

Effects of zinc fortification on the plasma fatty acid composition of Beninese school children: A randomised, double-blind controlled trial

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“Sango rinopa waneta, wadzungaira.”

(Ancient Shona Proverb – The hunting ground rewards one who has toiled and traversed it for long)

PREFACE

This dissertation is submitted for the degree Master of Science in Nutrition at the North West University. All the work presented was conducted at the Centre of Excellence for Nutrition (CEN) under the supervision of Drs Linda Malan and Jeannine Baumgartner. To the best of my knowledge unless referenced, work from this dissertation is original and unpublished. The dissertation will be presented in article format.

Tsitsi Chimhashu, presented preliminary results from this dissertation at the 26th Congress of the Nutrition Society of South Africa (NSSA), in Cape Town, South Africa on the 3rd of September, 2016. A version of the article in chapter two will be submitted to the Maternal and Child Nutrition journal for publication.

The co-authors of this article (see Chapter 3) gave permission that the article to be submitted for examination purposes (Table 1-1). The article is still to be submitted to the journal; therefore, no permission was obtained from the editor of the journal.

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ABSTRACT

Introduction

The lack of a specific and sensitive zinc (Zn) status biomarker is problematic. The linoleic acid: dihomono- γ -linolenic acid (LA:DGLA) ratio has been suggested by some researchers to be a Zn status marker. This is because Zn and fatty acids (FA) are known to interact, as Zn and FA deficient individuals present similar symptoms and physical manifestations. Although the mechanism by which Zn and FA interact is not fully understood, it is suggested that they interact through the FA synthesis pathway. Zn deficiency is suggested to impair the activity of desaturase enzymes, (desaturase enzymes are responsible for the conversion of essential fatty acids into longer-chain polyunsaturated FA) thereby causing a decrease in tissue long chain polyunsaturated metabolites. Zn supplementation in Zn deficient rats is known to affect their FA status, but little is known about effects of Zn fortification on the FA status in humans. There is also limited data on the FA status of African children as well as data on Zn and FA interactions in humans. Therefore the main aim of this study was to investigate whether there were associations between baseline plasma Zn and plasma total phospholipid FA composition, as well as to assess the effect of Zn fortified water on the plasma total phospholipid FA composition of rural Beninese school children aged between 6 and 10 years.

Methods

In a 20-week double blind randomised controlled trial, Beninese school children from a low-income rural setting aged between 6 and 10 years ($n = 185$) were randomly assigned to receive either a 300ml daily portion of Zn-fortified filtered water delivering 2.8 mg Zn (Zn+filter) or non-fortified filtered water (Filter). Plasma total phospholipid FA composition was determined using capillary gas chromatography and plasma Zn (PZn) analysis by atomic absorption spectrometry. Associations between Zn and FA were examined at baseline. Furthermore, the effect of Zn fortification on plasma FA composition was analysed in the total group, as well as in the Zn deficient and sufficient children and in girls and boys separately.

Results and discussion

At baseline, plasma Zn correlated positively with DGLA ($r = 0.209$; $p = 0.010$) and the DGLA:LA ratio ($r = 0.327$; $p < 0.001$). There was a significant inverse association between plasma Zn and linoleic acid (LA) ($r = -0.229$; $p = 0.005$) and the arachidonic: dihomono- γ -linolenic acid (ARA:DGLA) ratio ($r = -0.257$; $p < 0.001$). At baseline, LA ($p = 0.017$), eicosapentaenoic acid (EPA) ($p = 0.002$), n-3 docosapentaenoic acid (DPA) ($p = 0.020$), adrenic acid ($p = 0.010$) and the ARA:LA ratio ($p = 0.020$) differed between boys and girls. In Zn sufficient boys, gamma-

linolenic acid (GLA) was higher ($p = 0.020$) and the DGLA:GLA ratio tended to be higher ($p = 0.059$) than in Zn deficient boys. Zn fortification increased nervonic acid ($p = 0.048$) and tended to reduce LA ($p = 0.068$) in all children. Zn deficient children had a significantly higher nervonic acid composition ($p = 0.019$) after Zn fortification, whilst no significant effect was found in Zn sufficient children ($p = 0.382$). Fortification did not improve the plasma total phospholipid FA composition differently in boys and girls.

Conclusion

The findings from this research therefore supports that the LA:DGLA (or DGLA:LA) ratio could be a possible biomarker for Zn status. Our results further demonstrated that Zn filtered fortified water had an effect on the plasma total phospholipid FA composition of children, and even more so in Zn deficient children, thereby indicating that elongation and desaturation might be improved by Zn. The plasma total phospholipid FA composition was affected more by Zn deficiency in boys than in girls. However, further research is required to fully confirm these results, as well to examine the underlying mechanisms that exist between Zn and FA in humans.

Key words: fatty acid composition, plasma zinc, zinc fortification, children, fatty acid metabolism

OPSOMMING

Agtergrond

Die gebrek aan 'n spesifieke en sensitiewe sink status merker is 'n probleem. Die linoleïensuur tot dihomog- γ -linoleensuur (LS:DGLS) verhouding is al deur navorsers voorgestel as 'n sink status merker, omdat dit bekend is dat sink en vetsure 'n interaksie het. Beide sink en vetsuur gebrekkige individue toon soortgelyke simptome en fisiese manifestasies. Hoewel die meganisme van die interaksie nie ten volle verstaan word nie, is dit voorgestel dat die interaksie deur middel van die vetsuurbiosintese plaasvind. Dit word veronderstel dat 'n sink tekort die aktiwiteit van die desaturase ensieme benadeel (desaturase ensieme is verantwoordelik vir die metabolisme van essensiële vetsure na langer ketting poli-onversadigde vetsure) en sodoende 'n afname in weefsel lang ketting poli-onversadigde metaboliete veroorsaak. Sink aanvullings in rotte met 'n sink tekort is bekend om hul vetsuur status te beïnvloed, maar daar is min bekend oor gevolge van sink-gefortifiseerde water op die vetsuur status van die mens. Daar is ook beperkte data op die vetsuur status van kinders in Afrika, sowel as min inligting oor sink en vetsuur interaksies in die mens. Daarom was die hoofdoel van hierdie studie om vas te stel of daar 'n verwantskap is tussen die basislyn plasma sink en die plasma totale fosfolipied vetsuur samestelling, asook om die effek van sink-gefortifiseerde water op die plasma totale fosfolipied vetsuur samestelling van landelike Beninese skoolkinders tussen die ouderdomme van 6 en 10 jaar te bepaal.

Metodes

In 'n 20-week-lange dubbel blinde gerandomiseerde studie, is Beninese skoolkinders van 'n lae-inkomste landelike afkoms tussen 6 en 10 jaar ($n = 185$) ewekansig toegewys om óf 'n daaglikse 300 ml porsie sink-gefortifiseerde gefiltreerde water (met 2,8 mg sink) te ontvang (Zn + filter) óf nie-gefortifiseerde gefiltreerde water (filter). Die plasma totale fosfolipied vetsuur samestelling is bepaal met behulp van gas-chromatografie en plasma sink is ontleed met atoomabsorpsiespektrometrie. Assosiasies tussen sink en vetsure by basislyn is ondersoek. Verder is die uitwerking van sink fortifisering op die plasma vetsuursamestelling ontleed in die totale groep, sowel as apart in die kinders wat 'n sink tekort en voldoende sink gehad het asook in meisies en seuns.

Resultate en bespreking

By basislyn, het plasma sink positief gekorreleer met DGLS ($r = 0.209$; $p = 0.010$) en die DGLS:LS verhouding ($r = 0.327$; $p < 0.001$). Daar was 'n beduidende inverse verband tussen plasma sink en LS ($r = -0.229$; $p = 0.005$) en die aragidoonsuur tot DGLS (ARS:DGLS) verhouding ($r = -0.257$; $p < 0.001$). By basislyn het LS ($p = 0.017$), eikosapentanoësuur (EPS)

($p = 0.002$), n-3 dokosapentanoësuur (DPS) ($p = 0.020$), adreniese suur ($p = 0.010$) en die ARS:LS verhouding ($p = 0.020$) tussen seuns en meisies verskil. In seuns met voldoende sink, was γ -linoleensuur (GLS) verhoog ($p = 0.020$) en die DGLS:GLS verhouding was geneig om verhoog te wees ($p = 0.059$) in vergelyking met sink gebrekkige seuns. Sink fortifisering het nervoniese suur ($p = 0.048$) laat toeneem en 'n geneigdheid getoon om LS te laat afneem ($p = 0,068$) in alle kinders. Kinders met 'n sink tekort het 'n hoër nervoniese suur samestelling ($p = 0.019$) gehad na sink fortifisering, terwyl daar geen noemenswaardige effek was in die kinders met voldoende sink status nie ($p = 0.382$). Daar was geen onderskeid tussen die fortifiseringseffek van sink op die plasma totale fosfolipied vetsuur samestelling tussen seuns en meisies nie.

Gevolgtrekking

Die bevindinge van hierdie navorsing ondersteun die idee dat die LS:DGLS (of DGLS:LS) verhouding 'n moontlike merker vir sink status kan wees. Ons resultate demonstreer verder dat sink gefortifiseerde water 'n effek gehad het op die plasma totale fosfolipied vetsuur samestelling van kinders, en dat die effek groter was in kinders met 'n sink tekort; dus dat verlenging en desaturasie van vetsure kan verbeter word deur sink fortifisering. Die plasma totale fosfolipied vetsuur samestelling van seuns word meer beïnvloed deur sink tekort as die van dogters. Daar is egter verdere navorsing nodig om hierdie resultate ten volle te bevestig, asook om die onderliggende meganismes wat bestaan tussen sink en vetsure in die mens te ondersoek.

En sleuteltermes: vetsuur samestelling, plasma sink, sink fortifisering, kinders, vetsuur metabolisme

LIST OF ABBREVIATIONS

AAS	Atomic absorption spectrometry
AD	Alzheimer's disease
ALA	α -linolenic acid
ANCOVA	Analysis of covariance
ARA	Arachidonic acid
BMI	Body mass index
Ca ²⁺	Calcium
CDF	Cation Diffusion Facilitator
CE	Cholesterol ester
CEN	Centre of Excellence for Nutrition
CNS	Central nervous system
CRP	C-reactive protein
D5D	Delta-5 desaturase
D6D	Delta-6 desaturase
DGLA	dihomo- γ -linolenic acid (20:3 n-6)
DHA	Docosahexaenoic acid (22:6 n-3)
DRI	Dietary reference intake
EPA	Eicosapentaenoic acid (20:5 n-3)
ETHZ	Eidgenoessische Technische Hochschule Zurich
EAR	Estimated average requirement
EDTA	Ethylendiaminetetraacetic acid
EFA	Essential fatty acid
FA	Fatty acids
FADS	Fatty acid desaturase gene
FAO	Food and Agriculture Organization
Fe ²⁺	Ferrous iron
Fe ³⁺	Ferric iron
GC-MS/MS	Gas chromatography-tandem mass spectrometry
GLA	γ -linolenic acid (18:3n-6)
HAZ	Height-for-age z-score
IOM	Institute of Medicine

ISSFAL	International Society for the Study of Fatty Acids and Lipids
IZINCG	International Zinc Nutrition Consultative Group
LA	Linoleic acid (18:2n-6)
LCPUFA	Long-chain polyunsaturated fatty acid
LSF	LifeStraw®Family
Mg ²⁺	Magnesium ion
MUAC	Mid-upper arm circumference
MUFA	Monounsaturated fatty acid
mRNA	Messenger ribonucleic acid
n-3	Omega 3 fatty acids
n-6	Omega 6 fatty acids
n-3 DPA	Omega-3 docosapentaenoic acid (22:5 n-3)
n-6 DPA	Omega-6 docosapentaenoic acid (22:5 n-6)
NADP	Nicotinamide adenine dinucleotide phosphate
NEFA	Non-esterified fatty acids
NHANES	National Health and Nutrition Examination Survey
NWU	North-West University
PA	Phytic acid
PUFA	PUFA Poly-unsaturated fatty acids
PZn	Plasma/serum zinc
RCT	Randomised controlled trial
RDA	Recommended dietary allowance
SD	Standard deviation
SLC	Solute Linked Carrier
US EPA	United States Environment Protect Agency
WAZ	Weight-for-age z-score
WHO	World Health Organization
WHZ	Weight-for-height z-score
Zip	Zinc transporter, transports zinc into the cytoplasm
ZIP	Gene expressing for a Zip transporter
Zn	Zinc
ZnT	Zinc transporter, transports zinc out of the cytoplasm
Zrt	Zinc regulated transport

LIST OF SYMBOLS AND UNITS

r	correlation
°C	degrees Celsius
g	gram
g/d	gram per day
g/kg	gram per kilogram body weight
g/kg/d	gram per kilogram body weight per day
>	greater than/ above
↑	increased
kg	kilogram
<	less/ lower than
μmol/L	micromoles per litre
μm	micrometer
mg/d	microgram per day
mg/L	milligram per litre
mmol/L	mill moles per litre
–	negative
%	percentage
↓	reduced

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CHAPTER 1: INTRODUCTION

1.1 Background

Zinc (Zn) is a micronutrient important for the functioning of a wide variety of biological processes, such as linear growth, neurotransmission, reproduction and the maintenance of immune functions (Roohani *et al.*, 2013). Present in the divalent form (Zn⁺⁺), Zn typically binds to proteins, amino acids, peptides and nucleotides and it plays a critical role in the maintenance of protein structure, catalytic processes of enzymes and regulation of several biological processes (Shrimpton, 2001; Hambidge & Krebs, 2007). In the case of inadequate Zn intake, a wide range of clinical symptoms may occur, such as weakening of the immune system, failure of linear growth, limited cognitive capacity and a delay in the age of onset of puberty (Shrimpton, 2001). Zn deficiency has been found to be common among vulnerable populations in many developing countries and mainly affects children, pregnant women and their unborn children (Brown & Ruel, 2004). In developing countries, staple foods are usually rich in cereals and vegetables and poor in Zn-rich animal products and contain anti-nutritive substances that prevent the absorption of Zn in the small intestine (Mitchikpe *et al.*, 2009). There is insufficient data on the global prevalence of Zn deficiency even though national surveys have included the assessment of plasma Zn concentration in recent years. Zn deficiency is defined as a serum Zn concentration below 65 µg/L (Ghosh *et al.*, 2007). In that regard stunting levels and dietary Zn intake are used as a proxy to estimate Zn deficiency (Brown *et al.*, 2004). Estimated country-specific prevalence of inadequate dietary Zn availability based on the Food and Agriculture Organisation's (FAO) food balance sheets show that the prevalence of Zn deficiency in African countries like South Africa and Benin fall between 15-25%, with most African countries like Zambia and Mozambique having a prevalence of Zn deficiency even greater than 25% (Wessells & Brown, 2012). As shown by the results of a nutrition survey carried out in a community adjacent to the study area, there was an unexpected high dietary Zn contribution from the diet (Mitchikpe *et al.*, 2009). Results of a study conducted in 12 rural localities in the municipality of Natitingou in Benin showed that 52.8% of children aged 1-10 years had a serum Zn concentration below the cut-off (Galetti *et al.*, 2016). Furthermore, 50.7% were moderately stunted (<2SD), of which more than half were severely stunted. Even though limited data is available on the current Zn status in South African populations, recent studies conducted by the researchers at the Centre of Excellence for Nutrition (CEN) at the North-West University found that 50% and 24% of school children from Kimberley and the Valley of a Thousand Hills in KwaZulu-Natal,

respectively, were Zn-deficient, (Troesch *et al.*, 2011; Baumgartner, 2012). Zn supplementation and fortification are strategies that contribute to the improvement of Zn status in populations characterised by an inadequate intake of Zn (Brown & Ruel, 2004).

Besides the detrimental effects of inadequate Zn intake on clinical outcomes such as immune function, linear growth and cognitive functioning, low Zn intake may also affect the FA metabolism as Zn is a functional essential co-factor for the desaturase and elongase enzymes, in particular, the delta-6-desaturase which is responsible for the conversion of essential FA (EFA) into long chain polyunsaturated fatty acids (LCPUFA) (Huang *et al.*, 1982; Hulbert, 2008). Iron (Fe^{2+}) is the major component of the desaturase enzymes, but Zn has an ancillary function in relation to the nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate (NADP-NADPH) cycle, which is Zn dependent (Wiseman, 2013). In concordance, earlier animal studies showed similar results which supported the involvement of Zn in the FA metabolism (Bettger *et al.*, 1979; Bettger & O'Dell, 1981; Ayala & Brenner, 1982). In a recent study conducted by Reed *et al.* chickens (*Gallus gallus domesticus*) the authors showed that Zn deficiency decreased the expression of the hepatic delta-6 desaturase and increased the linoleic acid (LA; 18:2n-6) to dihomo-gamma-linolenic acid (DGLA; 20:3n6) ratio in erythrocyte membranes. The authors of this article went so far as to suggest that the erythrocyte LA:DGLA ratio may be a useful biomarker to assess dietary Zn manipulation. These findings have been recently supported by Knez and colleagues who found that that concentration of DGLA is decreased and that of LA: DGLA ratio is increased in people with lower dietary Zn intake (Knez *et al.*, 2016). Furthermore, it might be a possibility that some of the detrimental effects of Zn deficiency are mediated by impairing the LCPUFA status, especially regarding its role in impairing immune function.

Thus, if findings from the current study show that Zn fortification can affect FA status of Zn-deficient school children, this change in FA status could be advocated as a potential biological marker for Zn status. This is important because Zn analysis requires specific Zn-free blood collection tubes and acid-washed consumables for blood preparation, as well as a large amount of plasma/serum (2 ml), which makes it problematic to analyse especially in children.

1.2 Rationale of the study

It has been suggested that the LA:DGLA ratio may be used as a biomarker to assess Zn status and dietary Zn manipulation respectively (Reed *et al.*, 2014; Knez *et al.*, 2016). Furthermore, preliminary data from a recently conducted cross-sectional analysis in South African children indicate that there is a relationship between Zn status and n-3 long chain polyunsaturated fatty acids (LCPUFA) in membranes (unpublished data). These products of the desaturase and elongase enzymes are crucial for proper membrane functioning and play important roles in the development and functioning of the brain and immune system. To our knowledge, no study to date has investigated whether Zn fortification will have an effect on the LCPUFA status of children.

Therefore, this study will investigate whether there are associations between PZn and FA in order to establish whether the LA:DGLA can be used as Zn status biomarker. Furthermore, this study will assess whether the treatment of 6-10- y-old Beninese children in an area with a high prevalence of Zn deficiency with Zn-fortified water had an effect on their plasma total phospholipid FA composition. Moreover, this study will also give us the opportunity to assess the FA status of Beninese school children. This is important as data on FA status of African children is scarce and urgently needs further investigation.

1.3 Study site

The study was conducted in North-western Benin (Atacora province), in Natitingou district at the primary school of Kotopounga. In this particular community, plant staples contribute an estimated 87% of the daily Zn intake (Mitchikpe *et al.*, 2009). Based on results from a demographic health survey in Benin 25% of children under the age of 5 are stunted. Data derived from food balance sheets show that between 16.5–17.9% of the Beninese population are at risk of inadequate Zn intake. Benin is therefore, classified as a country at moderate risk of Zn-deficiency (Brown *et al.*, 2004; Wuehler *et al.*, 2005; Wessells & Brown, 2012).



Figure 1-1: Map showing the African continent with the study site Natitingou in Benin

<http://www.lahistoriaconmapas.com/atlas/country-map03/where-is-benin-in-africa-on-the-map.htm>) and (<http://www.lib.utexas.edu/maps/benin.html>)

1.4 Research aim

The aim of this study was to investigate the effect of Zn fortification on the plasma FA composition of rural Beninese school children between the ages of 6 and 10 years.

1.5 Research objectives

- i. To investigate whether there is a relationship between PZn and plasma total phospholipid FA composition of Beninese school children at baseline before the intervention.
- ii. To determine the effect of daily consumption of Zn-fortified water on the plasma total phospholipid FA composition in 6–10-year-old school children from Natitingou, Benin.

1.6 Ethical approval

Ethical approval for conducting the main study was granted by the Ethics committee of the Swiss Federal Institute of Technology Zurich and the National Committee for Ethics in Health Research of Benin (CNERES). Approval for this MSc sub-study was obtained from the Health Research Ethical committee of NWU.

1.7 Dissertation outline

This dissertation is presented in article format according to the North-West University (NWU) postgraduate manual. With the exception of Chapter three, all referencing used in this dissertation is in accordance with the NWU Harvard style. This dissertation is divided into four chapters as follows:

Chapter **One** is a brief introduction to this study and it details contributions made by the research team. Chapter **Two** is a detailed literature review of the available literature on Zn and FA. The review is divided into three sections. The first part of the literature review details Zn and the second section is on FA. The third section is dedicated to the interactions between FA and Zn as well as proposed mechanisms through which Zn and FA interact.

Chapter **Three** consist of a manuscript titled “Investigation into the relationship between Zn status and plasma total phospholipid FA composition: A randomised controlled trial with a cross-sectional baseline analysis.” This manuscript will be submitted for publication to the Maternal and Child Nutrition Journal. The headings, numbering, and reference style are according to the guidelines of the Maternal and Child Nutrition Journal.

Chapter **Four** summarises the main finding from this study and also states the limitations that were present. Conclusions and recommendations for further research are also given.

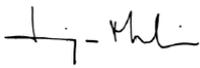
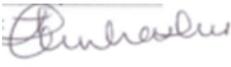
1.8 Research outputs

From this research, an article will be submitted to the Maternal and Nutrition Journal. Preliminary results from this project were presented at the 26th Congress of the Nutrition Society of South Africa (NSSA), in Cape Town. Further results will be presented at an international congress.

1.9 Research team

The contributions of the researchers listed as authors in the article and the contributions they made to this research are described in the table below.

Table 1-1: List of research team and their contribution to this research

Title, initials, and surname	Affiliation	Role in the study
Prof Dr. Michael Zimmermann 	Laboratory of Human Nutrition, Institute of Food, Nutrition and Health, ETH Zurich, Switzerland	The principal investigator of the main study (design, and planning of the study, approval of the final protocol, obtaining funding).
Dr. Diego Moretti 	Laboratory of Human Nutrition, Institute of Food, Nutrition and Health, ETH Zurich, Switzerland	Responsible for the scientific orientation of the research in Switzerland, designed main study, supervision of research activities in the main study as well as the implementation of the protocol.
Dr. Valeria Galetti 	Laboratory of Human Nutrition, Institute of Food, Nutrition and Health, ETH Zurich, Switzerland	PhD student, who designed the parent study, executed the Zn intervention study in Benin and Switzerland.
Dr. Linda Malan 	Centre of Excellence for Nutrition	Supervisor of T Chimhashu. Guided student with protocol and dissertation writing. Assisted with statistical analysis and interpretation of results.
Dr. Jeannine Baumgartner 	Centre of Excellence for Nutrition	Co-supervisor of T Chimhashu. Conceptualised and initiated the sub-study. Assisted with revising work and interpretation of results.
Dr. Paul van Jaarsveld 	Non-Communicable Diseases Research Unit (NCDRU) South African Medical Research Council	Analysis of total phospholipid fatty acids in the plasma samples, verifying the final fatty acid datasheet, guidance with fatty acid method reporting and writing.
Miss Tsitsi Chimhashu 	Centre of Excellence for Nutrition	MSc student. Responsible for protocol writing, compiling, and literature review, statistical analysis, interpretation of data and writing up of this dissertation.

