination of Zn Status. As was previously described,[11] blood samples were collected weekly from the wing vein (n = 15, ~150 μL) using microhematocrit heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA, USA). Samples were collected in the morning following an 8 h overnight fast. Serum Zn analysis: serum Zn concentrations were determined by an inductively coupled argon-plasma/atomic emission spectrophotometer (ICAP 61E Thermal Jarrell Ash Trace Analyzer, Jarrell Ash Co., Franklin, MA, USA) following wet ashing. Erythrocyte fatty acid analysis (LA/DGLA ratio): the LA/DGLA ratio is an emerging physiological biomarker reflecting dietary Zn intake and status.[11] Red blood cell fatty acid analysis was determined as described.[11] Gene expression analysis: the duodenal expression of Zn related proteins. These procedures were conducted as previously described.[11,14]

Morphological Examination of the Brush Border Membrane Intestinal Villi. As previously described,[22,23] intestinal samples (duodenal segment) from each treatment group were obtained at the conclusion of the study and fixed in fresh 4% (v/v) buffered formaldehyde, dehydrated, cleared, and embedded in paraffin. Sequential sections were cut at 5 μm and positioned on glass slides. Sections were deparaffinized in xylene, rehydrated in a graded alcohol sequence, stained with hematoxylin and eosin, and examined by a light microscopy. Morphometric measurements of villus height and width were performed with an Olympus light microscope using EPIX XCAP software. Villus surface area was calculated from villus height and width at half height. In addition, the number of goblet cells were counted per intestinal villi.

Goblet Cell Density. Morphometric measurements and counting of goblet cell (cell number per 10 intestinal villi) were performed with an Olympus light microscope using EPIX XCAP software.[22,24]

Isolation of Total RNA. Total RNA was extracted from 30 mg of duodenal (proximal duodenum, n = 15) and liver tissues (n = 15) using Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. All steps were carried out under RNase free conditions. RNA was quantified by absorbance at 260−280 nm. Integrity of the 28S and 18S rRNA was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining.

Gene Expression Analysis, Primer Design, and Real-Time qPCR Design. These procedures were conducted as previously described.[11] For further and specific details please refer to Supporting Information.

16S rRNA PCR (Polymerase Chain Reaction) Amplification and Sequencing. Microbial genomic DNA was extracted from cecal samples using the PowerSoil DNA isolation kit, as described by the manufacturer (MoBio Laboratories Ltd., Carlsbad, CA, USA). Bacterial 16S rRNA gene sequences were PCR-amplified from each sample using the 515F-806R primers for the V4 hypervariable region of the 16S rRNA gene, including 12-base barcodes, as previously published.[14] PCR procedure reactions consisted of 12.5 μLof KAPA HiFi HotStart ReadyMix (kit KK2601, Kapa Biosystems, Woburn, MA, USA), 10 μM of each primer, and 10−100 ng of DNA template. Reaction conditions consisted of an initial denaturing step for 3 min at 95 °C followed by 31 cycles of 20 s at 98 °C, 15 s at 60 °C, and 20 s at 72 °C. Triplicate PCR reactions were performed for each sample, which were combined and then purified with Ampure magnetic purification beads (Agencourt, Danvers, MA, USA). Purified PCR products were quantified using a Quant-iT PicoGreen dsDNA assay (Invitrogen, Carlsbad, CA, USA). Equimolar ratios of total samples were pooled and sequenced at the Faculty of Medicine of the Bar Ilan University (Safed, Israel) using a MiSeq Sequencer (Illumina, Madison, WI, USA).

16S rRNA Gene Sequence Analysis. For quality filtering of raw data, sequences with Phred <20 or shorter than 75% of expected length were discarded, as well as sequences containing primer mismatches, incorrect barcodes, ambiguous bases, or homopolymer runs in excess of 6 bases. The sequences that passed the quality filters were analyzed using the QIIME software package. Sequences were classified taxonomically using the Greengenes (GG) reference database at a confidence threshold of 80%. The GG taxonomies were used to generate summaries of the taxonomic distributions of operational taxonomic units (OTUs) across different levels (phylum, order, family, and genus). To standardize sequence counts across samples with uneven sampling, we randomly selected 22,450 sequences per sample (rarefaction) and used this as a basis to compare abundances of OTUs across samples. For phylogenetic tree-based analyses, each OTU was represented by a single sequence that was aligned using PyNAST. A phylogenetic tree was built with FastTree and used for estimates of α-diversity (within sample diversity, using Faith's phylogenetic diversity) and β-diversity (between sample diversity, using unweighted and weighted UniFrac). For Polygenetic Diversity (PD) measurements, means, and standard errors for given categories were calculated from 100 iterations using a rarefaction of 16,837 sequences per sample. Metagenome functional predictive analysis was carried out using PICRUSt software. Briefly, OTU abundance was normalized by 16S rRNA gene copy number, identified, and compared to a phylogenetic reference tree using the Greengenes database and was assigned functional traits and abundance based on known genomes and prediction using the Kyoto Encyclopaedia of Genes and Genomes (KEGG). Data representing significant fold-change differences in functional pathways between experimental groups were plotted.[14]

Statistical Analyses. All values are reported as the mean ± SEM. Statistical analysis was performed using SAS version 9.3 (SAS Institute, Cary, NC, USA). ANOVA was carried out to identify significant differences between the means of the experimental groups of birds, unless otherwise specified. Nonparametric factorial Kruskal−Wallis sum-rank tests was used to compare the relative abundance of

DOI: 10.1021/acs.jafc.8b01481
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distinct taxonomic units. Unweighted UniFrac, a phylogenetic measure of the degree of similarity between microbial communities, was used to assess phylogenetic diversity. The Spearman's rank correlation was employed to assess significant associations between bacterial groups and biomarkers of Zn status. Multivariate Association with Linear Models (MaAsLin) was used to identify potential correlations between OTUs abundance and host phenotype. Significant p-values ($p < 0.05$) associated with microbial clades and functions identified by LEfSe were corrected for multiple comparisons using the Benjamini and Hochberg false discovery rate (FDR) correction.