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CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

In developing countries, the prevalence of Zn deficiency is of public health concern as an estimated 25% of the global population is Zn deficient (Maret & Sandstead, 2006), and 17% are at risk of inadequate Zn intake (Wessels & Brown, 2012). Also, according to Briend and colleagues limited literature is available on the FA status, dietary intake in developing countries (Briend, 2011). Both, Zn and FA are essential for optimal growth, immune response, gene expression, visual development, neuro-transmission and cognition (Calder, 2015; De Mel & Suphioglu, 2014; Vallee & Falchuk, 1993).

The first two sections of this literature review will focus on the available literature on Zn and FA thus providing an overview of their metabolism and functions in the body. This will include a discussion on Zn water fortification as a strategy to increase dietary Zn intake as well as the lack of a suitable Zn concentration index. Furthermore, it will contain an investigation on the available literature on FA biomarkers that are used to assess FA status, intake and deficiencies. This is followed by a review that will focus on the relationship between the FA and Zn metabolism and the several suggested mechanisms by which they interact.

2.2 Zinc

Zinc (Zn) is an essential trace element which is indispensable in the biological system (Maret, 2013). This trace metal is a crucial component of plasma membranes and was first recognised to be essential to rats in 1934 and humans in 1963 (Prasad, 1983; Guthrie, 1989). It is the second most abundant trace element of the body's total trace mineral pool, only less abundant than iron (Simopoulos, 2002a; Smit *et al.*, 2004; Reed *et al.*, 2014), and more than nickel and cadmium (Crichton & Boelaert, 2001).

2.2.1 Zinc functions

The expansive biological functionality of Zn in human physiology is due to its stable chemical and physical properties (Vallee & Auld, 1990). Zn is known to be essential for cellular and physiological functions such as growth and development, immunity, receptor activity, gene expression and DNA metabolism in transcription factors, enzymatic catalysis, hormonal

storage and release, tissue repair, memory and visual processes (Vallee & Falchuk, 1993; De Mel & Suphioglu, 2014).

Zn is required for the proper development and function of the central nervous system (CNS) as it acts as a neurosecretory product or cofactor (Frederickson *et al.*, 2000). Endothelial integrity is Zn dependent and a deficiency in Zn causes severe impairment of the endothelial barrier function (Gimenez *et al.*, 2011). Adequate Zn nutrition is also necessary for normal pregnancy outcomes (Brenna *et al.*, 2009) such as embryogenesis, foetal growth, neurobehavioral development and milk secretion (Donangelo & King, 2012).

Three thousand Zn proteins are thought to be encoded by the human genome (Lönnerdal, 1998). Zn has numerous roles in DNA and RNA metabolism (Cousins, 1994) as it plays an important role in the regulation of a variety of genes such as those involved in nucleic acid metabolism (Hanas *et al.*, 1983; Johnston, 1987), cell signalling (Haase & Rink, 2007; Prasad, 2009), apoptosis (Beyersmann & Haase, 2001), cell proliferation and growth (MacDonald, 2000; Bao & Knoell, 2006). Zn influences the activity of multiple enzymes which act during replication and transcription such as DNA polymerase, thymidine kinase, DNA dependent RNA polymerase, terminal deoxyribonucleotidyl transferase and aminoacyl synthetase, as well as in Zn finger DNA binding proteins (Reed *et al.*, 2014).

Zn is considered to have catalytic, coactive and structural functions in enzymes (Vallee & Falchuk, 1993). To date Zn is known to be a cofactor of over 300 Zn metalloenzymes (Wallwork, 1986; Gaither & Eide, 2001). Furthermore, it is the only metal found in every one of the six enzyme subclasses (Holman, 1971). Examples of metalloenzymes that contain Zn are carbonic anhydrase and alcohol dehydrogenase (Adisa & Odutuga, 1999). Zn metalloenzymes also take part in the metabolism of molecules for instance proteins, lipids, and nucleic acids. They also participate in cellular differentiation and growth (Sandström, 2001).

Approximately 3% of all nucleic binding proteins contain Zn binding motifs (Crichton & Boelaert, 2001; Frederickson *et al.*, 2005). Zn finger motifs are a reoccurring pattern of amino acids with conserved residues of cysteine and histidine which facilitate the binding of a protein to another molecule such as DNA (Hill & Matrone, 1970; Gamsjaeger *et al.*, 2007). Zn also provides structural support for many proteins such as Zn clusters, and nuclear hormone receptors, many of which are crucial for cellular development and differentiation (Crichton & Boelaert, 2001).

In addition, microsomal Zn levels determine electron transfer from nicotinamide adenine dinucleotide (NADP) or nicotinamide adenine dinucleotide phosphate (NADPH) through the cytochromes B5 and P-450 to the terminal acceptor, such as desaturase enzymes. Desaturases and elongases are known to be such terminal acceptors and are dependent on this process for their function (Cunnane, 1988b; Jump, 2009).

Zn is stored in cells as part of the protein metallothionein (Kägi & Vallee, 1961). There is also evidence that suggests that Zn has an integral role in immune system functioning (Kruse-Jarres, 1989; Fraker *et al.*, 2000; Dardenne, 2002; Ibs & Rink, 2003). This is because Zn is considered to have anti-inflammatory properties. In experiments, endogenous Zn was found to inhibit lipopolysaccharide or IL-1 β -induced nitrogen oxide (NO) formation. Moreover Zn reduces the activity of smooth muscle cell NO synthase in Zn-sufficient rats that had been injected with lipopolysaccharide to induce inflammation. This illustrates one of the anti-inflammatory activities of Zn (Abou-Mohamed *et al.*, 1998).

An additional role of Zn is that it acts as a potent antioxidant. Two mechanisms by which Zn acts as an antioxidant are that it causes the protection of protein sulfhydryl groups against oxidation and inhibits the production of free radicals by transition metals. It achieves this by either displacing or competing with cupric or ferric (Fe³⁺) ions, which trigger the formation of free radicals (Bray & Bettger, 1990). It also reduces the formation of \cdot OH from H₂O₂ through the antagonism of these redox-active transition metals (Powell, 2000). Zn protects protein sulfhydryl groups by reducing sulfhydryl reactivity. It is thought to be as a result of Zn directly binding to sulfhydryl, or through the binding of Zn to some other protein site that is in close proximity to the sulfhydryl group thereby causing steric hindrance or a conformational change caused by Zn binding to some other site on the protein (Powell, 2000). Zn also plays an important role in antioxidant-induced death of cells (apoptosis), as it protects cells from the damaging effects of oxygen radicals (Calder, 2002).

2.2.2 Zinc homeostasis

About 1.5–2.5 g of Zn is present in the average adult (Shils & Shike, 2006). Although Zn is distributed throughout all tissues, it is concentrated in the skeletal muscle mass (1.4 g). Zn is also highly concentrated in the brain, in actual fact Zn is the most abundant trace metal in the human brain (De Mel & Suphioglu, 2014). The highest concentrations of Zn is found in the teeth, hair, and prostate and it is also found in significant amounts in the kidneys, pancreas, and liver (0.72g) (Galetti, 2014).

Unlike ferrous iron (Fe²⁺) and iodine, there is no real storage form of Zn in the body that can be easily mobilised during deficient times and dietary excess, thus regulation is highly

efficient (Sadhu & Gedamu, 1989; Weaver *et al.*, 2007; Fukada & Kambe, 2011). Homeostasis both at the cellular level and systemic level is important in Zn metabolism because Zn in excess is toxic and Zn deficiency is a problem (Weigand & Boesch-Saadatmandi, 2012). Zn homeostasis is maintained at the cellular level through import and export mechanisms. The primary mechanism for maintaining Zn homeostasis is through tightly regulated absorption and excretion of Zn, the gastrointestinal tract being the major site for this regulation (King *et al.*, 2000).

Zn molecules have a highly charged hydrophilic nature that makes them unsuitable for passive diffusion across cell membranes; hence Zn transport occurs through intermembrane proteins and Zn transporter proteins. Over the course of two decades, more than 20 Zn transporters have been identified and characterised (Kambe *et al.*, 2004). These transporters are mainly categorised into two metal transporter families; Zn transporters (ZnT, SLC-30), or Cation Diffusion Facilitator (CDF) and Zn-regulated transporter (Zrt) or iron-regulated transporter proteins like protein (ZIP, SLC-39) (Kambe *et al.*, 2004; Liuzzi & Cousins, 2004).

The ZIP-family facilitates the influx of Zn in cells or release from the intracellular vesicles thereby increasing the intracellular Zn concentrations (Kambe *et al.*, 2004). The main dietary Zn transporter is called ZIP-4 transporter and it is located in the apical membrane of the enterocyte (Wang *et al.*, 2002). As a mechanism for homeostasis under conditions of excess of Zn (McMahon & Cousins, 1998), the ZIP-1 transporter is thought to act as a backup system in dietary Zn uptake (Kambe *et al.*, 2004).

The ZnT transporter family facilitates efflux from the cell's cytoplasm to the extracellular environment or into the luminal compartments such as secretory granules, endosomes, and synaptic vesicles. An example of a ZnT transporter is ZnT-1 (De Mel & Suphioglu, 2014), it is localised in the basolateral membrane of enterocytes and renal tubular cells, where it facilitates the Zn efflux (Liuzzi *et al.*, 2001). It is, therefore, clear that the the gastrointestinal system, especially the small intestine is key to the maintenance of Zn homeostasis as it is responsible for processes such as absorption of exogenous Zn and excretion of endogenous Zn. Primary regulation of Zn in the body occurs in the liver and pancreas (De Mel & Suphioglu, 2014).

However, more research is required to establish the Zn homeostatic mechanisms and their control in the human body. This is because there are some factors that are at play in the host, such as dietary and environmental factors, which in some cases result in suboptimal Zn status of the individual (De Mel & Suphioglu, 2014).

2.2.3 Zinc sources and dietary intakes

The main food source of Zn is meat such as fish (shellfish, oysters), as well as beef, liver, kidney, heart and also cheese (Galetti, 2014). The highest concentration is found in oysters (160 mg/100 g). Plant proteins are also another good source of Zn. These include nuts, seeds, and legumes, such as beans, chickpeas, peas and lentils. Wholegrain cereals are another source of Zn, as Zn is found in the bran and germ tissues (Hunt, 2003; Lowe *et al.*, 2009). Moderate food sources of Zn include dairy products and poultry. Poor sources of Zn are fruits, tubers, fats, and oils (King *et al.*, 2001; Calder, 2002).

Zn can also be endogenously produced in the body. Pancreatic, biliary and gastrointestinal secretions are examples of Zn endogenous sources as well as the transepithelial flux or sloughing of mucosal cells (Van Biervliet, 2008).

The Estimated Average Requirement (EAR) is the median intake level for a nutrient for a specific sex and life stage group of healthy individuals at which the needs of 50% of the population will be met (Gibson *et al.*, 2008). As the needs of the other half of the population will not be met by the EAR, the EAR is increased by about 20% to arrive at the Recommended daily allowances (RDA). Since there is a lack of a sensitive Zn status biomarker (see section 2.2.6) the RDA for Zn (Table 2-1) is based on a number of different indicators of Zn nutritional status and represents the daily required intakes that are likely to prevent deficiency in nearly 97.5% of individuals in a specific sex and life stage group age (Brown *et al.*, 2004).

Table 2-1: Recommended daily allowances and Estimated average requirement for zinc by life stage and diet type as proposed by the IZiNCG

Age/life-stage	Sex	Reference body weight (kg)	Revisions suggested by IZiNCG for Zn (mg/d) RDA		Revisions suggested by IZiNCG for Zn (mg/d) EAR	
			Mixed/refined plant-based diets	Unrefined plant-based diets	Mixed/refined plant-based diets	Unrefined plant-based diets
6 – 11 months	M+F	9	4	5	3	4
1 – 3 years	M+F	12	3	3	2	2
4 – 8 years	M+F	21	4	5	3	4
9 – 13 years	M+F	38	6	9	5	7
14 – 18 years	M	64	10	14	8	11
14 – 18 years	F	56	9	11	7	9
Pregnancy	F	-	11	15	9	12
Lactation	F	-	10	11	8	9
> 18 years	M	65	13	19	10	15
> 18 years	F	55	8	9	6	7
Pregnancy	F	-	10	13	8	20
Lactation	F	-	9	10	7	8

Abbreviations: IZiNCG- International Zn Nutrition Consultative Group, RDAs- Recommended daily allowances, EAR- Estimated average requirement (Brown *et al.*, 2004)

If taken orally, Zn is considered to be relatively non-toxic, however, Zn toxicity may occur as a result of excess intake (Fosmire, 1990). This may be as a result of dietary Zn supplementation (Samman & Roberts, 1987), ingestion of contaminated Zn leaching from galvanized containers or piping (Brown *et al.*, 1964). In a review Zn has been linked by many scientific studies to cellular death (Plum *et al.*, 2010). An intake of more than 300 mg/d is associated with acute symptoms such as nausea, vomiting, epigastric pain, lethargy and fatigue (Fosmire, 1990). The body cannot tolerate excess amounts of Zn, and some studies, as reviewed by De Mel and Suphioglu, have shown that there is a relationship between Zn

neurotoxicity and degenerative diseases such as brain trauma, Alzheimer’s disease (AD) and epilepsy (De Mel & Suphioglu, 2014). For example, a 24-h exposure to high levels of Zn (40 µm) in mice neuronal cells was found to be sufficient to degenerate cells (Sheline *et al.*, 2000).

2.2.4 Zinc deficiency

Estimates by Maret suggest that Zn deficiency poses a serious risk to public health as it affects more than 25% of the world’s population (Maret, 2013). Zn deficiency is an important cause of morbidity in developing countries particularly among infants and young children (Gimenez *et al.*, 2011). The risk of Zn deficiency in individuals is classified into 3 categories as outlined in Table 2-2.

Table 2-2: Risk of zinc deficiency classification

	Inadequate Zn intake prevalence	Stunting prevalence
Category		
High risk	>25%	AND >20%
Moderate risk	>25%	OR >20%
Low risk	<25%	AND <20%

Moderate Zn deficiency may occur during infancy, childhood, adolescence and pregnancy as these are all periods of increased Zn requirement. Mild to moderate Zn deficiencies usually go undetected and undiagnosed because they usually do not present with specific organ pathologies and clinical symptoms (Gibson *et al.*, 2008).

A major cause of Zn deficiency is insufficient dietary Zn intake (Lönnerdal, 1998), as well a low bioavailability of Zn which is caused by Zn absorption inhibition by phytates (see section 2.2.5). In Vietnamese children, in whom 50% experienced protein energy malnutrition (PEM) during infancy, a link between a low protein intake and Zn deficiency was documented (Ninh *et al.*, 1996). Poverty, limited food availability or food choice that can be as a result of socioeconomic, cultural or religious constraint might also result in a low dietary intake of Zn (Arcanjo *et al.*, 2010). Strict vegetarians and, especially their children during rapid growth periods, may also develop Zn deficiency (Weigand & Boesch-Saadatmandi, 2012).

Conditional Zn deficiency might also be as a result of excess Zn loss due to gastrointestinal diseases such as diarrhoea, parasitic infections, celiac diseases, inflammatory bowel disease and Crohns disease (Prasad, 2012). Increased urinary Zn excretion in conditions

such as liver disease, cirrhosis, alcoholism, kidney diseases and diabetes mellitus has been reported in studies. Losses in Zn can also occur when there is excessive bleeding, through semen or an increased perspiration that occurs in a hot and humid climate or with excessive exercise (Gibson, 1994).

Increased physiological requirements during the life cycle may result in an increased risk of Zn deficiency. Increased Zn amounts occur during intense anabolic phases in organisms such as stress, trauma, obesity and rehabilitation after starvation (Tapola *et al.*, 2004). There is also an increase in Zn requirements during pregnancy as Zn accrual occurs in foetal and maternal tissues, in lactation as Zn is secreted in breast milk, and during catch-up growth for premature infants (Galetti, 2014). There is also an increased Zn requirement during weaning years in infants and children as well as in adolescents during puberty (Da Silva Rocha *et al.*, 2011).

Zn deficiency occurs not only as a result of a low intake of Zn rich foods or infection but can also be as a result of acrodermatitis enteropathica (AE) which is sometimes referred to as inherited Zn deficiency. AE is a rare genetic disease which is suggested to be as a result of a faulty gene that undergoes mutation in the SLC39A4 gene located on 8Q24 resulting in the recessive autoimmune disease. This disorder then results in the mutation of the ZIP4 Zn transporter (Wang *et al.*, 2001). As a result, affected individuals then have Zn absorption malfunctions, characteristic hyperpigmentation skin lesions, ophthalmic disorders, neuropsychiatric manifestations, anorexia, poor growth, delayed puberty, male hypogonadism and low plasma Zn levels, which may be a result of diarrhoea and poor Zn retention (Wapnir, 2000; Maverakis *et al.*, 2007). AE occurs worldwide with an incidence of 1 per 500'000 children with no apparent predilection for race or sex (Van Wouwe, 1989). Although under researched, there are data indicating that AE cases are present in African populations (Küry *et al.*, 2003; Kharfi *et al.*, 2010; Coromilas *et al.*, 2011; Engelken *et al.*, 2014). Some of the somatic consequences of AE can be reversed by vigorous Zn supplementation (Neldner & Hambidge, 1975). Without Zn therapy AE can be fatal (Kharfi *et al.*, 2010).

In animals, Zn deficiency has shown to have an effect on skin integrity (causes skin lesions), the gastrointestinal tract, immune, respiratory, skeletal and reproductive systems (Bhatnagar & Natchu, 2004). Zn-deficient individuals are characterised by a severely depressed immune function which causes frequent infections bullous pustular dermatitis, diarrhoea, alopecia and mental and emotional instability. Furthermore, severe Zn deficiency is known to induce anorexia, embryonic and post-natal growth retardation, difficulties in wound healing, and increased haemorrhage tendency (De Mel & Suphioglu, 2014). Other consequences of low

dietary Zn intake (100-300mg/d) include copper deficiency as well as adverse effects on the low-density to high-density lipoprotein (LDL/HDL) cholesterol ratio (Fosmire, 1990). Zn deficiency can also lead to mucosal dystrophy, which could in turn reduce absorption, not only of the polyglutamine forms of folate, but also of other nutrients. Furthermore, a poor Zn status can also affect the utilisation of vitamin A. This is because Zn-containing proteins are needed for the release of vitamin A from liver and for the tissue metabolism of vitamin A (Sandström, 2001).

A prolonged low Zn intake deprives the body of the potential beneficial effects of Zn such as interactions with oxidative free radicals and nitric oxide metabolism (Wapnir, 2000). All of these deficiency symptoms are also present in marginal deficiency. In addition to these symptoms, individuals with marginal deficiency may also present clinical signs which consist of impaired taste perception (hypogeusia), smell, the onset of night blindness (abnormal dark adaptation), impairment of memory, decreased spermatogenesis in males, reduced testosterone concentration, excess ammonia (hyperammonemia), decreased lean body mass, decreased natural killer cell activity and many more (Prasad, 1991).

Maternal (gestational) Zn deficiency which most likely leads to foetal Zn deficiency, is implicated in intrauterine growth retardation (IUGR) which in most cases results in depressed cell mediated immunity which may persist for years in the child (Clasen *et al.*, 2009). Maternal Zn deficiency has also been linked to infantile CNS abnormalities such as growth and development retardation of CNS tissues, peripheral neuropathy, spina bifida, accumulation of cerebrospinal fluid (hydrocephalus), absence of a major portion of the brain, skull and scalp (anencephalus) (Tako & Glahn, 2011), epilepsy and Pick's disease (Tako *et al.*, 2009).

Signs of severe Zn deficiency usually present in young children are development and growth retardation, a low appetite that results in depressed food intake, and an impaired immune response (Crichton & Boelaert, 2001; Weigand & Boesch-Saadatmandi, 2012). In recent years it has been discovered that there is a relationship between Zn deficiency and diarrhoea (Roohani *et al.*, 2013). During Zn deficiency the body is more susceptible to toxin-producing bacteria or enteroviral pathogens such as *Escherichia coli* that activate guanylate and adenylate cyclases. This stimulates chloride secretion, thereby causing diarrhoea and diminishing absorption of nutrients (Wapnir, 2000). In addition, in individuals with Zn deficiency there is a delayed termination of gastrointestinal disease episodes that are normally self-limiting thereby might impair the ability of the body to absorb water and electrolytes, delaying the termination of normally self-limiting gastrointestinal disease episodes (Wapnir, 2000).

In summary, Zn deficiency leads to stunted growth in children, a poor immune system, an increased risk of infection and possibly poor neurodevelopment (Gimenez *et al.*, 2011).

2.2.5 Bioavailability of zinc

There are factors that are known to influence Zn absorption, including the amount of Zn present in the intestinal lumen; food matrix composition; the presence of inhibitors (e.g., phytate, other minerals) or dietary promoters such as human milk and animal protein, Zn “status,” especially in relation to chronic Zn intake; and certain physiological states (De Mel & Suphioglu, 2014).

The most researched anti-nutritive factors (inhibitors) that hinder the bioavailability of Zn are phytate (inositol hexaphosphate or IP6) and fibre, which are present in frequently consumed grains and vegetables (Brown *et al.*, 2001). Phytate is the primary storage form of phosphorus and inositol in all grains and seeds (Cosgrove, 1966). On a global basis, plant-based diets with high phytate-to-Zn (PA:Zn) molar ratios are considered to be the major factor contributing to Zn deficiency (Arnold *et al.*, 1994). The negative effects of phytate in foods on human health are likely to be more pronounced in people in developing countries who live on marginal subsistence diets that consist mainly of seeds, grains or fruit and less on protein sources that are rich in Zn (Cosgrove, 1966).

Phytate is considered an anti-nutrient because it chelates the nutritionally important minerals calcium (Ca^{2+}), magnesium (Mg^{2+}), Zn^{2+} and Fe^{2+} (Reddy & Sathe, 2001). This then interferes with absorption by forming strong insoluble complexes that cannot be absorbed by the small intestines, thus decreasing bioavailability. These formed insoluble penta and hexa substituted salt complexes are difficult to hydrolyse during digestion because humans lack sufficient intestinal phytase to degrade the complexes. It has been discovered that fractional absorption of Zn is negatively associated with the phytate content (Sandström & Lönnerdal, 1989). When the complex includes peptides, the bioavailability of proteins and enzymatic activity may be reduced (Reddy & Sathe, 2001).

To predict poor Zn bioavailability from food, PA:Zn molar ratios are calculated (Mitchikpe *et al.*, 2008). As shown in Table 2-3 a PA:Zn molar ratio greater than 15 from unrefined diets or a diet that mainly composed of cereals and has a low dietary protein is estimated to have a low absorption of Zn (15%).

Phytate concentrations in cereal foods can be reduced to improve bioavailability by altering food preparation as well as processing methods. Soaking whole grains and pounding of grains, dehulling, malting and fermentation have all been shown by different studies to lower

the phytate levels of food (Mahgoub & Elhag, 1998; Hotz & Gibson, 2001; Lestienne *et al.*, 2005). More so, Zn absorption can be aided by the consumption of enhancers of Zn absorption such as low molecular weight chelators, such as ethylenediamine tetraacetic acid (EDTA), vitamin C, organic acids and meat (Mitchikpe *et al.*, 2008). In addition to increasing the absolute amount of Zn, the presence of animal protein can substantially enhance the efficiency of absorption (Sandström & Lönnerdal, 1989). This is because protein containing foods contain soluble, low-molecular-weight organic substances such as the sulphur-containing amino acids (histidine, methionine) and hydroxy acids that are able to bind Zn and facilitate its absorption (Oberleas *et al.*, 1966).

The presence of negative nutrient to nutrient interactions between Zn and minerals such as Fe^{2+} and Ca^{2+} (Oberleas *et al.*, 1966; Sandström, 2001) are known to decrease absorption of Zn (Lönnerdal, 2000). As proposed by Hill and colleague, these negative interactions may be as a result of the minerals sharing the same absorptive pathways (Hill & Matrone, 1970). High concentrations of one element may then affect the absorption of the other element. The second proposed mechanism by which trace element interactions occur is when the deficiency of one element affect the metabolism of the other element (Lönnerdal, 1998). Other factors that have been found to reduce the bioavailability of Zn are unabsorbed FA. These were found to chelate Zn ions, making its absorption impossible (Krebs *et al.*, 2000).

Table 2-3: Estimates of dietary zinc absorption, as developed by WHO, FNB/ IOM, and IZINCG

	WHO			IOM		IZINCG					
Diet type	Highly refined	Mixed	Unrefined	Mixed		Mixed			Unrefined		
Life stage, sex group	N/A	NA	NA	Children	>18y	1-18y, lactating (14-18yrs)	>18y M	>18y F, lactating (>18y)	1-18y, lactating (14-18yrs)	>18y	1-18y, lactating (14-18yrs)
PA:Zn molar ratio	< 5	5-15	>15	NA	NA	4-18			18-30		
Zinc absorption	50%	30%	15%	30%	41%	31%, 40%	26%	34%	23%, 32%	18%	25%, 35%

Abbreviations: WHO- World Health Organisation, FNB- Food and Nutrition Board, IOM- Institute of Medicine's, IZINCG- International Zinc Nutrition Consultative Group, PA:ZN- phytate to zinc ratio (Brown *et al.*, 2004)

2.2.6 Assessment of zinc status

2.2.6.1 Plasma / serum zinc concentration and other biochemical biomarkers

Discovery of a reliable, sensitive, and specific biomarker of Zn status has been the subject of considerable research in the past years. Nutritional biomarkers are either dietary, biochemical, anthropometric, or clinical indices and they allow the measuring of the level of nutrient intakes, exposures, nutrient status, or nutrient functional effects (Maberly *et al.*, 1981). For the assessments of Zn status, biochemical indices are mostly used.

The lack of suitable indices for measuring body Zn has led to uncertainties in establishing an RDA for Zn, establishing the global Zn status and difficulty in quantifying and categorising Zn deficiency (Sandström & Lönnerdal, 1989). Additionally, the task of establishing a Zn biomarker is made difficult by the homeostatic Zn mechanisms that are very effective in maintaining tissue and circulating serum/plasma Zn concentrations within a narrow range (King, 2011). The body tightly regulates the concentration of Zn in the body, maintaining the concentrations within strict limits even if the Zn intakes differ dramatically. As an example, small decrements in tissue Zn that lead to marginal Zn deficiency may not be measurable due to this tight regulation (Van Biervliet, 2008).

The paucity of a Zn status biomarker can also be attributed to the fact that most of the body Zn is located in the slow turn-over tissues such as muscles, bone, and skin (Lowe *et al.*, 2009). Only 0.2% of Zn circulates in the plasma and is associated with albumin and α 2-macroglobulin (King, 1990; Hambidge, 2003). Furthermore, difficulty in finding a biomarker may be also due to Zn's role in many biological and physiological functions such gene expression and growth (De Mel & Suphioglu, 2014).

Fortunately, the past years have resulted in the identification of a number of potentially useful biochemical biomarkers (Lowe *et al.*, 2009). A systematic review assessed the biomarkers that were used to measure the Zn status in healthy individuals in 46 publications who were part of randomised clinical Zn supplementation trials (Lowe *et al.*, 2009). The only efficient biological biomarkers were found to be plasma, serum, urinary, and hair Zn concentrations. All these indicators are, however, not consistent in assessing the Zn status in individuals since results from different studies are contradictory and inconsistent (Lowe *et al.*, 2009).

The widely used biomarker for Zn status is serum Zn concentration despite its poor sensitivity and imperfect specificity (Hambidge, 2003). Plasma Zn (PZn) concentration measures the level of exposure and plasma or serum have been shown to reflect short term

Zn intakes (Gibson *et al.*, 2008). At individual level, PZn is a poor biomarker because it is affected by infections, weight loss or starvation. However, studies have demonstrated that PZn and serum Zn respond to both depletion and repletion of Zn during severe dietary restriction and Zn supplementation (Baer & King, 1984; Ruz *et al.*, 1992). Never the less, serum and plasma Zn concentrations are used to reflect dietary Zn intake and also to predict functional responses to Zn interventions. PZn may not be a reliable biomarker of individual Zn status (ASSAf, 2013).

There are major confounding variables which affect the measurement of Zn status of individuals. These are age, sex, but also methodological factors such as time of day of blood sampling, blood withdrawal, specimen handling and fasting state of the subjects. Blood collection, separation and preparation of plasma specimen for Zn analysis should be considered in order to prevent the contamination of the sample with endogenous or exogenous Zn. Temperature, tourniquet time placement, time between blood collection and separation may also influence PZn concentration (IZiNCG), 2007).

Zn concentrations are age and sex related and differences are observed in some studies (Hotz *et al.*, 2003). King *et al.* found that during childhood up to the age of 10 years, females had lower serum Zn concentrations than males. Tissue demand for Zn is increased during growth, pregnancy and lactation to provide for additional Zn for tissue growth or milk synthesis (Galetti, 2014). As previously described in section 2.2.4 PZn can be decreased by acrodermatitis enteropathica (AE) and diarrhoea. Use of oral contraceptives, hormones or steroids, tissue injury in surgery and chronic alcoholism can also decrease PZn. Also, diurnal PZn concentrations cumulatively decrease after meals (Wallock *et al.*, 1993). Fasting results in increased PZn circulation (Hambidge *et al.*, 1989). Due to this, the IZiNCG recommends that the time of blood withdrawal and fasting status or time since the last meal consumption should be reported.

Other confounders of Zn status are systemic infections and inflammation that produce an acute-phase response that causes a decreased in plasma Zn concentrations. This may be due to the increased demand from the liver i.e. sequestration from the liver (Falchuk *et al.*, 1977). Zn homeostasis is altered during disease states in several ways that can be grouped into three groups: 1) decreased exogenous Zn uptake or excessive endogenous Zn losses from gastrointestinal tract or kidneys, 2) increased Zn requirements, 3) and increased body Zn redistribution (Krebs & Hambidge, 2001). Droke *et al.*, (2006) showed that an elevated C-reactive protein and leukocyte counts were associated with lower PZn levels in low-income American children with infection (Droke *et al.*, 2006). Contradictory, in a community-based cross-sectional study in Peruvian children a significant association between the presence of

infection and plasma Zn concentration was not found (Brown *et al.*, 1993). This could possibly be because infections were of variable magnitude and might have differed between the two population groups due to their nutritional status as well as prevalence and severity of infections (Brown *et al.*, 1993). Despite a high prevalence of childhood infections in developing countries, PZn concentration remains a useful indicator of population Zn status for these children (Brown, 1998).

The erythrocyte linoleic acid: dihomo- γ -linolenic acid (LA: DGLA) ratio is another possible biomarker that has been explored. In an *in vivo* model of *Gallus gallus* the LA: DGLA ratio was found to be significantly elevated in the Zn-deficient group compared to the Zn-sufficient group, thus leading to a conclusion that the erythrocyte LA: DGLA was able to differentiate Zn status between Zn adequate and Zn-deficient birds, and could be a sensitive biomarker that could be used to assess dietary Zn manipulation (Reed *et al.*, 2014). Recently in humans, Knez and colleagues were able to demonstrate that in apparently healthy adults the concentration of DGLA was decreased and the LA: DGLA ratio was increased in people with lower dietary Zn intake (Knez *et al.*, 2016).

Other potential Zn biomarkers are also being explored. Most recently, Zn metallothionein has been used as a Zn indicator. In humans, erythrocyte metallothionein (E-MT) levels are suggested to be better indicators of Zn depletion and repletion, as E-MT levels are sensitive to dietary Zn intake (Lee & Cousins, 1993; Zapata *et al.*, 1997). Although there is some evidence for the utility of enzyme assays such as alkaline phosphatase, copper-Zn superoxide dismutase and lymphocyte 5'-nucleotidase, the attempts to confirm the results often have been unsuccessful (Hambidge, 2003). Erythrocyte Zn has been shown to be both responsive and non-responsive to Zn depletion; it is often used to evaluate Zn status. (Baer & King, 1984). Other purported biomarkers of Zn include hair, urinary and faecal Zn (Baer & King, 1984; Lowe *et al.*, 2009).

A population has an elevated risk of Zn deficiency when a higher than expected percentage of the population has low serum Zn concentrations (Ravindran *et al.*, 1991). A prevalence of more than 20% of individuals in a population that have a low Zn concentration signifies an elevated risk of developing Zn deficiency (Ghosh *et al.*, 2007).

It is recommended that further high quality studies are required using the recommended biomarkers in children and adolescents, as studies on these age groups are scarce (Lowe *et al.*, 2009).

2.2.6.2 Reference data for zinc status

For children who are under the age of 10 years old, a low PZn concentration is defined as 65 µg/dL for morning non-fasting blood samples, <57 µg/dl for children under 10 years old (afternoon sampling). In males older than 10 years the lower cut-offs of fasting morning serum Zn concentration is 74µg/dL (11.3µmol/L) and for females, 70µg/dL (10.7µmol/L). For blood samples taken in the afternoon in children over the age of 10, the lower cut-offs are <61 µg/dl for males and <59 µg/dl for females Table 2-4 (ASSAf), 2013). Morning serum Zn concentration is used as a marker of Zn status, but the previously mentioned factors such as age, sex and fasting state should also be taken into account and treated as confounding variables (King, 1990).

Table 2-4: Suggested lower cut-offs for serum Zn concentration (µg/dl)¹ by age group, sex, time of blood collection and fasting status¹

Time of blood collection and fasting status	Time of blood collection and fasting status Suggested lower cut-offs for serum Zn		
	< 10 years	≥ 10 years	
	Male and Female	Females	Males
Morning, fasting for at least 8 hours	NA	70 (10.7)	74 (11.3)
Morning, non-fasting	65 (9.9)	66 (10.1)	70 (10.7)
Afternoon, non-fasting	57 (8.7)	59 (9.0)	61 (9.3)

¹ Concentration denoted in µg/dL and µmol/l in parenthesis. Conversion factor: µmol/l = (µg/dL)/6.54

2.2.7 Functional indicators of zinc status

In addition to the biochemical indicators of Zn status, there are functional population indicators of Zn status that are used as indirect methods of assessing the prevalence of Zn deficiency (Wessells & Brown, 2012). These include the percentage of the population with plasma/serum Zn concentrations below the suggested cut-offs, the prevalence of usual dietary Zn intakes below the EAR, and the percentage of children who are five years of age and under with height-for-age Z-scores less than -2 SD with respect to the WHO child

growth standards (Fischer Walker & Black, 2007; Hess *et al.*, 2007; Hotz, 2007; Gibson *et al.*, 2008).

2.2.8 Plasma zinc cutoff

A population has an elevated risk of developing Zn deficiency if 20% of the population have a serum Zn below the -2 standard deviation of the American NHANES II study (Ghosh *et al.*, 2007).

2.2.9 Stunting prevalence

According to WHO/ IOM and IZINCG, in a population, the risk of Zn deficiency can be assessed by initially consulting the stunting rates of children under the age of 5 years (Galetti *et al.*, 2016). Stunting prevalence in a population has been recommended as a functional indicator of a population's risk of Zn deficiency (Gibson *et al.*, 2008). This is because Zn deficiency may attribute to childhood stunting as it impairs growth (Caulfield LE *et al.*, 2004). Stunting refers to a shortness that is pathological, in such a way that it reflects a failure to reach a linear growth potential for a particular age (Lim *et al.*, 2006). Furthermore, a prevalence of low height-for-age in children under 5 years of age in a population reflects pre and post-natal nutritional conditions of young children such as a long-term cumulative socioeconomic, health and nutrition inadequacies, in particular, chronic malnutrition (Wessells & Brown, 2012).

When the prevalence of stunting is greater than 20% in children who are below the age of 5 years, the risk of Zn deficiency may also be elevated in the whole population and indicates a need for a public health intervention (Wessells *et al.*, 2012). Figure 2-1 illustrates the prevalence of childhood stunting as well as that of inadequate dietary Zn intake in the world.

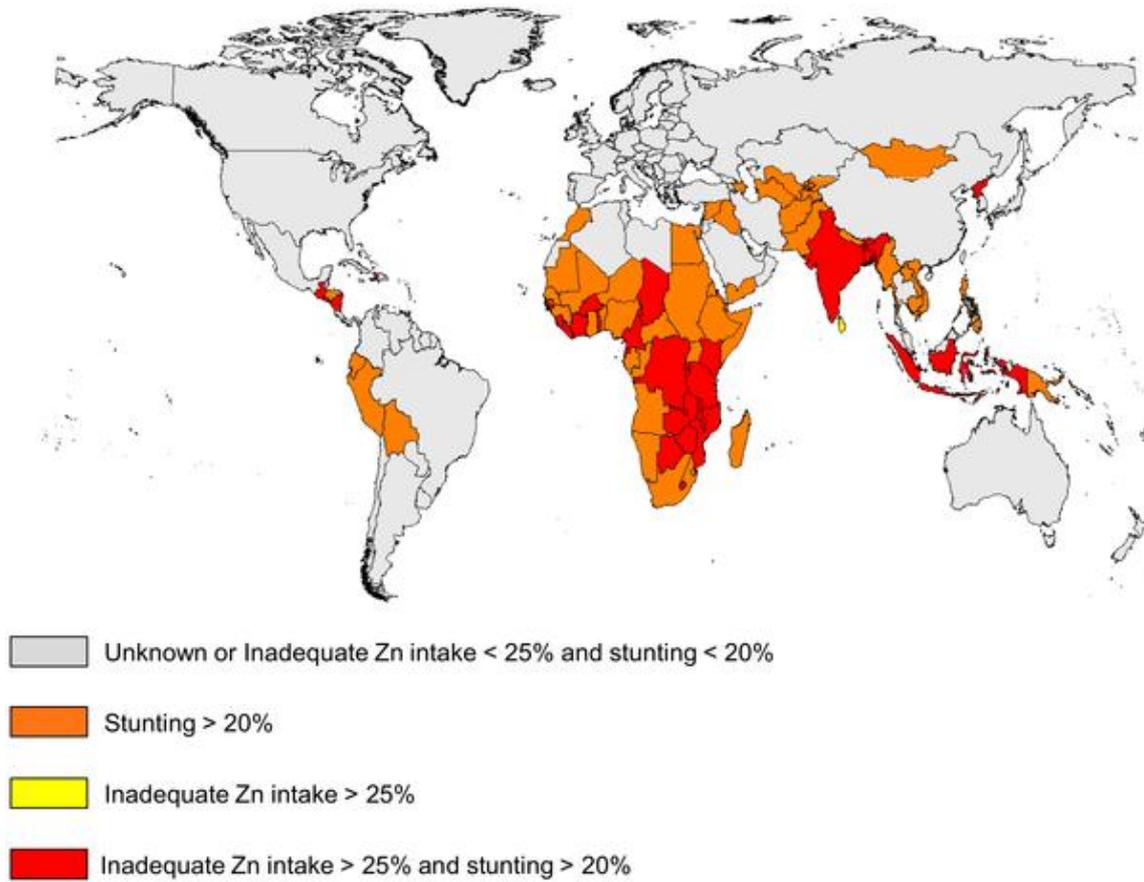


Figure 2-1: National risk of Zn deficiency based on the prevalence of childhood stunting

2.2.10 Dietary zinc intake

For dietary indicators, the prevalence of Zn intakes below the appropriate EAR is used. To determine Zn intake, quantitative dietary intake assessments such as weighed food records and or semi-quantitative methods like the 24-hour food recall are used (Gibson, 2005). Assessing dietary Zn intake is of importance because it identifies and quantifies the part of a population that is at risk of inadequate Zn intake. It also provides information on dietary patterns that may contribute to Zn inadequacy (de Benoist *et al.*, 2007).

Different sets of EAR cut-offs have been suggested by the WHO, FNB/IOM and IZiNCG and inconsistencies have been reported when these were compared (Wessells *et al.*, 2012). Consistent with these results Galetti *et al.* found a variation in the risk of Zn deficiency when the different EARs were applied (Galetti *et al.*, 2016). When applying the WHO EAR cutoff, 80% of the school aged children were at risk of Zn deficiency. Whereas when the IZiNCG cutoff was applied, 11% were below the EAR. A possible explanation for this could be that these cutoffs are based on different age classifications, body weights and Zn excretion assumptions (Galetti *et al.*, 2016).

Where the prevalence of inadequate intake of Zn is greater than 25% the risk of Zn deficiency is considered to be elevated (Ghosh *et al.*, 2007). By using both food balance sheet information and the prevalence of stunting, it may be possible to estimate the risk of Zn deficiency in the whole population, including older children and preschool children as well as adults (de Benoist *et al.*, 2007; Wessells *et al.*, 2012).

Ideally, all of these indicators together with the recommended biochemical biomarkers should be used together to obtain a better picture and understanding of the risk of Zn deficiency in a population and to identify specific subgroups with an elevated risk that would require a public intervention (de Benoist *et al.*, 2007).

2.2.11 Global zinc deficiency prevalence

At national level, only a few countries have information on their country's Zn status based on PZn (Da Silva Rocha *et al.*, 2011). It is estimated that 17.3% of the global population is at risk of inadequate Zn intake (Wessells & Brown, 2012). In high-income countries the prevalence of inadequate Zn intake is estimated to be 7.5% and 30% in low-income countries in South Asia. It is estimated that countries in the Global South have the greatest risk of inadequate Zn intake see Figure 2-1 (Wessells *et al.*, 2012). The prevalence of Zn deficiency is higher in children in low- and middle-income countries who are under five years of age than in the general population. This is because children in general have higher

nutrient density needs and in those particular countries rates of infection among infants and young children are high (Wessells *et al.*, 2012). Data based on Zn availability from national food supplies, countries in South and Southeast Asia, Sub-Saharan Africa and Central America which have been identified as being at highest risk of inadequate Zn intake and should be prioritized for biochemical and dietary assessments of population Zn status (Wessells *et al.*, 2012).

2.2.12 Supplementation and fortification of food as strategies to improve zinc status

Zn is special among individual nutrients since it has been designated as a “problem” nutrient of which adequate intake is difficult to obtain from complementary foods without fortification (Hambidge & Krebs, 2007). To increase dietary intake, several developing countries are fortifying food with Zn. This is because individuals with poor Zn status were found to be more responsive to Zn interventions than those with a normal status because they seemed to absorb Zn efficiently (Crichton & Boelaert, 2001). In populations at risk of Zn deficiency, preventive Zn supplementation has been found to reduce the incidence of premature delivery, decrease morbidity from childhood diarrhoea and acute lower respiratory infection, lower all-cause mortality, and increase linear growth and weight gain among infants and young children (Brown *et al.*, 2009; Hess & King, 2009).

Fortified foods with increased Zn lead to a higher total absorption in Zn when compared to conventional foods (Elnagar *et al.*, 1997). Furthermore, fortification can reach a vast population, without changing its dietary pattern. Staple food grains such as wheat, millet and maize are often used as fortification vehicles in numerous studies (Tripathi & Platel, 2010). A systematic review that assessed the efficacy of Zn fortification found that in 11 studies (n = 771), Zn fortification was associated with significant improvements in plasma Zn concentrations [standard mean difference (SMD) 1.28, 95% CI 0.56, 2.01]. However, information on the overall effectiveness of Zn fortification is limited (Das *et al.*, 2013). Table 2-5 shows six randomised controlled trials that investigated the effects on Zn fortification on Zn status as well as on immunity and growth. These trials differ in length but all seem to show that Zn fortification improved serum/plasma Zn concentration.

Table 2-5: Effect of zinc fortification trials in children

Author, year	Country, age	Study design	Fortification vehicle	Dose, type	Duration (months)	Findings
Hambidge, 1979	USA, 2- to 7-year-old healthy children	RCT	ready-to-eat breakfast cereals	2.57 mg/d, ZnO	9	↑ PZn, NS food intake or growth Velocity, plasma copper and serum cholesterol.
Hettiarachchi, 2004	Sri Lanka, 7- to 10 - year-old	RCT	rice	6 mg/100g, ZnO	1	↑ PZn
Galetti, 2015	Benin, 6-10 years	RCT	water	2.8mg, ZnSO ₄	6	↑ PZn (TTI), NS diarrhoea or growth
Kiliç, 1998	Turkey, 7- to 11-year-old school children	RCT	bread	2 mg/kg/d, Zn(O ₂ CCH ₃) ₂	3	↑ serum and leukocyte Zn, weight, serum albumin, immune functions, phosphatase
Kujinga, 2016 ²	Kenya, 2-6 year	RCT	water	2mg/d, ZnSO ₄	6	NS in PZn, improvement on overall morbidity (TTI)
Ohiokpehai, 2009	Kenya, 6- to 9- year-old	quasi	corn-soy blend porridge	5.0mg/100g.	3	↑ mean serum zinc

Abbreviations: RCT- randomised controlled trial, IG- Intervention group, C- control group, NS- not significant, Zn(O₂CCH₃)₂- zinc acetate, quasi-quasi-RCT, TTI- time by treatment interaction

¹children with asymptomatic zinc deficiency, ² unpublished

2.2.13 Water fortification

Fortification of water is not a new concept; water has been fortified with minerals such as iodine (Maberly *et al.*, 1981; Elnagar *et al.*, 1997; Lim *et al.*, 2006), fluorine (McDonagh *et al.*, 2000) and Fe^{2+} , (Arcanjo *et al.*, 2010; Da Silva Rocha *et al.*, 2011) vitamins B₆, B₁₂, and D and Ca^{2+} (Tapola *et al.*, 2004). Water as a fortificant vehicle has the advantage of being stable, having a long shelf life, and a high bioavailability of the fortificant due to absence of the food matrix (Polaki & Yarla, 2014).

2.2.13.1 Lifestraw Family filtering device

LifeStraw® Family 1.0 (LSF) (Figure 2-2) is a point of use water filtration device that is intended for household use. The LSF filter meets the microbiological performance specifications by United States Environment Protect Agency (US EPA) and WHO (USEPA, 1987) and It is designed to treat water of high levels of turbidity and of unknown microbiologic quality, making it suitable to use in low-income settings (Clasen *et al.*, 2009; Naranjo & Gerba, 2011). The LSF filter can filter up to 18,000 litres of water. Untreated water is put into the upper bucket (2.5L) containing an 80- μm -textile pre-filter that is used for separation of larger particles (80 μm). Water runs through a 1metre-long, PVC connection hose which creates enough pressure to force water into the ultrafiltration cartridge. This water then runs through a halogen chamber that has a slow-eluting solid chlorine tablet that helps prevent biofilm formation on the ultrafiltration membranes. The ultrafiltration cartridge is a plastic cylinder which has several hollow fibres with a 20-nm-pore size that form the microbiological barrier. Clean water can then be accessed through the outlet hose by opening the valve that is mounted on the side of the filtration cartridge. To backwash the membrane one has to squeeze the red backwash bulb three times with both taps closed and then release the dirty water from the red tape (Galetti, 2014; Kujinga *et al.*, 2016).

A modified LSF filter that has a Zn fortification chamber next to the filtration cartridge was developed. This Zn LSF filter has a nutrient chamber containing two soluble Zn based glass plates adjacent to the ultrafiltration chamber. Fortification of water occurs via one or two Zn oxide-embedded sodium phosphate-based soluble glass plates that are mounted into the fortification chamber (Giltech Ltd, UK). Once in contact with water, the plate slowly dissolves in a controlled manner and releases Zn^{2+} ions into water. In Beninese children aged between 6-10 years, Galletti and colleagues were able to show that consumption of Zn-fortified water with 2.8mg of Zn was able to maintain higher plasma Zn levels compared to non-fortified water (Galetti *et al.*, 2015).