RESULTS

Phenotypic Alterations after the Consumption of Zn Biofortified Diet. Supplementary Table 1 presents the composition of the BZn and CZn diets. Supplementary Table 2 presents the measured Zn status indicators which defined two distinct Zn levels between treatment groups. Because of the absence of a particular marker of Zn intake and deficiency,[14] we chose to use an array of biological indicators of Zn status including growth (bodyweight), immunological (hepatic mRNA expression of cytokines), and physiological (tissue Zn, serum Zn, and the erythrocyte LA/DGLA ratio) parameters to confirm the relative Zn deficiency in the CZn treatment group. Results of the Zn status biomarkers used in this study were first presented in our recent publication.[11] As we demonstrated previously, all measured Zn-dependent physiological parameters were significantly different between the two groups, demonstrating relative Zn adequacy in the BZn group and relative Zn deficiency in CZn group ($p < 0.05$). Duodenal mRNA expression of various Zn transporters demonstrated greater expression in the CZn group ($p < 0.05$), signifying a compensatory mechanism for the relatively low Zn concentration in this group's diet.[11] Similarly, the expression of Fe related transporters (DMT1, FPN1, and Dcytb) was statistically different among the groups with the higher expression measured in the CZn group ($p < 0.05$). Altogether, the results of these Zn status parameters indicate that, by the end of the study, animals in the CZn were mildly Zn deficient, whereas animals in the BZn group were Zn adequate.

Morphological Differences in Villus Epithelial Cells in the BZn versus CZn Group. Villus surface area was increased in the BZn versus the CZn group; however, this increase did not achieve statistical significance (Table 1, $p > 0.05$). Supplementary Figure 1 demonstrates the representative histological differences observed in villus epithelial cells between the two groups. There was a significant increase in the goblet cell density, defined as the average number of goblet cells per 10 intestinal villi, in the BZn group versus CZn group ($p < 0.05$, Table 1). These results mirror those of previous studies, indicating that Zn deficiency directly affects the differentiation of enterocytes and intestinal goblet cells.[24,25]

Table 1. Morphological Differences in Villus Epithelial Cells between the Two Groups[a]

treatment group	villus surface area (cm^2)	goblet cell density (per 10 villi)
BZn	3.15 ± 0.15 a	105.9 ± 11.38 a
CZn	2.98 ± 0.12 a	84.85 ± 3.36 b

[a]Within a column and for each parameter, means without a common letter are significantly different ($n = 15$, $p < 0.05$). Values are the means ± SEM.

β-diversity but Not α-Diversity of the Intestinal Microbiota Is Significantly Altered by the Zn Biofortified Diet. Cecal content samples from the CZn and BZn treatment groups were collected and used for bacterial DNA extraction and sequencing of the V4 hypervariable region in the 16S rRNA gene. The cecum contains highly diverse and abundant microbiota and represents the primary site of bacterial fermentation in *Gallus gallus*.[26] The diversity of the cecal microbiota between the two treatment groups was assessed initially through measures of α and β-diversity. The Chao1 index, used to assess α-diversity (Figure 1A), was not significantly different between the CZn and BZn groups ($p > 0.05$). No difference was obtained in the number of observed species between groups (Figure 1B, $p > 0.05$). We utilized unweighted UniFrac distances as a measure of β-diversity to assess the effect of the Zn biofortified diet on between-individual variation in bacterial community composition (Figure 1C). Principal coordinate analysis showed a statistically significant difference in clustering between the CZn and BZn groups, suggesting that individual samples were more similar to other samples within the same group, as opposed to samples of the other group ($p < 0.05$). Additionally, individual samples of the CZn group clustered significantly closer to each other than did members of the BZn group ($p < 0.05$).

Effects of a Zn Biofortified Diet on the Composition of the Intestinal Microbiota. We conducted a taxon-based analysis of the cecal microbiota. 16S rRNA gene sequencing revealed that >98% of all bacterial sequences in both treatment groups were dominated by three major phyla: Firmicutes, Actinobacteria, and Proteobacteria, whereas sequences of Bacteroidetes, Fusobacteria, and Verrucomicrobia were also identified but in much lower abundance. The difference in abundance between the three dominant phyla was not significant between treatment groups (Figure 2A, $p = 0.300$, $p = 0.300$, and $p = 0.701$). After FDR correction, no significant differences between groups at the genus level were identified (Figure 2B). As in the human gut,[27] the Firmicutes phylum vastly predominated in the *Gallus gallus* cecum.[28]

We next investigated whether these taxonomic shifts were associated with the host phenotype as defined by the measured physiologic markers of Zn status, specifically gene expression of the various ZnT and ZIP family transmembrane proteins. In general, ZIP members facilitate Zn influx into the cytosol from extracellular fluid or from intracellular vesicles, while ZnT transporters lower intracellular Zn by mediating Zn efflux from the cell or influx into intracellular vesicles.[29] These proteins are widely transcribed in the brush-border of the small intestine.[30] When dietary Zn is low, enterocytes increase ZIP expression with more ZIPs localized to the apical membrane, while ZnT members are downregulated in an attempt to restore Zn homeostasis during depleted intestinal Zn conditions. However, both increased and decreased ZnT expression has been demonstrated in response to Zn deficiency.[31,32] Figure 3A demonstrates that animals with increased ZnT7 expression cluster more closely compared to those with lower expression ($q = 0.028$). Figures 3B shows 2 genera, *Lachnospiraceae* and *Erysipelotrichaceae*, depleted in animals with increased ZnT7 expression, while Figure 3C shows 2 genera, *Phascolarctobacterium* and *Veillonella*, enriched in animals with increased ZnT7 expression. Both *Veillonella* and members of the family *Erysipelotrichaceae* have been shown to increase in abundance following therapeutic levels of Zn oxide in porcine models, but their interaction with ZnT transporters at more physiologic

DOI: 10.1021/acs.jafc.8b01481
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

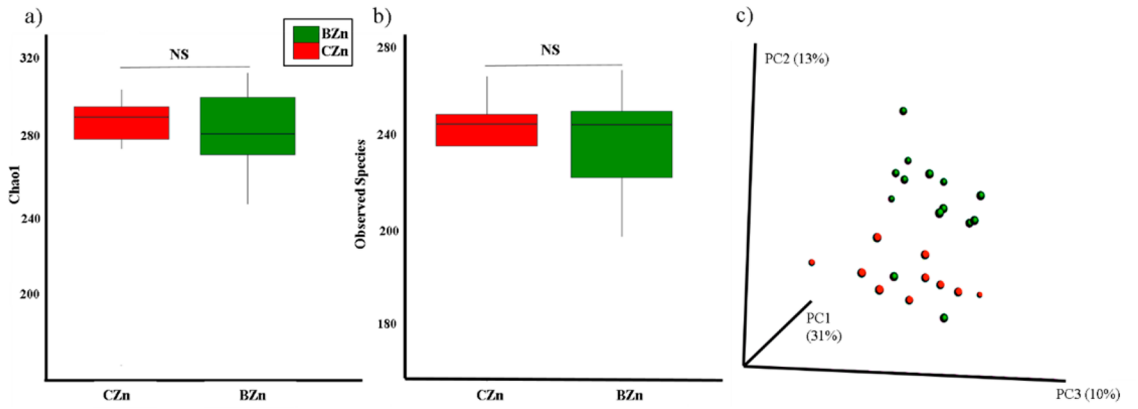

Figure 1. Microbial diversity of the cecal microbiome. (a) Measure of α-diversity using the Chao1 Index and (b) α-diversity using Observed Species Index; (c) Measure of β-diversity using unweighted UniFrac distances separated by the first three principal components (PCoA). Each dot represents one animal, and the colors represent the different treatment groups.

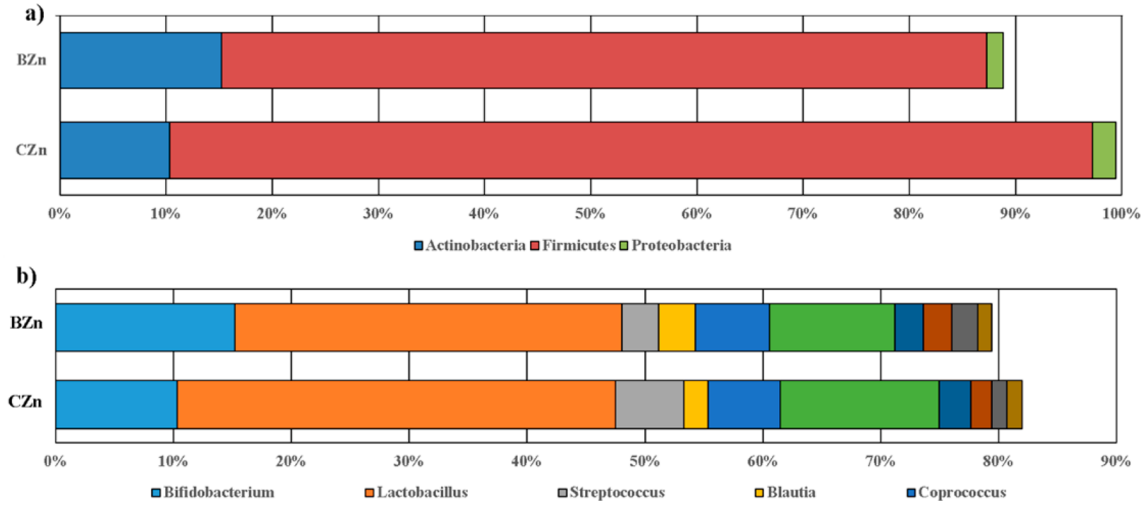

Figure 2. Compositional changes of gut microbiota in response to a biofortified diet. (a) Phylum level changes in the CZn and BZn groups as measured at the end of the study (day 42). Only phyla with abundance ≥1% are displayed. (b) Genus level changes in the CZn and BZn groups as measured at the end of the study (day 42). Only genera with abundance ≥5% are displayed.

doses of Zn needs to be further clarified in this animal model.[33,34]

We utilized MaAsLin to identify a significant positive association between the *Ruminococcus* genus and the BZn group (q = 0.036, Figure 3D−F). Additionally, Δ^6 desaturase, a Zn-dependent enzyme that catalyzes fatty acid desaturation,[21] and ZIP9 were also positively correlated with the *Ruminococcus* genus (q = 0.035 and q = 0.012, respectively).