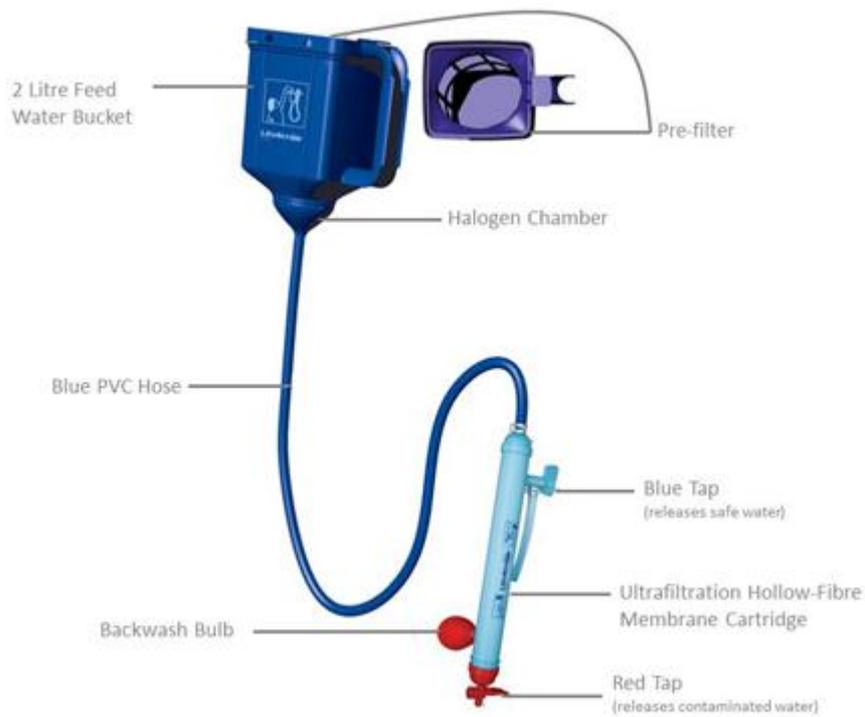


Figure 2-2: The LifeStraw® Family Filter 1.0, a point of use water filter by Vestergaard Frandsen

(<http://vestergaard.com/index.php/lifestraw-family-1-0>last accessed 03112016)

2.3 Fatty acids

2.3.1 Metabolism of long chain polyunsaturated fatty acids (LCPUFA)

2.3.1.1 Biochemistry

Fatty acids (FA) are carboxylic acids comprising of a hydrophobic hydrocarbon chain with a terminal hydrophilic carboxyl group R (-COOH). Where -COOH is the structure of carbon, oxygen, and hydrogen atoms that comprise the carboxyl group and R is the remainder of the molecule (Nelson *et al.*, 2008). FA are named according to their chain length. Short chain FA have two to six carbons, medium chain FA have 8-12 carbons, whilst long-chain fatty acids (LCFA) have greater than or equal to 12 carbon atoms. FA are further divided into saturated (no double bonds), monounsaturated (one double bond) and polyunsaturated fatty acids (PUFA) that have two or more double bonds in the carbon chains. The PUFA are further divided into two families, the n-6 and n-3 series, depending on the place of the first double bond. Long-chain polyunsaturated fatty acids (LCPUFA) are a class of PUFA that contain 14 or more carbon atoms with at least two double bonds (Abedi & Sahari, 2014).

Linoleic acid (LA, 18:2n-6) and α -linolenic acid (ALA, 18:3n-3) are the two parent EFA (Calder, 2015). The term 'essential' means that they must be supplied from the diet because they are required by the human body and cannot be endogenously synthesised (Decsi & Koletzko, 2000). They are also called essential since both families are needed for normal development and biological function, even though it is their longer-chain derivatives that are of more functional importance (Uauy *et al.*, 2001b; Van Biervliet, 2008).

LA and ALA are synthesised only by plants from oleic acid (18:1n-9), this is because plants possess the delta-12 and -15-desaturases that introduce double bonds beyond carbon 9 towards the methyl end (Calder, 2013b). The enzyme system responsible for this property is missing in animals including humans but once LA and ALA are supplied to animals and humans in the diet, their longer chain PUFA derivatives are formed by a series of biochemical reactions catalysed in the microsomal peroxisomes and endoplasmic reticulum of the liver (Tocher *et al.*, 1998).

2.3.1.2 Biosynthesis of long chain polyunsaturated fatty acids

The liver contains enzymes called elongases and desaturases. The elongase enzymes add two carbon atoms at the carboxyl end of a FA, while the desaturase enzymes remove two hydrogen atoms from the hydrocarbon chain, thus creating a double bond. In humans three desaturase enzymes are known, these are delta-9, -6 and -5. Mono-unsaturated fatty acids (MUFA) require

delta-9 for synthesis, whereas delta-6 and -5 are imperative for the synthesis of LCPUFA (Tocher *et al.*, 1998). As shown in Figure 2–3, the parent FA is converted by elongase and desaturase in a multistage bioconversion process to form unsaturated LCPUFA of 20 and 22 carbon atom lengths (Uauy *et al.*, 2001a). Fatty acid desaturase (FADS) 1 and 2 genes encode delta-5 and -6 respectively (Burdge & Calder, 2014). Delta-6-desaturase is known to be the rate-limiting step in the metabolism of EFA (Das, 2010).

For the normal activity of delta-6-desaturase, co-factors such as Mg^{2+} , Zn^{2+} , and Vitamin B₆ are required and a deficiency of these minerals are thought to reduce activity of desaturase and elongase enzymes (Johnson *et al.*, 1989; Prasad, 2013). Desaturases and elongases are dependent on the electron transfer pathway. Microsomal Zn levels directly determine electron transfer from nicotinamide adenine dinucleotide (NADP) or nicotinamide adenine dinucleotide phosphate (NADPH) through the cytochromes B5 and P-450 to the desaturase (and elongase) enzymes which is the terminal electron transfer (Cunnane, 1988b). Studies have shown that the activity of delta-6-desaturase is also modulated by genetic and dietary factors (Hill *et al.*, 1963). In addition, the activities of delta-5 and -6-desaturase are regulated by nutritional status, hormones and by feedback inhibition by end products (Burdge & Calder, 2014).

Irrespective of diet, gender affects the activity of desaturase enzymes. Female rats of reproductive age have a higher level of mRNA that code for delta-5 and delta-6-desaturases. (Ma *et al.*, 1995; Childs *et al.*, 2008; Childs *et al.*, 2010). Polymorphisms in the FADS1 and 2 are thought to be present in individuals at risk to atopic and cardiovascular disease, as differences in n-6 and n-3 PUFA statuses occur in individuals who are suffering from these diseases (Calder, 2015). A recent review noted that activities of delta-6 and -5-desaturases are inhibited by hyperglycemia, vitamin A, cholesterol, phytochemicals, alcohol, EFA excess and fasting (Bhagat & Das, 2015). The activity of delta-5- and 6-desaturase is also known to fall with age (Horrobin, 1981).

As illustrated in Figure 2–3 the n-3 PUFA, ALA, is converted to stearidonic acid (18:4n-3) and eicosatetraenoic acid (20:4n-3) and then to eicosapentaenoic acid (EPA; 20:5n-3) by delta-6-desaturase and elongase enzymes. Further metabolism of EPA to docosahexaenoic acid (DHA, 22:6n-3), as proposed by Sprecher and colleagues involves two sequential elongations of EPA to docosapentaenoic acid (22:5n-3) and tetracosapentaenoic acid (24:5n-3) (Sprecher, 2000). This is followed by transport to the peroxisomes where DHA is formed from tetracosahexaenoic (24:6n-3) through chain shortening by two carbon units during one cycle of the peroxisomal pathway, and then a single cycle of beta-oxidation to yield DHA, which is then transported back to the microsomes for incorporation into glycerolipids. Another synthesis pathway was proposed

by Infante and Huszagh, they suggested that synthesis of DHA and n-6 docosapentaenoic acid (DPA, 22:5n-6) occurs in the mitochondria through specific carnitine-dependent pathways, and utilises separate enzymes for the n-6 and n-3 PUFA (Infante & Huszagh, 2001). However, the most accepted pathway is the Sprecher shunt.

Like ALA, LA is converted to gamma-linolenic acid (GLA; 18:3n-6), and dihomogamma-linolenic acid (DGLA, 20:3n-6) and then to the key and predominant n-6 LCPUFA, ARA, which is further metabolised to n-6 DPA by the same desaturase and elongase enzymes as those involved in n-3 FA synthesis (Figure 2–3). Quantitatively, the n-6 equivalent of DHA is ARA as they are both main constituents of membranes (Plourde & Cunnane, 2007). ARA and EPA are the principal precursors for production of eicosanoids such as prostaglandins, leukotrienes and other lipoxygenase or cyclooxygenase (Calder, 2015) (see section 2.3.4.3). Similarly palmitic acid (16:0) is converted to stearic acid (18:0), and to oleic acid. A three step elongation system of oleic acid to gadoleic acid (20:1n-11) and erucic acid (22:1n-9) ensues to produce nervonic acid (24:1) (Hale *et al.*, 1930).

The FA of the n-6 and n-3 series compete for the same elongase and desaturase enzymes which may result in a decrease of the PUFA production of the competing series FA (Van Biervliet, 2008), therefore, the balance between n-3 and n-6 FA in the diet is important (Smit *et al.*, 2004). However, it has now been established that the desaturase enzymes show a preference for the different FA series in the order n-3>n-6>n-9 (Innis, 1991). Thus, ALA is the preferred substrate for delta-6-desaturase, followed by LA and then oleic acid. The metabolism of n-6 FA is quantitatively more important because LA is much more prevalent than ALA in most human diets (approximately 6- to 11-fold more) (Burdge & Calder, 2014), thus high concentrations of LA negatively affects the conversion of ALA to EPA (Liou & Innis, 2009). Therefore, a low intake of LA relative to ALA is crucial for normal metabolism in the body (Simopoulos, 1999).

Due to the competition for the same enzyme between n-6 and n-3 FA (Figure 2–3), conversion of the non-essential FA oleic acid to mead acid (20:3n-9) occurs only at relatively low levels compared to both ALA and LA and has, therefore, been regarded as a marker for dietary EFA deficiency (EFAD) (Innis, 1991). Although being in the same PUFA family, both n-3 and n-6 PUFA are metabolically and functionally distinct and have opposing physiological effects (De Mel & Suphioglu, 2014).

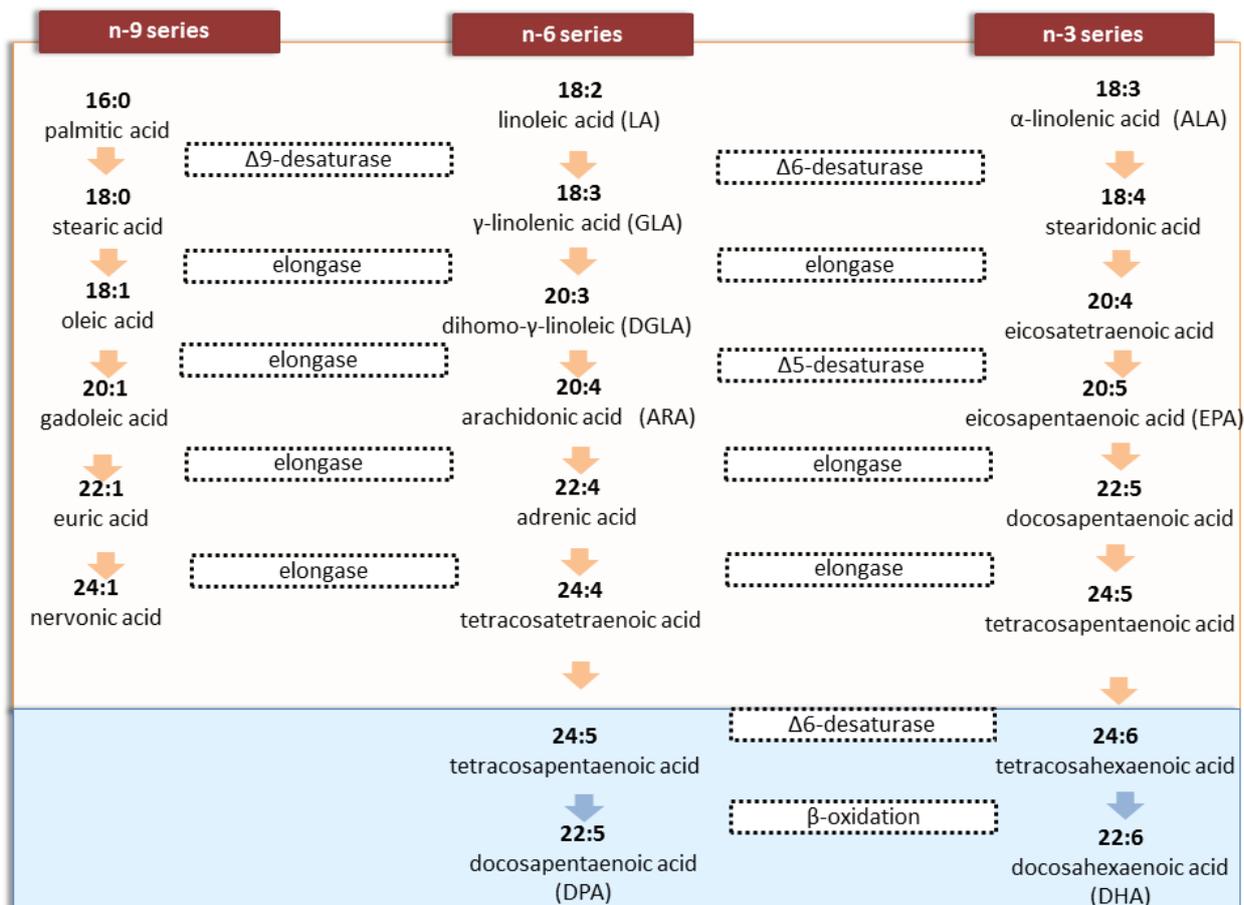


Figure 2-3: Schematic illustration of the synthesis of EFA to n-3, n-6 and n-9 LCPUFA (Sprecher pathway)

Adapted from (Baumgartner, 2012) and (Shmeeda *et al.*, 2002)

2.3.2 Recommended fatty acid intake in children

In older (2-18-year-olds) children it is recommended that total fat intake should be between 25–35% of total energy (TE). Saturated FA intake should not be more than 10% of the TE intake and total PUFA intake for this age group should be 11%E (percentage of energy supply). EPA+DHA intake for children who are between the ages of 6-10 years should be 200-250 mg. The FAO report also advises that total trans-FA intake should be less than 1% E. It is also suggested that 2-3 x 100g portions of oily fish should be consumed on a weekly basis since the conversion of EFA to their LCPUFA derivatives is not highly efficient (FAO, 2010). Mackerel, salmon, herring, tuna and pilchards are good examples of fish that are rich in n-3 LCPUFA (Chow, 2007).

2.3.2.1 Fatty acid intake in low income countries

Data on fat and FA intake dietary in low-income countries is scarce. This might be because the complexity of fat makes it one of the most difficult dietary components to measure (Arab, 2003). One might also attribute this to a lack of information on the FA composition of diets in low-income countries, as food composition tables are usually incomplete.

In low-income countries, the majority of the population is of a low economic status. The availability of fat, especially of the n-3 and n-6 PUFA series in the food supply is often very low and below the recommended dietary intakes (Wolff *et al.*, 1984). In these countries, the commonly eaten staple foods are rice, wheat and maize, all of which are poor sources of n-3 PUFA. Legumes such as soybean are sometimes consumed. These may be a good source of FA in vulnerable populations that don't have access to animal products (Michaelsen *et al.*, 2011). Known PUFA sources in vulnerable communities are vegetable oils, the commonly used oils being palm oil, sunflower oil and sesame seed oil (Michaelsen *et al.*, 2011). Animal food sources are also an important source of n-3 and n-6 PUFA. In poor communities the intake of meat and fish are rare. However, in these communities, chicken and eggs are a more accessible meat source (Zhang *et al.*, 2013). The known food sources of individual FA are shown in Table 2-6.

Intake of LCPUFA such as DHA and ARA is very small (5%) compared to that of EFA (Simopoulos, 1991). *In vivo* conversion of the parent FA's (LA and ALA) to the LCPUFA such as EPA, ARA and DHA has been shown to be below 10% and below 0.1% respectively, this usually then results in low levels of the LCPUFA (Burdge & Calder, 2005). This theorem is supported by different studies as they ascribe low LCPUFA levels in tissues to impaired conversion of parent EFA to LCPUFA, rather than to a diminished intake of LCPUFA or their precursors (Vajreswari *et al.*, 1990; Decsi *et al.*, 1995). Stable-isotope tracer studies also have been able to demonstrate that less than 1% of LCPUFA is metabolised in humans (Pawlosky *et al.*, 2001). Therefore, there should be an emphasis on the intake of rich preformed PUFA sources such as fish. Furthermore, intake of PUFA sources is also important because FA levels in tissues are highly influenced by dietary FA composition (Sontrop & Campbell, 2006). Low fat intake may adversely affect the status of the fat-soluble vitamins A, D and E, which in turn could impair LCPUFA status (Smit *et al.*, 2004b).

Table 2-6: Fatty acid food sources

Fatty Acid Name	Food Sources
Oleic acid	Endogenous desaturation of stearic acid; milk; eggs; animal fats; meat; cocoa butter; most vegetable oils, and especially olive oil
Linoleic acid	Some milk; eggs; animal fats; meat; most vegetable oils, and especially corn, sunflower, safflower and soybean oils; green leaves (Cannot be synthesised in mammals)
α -Linolenic acid	Green leaves; some vegetable oils- and especially rapeseed (canola), soybean, perilla oil, flaxseed and linseed oils (Cannot be synthesised in mammals)
Arachidonic acid	Synthesised from LA via γ -linolenic and dihomo- γ -linolenic acids; meat
Eicosapentaenoic acid	Synthesised from ALA; fatty fish (salmon, herring, anchovy, smelt and mackerel); fish oils
Docosapentaenoic acid	Synthesised from ALA via EPA; fatty fish (salmon, herring, anchovy, smelt and mackerel); fish oils
Docosahexaenoic acid	Synthesised from ALA via EPA; fatty fish; (salmon, herring, anchovy, smelt and mackerel) fish oils

Abbreviations: ALA- α -Linolenic acid, LA- Linoleic acid, EPA- Eicosapentaenoic acid
Adapted from Burdge and Calder (Calder, 2015)

2.3.3 Transport and storage of fatty acids

The hydrophobic nature of FA presents a challenge to their transport in the body as blood is in aqueous form. Therefore, FA are transported non-covalently bound to albumin and are known as non-esterified FA (NEFA). Small amounts of FA are bound to albumin (1–2.5 mg/L) which corresponds to 8-10% of the total FA in plasma (Rise *et al.*, 2007). Complex lipids are transported in lipoproteins such as chylomicrons and very-low density lipoprotein (VLDL) (Glatz, 2011). Additionally, FA are bound to phospholipids *de novo* (Kennedy & Weiss, 1956).

2.3.4 Importance of fatty acids

The importance of ALA and LA became apparent in the 1930s when animals fed a rigid fat-free diet developed growth retardation, dermatitis and infertility (Adisa & Odutuga, 1999). EFA supplementation in the diets of the EFA-deficient rats caused a reversal of the pathological conditions that were created by the absence of fat in the rats' diet (Ma *et al.*, 1995). The importance of FA in the human diet was later recognized in early 1980's (Adisa & Odutuga, 1999; Kuwahara *et al.*, 2000).

Dietary lipids were once thought to be only energy sources but recent research has shown that FA and lipids are key nutrients (Calder, 2015). As a result, LCPUFA have been under the spotlight in the scientific world for their proposed benefits and functions in the human body. FA and their products are now known to have regulatory and structural functions. Additionally, FA are known to influence health, early growth, and development (Wilkens & Lee, 1998). Structural variation among complex lipids and FA give rise to functional differences that result in a difference on their impact upon metabolism, cell and tissue responses (Burdge & Calder, 2014).

2.3.4.1 As energy fuels

FA are components of triglycerides (TAG), which are major sources of energy production and storage (Turner *et al.*, 2014). TAG hydrolysis releases FA that are used as an energy source in the FA β -oxidation pathway in mitochondria and peroxisomes (Burdge & Calder, 2014).

2.3.4.2 As structural components

Through esterification FA become part of lipoprotein bio-membranes. Different proportions of plasma FA are esterified into various components of lipoproteins, i.e. in different lipid classes, namely in phospholipids; triacylglycerols (TG) and cholesteryl esters (CE) (Rise *et al.*, 2007).

Phospholipids are the most abundant class of lipids within biological membranes (Berg *et al.*, 2002). Phospholipids are FA with phosphorus-containing polar head groups and contribute to the physiochemical properties of the bilayer membrane (Figure 2-4). The FA tails of the phospholipids are responsible for the hydrophobic nature of membrane bilayers. The core of a phospholipid is a glycerol molecule to which FA are attached through ester bonds. Other groups can be attached to the glycerol group of the phospholipid such as choline, ethanolamine, serine, glycerol or inositol. Thus giving rise to different classes of phospholipid, phosphatidylcholine (Sellmayer *et al.*, 1996) phosphatidylethanolamine (PE), phosphatidylserine (Hodge *et al.*, 2007), phosphatidylglycerol and phosphatidylinositol (PI) (Calder, 2015).

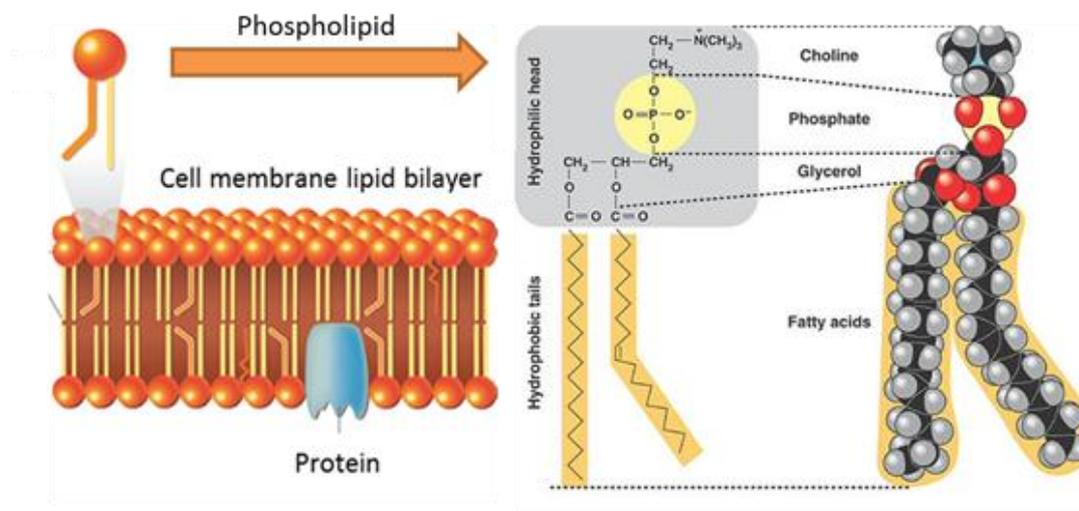


Figure 2-4: Schematic of lipid bilayer and phospholipid molecule. Figure adapted from

(<http://bioapwikispaces.com/Ch+5+Collaboration+2010>. Last access 03.11.2016)

The phospholipid membrane components influence the conformation and many membrane functions such as ion channeling and transport, endo- and exocytosis and the functions of membrane associated receptors and enzymes (Prasad, 2000; Gimenez *et al.*, 2011). The phospholipids in some cases can be substrates for the production of lipid second messengers in signal transduction pathway (Burdge & Calder, 2014). In addition, the FA composition of the lipid bilayer determines membrane fluidity and flexibility (Van Biervliet, 2008). A change in the composition can then modify the activities and interactions of integral proteins and cell function.

2.3.4.3 Role in signal transduction

The n-6 LCPUFA ARA, DGLA and EPA are precursors of biologically active molecules named eicosanoids. Eicosanoid derived from ARA are more biologically active compared to those produced from DGLA and EPA. After release from the membrane by phospholipase A2, they either enter the pathway of the cyclooxygenases (COX) (Nelson *et al.*, 2008) resulting in prostaglandins and thromboxanes (TAX) or the pathway of 5,12-/ 15- lipoxygenase (LOX) resulting in leukotrienes and lipoxins (Burdge & Calder, 2014). Release from membrane phosphatidylinositol-4, 5 biphosphates is catalysed by phospholipase C and triacylglycerol lipase (Dieck *et al.*, 2005; Burdge & Calder, 2014). The eicosanoids are important regulators of inflammation, atherogenesis and prothrombosis (Calder, 2013a).

The balance between the different PUFA in the cell membranes is of paramount importance as this influences the severity of inflammation (Van Biervliet, 2008). Increased n-6 FA concentrations in the human body increase biological factors that are implicated in health conditions such as C-reactive proteins, leukotriene B₄, IL-1, IL-6 and tumour necrosis factor (TNF). All of which are reduced in the presence of n-3 FA, such as DGLA and EPA. This is because n-3 and n-6 FA compete for metabolism resulting in the reduction of COX and LOX products from ARA such as prostaglandin E₂ series (PGE₂) (De Mel & Suphioglu, 2014). The PGE₂-series is also responsible for growth promoting effects, growth-related early gene expression factors and Ca²⁺ metabolism (Sellmayer *et al.*, 1996; Kruger & Horrobin, 1997).

As such, a diet rich in n-6 FA tends to shift the physiological state to one that is pro-thrombotic and pro-aggregatory. With this the viscosity of blood is increased, as well as vasospasms and vasoconstriction as well as decreases in bleeding time (Simopoulos, 1999).

2.3.4.4 Role in health and disease

A large body of research has shown that n-3 FA have a role that they play in the prevention of coronary heart disease, rheumatoid arthritis, hypertension, ulcerative colitis, Crohn's disease as well as type 2 diabetes (Simopoulos, 2002b). Their role in these diseases is attributed to the n-3 FA ability to be antiarrhythmic, anti-thrombotic, anti-inflammatory, hypolipidemic, and vasodilatory (Simopoulos, 1999; Rise *et al.*, 2007; De Mel & Suphioglu, 2014; Colussi *et al.*, 2016). Additionally, n-3 FA (EPA and DHA) lower the risk of coronary heart disease, this is because they have the ability to lower serum triacylglycerol concentrations (De Mel & Suphioglu, 2014).

N-3 PUFA are also essential in early postnatal development processes such as cellular differentiation, photoreceptor membrane biogenesis and active synaptogenesis (Longo *et al.*, 2003). The central nervous system (CNS) of vertebrates is characterised by a high concentration of n-3 FA, mainly DHA. About 40% of the brain is comprised of DHA. In the brain, DHA mainly concentrated in membrane phospholipids at synapses and retinal photoreceptors (Weiser *et al.*, 2016).

Evidence from studies has shown that postnatal DHA status in preterm infants is related to visual functions and neurodevelopment (SanGiovanni *et al.*, 2000). The brain and the CNS are very rich in DHA. Due to this, it is then logical that a deficiency in n-3 PUFA will lead to lower cognitive functions in children and neuronal defects such as AD in older people (Innis, 1991; Uauy *et al.*, 1999; Uauy *et al.*, 2001b). In an effort to prove this, a vast majority of studies have

investigated this. Epidemiological studies suggest that there is a link between increased n-3 PUFA intake in the form of fish and reduction in AD risk (De Mel & Suphioglu, 2014).

Observational and experimental research indicates that an association exists between n-3 PUFA and depression. This might be explained by the low levels of the DHA that predict low levels of cerebrospinal fluid 5-hydroxyindolacetic acid, which is the major metabolite of serotonin. The 5-hydroxyindolacetic acid is known to be protective against depression (Hibbeln *et al.*, 1998). Furthermore, n-3 FA also plays an essential role in immune responses, gene expression and intracellular communications (De Mel & Suphioglu, 2014). A study demonstrated that LCPUFA also have an effect on blood pressure, suggesting that early exposure to dietary LCPUFA might have lasting effects on reduced blood pressure and cardiovascular risk (Koletzko *et al.*, 2008).

The 2008 FAO report on fats and FA in humans concluded that there was promising evidence from research that PUFA alter the indices that are related to the metabolic system such as diabetes (FAO, 2010). However, there is a need for additional research to be conducted to have accurate data on the effects of FA as a whole in health and disease.

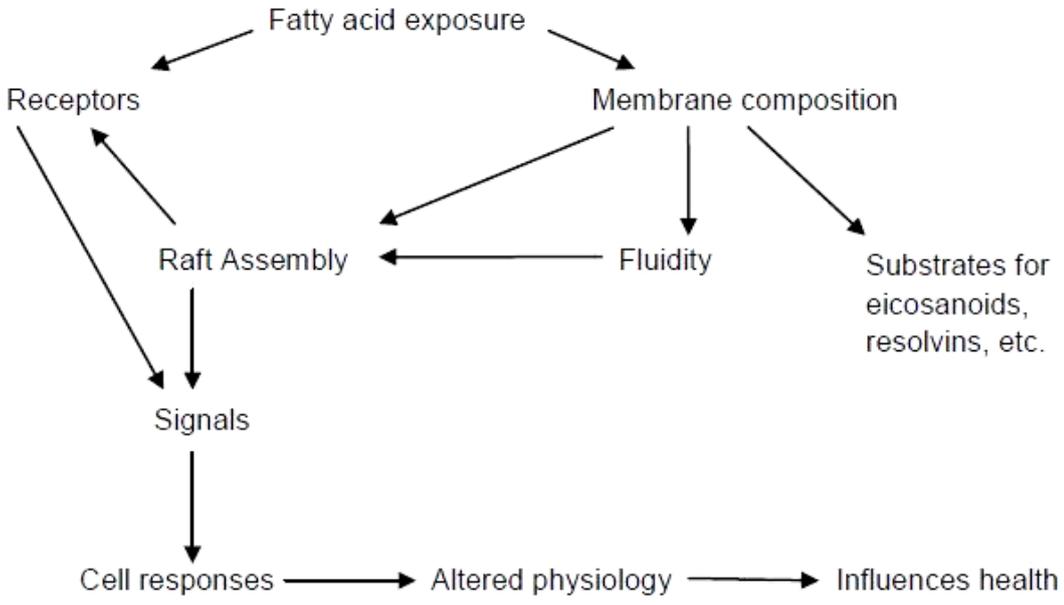


Figure 2-5: Overview of the mechanism by which fatty acid exposure affects health outcomes

(Adapted from Calder, 2012).

2.3.5 Assessment of general fatty acid status and intake and of specific fatty acid deficiencies

Efficient biomarkers of EFA and LCPUFA are important to understanding which indicators truly reflect FA status. Dietary fat biomarker interpretation is not always direct and no biomarkers reflect absolute fat intake (Arab, 2003). This is because the dietary assessment methods used, population characteristics, and biomarker concentrations are different between individuals and between studies. In addition, non-dietary factors also affect the concentrations as well as the compositions of FA in tissues and plasma. These include: absorption, metabolism, genetic and lifestyle determinants. In addition to this, FA compositions are affected by the endogenous synthesis of some of the FA, different physiologic functions of the FA in different blood fractions as well as the different roles of plasma and red blood cells as vehicles for FA transport (Sun *et al.*, 2007).

Fortunately, there has been a rise in research over the past years on diet and the development of chronic diseases that has resulted in extensive literature on biomarkers for dietary FA (Willet, 2013). Several EFA and LCPUFA biomarkers have been investigated and are used randomly in studies. In interventions, biomarkers of FA intake are used to validate FFQ's, evaluate compliance with dietary interventions, or predict the risk of disease (Sun *et al.*, 2007). FA can be measured in adipose tissue, cheek cells, whole blood, erythrocyte membranes, platelets, plasma as well as different fractions of serum or plasma including and not limited to CE, phospholipids, TAGs, and free FA (Baylin *et al.*, 2005; Sun *et al.*, 2007).

2.3.5.1 Biomarkers of fatty acid dietary intake

The distribution of FA varies considerably between the different tissues, reflecting their different metabolic changes and different physiologic roles. Due to differences of FA incorporation, adipose tissues are now thought to reflect dietary FA intakes over a much longer period of time than any other blood fractions. However, whole blood, plasma and RBC can be equally valid as they require minimum sample preparation and processing as well as being easily available and accessible (Baylin *et al.*, 2005). Researchers also suggest that RBC, platelet membrane phospholipid and plasma can be used to monitor short term dietary intake (Frederickson *et al.*, 2000).

Additionally, the superiority of RBC over plasma in assessing long-term dietary intake has been established. This is because the RBC turnover is much slower than that of plasma or platelet lipids and less sensitive to recent FA intake from the diet (Sun *et al.*, 2007). Plasma phospholipid and CE and free FA compositions are responsive to total dietary fat content and

are sometimes used as biomarkers of fat intake. Using EPA as a biomarker for fish oil intake, Katan and colleagues found that the incorporation half-lives of EPA into the human body are about 5 days for serum CE, almost a month for RBC, and longer than a year for subcutaneous fat tissue (Katan *et al.*, 1997).

The use of plasma to reflect FA dietary intake as shown in n-3 FA supplementation trials, is made difficult by the presence of exchanges of FA between lipid pools in plasma and circulating cells (Willett, 2013). Nevertheless, the use of plasma FA as an assessment tool of FA status in an individual is widely used. The FA composition of plasma is considered a reflection of short-term or recent fat intake (Wolters *et al.*, 2014). Additionally, the FA composition of serum cell membrane PL has become a reliable biomarker for assessing nutrition status and is used to predict dietary fat intakes and *de novo* lipid synthesis (Hjartåker *et al.*, 1997). The measurement of plasma phospholipid FA composition is further useful in that it has the potential to function as an alternate measure of the possible effects of diet on a whole range of cell membrane lipids. This is because the FA composition of the phospholipid fraction of plasma is closely related to the FA composition in RBC as well as in platelet membrane phospholipid (Holman, 1986).

2.3.5.2 Biomarkers of general fatty acid status

RBC, plasma, whole dried blood spots and white blood cells can be used to assess FA status as well as phospholipids and CE. Phospholipids have physical and biological properties which enhance their specific functions (Vance & Vance, 1996). Phospholipids and CE have the advantage over FA and TAGs because they have a slow turnover thus making them a more reliable estimate of PUFA and EFA status (Glew *et al.*, 2002; Fekete *et al.*, 2009). In their study Rise *et al.* determined the FA distribution in the lipid classes. Their results showed that 50% of total FA in plasma and RBCs are characterised by the highest percentage of FA incorporated in phospholipids whereas small amounts of FA are incorporated in CE (Rise *et al.*, 2007). Furthermore, phospholipids are used to assess the FA status of individuals because they are the major structural components of membranes and the FA pattern of plasma phospholipids reflect tissue phospholipid (Knutsen *et al.*, 1988).

Current understanding of the origin and relative affinity of FA incorporated in plasma and RBC suggests that RBC LCPUFA content is a more reliable parameter for long-term LCPUFA status than plasma. Rise *et al.* recommend the use of whole blood for FA analysis to determine FA status during assessment (Rise *et al.*, 2007). This is because there are dietary induced changes that occur in the FA composition of plasma, platelet and erythrocyte lipids (Skeaff *et al.*, 2006). The profile of plasma/serum or red blood cell phospholipid is therefore considered suitable to show the overall FA status of a given individual (Van Biervliet, 2008).

2.3.5.3 Individual fatty acids as status markers of specific deficiencies

LCPUFA 'shortage markers' are biomarkers of FA deficiency. The absence of LCPUFA such as DHA that are required meet the physiological requirements of the body causes the body to synthesise certain FA of comparable chain length and degree of unsaturation, that under normal conditions are not present. If there is a shortage of DHA, production of the n-6 LCPUFA n-6 DPA occurs. Therefore, the ratio between DHA and n-6 DPA is a reliable indicator of the DHA status (Hornstra, 2001).

Mead acid/eicosatrienoic acid (20:3n-9) is considered an indicator of EFA deficiency because n-3, n-6, and n-9 unsaturated FA with 18 carbons (ALA, LA, and oleic acid) compete for the same elongase and desaturase enzymes for LCPUFA biosynthesis (Begin *et al.*, 1989). Reduced availability of n-3 and/or n-6 FA then leads to enhanced rate of the desaturation and chain elongation of n-9 FA (Decsi *et al.*, 1995). Under these conditions, oleic acid will be desaturated and elongated to form mead acid and di-homo-mead acid (22:3n-9). The presence of mead acid is regarded as an indicator EFA (Van Biervliet, 2008).

The trienoic/tetraenoic ratio (20:3n-9/20:4n-6) is also proposed as an index of essential FA deficiency (Mocchegiani *et al.*, 1995). It should be pointed out that these 'functional' status markers are based on biochemical pathways and measurements, and need validation with respect to physiological functions (Hornstra, 2001).

2.3.5.4 Essential fatty acid deficiency (EFAD)

EFAD in the very strict sense of the word is defined as a deficiency of the parent EFA (Smit *et al.*, 2004) and is a type of malnutrition (Houssaïni *et al.*, 2001). EFAD is characterised by low LA, often in combination with low long chain metabolites such as ARA and DHA, and high oleic and mead acid (Smit *et al.*, 2004). EFA-deficient individuals have the following signs that include reduced brain and body weight, skin changes, infertility, loss of muscle tone, degenerative changes in kidneys, lungs and liver, increased susceptibility to infections and behavioural changes (Smit *et al.*, 2004). During EFAD there is an up regulation in the delta-6 and delta-9-desaturase activity and a down regulation of delta-5-desaturase activity (Bhagat & Das, 2015). To date, some researchers now consider DHA deficiency to be a major cause of many disorders such as depression, inability to concentrate, bipolar disease, anxiety, cardiovascular diseases, dry skin, and diabetes among others (De Mel & Suphioglu, 2014).

2.4 Zinc and fatty acid interactions

An interaction between Zn and EFA metabolism was initially proposed by Bettger and co-workers in rats. Both Zn and EFAD present similar signs, symptoms and physical manifestations (Bettger *et al.*, 1979). Thus, a close link between FA metabolism and Zn status has been suggested and much research has since been conducted in animal models (Gimenez *et al.*, 2011). A significant interaction between Zn and PUFA was also demonstrated in Zn-deficient chickens, and some of the symptoms observed were similar to those observed in rats (Bettger *et al.*, 1979; Reed *et al.*, 2014). Notably, the tissue FA profiles in the chickens correlated with the severity of skin lesions and leg abnormalities. The studies by Bettger *et al.* were able to demonstrate that Zn deficiency intensified the effects of EFAD (Bettger *et al.*, 1979; Wauben *et al.*, 1999).

Other experimental reports conducted in Zn deficiency and EFAD induced animals indicated that both these dietary deficiencies cause, amongst other problems, stunted growth, altered sexual functions such as delayed sexual maturation and infertility, dermal lesions which may be caused by decreased wound healing and other related skin problems as well as alopecia (Hurley & Swenerton, 1971; Odutuga, 1982; Leat *et al.*, 1983; Leat, 1983; Prasad, 1983; Knutsen *et al.*, 1988; Sanders, 1988; Oloyede *et al.*, 1992; Ahn & Koo, 1995). Eder and Kirchgessner as well as Bettger *et al.* found that double deficiency of EFA and Zn aggravated the skin lesions. Contrary to this hypothesis, short-term Zn deficiency did not exacerbate EFAD in rat liver phospholipids (Wauben *et al.*, 1999).

Observation of shared pathologies led to numerous animal studies which successfully demonstrated that dietary Zn deficiency alters the FA composition of phospholipid of rat liver, plasma and RBC (Bettger *et al.*, 1979; Clejan *et al.*, 1982; Cunnane, 1988a; Kudo *et al.*, 1990; Eder & Kirchgessner, 1994b; Eder & Kirchgessner, 1994a). An overview of the effect of Zn deficiency on FA composition in various rat tissues is shown in Table 2-7. Findings of these studies, however, have been inconsistent (Clejan *et al.*, 1982; Cunnane, 1988a). For example, some studies have reported higher levels of LA (Ayala & Brenner, 1982; Cunnane *et al.*, 1984; Kudo *et al.*, 1990; Faure *et al.*, 1995) whilst some have reported lower levels of ARA (Ayala & Brenner, 1982; Clejan *et al.*, 1982; Cunnane *et al.*, 1984; Kramer *et al.*, 1984; Dib *et al.*, 1989; Wauben *et al.*, 1999), and high or normal levels of ARA in tissue phospholipid of Zn-deficient animals.

Zn and FA are now known to interact metabolically, even though the mechanism by which this occurs is not yet been unequivocally identified. However, there are proposed mechanisms that

are thought to induce these metabolic interactions. These include the role Zn has in modulating intestinal lipid transport, its role in prostaglandin metabolism as well as the maintenance of membrane structural and functional integrity (Adisa & Odutuga, 1999). As observed by Reed *et al.* the availability of information as well as research that looks at the relationship between membrane FA accumulation in relation to Zn homeostasis is little, even though Zn is thought to have a crucial role as a cofactor in desaturase and elongase enzymes (Reed *et al.*, 2014).

Zn is a component of bio-membranes and is suggested to be imperative in stabilising the membrane structure and maintaining the integrity of the cell as well as its function (Bettger & O'Dell, 1981). When compared against controls, RBCs from Zn-deficient rats have an increased osmotic fragility (O'dell *et al.*, 1987), this increase suggests that there is a structural defect that occurs in the plasma membrane (Johanning & O'Dell, 1989). Zn deficiency alters the FA patterns of PL in various rat tissues and serum (Fogerty *et al.*, 1985; Cunnane, 1988a), an effect that may be due in part to food restriction (Kramer *et al.*, 1986). In a study with the main purpose to determine the combined effect of Zn deficiency on RBC membrane components and increase in membrane fragility, the PL FA composition was altered by Zn deficiency in immature rats (Johanning & O'Dell, 1989). In this research it was concluded that EFAD in rats gave rise to more fragile erythrocytes as well as decreased proportions of LA and ARA (O'dell *et al.*, 1987).

Table 2-7: Results on the effect of Zn deficiency on the levels of fatty acids in tissues of animals

Author, year	Animal model	Tissue	Lipid fraction	Duration (days)	Effect of Zn deficiency on fatty acids			
					n-6	n-3	n-9	n-7
Ayala,1983	Rats, weaned	Liver and testes	TL	60	↑LA ↓ ARA	ND	ND	ND
Clejan, 1982	Rats	plasma	PL	180	↓ ARA	ND ³	ND	ND
Cunnane, 1984	Rats, weaned	Liver, testes, plasma, skin	TPLP	-	↑ LA ↓ ARA	ND	ND	ND
Eder, 1994a	Rats	liver	PL,PC	10	↓	↑	↓	↑
Eder, 1994	Rats	RBC	PC, PE, PS,	10	ND	↑ DHA ^{1,2}	ND	ND
Eder, 1996	Rats, weaned	liver	PL	10	↑LA, DGLA ↓ PUFA	ND	ND	ND
Fraure, 1995	Rats	platelets	TL	60	↑ LA, ↓ ARA	ND	ND	ND
Kramer, 1984	Rats, weaned	liver	TPLP	21	↑ LA, 22:5	ND	ND	ND
Kramer, 1986	Rats, weaned	Liver, spleen, thymus, serum	TPLP	21	↑LA, GLA, DGLA, ↓ARA, n-6 DPA, ↑ n-6	↑ total n-3	↑ total n-9	ND
Kudo,1990	Rats, weaned	liver	TPLP	-	↑ LA, ARA	ND	↓ 18:1, 20:3	ND
Wauben, 1999	Rat pups	Liver, brain	PC,PE	16	↓ARA ³ , ↑22:5 ⁴	↓ DHA ³	ND	ND

Abbreviations: TL- total lipids TPLP- total phospholipids, PE – phosphatidylethanolamine, PC – phosphatidylcholine, NC- no change, ND- not determined, RBC- red blood cells, PL- plasma, PLP- phospholipid; PL TPLP- plasma total phospholipid

¹ in PC, PE, PS total RBC membrane FA of rats fed coconut oil

² in PC of rats fed fish oil

³ in liver of EFA adequate pups

⁴ in brain PE of EFA adequate pups

2.4.1 Desaturase Enzymes

A low Zn intake and status are thought to cause a reduction of the activity of delta 5- and delta 6-desaturase enzymes (Ayala & Brenner, 1982; Clejan *et al.*, 1982; Fogerty *et al.*, 1985; Bekaroğlu *et al.*, 1996; Reed *et al.*, 2014).

The reduction of these enzyme activities has been described in Zn-deficient animals (Clejan *et al.*, 1982). An early study by Ayala *et al.* showed that Zn deficiency in rats decreased delta-5 and -6 desaturase activity in liver and testes and impaired the synthesis of PUFA (Ayala & Brenner, 1982). Dieck *et al.* also observed that changes in the composition of liver phospholipids of Zn-deficient rats were present. They observed that proportions of cis-9-oleic, cis-11-vaccenic, caprylic, myristic, LA and EPA increased whilst the composition of stearic and ARA decreased (Dieck *et al.*, 2005). A study conducted by Reed *et al.* in chickens (*Gallus gallus domesticus*) fed on either a Zn adequate or Zn-deficient diet for 28 days, showed that Zn deficiency significantly reduced the expression of the hepatic delta-6-desaturase and increased the LA to DGLA ratio in erythrocyte membranes. These results were indicative of a disturbed precursor to product ratio and thus impairment of delta-6-desaturase activity (Reed *et al.*, 2014).

Recently in healthy human volunteers, who were between the ages of 25-55 years old, Knez *et al.* demonstrated that the activities of the delta 6- and 9-desaturase were reduced by a low intake of Zn and thus sensitive to Zn intake (Knez *et al.*, 2016). This is because desaturase enzymes are coupled to the NADP/NAPH-cytochrome b5 electron transfer chain and a low dietary Zn intake subsequently affect the electron transferring chain thereby affecting the activities of desaturase enzymes (Cunnane, 2005). Therefore, Zn deficiency, even early stage Zn deficiency, leads to inconsistencies in the ratio of desaturase precursors and products, such as the LA to DGLA ratio (Knez *et al.*, 2016). Figure 2-6 shows a diagram illustrating the relationship between delta 6-desaturases and Zn deficiency and how Zn deficiency impairs the bioconversion of FA (Reed *et al.*, 2014).