Significant Bacterial Biomarkers Can Discriminate the Intestinal Microbiota of the BZn versus CZn Groups. For the investigation of relative abundances at all taxonomic levels, we used the linear discriminant analysis effect size (LEfSe) method to investigate significant bacterial biomarkers that could identify differences in the gut microbiota of the BZn and CZn groups.[35] Figure 4A and B present the differences in abundance between groups at the various taxonomic levels, with their respective LDA scores. We observed a general taxonomic delineation between the BZn and CZn groups, whereby the SCFA-producing Firmicutes predominated in the BZn group. Specifically, *Lactobacillus reuteri* (LDA score = 4.94, p = 0.024) and members of the *Dorea* (LDA score = 4.04, p = 0.010), *Clostridiales* (LDA score = 3.39, p = 0.008), *Ruminococcus* (LDA score = 3.35, p = 0.001), and *Lachnospiraceae* genera (LDA score = 3.21, p = 0.015) were significantly enriched in the BZn group. In the CZn group, however, members of the Verrucomicrobia and Bacteroidetes were the predominantly enriched phyla. Specifically, *Akkermansia muciniphila* (LDA score = 4.17, p = 0.022), *Lactococcus* (LDA score = 3.57, p = 0.021), and members of the *Verrucomicrobium* (LDA score = 4.17, p = 0.021), *Bacteroides* (LDA score = 3.05, p = 0.010), and *Bacteroidales* (LDA score = 3.46, p = 0.014) genera were significantly enriched in the CZn group.

Zn Biofortified Diet Alters the Metagenomic Potential of the Intestinal Microbiota. We investigated whether the biofortified Zn diet influenced the genetic capacity of the microbiota. We recently demonstrated that metagenomic perturbations of the gut microbiota in *Gallus gallus* influence the severity Zn deficiency provided by an elemental diet via, among other pathways, decreasing the capacity of resident bacteria to produce beneficial SCFAs for optimal Zn absorption by the host.[14] However, the clinical significance of alterations in the metabolic or functional capacity of the host microbiome from the consumption of a more realistic, Zn biofortified diet has not previously been explored, even though Zn biofortified diets are consumed on a population-wide level.

DOI: 10.1021/acs.jafc.8b01481
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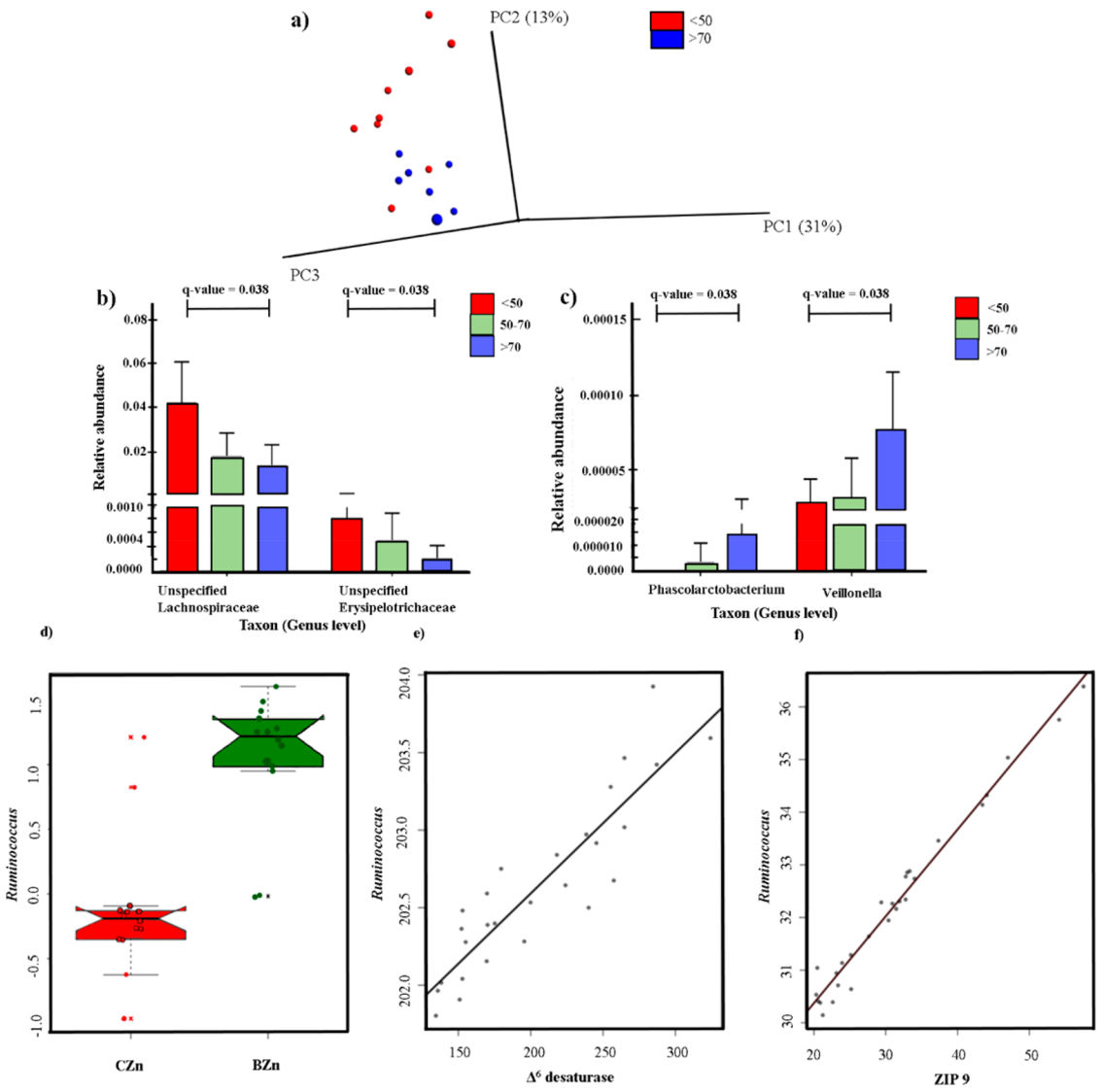