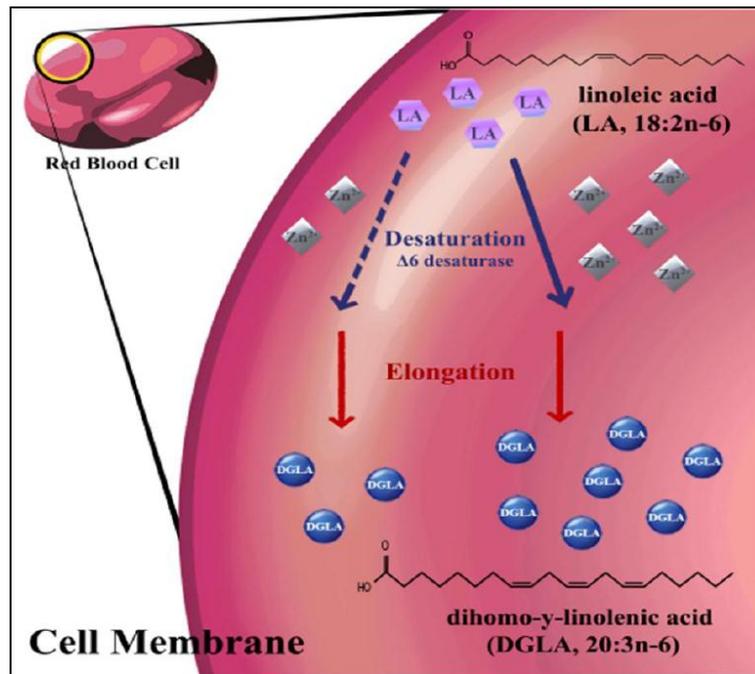


Figure 2-6: Systematic diagram showing the effect that Zn deficiency has on delta-6-desaturase

Adapted from (Reed *et al.*, 2014)

However, as shown in Table 2-8, conclusions from some other studies do not support this hypothesis. Eder and Kirchgessner found greater levels of n-3 LCPUFA in liver PL of Zn-deficient young adult rats (Eder & Kirchgessner, 1994a) and Kudo *et al.* found greater ARA levels in liver and plasma phospholipids of Zn-deficient adult rats (Kudo *et al.*, 1990). These studies did, however, not assess the activity of desaturases in the microsomes but rather looked at the changes in the FA composition and or concentration over the research period (Kudo *et al.*, 1990; Eder & Kirchgessner, 1994b; Eder & Kirchgessner, 1994a).

These contradictory results may be due to the diet given to the animals, which is a component sometimes overlooked by researchers. In a study, a fat-free diet was used by Eder and Kirchgessner *et al.* They demonstrated that the type of dietary fat influences the effects of Zn deficiency on FA composition of liver lipids (Eder & Kirchgessner, 1994a). Other researchers argue that results from these experiments may also be explained by the reduced food intake resulting from Zn deficiency. Zn deficiency is known to affect appetite and taste thus reducing intake which in itself may affect EFA metabolism (Kramer *et al.*, 1984).

Table 2-8: Results from Zn deficiency studies on desaturase enzyme activity

Author, year	Animal model	Tissue	Lipid fraction	D6D	D5D	D9D	Other
Ayala, 1983	Rats, weanling	liver, testes	PLP	↓	↓	↑	↓ ARA
Clejan, 1982	Rats, weanling	Testes	PLP	↓	↓	ND	↓ ARA
Eder 1995*	rats	Liver	PL, microsomes	NC	NC	↓*	-
Reed, 2014	chickens	Liver	Serum	↓	ND	ND	↑ LA: DGLA ratio

Abbreviations: NC- no change, ND- not determined, PL- plasma, PLP- phospholipid; * rats were fed coconut oil

2.4.2 Transport-Binding of fatty acids to albumin

One of the notable features of albumin is its ability to bind reversibly and transport a vast array of small molecules, such as FA, metal ions, toxic metabolites, hormones and drugs (Peters Jr, 1995; Fanali *et al.*, 2012). Most of the Zn in blood is bound to albumin (19 μ M), as well one or two molecules of LCPUFA. Of the eight known FA binding sites, two are thought to be located in domain III and one is thought to be located in domain I. FA site 2 is thought to be a low-affinity site which becomes populated only when FA anions are highly abundant (Stewart *et al.*, 2003).

A molecular interaction of Zn and FA as reviewed recently by Barnett and colleagues showed that through isothermal titration calorimetry (ITC) experiments, FA modulate the affinity of albumin toward Zn at all physiological levels. The binding of FA to albumin serves as an allosteric switch that modulates Zn binding to albumin in blood plasma (Figure 2–7). Therefore, the binding of Zn and LCPUFA to albumin is interactive and that albumin is a molecular link between the levels and distribution of FA and Zn in plasma as well as adjacent tissues (Barnett *et al.*, 2013).

On the other hand a possible mechanism by which FA influences the transport and delivery of Zn in blood has been proposed, analysis of the x-ray structures confirmed that FA binding to site 2 of albumin triggered a spring-lock mechanism, which disengaged the upper (His-67Asn-99) and lower (His-247Asp-249) halves of the metal site (Stewart *et al.*, 2003).

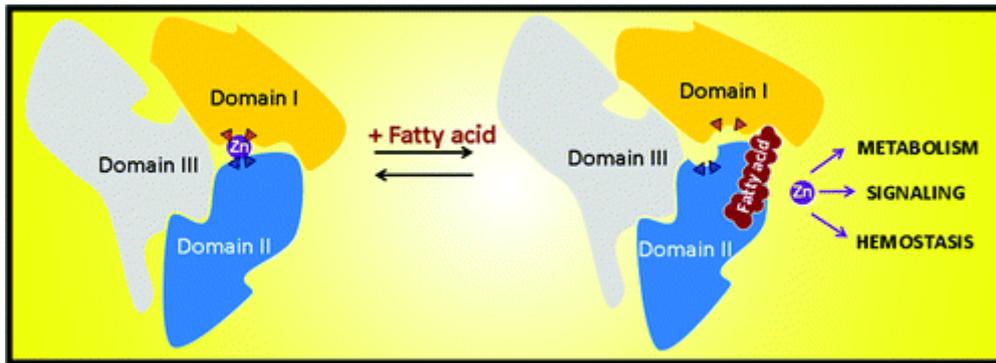


Figure 2-7: Diagram showing how fatty acids affect the albumin sites of Zn

DHA is said to do this through its interaction with Zn transporters in the brain, namely the ZnT3 transporter. Evidence from cultured human neuronal cells suggests that there is a Zn-transporter-expression-level dependent mechanism which causes DHA to possess neuroprotection properties. DHA is suggested to reduce ZnT3 transporter expressions level in human neuronal cells, which leads to a reduction in Zn influx as well as free Zn (which in excess causes neurodegeneration) and caspase-3 (a critical proteolytic enzyme that is a central component of apoptotic cell death) (De Mel & Suphioglu, 2014).

This theorem is also supported by Suphioglu *et al.* who established that DHA-treated M17 cells had a decreased Zn uptake (65%) as well as ZnT3 mRNA and protein levels in comparison with DHA-depleted cells (Suphioglu *et al.*, 2010). Similarly, in adult male Sprague–Dawley rats that were raised and maintained on a control diet that contained n-3 PUFA or a diet deficient n-3 PUFA, Zn deficient rats had increased expression of ZnT3 in the brain causing an increase in the level of free Zn in the hippocampus. These animals also had decreased plasma Zn levels compared with n-3 PUFA adequate animals. The authors concluded that an overexpression of ZnT3 due to a perinatal n-3 PUFA deficiency caused abnormal Zn metabolism in the brain of the rats (Jayasooriya *et al.*, 2005).

2.4.3 Role of Zn in the absorption of lipids and lipid soluble substances

Koo and Turk, were among the first to report impaired absorption of lipids (triacylglycerol) in Zn deficiency (Koo & Turk, 1977). Since then, research in animal models has been able to show the role of Zn in the absorption of other lipid classes and lipid soluble substances (Gimenez *et al.*, 2011). As reviewed by Gimenez *et al.* a reduction in lipid absorption in Zn-deficient rats is thought to be attributed to by improper lipoprotein assemblage that causes impaired lipid transport across the intestinal mucosa as well as in the blood stream or the combination of both (Adisa & Odotuga, 1999). Zn-deficient rats had an impaired luminal hydrolysis of phosphatidylcholine to lisophosphatidylcholine by phospholipase A2, all of which lead to an impedance in the absorption

of fat and fat-soluble vitamins (Noh & Koo, 2001). Others have postulated that Zn influences the incorporation of FA into the PL, thereby explaining the differences in FA compositions observed in Zn-deficient and sufficient rats (Prasad, 2013).

2.5 Effect of Protein Energy Malnutrition (PEM) on fatty acid composition in humans

Under nutrition refers to an overall deficiency of nutrients (carbohydrates, proteins, fats, vitamins, and minerals) that is due to an inadequate dietary intake. Malnutrition during the first years of life is a major health issue in low-income countries (de Souza *et al.*, 2011). Severe childhood malnutrition comprises three clinical syndromes: nutritional dwarfism or stunting in height, marasmus or wasting, and, oedematous malnutrition or kwashiorkor (Golden, 1997). Children who are at risk of under nutrition are at risk of n-3 FA deficiency as well as micronutrient deficiencies such as Zn and Fe²⁺. Some PEM clinical symptoms include skin changes, impaired resistance to infections, impaired growth rate and disturbed development all of which are characteristics of EFAD and Zn deficiency (Smit *et al.*, 2004).

Research has shown that severe acquired Zn deficiency coexists with severe protein-energy malnutrition (PEM) (Bhaskaram, 2002). FA deficiency is also present in malnourished children this is because lipid metabolism is disturbed in malnutrition. As illustrated in Figure 2-8, several factors may attribute to EFAD in malnourished children. These include, inadequate dietary intake which cause micronutrient deficiencies such as Zn deficiency, malabsorption (that may be caused by recurrent gastro-intestinal infections such as diarrhoea), low insulin levels, impaired hepatic desaturation and elongase activity especially that of the rate limiting step in FA synthesis delta-6 and rapid turnover of RBC (Smit *et al.*, 2004).

Other schools of thought have suggested that PEM can also cause EFAD because of impaired digestive and transport system that results in a reduced EFA supply (digestion, and transport), as well as high EFA expenditure (b-oxidation and peroxidation) that may occur in a malnourished individual. In addition, malnourished children are thought to utilise most of their body lipid stores, which normally would be compensated for by an adequate dietary intake (SanGiovanni *et al.*, 2000). EFAD in malnourished individuals may be worsened by an increased oxidative decomposition of PUFA that can be due to deficiency of vitamin E, selenium, or both, may be contributing factors (Franco *et al.*, 1999; SanGiovanni *et al.*, 2000). The continuance of EFAD malnourished individuals results in a vicious cycle (Smit *et al.*, 2004).

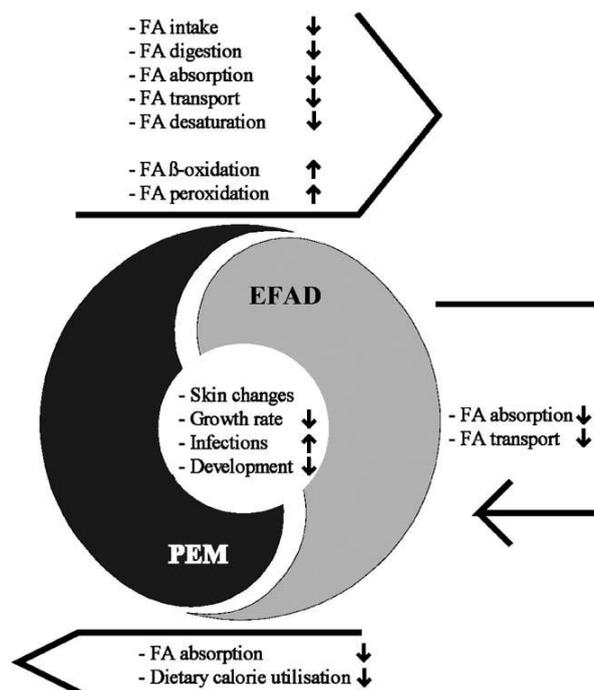


Figure 2-8: Diagram showing the protein energy malnutrition (PEM) essential EFAD vicious cycle

Figure adapted from (Smit *et al.*, 2004a)

As detailed in Table 2-9, when compared against healthy children, PEM be it severe malnutrition, marasmus or kwashiorkor in children is associated with markedly reduced percentages of PUFA and some of its functionally important PUFA metabolites such as GLA, C20:3 and ARA, in RBC's and plasma phospholipids as well as sterol esters as well compared with healthy control subjects (Holman *et al.*, 1981; Wolff *et al.*, 1984; Koletzko *et al.*, 1986; Babirekere-Iriso *et al.*, 2016). Studies have also shown a decrease in plasma LA and an increase in plasma SFA and MUFA that are non-essential in the children that are malnourished (Franco *et al.*, 1999). The pool of C18 parent EFA cannot be elongated and unsaturated, which leads to the production of "shortage markers" such mead acid to be observed in tissues. (Borno *et al.*, 1999).

Proposed mechanisms by which PEM causes EFAD are supported by studies conducted in different parts of the world (Table 2-9). In plasma and/or RBC of children from Pakistan (Smit *et al.*, 1997), Nigeria (Koletzko *et al.*, 1986), Peru (Wolff *et al.*, 1984). Argentina (Holman *et al.*, 1981; Marín *et al.*, 1991) and Romania, including children suffering from HIV (Decsi *et al.*, 1995) showed a deficiency of LA and its derivatives. The n-3 FA in plasma phospholipids were found to be either slightly increased or decreased (Holman *et al.*, 1981; Koletzko *et al.*, 1986; Decsi *et al.*, 1995). In their study, Marin *et al.* discovered that a decrease in n-6 FA was partially compensated

by n-9 FA incorporated into RBC and plasma phospholipids of infants. Notably, there were significant correlations between the deficiency state and the severity of malnutrition. Therefore infants with grades II and III protein-caloric malnutrition had an increase in n-9 FA and a reduced n-6 FA (Marín *et al.*, 1991).

Table 2-9: Fatty acid status of malnourished children

Author, year	Country	Average age (months)	Analysed Tissue	Effect of malnutrition
Babirekere-Iriso, 2016	Uganda	6-59	WB	↓Total all n-6 PUFA, n-3 PUFA, n-6:n-3 PUFA ratio
Desci et al, 1995	Austria	29	PL	↓ARA ↓DHA
Dijkstra et al, 1997	Pakistan	4-59	RBC	↓n-6, ↓ n-3, ↑ n-9
Franco et al,	Brazil	12	PL	↓LA
Golden, 1981	Argentina	12	PL	↓ n-6, ↑n-9 metabolites

Abbreviations: PL- phospholipid, PUFA- polyunsaturated fatty acids, RBC- red blood cells, LA- linoleic acid, WB- whole blood

2.6 Study site analysis of Benin

Benin is a country that is in West Africa and is bordered by Togo to the west, Nigeria to the east, and Burkina Faso and Niger to the north. Benin’s climate is subequatorial/tropical savanna with temperatures that range between 17 °C and 35 °C. During the dry season temperatures are increased to between 20–40 °C with the approaching rainy season. Benin climate is also characterised by one rainy season (May-June to September-October) and one dry season (October-November to April-May) (Galetti, 2014; Galetti *et al.*, 2016) .

Along with under nutrition and micronutrient deficiencies, obesity is also an emerging public health problem in Benin (Sodjinou *et al.*, 2009). A Multi-Indicator Cluster Survey showed that the prevalence of malnutrition in Benin was 44.6% in children under 5 (WHO, 2014). Benin is classified as a country that has a population that is moderately Zn-deficient, this is because, according to data derived from food balance sheets and demographic data, 16.5-17.9% and 25% of the population is at risk of inadequate Zn intake (Brown *et al.*, 2004; Wuehler *et al.*, 2005; Wessells & Brown, 2012). Benin also has a high anaemia prevalence in both women of reproductive age and young children (>60%). Vitamin A deficiency in Benin is estimated to affect 70.2% of children who are between the ages of 12 and 71 months of age (FAO, 2011). A survey

conducted by the Benin government in 2011 found that 33.6% of Benin households are food insecure (WHO, 2014).

The main food dishes in Benin are derived from high-phytate crops such as cereals (maize, sorghum, millet and rice), roots and tubers (cassava, yams, cocoyam and sweet potato) and legumes (cowpeas, mung beans) and are thus low in micronutrients. At household level, staple foods provide the majority of energy intake of household members and are sometimes meals are complemented with animal products (fish, goat, beef, pork, chicken and guinea fowl) (Sodjinou, 2006). A literature review on the contribution of fish intake, aquaculture, and small-scale fisheries to improve food and nutrition security named Benin to be one of the African countries where fish contributes more than 30% of the total animal protein supply. It is stated that Benin relies on fish protein (31.8%) as its main animal protein source (Kawarazuka, 2010). This, however, contradicts interpreted statistics of animal source foods from the Food and Agriculture Organization (FAO) statistical database that show that many African countries are in the bottom quartile for consumption of meat plus fish combined. These countries include Benin, Morocco, Cameroon, Somalia, Zambia, Sudan, Sierra Leone, Algeria, Kenya, Angola, Côte d'Ivoire, Djibouti, Guinea (Speedy, 2003). Mitchikpe *et al.* found that the contribution of fat to the energy intake of the school-age children in north-western Benin was 15%; a low percentage that suggests that the diet is not balanced (Mitchikpe *et al.*, 2009).

The Atacora Province is in northern Benin. The population density is low with 21 inhabitants/km². In Nattingou, Benin, the prevalence of <5-y stunting and the prevalence of low PZn of Beninese children was recently found to be 51.3%. and 45.7% (Galetti *et al.*, 2016). The main economic activity is farming and is strongly dependent on rainfall (Mitchikpe *et al.*, 2009). The socio-economic status of the population is poor and is mainly characterised by low income, poor sanitation, low school attendance and high illiteracy rate (Van-Liere, 1993). Data originating from the food frequency questionnaire in school-age children showed that the major source of fat in that region was from shea butter. This same study also showed that dried fishes are the highest contributor to animal proteins, although is rarely eaten by the population (Mama 2003, in French). Similarly, a dietary survey (3-d weighed food record) conducted in Nattingou Benin showed that contribution of fat to the energy intake of the children was 7.5 % grams of fish and shea butter per day). The same survey also showed that the population in Benin mainly consumes dried small fishes, usually *Pellicotrisa* species or small sardines. This is not eaten as a whole fish but is used to season their dishes, to give them a savoury taste (Ategbro 2014, in French). Shea butter is essentially composed of TAGs with oleic, stearic, linoleic, and palmitic FA (Maranz *et al.*, 2004) and the *Pellicotrisa* species or small sardines have high concentrations of n-3 FA (Opperman & Benade, 2013), but research in fish shows that drying alters their PUFA composition (Telahigue *et al.*, 2013).

2.7 Summary of Literature review

In this literature review an effort was made to provide an in depth investigation on Zn and FA food sources, metabolism, functions in the body and methods of assessment. Furthermore, the presence of interactions between Zn and FA were discussed and Table 2-7 shows that Zn sufficiency in rats generally caused an increase in FA synthesis of the precursor FA (LA, ALA) to their longer chain derivatives (ARA, DHA) and the opposite in Zn-deficient rats. The proposed mechanisms that are thought to induce the metabolic interactions between Zn and FA include the role Zn has in the FA desaturase enzymes, modulating intestinal lipid transport, prostaglandin metabolism as well as the maintenance of membrane structural and functional integrity (Adisa & Odotuga, 1999). However, further investigation is required to fully determine the exact mechanisms behind this interaction. In an effort to illustrate the interactions that exist between Zn and FA, Beninese children were given Zn-fortified water whereafter their FA status was assessed. The results from this intervention are reported in the article (Chapter 3).

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CHAPTER 3: MANUSCRIPT 1

3.1 Sensitivity of fatty acid synthesis to zinc status

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Abstract

There is evidence that zinc (Zn) and fatty acids (FA) interact, although the mechanisms involved are not understood. Zn supplementation affects the FA status of Zn-deficient rats, but little is known about the corresponding effect in humans. We evaluated the associations between baseline Zn status and plasma total phospholipid FA composition, as well as the effect of daily consumption of Zn-fortified water on FA status in Beninese schoolchildren. A 20-week, double-blind randomised controlled trial was conducted in 277 children aged between 6 and 10 years. The children were randomly assigned to receive a daily portion of Zn-fortified, filtered water delivering 2.8 mg Zn (Zn+filter) or non-fortified filtered water (Filter). Plasma total phospholipid FA composition was determined using capillary gas–liquid chromatography and plasma Zn analysis by atomic absorption spectrometry. At baseline, plasma Zn correlated positively with DGLA ($r = 0.209$; $p = 0.010$) and the DGLA:LA ratio ($r = 0.327$; $p < 0.001$) and negatively with LA ($r = -0.229$; $p = 0.005$) and the ARA:DGLA ratio ($r = -0.257$; $p < 0.001$). Zn fortification increased nervonic acid ($B: 0.109$; 95% CI: 0.001, 0.216) in all children. Zn-deficient children had a significantly higher nervonic acid composition ($B: 0.234$; 95% CI: 0.040, 0.428) and a low ALA composition ($B: -0.1260$; 95% CI: -0.203 , -0.048) after Zn fortification. These results support the notion that the LA:DGLA ratio can be used as a biomarker for Zn status in humans. In addition, we demonstrated that Zn filtered fortified water elevated nervonic acid composition in the plasma total phospholipid of children, and even more so in Zn-deficient children. Zn therefore plays an important role in FA synthesis.

Key words: fatty acid composition, plasma zinc, zinc fortification, children, fatty acid metabolism

Introduction

Zinc (Zn) and fatty acids (FA) are essential for optimal growth, immune response, gene expression, visual development, neurotransmission and cognition (Calder, 2015; De Mel & Suphioglu, 2014; Vallee & Falchuk, 1993). The high prevalence of Zn deficiency is therefore of public health concern as fully a quarter of the world's population is estimated to be Zn-deficient (Maret & Sandstead, 2006), and 17% is at risk of inadequate Zn intake (Wessells & Brown, 2012). The Beninese population is classified as one that is moderately Zn-deficient. According to data derived from food balance sheets and demographic data, 17–25% of the population is at risk of inadequate Zn intake (Wessells & Brown, 2012; Wuehler, Peerson, & Brown, 2005).

A relationship between Zn and FA metabolism has been confirmed in animal studies (Gimenez, Oliveros, & Gomez, 2011). Zn is thought to be a cofactor in desaturase and elongase enzymes, most likely through the microsomal cytochrome NADH/NAPH mechanism (Cunnane, 1988a; Prasad, 2013). The desaturase and elongase enzymes metabolise the essential fatty acids (EFA) linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3) to their long-chain polyunsaturated FA (LCPUFA) metabolites, as well as the saturated fatty acids (SFA) palmitic acid (16:0) to nervonic acid (24:1n-9) (Nakamura & Nara, 2004). It has also been shown that Zn facilitates and affects the incorporation of FA into phospholipids (Gimenez et al., 2011).

The lack of a suitable biomarker for measuring body Zn concentrations that is sensitive, reliable and specific is a troubling problem (Lowe, Fekete, & Decsi, 2009). In an effort to address this concern, recent studies in chickens (*Gallus gallus*) and in humans have suggested that the ratio of linoleic acid (LA; 18:2n-6) to dihomo- γ -linolenic acid (DGLA; 20:3n6), to be a potential biomarker of Zn status (Knez et al., 2016; Reed et al., 2014). This is because the conversion of LA to DGLA encompasses desaturase and elongase enzymes. Reed *et al.* (2014) and Knez *et al.* (2016), respectively, found that Zn deficiency increased the LA:DGLA ratio in erythrocyte and plasma membranes. In the latter study, a lower dietary Zn intake increased the LA:DGLA ratio in apparently healthy 28-55-year olds adults

Zn supplementation is effective in increasing dietary Zn intake and status (Brown & Ruel MT, 2004). Although the results from fortification studies are inconsistent (Das, Kumar, Salam, & Bhutta, 2013), Zn fortification of staple food grains such as wheat, millet and maize is practised in an effort to improve dietary Zn intake (Tripathi & Platel, 2010). However, the bioavailability of Zn from these grains is low as they have high phytate to Zn molar ratios (Moretti, Biebinger, Bruins, Hoefl, & Kraemer, 2014). Phytic acid is an anti-nutritive factor that reduces micronutrient absorption (Sian, Hambidge, Westcott, Miller, & Fennessey, 1993). Fortified water when used as

a fortification vehicle has the benefit of being free of anti-nutritive factors of absorption, making it an ideal source of Zn (Polaki & Yarla, 2014). In the main study, Galetti and colleagues established that water from a modified LifeStrawFamily (LSF) water filter, which was configured with glassy Zn phosphate-based plates, was efficient in improving the Zn status of 6-10 year old Beninese children (Galetti et al., 2015).