Figure 3. Taxonomic shifts associated with host phenotype. (a) Unweighted UniFrac distances separated by the first three principal components (PCoA) according to ZnT7 expression. Each dot represents one animal, and the colors represent the expression level of ZnT7 (red = low ZnT7 expression; blue = high ZnT7 expression). (b) Kruskal–Wallis sum-rank tests demonstrate depletion and (c) enrichment of four genera associated with increased levels of ZnT7 expression; (d) positive correlation between the *Ruminococcus* genus and the BZn group (AU); (e) positive correlations between Δ^6 desaturase and AU; (f) ZIP9 expression and the *Ruminococcus* genus (AU).

Using PICRUSt,[36] metagenome functional predictive analysis revealed that in the BZn group, 157 of the 240 (~65%) KEGG metabolic pathways analyzed were differentially enriched as compared to the CZn group ($p < 0.05$). After FDR correction, 6 of the 240 (~3%) KEGG metabolic pathways analyzed were differentially enriched as compared to the CZn group (Figure 5).

DISCUSSION

Our recently published efficacy trial demonstrated that Zn present in a Zn- biofortified wheat variety, the identical variety used in this study, is highly bioavailable.[11] The increased amount of Zn in the biofortified wheat contributed to a greater enterocyte uptake of Zn and physiologically significant improvement in overall Zn status. The gut microbial environment has been only recently recognized as a significant and vital organ in the absorption and utilization of Zn from the diet.[12,13] However, to our knowledge, characterizations of potential alterations in the gut microbiota following consumption of a Zn biofortified staple food crop based diet has not yet been investigated, although intake of Zn biofortified staple food crops is projected to grow substantially due to increasing application of population-wide biofortification strategies.[9] Therefore, the present study investigated how a Zn biofortified wheat diet, using identical biofortified wheat consumed by the targeted human populations, impacted the gut microbiota in a representative animal of human nutrition.

Similar to other animal models of human nutrition, *Gallus gallus* harbors a complex and active gut microbiota,[37] influenced by host genetics, geography, and diet.[38] Compositional similarity at multiple taxonomic levels is observed between the gut microbiota of *Gallus gallus* and humans, with Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria representing the four dominant bacterial phyla in both.[39,40] Its fast maturation and well-characterized phenotype during chronic micronutrient deficiency make it an ideal model of human nutrition, specifically with respect to dietary Zn and Fe deficiencies.[19] It represents a clinically validated tool to assess

DOI: 10.1021/acs.jafc.8b01481
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

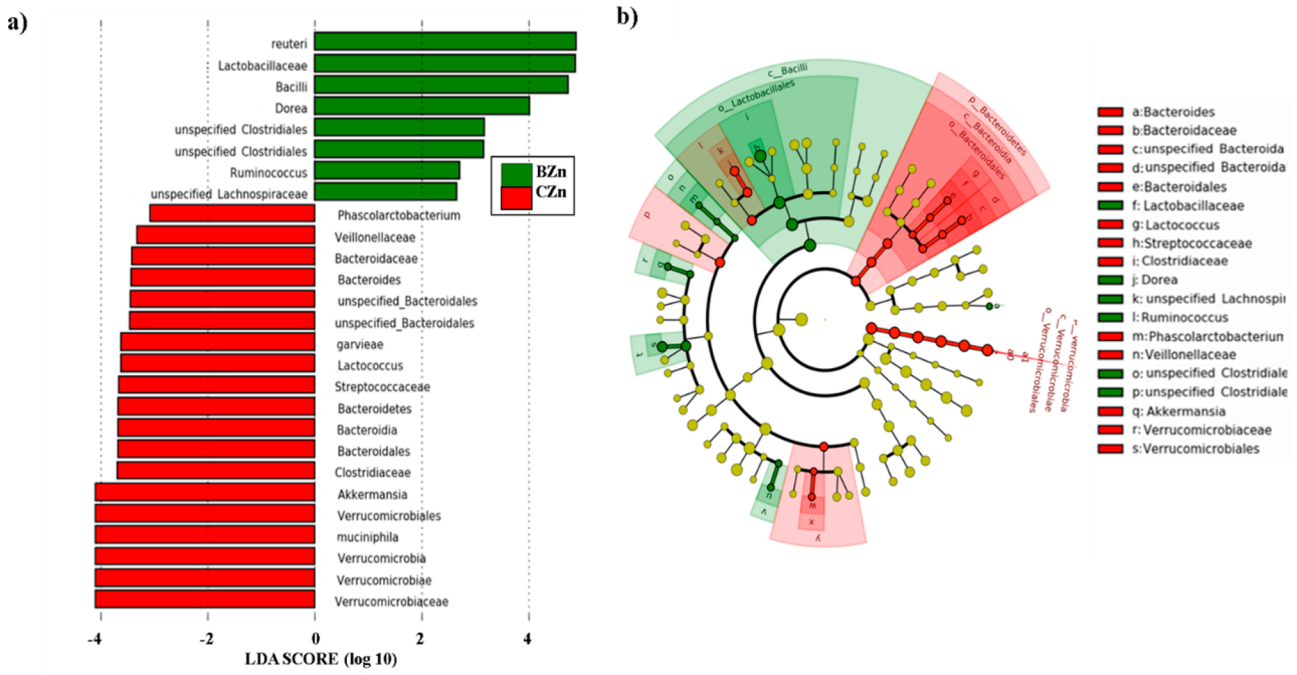

Figure 4. LEfSe method identifying the most differentially enriched taxa in the BZn and CZn groups. (a) Taxonomic cladogram obtained using LEfSe analysis of the 16S rRNA sequences. Treatment groups are indicated by the different colors with the brightness of each dot proportional to its effect size. (b) Computed LDA scores of the relative abundance difference between the CZn and BZn groups. Negative LDA scores (red) are enriched in the CZn group, while positive LDA scores (green) are enriched in the BZn group.

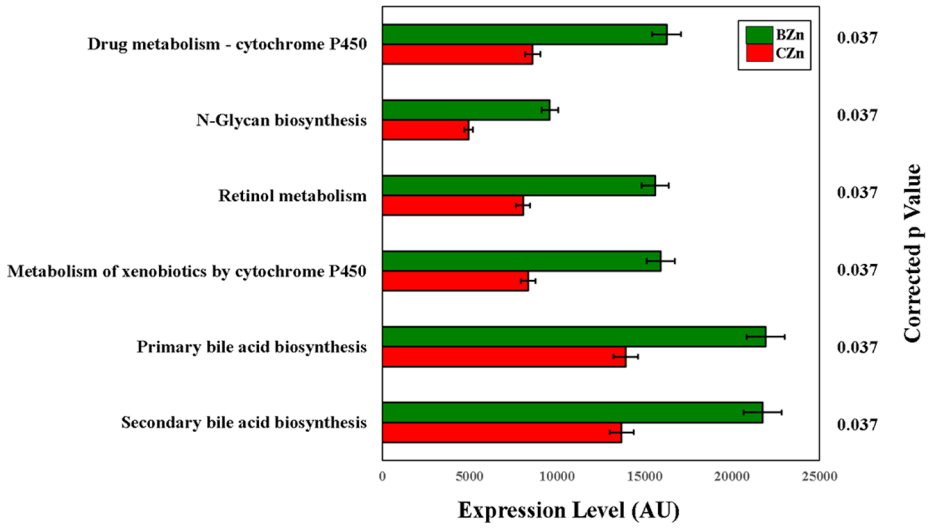

Figure 5. Functional capacity of the gut microbiota is altered following a Zn biofortified diet. Relative abundance of differentially enriched KEGG microbial metabolic pathways in the microbiota. Treatment groups are indicated by the different colors, and *p* values are displayed on the *y*-axis.

physiological outcomes of low dietary Zn in efficacy trials using micronutrient biofortified staple food crops.[11,19,39]

Our data are the first to demonstrate that chronic consumption of a Zn biofortified wheat based diet significantly alters the gut microbiota. Differences in β-diversity may be largely due to the differential Zn content between the two groups. As our group previously demonstrated, Zn is an essential micronutrient for bacteria,[14] and resident microbes compete with each other and their host for Zn and other nutrients.[41] Therefore, varying bioavailable Zn levels in the intestinal lumen can reshape the intestinal microbial populations by enabling establishment of bacteria that can effectively compete for Zn. Because previous studies, including ours, have investigated the Zn deficiency−microbiota interaction using purified or elemental diets, comparison of our current data using a Zn biofortified diet is novel and remains challenging. To that end, we anticipated to observe a more significant discrepancy in taxonomic change in the CZn relative to the BZn group, especially as it relates to the expansion of pathogenic bacteria, since Zn deficiency decrease global gut diversity and can induce dysbiosis.[14]

One potential explanation for why this was not observed in the control group is that a more realistic biofortified diet provided higher quality nutrition and bioaccessible Zn from food versus Zn carbonate or some other elemental form. This could influence the interaction between Zn and the micro-

DOI: 10.1021/acs.jafc.8b01481
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biome, as several dietary compounds such as phytate and phenolic compounds present in whole foods are known to positively modulate microbial composition in the intestines (i.e., these compounds favor the growth of certain gut bacteria, which have direct health benefits to the host).[42,43] The BZn diet did contain higher amounts of phytate compared to those in the CZn diet; however, this difference was not statistically significant. Additionally, the differential in Zn concentration between the two diets was not as wide as that in previous studies (46.5 μg/g vs 32.8 μg/g in the BZn vs CZn group, compared with a previously published 42 μg/g vs 2.5 μg/g in a Zn replete vs Zn deficient cohort).[14] Although physiologically the CZn group was Zn deficient, this deficiency was not as profound as that in previous studies, and therefore, taxonomic variations between the two groups should not be expected to be as significantly divergent as demonstrated in previous studies.