In this complementary study we sought to provide evidence that plasma Zn and plasma total phospholipid FA composition interact, as well as address the question of whether the LA:DGLA ratio is a potential Zn status biomarker. Furthermore, we show the effect Zn-fortified water has on the plasma total phospholipid FA composition of Beninese school children aged between 6-10 years.

Methodology

Study design, site and subjects

This study was part of a 20-week double-blind randomised trial that was conducted in rural Benin in 6–10-y old school children between the months of February and June 2013. Its main objective was to determine Zn bioavailability from fortified filtered water and the efficacy of Zn-fortified water (2.8 mg/d) in improving Zn status (Galetti et al., 2015). This secondary-study aimed to investigate whether there were associations between plasma Zn and plasma total phospholipid FA composition, as well as assess the effect of Zn fortification on the plasma total phospholipid FA status composition and status in these children. The study was conducted at the primary school of Kotopounga, a rural town in north-western Benin, where the climate is a sub-equatorial/tropical savanna with one rainy season (May-June to September-October) and one dry season (October-November to April-May). Study procedures were translated into the local Waama and Otamari languages. Children (n=277) were allocated randomly at enrollment to one of three treatment arms, namely a Zn+filter group, filter group and pump group. This secondary study utilised the Zn+filter and filter groups. Plasma total phospholipid FA composition was determined in 186 subjects (Figure 1). Children were excluded from the study if they had: 1) severe anaemia (defined as haemoglobin < 7 g/dL), 2) major chronic diseases, 3) long-term medication use, or 4) Zn supplementation. The Zn-fortified water (300 mL serving) contained a median (IQR) Zn dosage of 2.4 (2.0, 3.2) mg, which resulted in a daily Zn intake of 4.3 (3.5, 5.2) mg on school days, and averaged over the entire study an overall daily Zn intake of 2.8 (0.0, 4.5) mg.

Sample size

No similar previous study has been conducted. We, therefore, based the sample size calculation on an independent t-test analysis in G * power (Faul, Erdfelder, Buchner, & Lang, 2009). A sample size of 52 per group was needed to give the study 80% power to detect a large effect size (0.4) of the intervention if significance was set at 0.05.

Intervention

Water was either filtered with a LSF equipped with a Zn fortification chamber (Zn+filter) or via an LSF with a placebo chamber (filter) and hygienically stored in tanks until consumption. The Zn concentration in water was monitored daily, both with a rapid assessment method (Aquaquant; Merck) in the field and then, after the intervention by flame atomic absorption spectrometry (Varian Inc, California).

From Monday to Friday, children each received a morning and an afternoon serving of 300 ml (during the 10h00 and the 15h00 breaks), with the exception of Wednesdays, when they consumed only the morning serving.

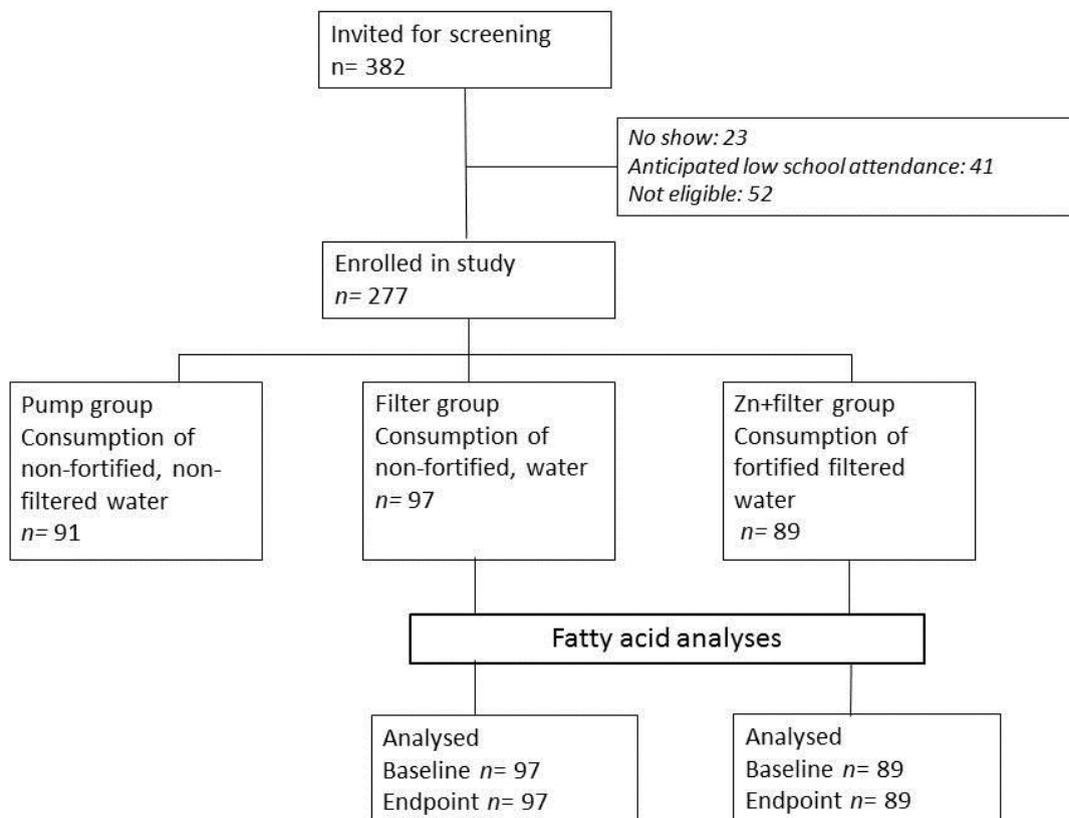


Figure 1: Flow diagram of the secondary study in context of the main study.

Blood sampling and biochemical analysis

Plasma was divided into aliquots in acid-washed test tubes (Eppendorf AG) and transported in a refrigerated cooler box to the laboratory unit, where it was stored at -20°C within 5 h. The specimens were shipped to ETH Zurich and to a laboratory in Freiburg, Germany, on dry ice with an express international courier. In Zurich, plasma Zn (PZn) was measured (Galetti et al., 2015). Briefly, standardised protocols by the International Zn Nutrition Consultative Group (IZnG) were followed during sample collection, handling, and analysis in order to avoid exogenous or endogenous Zn contamination (IZiNCG, 2007). Two morning (mean time: 1024 ± 1 h 18 min) fasting (subjects in non-fasting state were 5%, 4.3%, and 3.3% at baseline, midpoint, and endpoint sampling, respectively) venous whole-blood samples from each study participant was collected directly into trace element-free lithium heparin tubes for PZn analysis. The blood samples were refrigerated at 4-8°C immediately after collection, centrifuged within 1 h (3000 x g for 10 min at room temperature), divided into aliquots in acid-washed plastic vials (Eppendorf AG), and frozen at -20°C for later analysis of PZn and CRP. PZn was measured by flame atomic absorption spectrometer (AA240FS; Varian Inc.,) using a commercial aqueous standard (Titrisol 1.009953.0001; Merck) for external calibration, and Seronorm Trace Elements Serum L-2 (Sero AS) as reference material, which delivered values within acceptable ranges as specified by the manufacturer. Instrumental parameters were set at 213.9 nm for wavelength, 1 cm for slit width, and no background correction.

In Freiburg, CRP and AGP were analysed by using a sandwich ELISA (Erhardt, Estes, Pfeiffer, Biesalski, & Craft, 2004). Liquicheck Trilevel (Bio-Rad Laboratories Inc.) was used as control material with each run of analysis, and measured values were within acceptable ranges as specified by the manufacturer. Haemoglobin was assessed with a portable HemoCue 201+ photometer (HemoCue AB). Samples were immediately stored in a refrigerated cooler box and centrifuged within 1 h (3000 x g for 10 min at room temperature) on an E8F Portafuge portable centrifuge.

The prevalence of Zn deficiency was calculated by using sex- and age-specific PZn lower cutoffs suggested by the IZiNCG (Hess, Peerson, King, & Brown, 2007). Subclinical inflammation was defined as equal to or above 5 mg/L for CRP and/or 1 g/L for AGP. Severe anaemia was defined as haemoglobin equal to or below 7 g/dL (WHO/UNICEF/UNU, 2001).

Baseline and endpoint (week 20) Zn status in the population was monitored by measuring PZn. Acute infections and the inflammation markers α 1-acid glycoprotein (AGP) and CRP were assessed because they can confound the interpretation of PZn (Thurnham, Mburu, Mwaniki, & De Wagt, 2005). Anthropometric measures were also taken as described previously (Galetti et al., 2015).

Plasma total phospholipid fatty acid analysis

Baseline and endpoint samples for each subject were prepared and analysed with gas liquid chromatography (GC) together in the same batch (pair-wise analysis) at the Non-Communicable Diseases Research Unit (NCDRU) of the South African Medical Research Council (SAMRC). In short, lipids were extracted from plasma with chloroform: methanol (2:1 vol: vol; containing 0.01% butylated hydroxytoluene) by using a modification of the Folch et al. (Hon et al., 2009). Lipid extracts were concentrated, and the neutral lipids were separated from the total phospholipid fraction by using thin-layer chromatography (silica gel 60 plates, 10 x 10 cm; Merck) and eluted with diethyl ether: petroleum ether: acetic acid (30:90:1; vol: vol: vol). The lipid band that contained total phospholipids (application origin) was removed from the thin-layer chromatography plate and trans-methylated with methanol:-sulphuric acid (95:5 vol: vol) at 70°C for 2h to yield fatty acid methyl esters (FAMES). The resulting FAMES were extracted with water and hexane. The organic layer was evaporated, redissolved in hexane, and analysed (sample volume 1 µL) by using gas liquid chromatography on a Finnigan Focus Gas Chromatograph (GC) equipped with flame ionisation detector (Thermo Electron Corporation, Austin, TX, USA) and a 30m capillary column of 0.32mm internal diameter; BPX70 0.25µm (SGE International Pty Ltd, Ringwood, Victoria, Australia). Gas flow rates were: N₂ (make up gas), 25 ml/min; air, 250 ml/min; H₂ (carrier gas), 25 ml/min, with a split ratio of 1:20. GC-oven temperature programming was linear at 4.5 C/min, initial temperature 140 C (hold-time 1 min), final temperature 220 C (hold-time 5 min), injector temperature 220°C, and detector temperature 250°C. The FAMES were identified by comparison of the retention times with those of a standard FAME mixture (27 FAMES; NuChek Prep Inc., Elysian, MN, USA). Relative percentages of FA were calculated by taking the area count (area under the curve) of a given FAMES as a percentage of the total area count of all FA identified in the sample.

It is not possible to measure the activity of desaturase enzymes in microsomes directly, therefore, product-precursor ratios were used to reflect enzyme activity. The GLA:LA (although not widely used since GLA is rapidly converted to DGLA), ARA:DGLA and DGLA:LA ratios were used to estimate delta -5 and delta -6-desaturase activities (Fan & Chapkin, 1998; Warensjö et al., 2009).

Statistical analysis

The software package SPSS, 22 (IBM, Chicago IL, USA), together with Excel, version 14 (Microsoft Office, Seattle WA, USA) were used to analyse the data of this study. All data were checked for normality using histograms and the Shapiro Wilks test. When data were not normally distributed, values were either logarithmically or square root transformed before statistical analysis. Values in the text and in the tables are represented as mean ± SD for normally

distributed data, median (IQR) for non-normal data, and percentages for categorical variables ($n/N \times 100$, where n = number of relevant cases, N = number of total cases). Baseline differences in participant characteristics between the groups were tested by using independent t-tests for continuous variables and Pearson chi-square test for binary outcomes. Partial correlation coefficients, adjusted for sex, BMI, age, weight and height, were calculated to determine associations between FA and PZn concentrations. The effects of Zn fortification on plasma total phospholipid FA composition were analysed with one-way ANCOVA by using respective baseline FA values, sex and BMI as individual level covariates, in the total groups as well as stratified by baseline Zn status (deficient versus sufficient) and by sex (boys versus girls). Differences at baseline between Zn-sufficient and deficient groups were analysed by one way ANCOVA, adjusted for age and sex in all children and separately in boys and girls, adjusted for age. Significance was set at $P < 0.05$.

Ethical considerations

The ethical committees of the North-West University, Potchefstroom, South Africa (NWU-00086-15-A1), the Beninese National Ethics Committee for Health Research (No. 029; 19 October 2012), and ETH Zürich, Switzerland (KEK-ZH-No.2012–0168), approved the study protocol. Before the screening, parents gave informed consent by either a written signature or a fingerprint.

Results

Study participant characteristics

Of the 262 children who completed the Zn efficacy study, 186 had their plasma total phospholipid FA analysed, as we did not include the pump group from the main study as part of the secondary study (Figure 1). Table 1 summarises the demographic and characteristics of the study participants. Haemoglobin was significantly higher in the Zn-filter group than in the filter group at baseline ($p = 0.030$). Anthropometric measures between groups at baseline did not differ. Five of the PZn concentration outliers were not included in analysis among the school-age children (range: 114–160 mg/dL) (Galetti et al., 2015). Median (IQR) PZn concentration was 70.3 $\mu\text{g/dL}$ (62.7, 77.3) for the Zn+filter group and 68.6 $\mu\text{g/dL}$ (62.1, 76.9) for the filter group at baseline, and a prevalence of Zn deficiency in all the children was 34% (n/N 63/184). There were no differences at baseline between the plasma total phospholipid FA compositions of the treatment groups except for 20:2n-6 ($p = 0.040$) and the ARA:DGLA ratio ($p = 0.030$) that was higher in the Zn+filter group as seen in

Table 4.

Table 1: Baseline anthropometric measurements and biochemical characteristics of children by treatment group ¹

Characteristics	Zn+filter (n = 88-89)	Filter (n = 96-97)
Male	51.6 (46/89) ^c	57.7 (56/97)
Female	48.3 (43/89)	42.3 (41/97)
Age, y	8.10 (6.64, 9.59)	8.52 (7.33, 9.58)
Height, cm	121.94 ± 10.75 ^a	121.03 ± 9.91
Weight, kg	21 (18.00, 23.00) ^b	19.25 (17.46, 23.38)
BMI, kg/m²	13.77 ± 1.11	13.77 ± 1.07
Midupper arm circumference, mm	171.98 ± 16.12	170.09 ± 15.25
HAZ	-1.22 (-1.84, -0.41)	-1.47 (-2.01, -0.74)
Moderate stunting (HAZ < 2), %	13.48 (12/89)	3.09 (3/97)
Severe stunting (HAZ <3), %	5.61 (5/89)	8.24 (8/97)
WAZ	-1.76 ± 1.05	-1.92 ± 0.97
Moderate underweight (WAZ < 2), %	18 (18/89)	23 (23/97)
Severe underweight (WAZ <3), %	8 (8/89)	10 (10/97)
BAZ	-1.41 (-2.21, -0.97)	-1.62 (-2.21, -0.99)
Moderate thinness (BAZ <2), %	19.1 (17/89)	24.74 (24/97)
Severe thinness (BAZ <3), %	7.8 (7/89)	6.18 (6/97)
PZn, µg/dL	70.30 (62.65, 77.3)	68.60 (62.1, 76.85)
Low PZn² (%)	31.82 (28/88)	36.50 (35/96)
PF, µg/L	53.12 (36.49, 105.40)	56.03 (56.03, 83.74)
Iron deficiency (PF < 30 µg/L), %	20.22 (18/89)	16.49 (16/97)
Haemoglobin, g/dl*	13.30 ± 1.01	12.96 ± 1.11
Anaemia, %	4.49 (4/89)	10.3 (10/97)
CRP, mg/L	0.29 (0.15, 1.29)	0.48 (0.19, 1.11)
Inflammation (CRP ≥ 5 mg/L), %	10.11 (9/89)	6.18 (6/97)
AGP, g/L	0.87 (0.69, 1.12)	0.88 (0.71, 1.09)
Inflammation (AGP >1 g/L), %	33.7 (30/89)	31.95 (31/97)

Abbreviations: AGP, a1-acid glycoprotein; BAZ, BMI-for-age z score; CRP, C-reactive protein; HAZ, height-for-age zscore; PF, plasma ferritin; WAZ, weight-for-age z score; PZn, plasma zinc

¹ Differences between the Zn+filter group and filter groups were examined by using independent t-tests for continuous variables and by using the chi-square test for categorical variables.

* Hb was significantly higher in the Zn+filter group (p= 0.030)

² Low PZn concentration defined as PZn ≤ 65 mg/dL for children aged 10 y (morning sampling), 57 mg/dL for children aged 10 y (afternoon sampling), 70 mg/dL for females aged 10 y (morning fasting sampling), 59 mg/dL for females aged 10 y (afternoon sampling), 74 mg/dL for males aged 10 y (morning fasting sampling), and 61 mg/dL for males aged 10 y (afternoon sampling) (Ghosh A & C., 2007)

^a Data are mean ± SD (all such values), ^b median [IQR] (all such values), or ^c percentage (% , n/N* 100) (all such values)

Baseline associations of plasma zinc with plasma total phospholipid fatty acid composition

Table 2 gives the corrected correlation coefficients for the associations between PZn status and plasma total phospholipid FA composition, adjusted for age, sex, BMI, height and weight. The strongest positive correlations were found between plasma Zn and the DGLA:LA ($r = 0.296$, $p \leq 0.001$), and the GLA:LA ($r = 0.324$, $p = 0.054$) ratios. PZn also correlated positively with DGLA ($r = 0.199$, $p = 0.014$), and negatively with LA ($r = -0.208$, $p = 0.010$), and the ARA:DGLA ratio ($r = -0.176$, $p \leq 0.030$).

Table 2: Baseline correlations of plasma zinc with plasma total phospholipid fatty acid composition¹

Fatty Acid	All children		
	n=39 - 186	r-value	p-value
18:1n-9 (OA) ⁴	13.44 (12.56, 14.46) ²	0.023	0.780
24:1n-9 (Nervonic)	1.39 ± 0.52 ³	0.054	0.506
18:2n-6 (LA) ⁴	15.68 (14.46, 17.1)	-0.208	0.010
18:3 n-6 (GLA)	0.18 ± 0.104	0.257	0.130
20:2n-6 (Eicosadienoic) ⁴	0.39 (0.34, 0.45)	-0.012	0.890
20:3n-6 (DGLA) ⁵	2.31 (1.85, 2.70)	0.199	0.014
20:4n-6 (ARA)	7.60 ± 1.84	0.007	0.933
22:4 n-6 (Adrenic)	0.46 ± 0.15	0.054	0.560
22:5n-6 (n-6 DPA)	0.50 ± 0.18	0.130	0.154
18:3n-3 (ALA) ⁵	0.21 ± 0.19	0.248	0.165
20:5n-3 (EPA)	0.19 ± 0.07	-0.131	0.340
22:5n-3 (n-3 DPA) ⁴	0.34 (0.26,0.41)	0.077	0.422
22:6n-3 (DHA)	1.63 ± 0.58	0.085	0.300
Total n-6 LCPUFA	9.94 ± 2.17	-0.097	0.234

GLA: LA ratio	0.01 ± 0.01	0.324	0.054
DGLA:GLA ratio	17.71 ± 7.79	-0.069	0.912
DGLA:LA ratio	0.15 ± 0.04	0.296	< 0.000
ARA:DGLA ratio	3.37 ± 0.89	-0.176	0.030
ARA:LA ratio	0.49 ± 0.13	0.130	0.110
n-6 DPA:DHA ratio	0.29 ± 0.09	0.069	0.453

Abbreviations: PUFA, polyunsaturated fatty acids; LCPUFA, long chain polyunsaturated fatty acids; LA, linoleic acid; ARA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. DGLA, dihomo- γ -linolenic acid, DPA, docosapentaenoic acid; GLA-gamma-linolenic acid; OA, oleic acid

Correlations were determined by using linear regression analysis, adjusted for sex, BMI, age and partial correlation coefficients are shown.

² median (IQR) (all such values)

³ mean ± SD (all such values)

⁴ Data were log transformed to perform ANCOVA.

⁵ Data were squared to perform ANCOVA.

There were no significant differences at baseline in plasma total phospholipid FA composition between the Zn-sufficient (n = 24 –112) and Zn-deficient (n = 15 – 54) groups (data not shown). At baseline, boys had lower plasma total phospholipid compositions for LA (p = 0.017), EPA (p = 0.002), N-3 DPA (p = 0.020), adrenic acid (p=0.010) and in ARA:LA (p = 0.02) than girls (data not shown). However, at baseline (Table 3) there was a significant difference between the FA composition of Zn-sufficient and Zn-deficient boys; Zn-sufficient boys had a higher plasma total phospholipid composition for GLA (p = 0.020) and tended to have a higher DGLA:GLA ratio (p = 0.059).

Table 3: Baseline plasma total phospholipid fatty acid composition of zinc deficient and sufficient boys¹

Fatty Acid	Sufficient	Deficient ²	<i>B</i> -value ⁵
	9-58	6-32	
18:1n-9 (OA)⁶	13.43 (12.70,14.44) ³	14.15 (12.57, 13.48)	0.106 (-0.638, 0.851)
24:1n-9 (Nervonic)	1.35 ± 0.50 ⁴	1.43 ± 0.57	-0.073 (-0.306, 0.159)
18:2n-6 (LA)⁶	15.34 (13.84, 16.74)	18.86 (14.72, 17.11)	-0.025 (-0.056, 0.006)
18:3 n-6 (GLA)	0.14 ± 0.32	0.28 ± 0.12	-0.107 (-0.200, -0.013)
20:2n-6 (Eicosadienoic)⁶	0.39 (0.35, 0.44)	0.40 (0.34, 0.49)	-0.010 (0-.059, 0.039)
20:3n-6 (DGLA)⁷	2.36 (1.84, 2.73)	2.27 (1.82, 2.76)	0.009 (-0.079, 0.097)
20:4n-6 (ARA)	7.81 ± 2.17	7.73 ± 1.42	0.138 (-0.694, 0.969)
22:4 n-6 (Adrenic)	0.51 ± 0.14	0.47 ± 0.17	0.034 (-0.041, 0.110)
22:5n-6 (n-6 DPA)	0.53 ± 0.17	0.50 ± 0.20	0.043 (-0.045, 0.132)
18:3n-3 (ALA)	0.23 ± 0.14	0.23 ± 0.15	0.157 (-0.524, 0.838)
20:5n-3 (EPA)	0.23 ± 0.81	0.22 ± 0.86	0.008 (-0.062, 0.078)
22:5n-3 (n-3 DPA)⁶	0.38 (0.30, 0.53)	0.36 (0.25, 0.41)	0.057 (-0.039, 0.153)
22:6n-3 (DHA)	1.70 ± 0.64	1.51 ± 0.51	0.212 (-0.041, 0.465)
Total n-6 LCPUFA	10.14 ± 2.54	10.04 ± 1.75	0.164 (-0.820, 1.148)
GLA:LA ratio	0.01 ± 0.00	0.02 ± 0.00	-0.006 (-0.013, 0.002)
DGLA:GLA ratio	20.11 ± 6.54	12.94 ± 7.43	7.397 (-0.535, 15.330)
DGLA:LA ratio	0.16 ± 0.04	0.15 ± 0.04	0.010 (-0.010, 0.029)
ARA:DGLA ratio	3.44 ± 0.91	3.53 ± 0.96	-0.073 (-0.479, 0.333)
ARA:LA ratio	0.52 ± 0.14	0.49 ± 0.11	0.030 (-0.026, 0.086)
n-6 DPA:DHA ratio	0.30 ± 0.80	0.31 ± 0.11	-0.016 (-0.057, 0.026)

Abbreviations: PUFA, polyunsaturated fatty acids; LCPUFA, long chain polyunsaturated fatty acids; LA, linoleic acid; ARA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid, DGLA, dihomog-γ-linolenic acid, DPA, docosapentaenoic acid; OA, oleic acid.