The six bacterial biosynthetic pathways, responsible for bile acid production, cytochrome p450 activity, and glycan metabolism, significantly depleted in the CZn group most likely reflect the decreased concentration of bioavailable Zn in the luminal milieu provided by the control diet. The effects of dietary Zn deficiency on the biosynthesis of various steroid hormones has been well characterized,[44−46] whereby Zn deficiency significantly decreases the activity of the metabolic capacity of cytochrome p450 isozymes responsible for their biosynthesis.[47,48] Bacterial surface glycan synthesis is an important biological property for colonization and survival by both commensal and pathogenic microbes.[49] Certain bacteria, such as members of the *Campylobacter* genus, will remove N-linked glycans via downregulation of glycan biosynthesis in response to low luminal Zn conditions in the intestines.[41,50−52] Additionally, these glycan moieties are recognized by Zn-dependent processes of the adaptive and innate immune system.[53] Hence, depletion of this bacterial pathway may be explained by the lower amounts of available Zn in the CZn group. Altogether, the observed metagenomic differences between the two groups demonstrate that decreased bioavailable Zn provided by the control diet selectively depleted bacterial biosynthetic pathways with a fundamental requirement of dietary Zn.[14]

As demonstrated in Figure 3, the taxonomic shifts associated with the Zn adequate phenotype are similar to our previously published observations, whereby *Ruminococcus* is strongly correlated with Zn adequacy and elevated Δ^6 desaturase activity (a Zn-dependent, rate limiting enzyme of fatty acid desaturation and biomarker of Zn deficiency).[14] *Ruminococcus* contribute to a significant bulk of gut SCFA production such that its absence significantly reduces dietary insoluble carbohydrate fermentation.[54] As we have previously described, SCFAs have the potential to increase Zn solubilization and utilization, thereby improving host Zn status.[14] With this new data, increasing evidence now implicates the *Ruminococcus* genus as an indicator of and potential contributor to improved host Zn status. Further longitudinal trials are needed to identify the specific role of *Ruminococcus* in host−microbiota Zn balance.

LEfSe analysis revealed that the BZn group was enriched in Bacilli and the lower *Lactobacillus*. Members of the *Lactobacillus* genus, such as *L. reuteri* and other lactic acid bacteria, have been shown to improve gut health in humans and a variety of animal models by increasing villus surface area, goblet cell number per villi, and decreasing colonization of pathogenic microorganisms like *Salmonella* and enteropathogenic *E. coli*.[55,56] A major mechanism by which they benefit the host is via improved mucosal barrier integrity from the production of SCFAs.[57] As we and others have previously demonstrated,[14,58] dietary Zn deficiency has a dramatic effect on limiting gut SCFA production, which can further perpetuate a Zn deficient phenotype.

The CZn group, however, was dominated by Bacteroidetes, Verrucomicrobia, and the lower *Akkermansia muciniphilia*, a novel mucin-degrading bacterium.[59] Increases in Verrucomicrobia and other mucin-degrading bacteria have been demonstrated after ingestion of certain dietary products (i.e., ellagitannins and other polyphenolic compounds).[60] Conflicting data exist for this group of bacteria as it pertains to Zn deficiency; some animal studies show that therapeutic levels of Zn (specifically Zn oxide) increase Verrucomicrobia,[34] while others show an increase in abundance during Zn deficiency.[61] Clearly, the significance of this group in Zn deficiency is not known, and additional research is needed to explain the conflicting compositional changes from dietary increases in Zn through biofortification versus supplementation.[62]

Recent studies demonstrate that both Verrucomicrobia, specifically *Akkermansia muciniphilia*, and members of the Bacteroidetes possess a significant capacity for lateral gene transfer of Zn-metallopeptidase enzymes.[63] A relative enrichment of these groups in the CZn versus BZn group may illustrate a pertinent example of host−microbe interplay; bacteria with the capacity to degrade mucin, an important nutrient contributor to overall mucosal homeostasis, can transfer vital Zn-dependent metalloproteinases when conditions of low luminal Zn exist and production of these enzymes are naturally suppressed. This interplay is highlighted further in the CZn group, where KEGG analysis revealed a significant decrease in bacterial cyp450 production. This enzyme family is responsible for producing the Zn-dependent superoxide dismutase enzyme, which has demonstrated a central role in modulating Zn deficiency-induced reduction of crypt cell proliferation and villus surface area, and diminished brush border disaccharidase activity.[57] Therefore, in a mutualistic fashion, the observed proliferation of these bacteria may be explained as a compensatory mechanism to a mucin-rich, Zn depleted gut. Using these findings, future studies should focus on the specific mechanisms whereby these bacteria may be attempting to restore Zn homeostasis during dietary Zn restriction.

To summarize, the presented findings exhibit a significant remodeling of the intestinal milieu that occurs in animals receiving a clinically relevant Zn biofortified wheat based diet. This study is the first to report on how Zn biofortified wheat affects the composition and metagenome of the intestinal microbiota. Animals who consumed the Zn biofortified wheat-based diet had increased microbial β-diversity, with concomitant increases in SCFA-producing lactic acid bacteria. Jointly, these observations deliver indication that ingesting a Zn biofortified wheat-based diet positively restructures the gut microbiota. As the consumption of Zn biofortified diets rises due to the increasing application of biofortification strategies, exploring the effects on the gut microbiota from Zn biofortified staple food crops remains an important strategy to further advance the efficacy and safety of this method.

DOI: 10.1021/acs.jafc.8b01481
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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b01481.

Supplementary methods, composition of the experimental diets, physiologic indicators of Zn status in treatment groups, representative histological differences observed in villus epithelial cells between the two groups, and supplementary reference (PDF)

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Notes

The authors declare no competing financial interest.

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DOI: 10.1021/acs.jafc.8b01481
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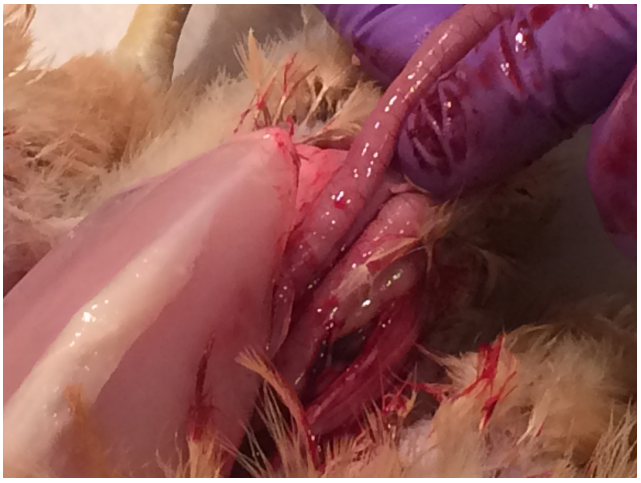
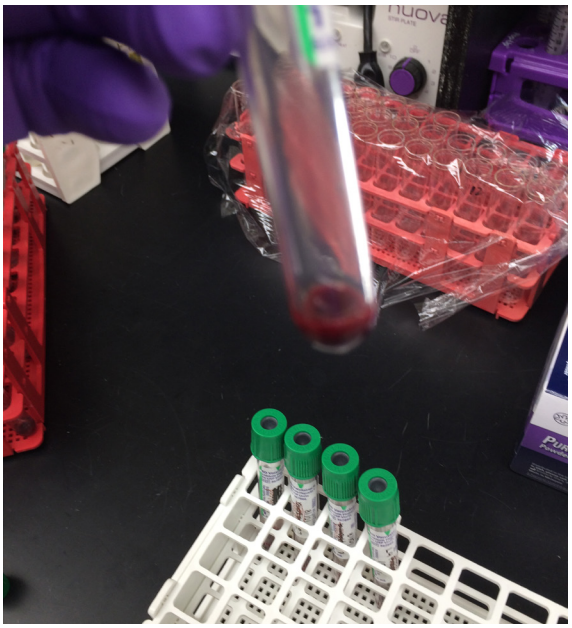
Appendix 3

Photo Gallery

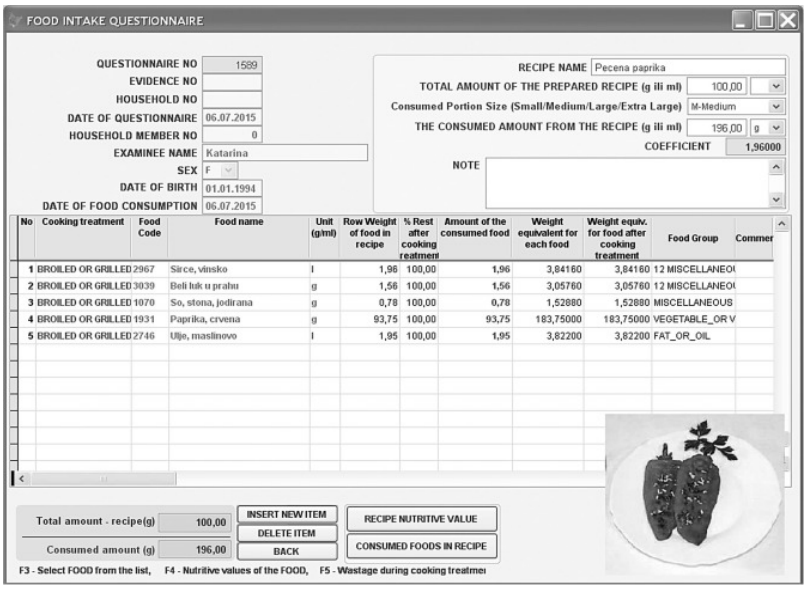

LOW 5




mL 0
±5%
100
200




VACUE
Ward
Dep
454092


FOOD INTAKE QUESTIONNAIRE
QUESTIONNAIRE NO 1589
EVIDENCE NO
HOUSEHOLD NO
DATE OF QUESTIONNAIRE 06.07.2015
HOUSEHOLD MEMBER NO 0
EXAMINEE NAME Katarina
SEX F
DATE OF BIRTH 01.01.1994
DATE OF FOOD CONSUMPTION 06.07.2015
RECIPE NAME Pecena paprika
TOTAL AMOUNT OF THE PREPARED RECIPE (g ili ml) 100,00
Consumed Portion Size (Small/Medium/Large/Extra Large) M-Medium
THE CONSUMED AMOUNT FROM THE RECIPE (g ili ml) 196,00 g
COEFFICIENT 1,96000
NOTE
No Cooking treatment Food Code Food name Unit (g/ml) Row Weight of food in recipe % Rest after cooking reatment Amount of the consumed food Weight equivalent for each food Weight equiv. for food after cooking treatment Food Group Comme
1 BROILED OR GRILLED 2967 Sirce, vinsko l 1,96 100,00 1,96 3,84160 3,84160 12 MISCELLANEO
2 BROILED OR GRILLED 3039 Beli luk u prahu g 1,56 100,00 1,56 3,05760 3,05760 12 MISCELLANEO
3 BROILED OR GRILLED 1070 So, stona, jodirana g 0,78 100,00 0,78 1,52880 1,52880 MISCELLANEOUS
4 BROILED OR GRILLED 1931 Paprika, crvena g 93,75 100,00 93,75 183,75000 183,75000 VEGETABLE_OR V
5 BROILED OR GRILLED 2746 Ulje, maslinovo l 1,95 100,00 1,95 3,82200 3,82200 FAT_OR_OIL
Total amount - recipe(g) 100,00
Consumed amount (g) 196,00
INSERT NEW ITEM
DELETE ITEM
BACK
RECIPE NUTRITIVE VALUE
CONSUMED FOODS IN RECIPE
F3 - Select FOOD from the list, F4 - Nutritive values of the FOOD, F5 - Wastage during cooking treatmer