¹ Intervention effects estimated by one way ANCOVA adjusted for age, and BMI.

² Low PZn concentration defined as PZn ≤ 65 mg/dL for children aged 10 y (morning sampling), 57 mg/dL for children aged 10 y (afternoon sampling), 70 mg/dL for females aged 10 y (morning fasting sampling), 59 mg/dL for females aged 10 y (afternoon sampling), 74 mg/dL for males aged 10 y (morning fasting sampling), and 61 mg/dL for males aged 10 y (afternoon sampling) (Ghosh A & C., 2007).

³ Median (IQR) (all such values).

⁴ Mean ± SD (all such values).

⁵ *B*-value; 95% CI in parenthesis (all such values).

⁶ Data were log transformed to perform ANCOVA.

⁷ Data were squared to perform ANCOVA.

Effect of zinc Intervention on plasma total phospholipid fatty acid status

The effects of Zn fortification on the plasma total phospholipid FA composition of all the children are given in Table 4. Zn fortification increased nervonic acid ($p = 0.048$). Furthermore, Zn fortification showed a trend towards reducing ALA ($p = 0.097$) (Table 4).

Table 5 shows the effect of Zn fortification on the plasma total phospholipid FA composition of the Zn-deficient children only. Zn fortification significantly increased nervonic acid ($p = 0.019$) and decreased ALA composition in the Zn-deficient group ($p = 0.009$). There were no intervention effects in the Zn-sufficient group (data not shown), as well as no differences between the intervention effects in boys and girls (data not shown).

Table 4: Effects of 20 weeks of zinc fortification on plasma total phospholipid fatty acid composition of 6-10y school aged Beninese children ¹

Fatty Acid	Time points	Zn+filter	Filter	Intervention effect²
		n= 18 – 89	n= 21 - 97	
18:1n-9 (OA)⁶	Baseline	13.28 (12.47,14.46) ³	13.45 (12.68,14.46)	
	Endpoint	12.81 (11.7,14.02)	12.8 (11.7,13.95)	0.002 (-0.013, 0 .017) ⁵
24:1n-9 (Nervonic)	Baseline	1.41 ± 0.53 ⁴	1.36 ± 0.51	
	Endpoint	1.37 ± 0.42	1.25 ± 0.45	0.109 (0 .001, 0.216)
18:2n-6 (LA)⁶	Baseline	16.31 (14.61, 17.42)	15.36 (14.4, 16.87)	
	Endpoint	18.01 (16.41, 18.98)	17.18 (16.1, 18.48)	0.015 (-0.001, 0.031)
18:3 n-6 (GLA)	Baseline	0.20 ± 0.13	0.16 ± 0.07	
	Endpoint	0.33 ± 0.47	0.20 ± 0.06	0.137 (-0.240 ,0.514)
20:2n-6 (Eicosadienoic)⁶	Baseline	0.40 (0.37, 0.45)	0.37 (0.31, 0.45)	
	Endpoint	0.39 (0.34, 0.44)	0.37 (0.31, 0.44)*	0.019 (-0.014, 0.050)
20:3n-6 (DGLA)⁷	Baseline	2.38 (1.92, 2.92)	2.24 (1.84, 2.65)	
	Endpoint	2.42 (1.87, 2.90)	2.23 (1.75, 2.66)	0.041 (-0.013, 0 .094)
20:4n-6 (ARA)	Baseline	7.47 ± 1.90	7.73 ± 1.78	
	Endpoint	8.31 ± 1.63	8.35 ± 2.19	0.145 (-0.349, 0.639)
22:4 n-6 (Adrenic)	Baseline	0.47 ± 0.14	0.46 ± 0.15	
	Endpoint	0.46 ± 0.13	0.48 ± 0.14	-0.020 (-0.070, 0.020)
22:5n-6 (n-6 DPA)	Baseline	0.49 ± 0.16	0.52 ± 0.19	
	Endpoint	0.49 ± 0.21	0.49 ± 0.2	0.031 (-0.031, 0.073)
total n-6 LCPUFA	Baseline	9.87 ± 2.29	10.00 ± 2.07	
	Endpoint	10.72 ± 1.98	10.61 ± 2.48	0.249 (-0.298, 0 .795)
18:3n-3 (ALA)	Baseline	0.21 ± 0.19	0.23 ± 0.31	
	Endpoint	0.33 ± 0. 51	0.22 ± 0.13	-0.030 (-0.190, 0.130)
20:5n-3 (EPA)	Baseline	0.20 ± 0.07	0.18 ± 0.06	
	Endpoint	0.22 ± 0.11	0.24 ± 0.11	0.030 (-0.040, 0.100)
22:5n-3 (n-3 DPA)⁶	Baseline	0.35 (0.24, 0.40)	0.36 (0.29, 0.44)	

	Endpoint	0.38 (0.28, 0.45)	0.42 (0.31, 0.52)	0.021 (-0.031, 0.073)
22:6n-3 (DHA)	Baseline	1.67 ± 0.61	1.59 ± 0.55	
	Endpoint	1.72 ± 0.53	1.63 ± 0.59	0.070 (-0.068, 0.208)
GLA: LA ratio	Baseline	0.01 ± 0.01	0.01 ± 0.00	
	Endpoint	0.02 ± 0.03	0.01 ± 0.00	0.009 (0.009, 0.034)
DGLA:GLA ratio	Baseline	17.24 ± 8.12	18.14 ± 7.64	
	Endpoint	16.90 ± 12.8	15.93 ± 6.73	0.997 (-11.650, 13.645)
DGLA:LA ratio	Baseline	0.16 ± 0.05	0.15 ± 0.04	
	Endpoint	0.02 ± 0.01	0.021 ± 0.01	0.001 (-0.002, 0.003)
ARA:DGLA ratio	Baseline	3.22 ± 0.87	3.52 ± 0.88	
	Endpoint	3.61 ± 0.96 ^a	3.86 ± 1.18 [*]	-0.153 (-0.473, 0.167)
ARA:LA ratio	Baseline	0.48 ± 0.13	0.50 ± 0.12	
	Endpoint	0.47 ± 0.11	0.49 ± 0.14	-0.007 (-0.043, 0.028)
n-6 DPA:DHA ratio	Baseline	0.28 ± 0.10	0.30 ± 0.10	
	Endpoint	0.28 ± 0.11	0.28 ± 0.11	-0.006 (-0.036, 0.024)

Abbreviations: PUFA, polyunsaturated fatty acids; LCPUFA, long chain polyunsaturated fatty acids; LA, linoleic acid; ARA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid, DGLA, dihomo- γ -linolenic acid, DPA, docosapentaenoic acid, OA, oleic acid.

¹ Values with a * changed significantly from baseline to endpoint ($p < 0.001$) and were analysed with dependent t-tests.

² Intervention effects were estimated by one way ANCOVA, adjusted for respective baseline value, sex, and BMI.

³ Median (IQR) (all such values).

⁴ Mean \pm SD (all such values).

⁵ *B value*; 95% CI in parenthesis (all such values),

⁶ Data were log transformed to perform ANCOVA.

⁷ Data were squared to perform ANCOVA.

Table 5: Effect of zinc fortification on plasma total phospholipid fatty acid composition in zinc deficient subjects^{1,2}

Fatty Acid	Timepoints	Zn + filter	Filter	Intervention effect ²
		N = 6-24	N = 9-32	
18:1n-9 (OA)⁶	Baseline	13.58 (12.55, 14.54) ³	13.44 (12.72, 14.27)	
	Endpoint	13.09 (11.83, 14.13)	12.99 (11.67, 14.21)	0.008 (-0.019, 0.036) ⁵
24:1n-9 (Nervonic)	Baseline	1.35 ± 0.48 ⁴	1.41 ± 0.51	
	Endpoint	1.44 ± 0.50	1.29 ± 0.42	0.234 (0.040, 0.428)
18:2n-6 (LA)⁶	Baseline	15.74 (13.76, 17.06)	15.32 (14.28, 16.68)	
	Endpoint	18.16 (15.37, 18.67)	17.06 (15.99, 18.55)	0.018 (-0.013, 0.049)
18:3 n-6 (GLA)	Baseline	0.20 ± 0.14	0.19 ± 0.07	
	Endpoint	0.46 ± 0.65	0.18 ± 0.04	0.002 (0.009, 0.095)
20:2n-6 (Eicosadienoic)⁶	Baseline	0.39 (0.35, 0.43)	0.38 (0.32, 0.47)	
	Endpoint	0.39 (0.34, 0.42)	0.35 (0.30, 0.44)	0.024 (-0.034, 0.081)
20:3n-6 (DGLA)⁷	Baseline	2.32 (1.89, 2.95)	2.30 (1.83, 2.69)	
	Endpoint	2.42 (1.94, 2.78)	2.29 (1.90, 2.87)	0.001 (-0.084, 0.086)
20:4n-6 (ARA)	Baseline	7.51 ± 2.11	8.00 ± 1.72	
	Endpoint	8.39 ± 1.55	8.54 ± 2.13	-0.082 (0.986, 0.822)
22:4 n-6 (Adrenic)	Baseline	0.50 ± 0.15	0.49 ± 0.14	
	Endpoint	0.46 ± 0.1	0.48 ± 0.13	-0.040 (-0.118, 0.039)
22:5n-6 (n-6 DPA)	Baseline	0.49 ± 0.14	0.54 ± 0.2	
	Endpoint	0.49 ± 0.2	0.48 ± 0.18	-0.016 (-0.139, 0.106)
total n-6 LCPUFA	Baseline	9.86 ± 2.57	10.31 ± 1.97	
	Endpoint	10.78 ± 1.78	10.88 ± 2.35	-0.071 (-1.024, 0.882)
18:3n-3 (ALA)	Baseline	0.15 ± 0.12	0.21 ± 0.11	
	Endpoint	0.11 ± 0.03	0.23 ± 0.18	-0.126 (-0.203, -0.048)
20:5n-3 (EPA)	Baseline	0.24 ± 0.08	0.20 ± 0.07	
	Endpoint	0.25 ± 0.14	0.23 ± 0.07	0.030 (-0.081, 0.140)
22:5n-3 (n-3 DPA)⁶	Baseline	2.32 (0.25, 0.40)	2.30 (0.31, 0.49)	
	Endpoint	0.40 (0.31, 0.47)	0.45 (0.33, 0.53)	0.024 (-0.065, 0.114)
22:6n-3 (DHA)	Baseline	1.62 ± 0.67	1.64 ± 0.54	
	Endpoint	1.63 ± 0.51	1.62 ± 0.55	0.056 (-0.170, 0.283)
GLA: LA ratio	Baseline	0.01 ± 0.00	0.01 ± 0.00	
	Endpoint	0.02 ± 0.04	0.01 ± 0.00	1.413 (-0.007, 0.007)
DGLA:GLA ratio	Baseline	17.48 ± 9.63	16.67 ± 6.74	
	Endpoint	12.31 ± 6.40	17.64 ± 5.66	-2.496 (-7.992, 3.000)
DGLA:LA ratio	Baseline	0.15 ± 0.05	0.15 ± 6.74	
	Endpoint	0.02 ± 0.00	0.02 ± 0.00	-0.001 (-0.004, 0.003)
ARA:DGLA ratio	Baseline	3.31 ± 0.91	3.58 ± 0.93	
	Endpoint	3.67 ± 1.00	3.91 ± 1.35	-0.161 (-0.760, 0.438)
ARA:LA ratio	Baseline	0.50 ± 0.14	0.52 ± 0.11	
	Endpoint	0.48 ± 0.10	0.50 ± 0.14	-0.031 (-0.099, 0.036)
n-6 DPA:DHA ratio	Baseline	0.29 ± 0.08	0.30 ± 0.08	
	Endpoint	0.29 ± 0.1	0.28 ± 0.10	-0.030 (-0.030, 0.024)

Abbreviations: PUFA, polyunsaturated fatty acids; LCPUFA, long chain polyunsaturated fatty acids; LA, linoleic acid; ARA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. DGLA-dihomo- γ -linolenic acid, DPA, docosapentaenoic acid; OA- oleic acid

¹ Intervention effects estimated by one way ANCOVA adjusted for respective baseline value, sex, and BMI

² Low PZn concentration defined as PZn \leq ,65 mg/dL for children aged 10 y (morning sampling), 57 mg/dL for children aged 10 y (afternoon sampling), 70 mg/dL for females aged 10 y (morning fasting sampling), 59 mg/dL for females aged 10 y (afternoon sampling), 74 mg/dL for males aged 10 y (morning fasting sampling), and 61 mg/dL for males aged 10 y (afternoon sampling) (Ghosh A & C., 2007)

³ Median (IQR) (all such values).

⁴ Mean \pm SD (all such values).

⁵ *B value*; 95% CI in parenthesis (all such values).

⁶ Data were log transformed to perform ANCOVA.

⁷ Data were squared to perform ANCOVA.

Discussion

In rural 6-10-year-old Beninese children, an elevated plasma Zn status was positively correlated with plasma total phospholipid FA composition at baseline for DGLA, DGLA:LA and GLA:LA ratios. Thus, confirming the presence of an association between Zn status and the total plasma phospholipid FA composition. Furthermore, the results show that an increase in plasma Zn concentration was associated with improved FA desaturase and/or elongase activity. The most interesting finding was that Zn fortification increased the longer-chain monounsaturated n-9 FA nervonic acid in plasma total phospholipids in the group as a whole, and particularly in children that were Zn-deficient at baseline. This result was unexpected as it has been shown previously that Zn deficiency mainly affects n-6 FA composition. This could be as a result of increased plasma Zn concentrations that also might have improved elongase activity. In Zn-deficient children, Zn decreased ALA in the Zn+filter group. Our results suggest that the availability of Zn in the intervention group possibly increased the conversion of ALA to its longer chain metabolites (Prasad, 2013). Furthermore, the FA composition in the boys indicated that they were more affected by Zn deficiency than girls.

In this study, an increase in plasma Zn status was associated with an enhanced conversion of precursor FA to long-chain FA, as we found a positive significant association between Zn status and the DGLA:LA ratio. Similarly, we showed that plasma Zn correlates with DGLA. An inverse relationship between Zn status and LA also supports the theory that a higher availability of Zn results in a more efficient conversion of LA, an essential FA, to longer-chain metabolites. Although not significant, there was a strong association between plasma Zn status and the GLA:LA ratio (Table 2). This might be because GLA is rapidly elongated to DGLA, thus resulting in relatively low GLA composition (Fan & Chapkin, 1998). An increase in Zn status would be

thought to result in a positive association between plasma Zn and the ARA:DGLA ratio; interestingly, this study found an inverse relationship between the two. Contrary to findings from the study conducted by Reed and colleagues, we found that, when compared against the Zn-sufficient group, there was no significant difference in the DGLA:LA ratio in the Zn-deficient groups (0.15 ± 0.43 , and 0.14 ± 0.42 , respectively, $p = 0.217$). Therefore, our study was able to support the value of the LA:DGLA/DGLA:LA ratio as an emerging biomarker for Zn status, as proposed by Knez *et al.* (2016) and Reed *et al.* (2014).

To our knowledge, this is one of the first human studies to investigate the effect of Zn fortification on FA status. The majority of previous such studies were on animals (Cunnane, 1988a; Cunnane, Horrobin, & Manku, 1984; Faure, Durand, Blache, Favier, & Roussel, 1995; Kudo, Nakagawa, & Waku, 1990). These studies found lower n-6 LCPUFA concentration/composition in the blood/tissue of Zn-deficient animals and higher LA, indicating a disruption in desaturation and/or elongation of FA (Ayala & Brenner, 1982; Cunnane, 1988a; Cunnane *et al.*, 1984; Dib, Clavel, & Carreau, 1989; Kramer, Briske-Anderson, Johnson, & Holman, 1986).

Zn is described as a cofactor for desaturase enzymes (Horrobin, 1981), and Zn deficiency is suggested to result in a disruption of the rate-limiting delta-6-desaturase step in FA synthesis (Tang, Cho, Nakamura, & Clarke, 2003). The overall reaction for PUFA synthesis involves four enzymes and utilises two-carbon units donated from malonyl-CoA, and nicotinamide adenine dinucleotide phosphate (NADPH) as a reductant, and fatty acyl coenzyme A (CoA) as substrates. Zn deficiency and low dietary Zn intake are known to affect the electron transfer pathway (Knez *et al.*, 2016). Microsomal Zn levels directly determine the electron transferrin chain activity. This is because the terminal electron transfer of electrons from nicotinamide adenine dinucleotide (NADP) or NADPH through the cytochromes b5 and P-450 to the desaturase protein is Zn sensitive (Cunnane, 1988b). From our results, NADPH, seems to be more affected by Zn deficiency and/or Zn fortification. Zn-containing enzyme called microsomal pyrophosphatase that is capable of metabolising both NADPH and NADH is activated by Zn (Jeffery, 1983). In Zn-deficient rats P-450 activity is reduced. This then results in an inhibition in the terminal desaturase reaction (Cunnane, 1988b). Thus, FA elongases, which function with FA desaturases' to generate many of the long chain mono- and polyunsaturated FA, can be expected to also be affected by Zn status (Jump, 2009).

The proposed molecular interaction, in which Zn affects the activity of desaturase and/or elongase enzymes, was supported by results from this study. Even with a low dosage of Zn and a small intervention effect (Galetti *et al.*, 2015), Zn fortification had a detectable effect on the FA composition of Zn-deficient children. Compared with the filter group, nervonic acid was also

elevated at the endpoint in the Zn+filter group (Table 4), thus supporting the concept that the availability of Zn in children results in the increase in activity of desaturase and/or elongase enzymes. As shown in Table 5, ALA was significantly increased in the filter group relative to the Zn+filter group. This result further supports the notion that the desaturase enzyme activity in the Zn+filter group was elevated, resulting in an increase in the conversion of ALA to LCPUFA metabolites.

The novel finding that nervonic acid was increased the most by Zn fortification may be important due to the important role of this FA in brain function (Table 2) (Sargent, Coupland, & Wilson, 1994). Nervonic acid, a mono-unsaturated FA, is a product of a three-step elongation process involving oleic acid (18:1n-9), gadoleic acid (20:1n-11) and erucic acid (22:1n-9). Nervonic acid is suggested to play a vital role in brain function as it is important for the biosynthesis of sphingomyelin and is found in the white matter of the human brain (El-Agamy, 2009). There is an apparent relationship that has been discovered during demyelination between nervonic acid levels and the diseases adrenoleukodystrophy (ALD) and multiple sclerosis (Coupland, 1999). The sphingolipids from post mortem ALD demyelated brain tissue have been found to have decreased levels of nervonic acid (Sargent et al., 1994). Apart from increasing desaturase activity, the availability of Zn in the Zn+filter group might also have resulted in an increased activity of elongase enzymes that are involved in the n-9 synthesis pathway, resulting in elevated nervonic acid in the plasma total phospholipids.

The small intervention effects observed in the composition of the majority of the FA might have been due to this study being a complementary study to a study whose main aim was to evaluate the efficacy of simultaneous Zn fortification and water filtration on Zn status in Beninese children (Galetti et al., 2015). Thus, the original study were not designed and powered for the purpose of this complementary study. Some Zn fortification studies have used the same Zn fortificant levels such as those used by Galetti *et a.* (2015) and showed an improvement in Zn status (Hambidge, Chavez, Brown, & Walravens, 1979; Ohiokpehai, David, & Kamau, 2009). The efficacy of Zn fortification to improve Zn status is, however, inconsistent (Das et al., 2013). Plasma Zn in the intervention group was maintained at baseline values rather than improved throughout the duration of the study in Beninese children (Galetti et al., 2015). Zn supplementation in children, in contrast, has consistently showed large increases in mean serum Zn concentration (Hess & Brown, 2009). Furthermore, we cannot exclude the possibility that an increase in Zn concentrations and duration might have resulted in a more pronounced difference in the FA composition of the children in the Zn-filter group.

Another noteworthy observation was that the sex of our subjects had an influence on the FA composition. LCPUFA of plasma total phospholipids were increased in Zn-sufficient boys, GLA was significantly higher in Zn-sufficient boys than those with a deficiency. This could indicate that the activity of desaturase and elongase enzymes is mostly affected by Zn deficiency in boys. However, Zn intervention did not have a significant effect on the difference in the FA composition between the sexes. These results are, however, not consistent with findings from other studies. In a systematic review, it was found that men had a less efficient conversion of EFA to LCPUFA, thus resulting in them making a smaller contribution of ARA and DHA to plasma total lipids and plasma phospholipids than women. (Lohner, Fekete, Marosvölgyi, & Decsi, 2013).

This study had limitations. First, although the baseline FA composition was similar for the different groups, indicating similar intake, and that we corrected for baseline FA composition, the quality of results might have been affected by the lack of dietary fat intake data at baseline. Dietary intake is known to correlate with FA plasma composition (Hodge et al., 2007). Eder and colleagues argued that the dietary fat intake should be considered when investigating the effect of Zn deficiency on essential FA metabolism (Eder & Kirchgessner, 1994), since an increased concentration of dietary LA may affect the LA:DGLA ratio irrespective of Zn status (Reed et al., 2014). Huang et al (1982) found that increasing dietary GLA was effective in reversing the biological effects of Zn deficiency as supplementing with GLA had an effect on membrane EFA composition. Similarly, Tsai *et al.* (1983) showed that delta-6-desaturase activity in Zn-deficient rats was normalised after subcutaneous injection of safflower oil (high in LA). People in Benin consume Shea butter, a source of n-6 FA such as LA, GLA and ARA (Di Vincenzo et al., 2005; Dubois, Breton, Linder, Fanni, & Parmentier, 2007; Maranz, Wiesman, Bisgaard, & Bianchi, 2004; Okullo et al., 2010; Tchobo et al., 2007).

Second, our study was limited by the use of a single type of blood tissue sample for the outcome measure, as was noted by other researchers (Smit, Muskiet, & Boersma, 2004). The blood pools or tissue lipids in which FA biomarkers are measured differ in their delay of response to dietary changes. As our study was conducted over 20 weeks, the use of plasma as an FA biomarker might not have been ideal. Plasma and serum reflect short term intake (Katan, Deslypere, Van Birgelen, Penders, & Zegwaard, 1997). Red blood cells are superior to plasma for analyses of FA biomarkers, as they are able to reflect long-term FA intake and have a slower turnover rate (120 days), which reflects intake over weeks (Sun, Ma, Campos, Hankinson, & Hu, 2007). Additionally, red blood cells reflect FA composition of other tissues better in the body. The collection of plasma is easier, but its FA composition may not reflect the overall tissue FA status. In order to assess long-term FA intake and although difficult to obtain, adipose tissue is the best FA biomarker (Baylin & Campos, 2006). Plasma FA composition reflects the last 1–2 weeks of dietary intake,

while red blood cell FA composition reflects the last 1–2 months; adipose tissue FA composition reflects the previous year's dietary intake (Arab, 2003; Katan et al., 1997). Our study had the advantage of having the FA composition being measured in the plasma total phospholipids. Phospholipids can be used to assess desaturation as they contain a large portion of unsaturated FA that are derived from EFA (Eder & Kirchgessner, 1996).

Lastly, the study population was malnourished, as there was a high prevalence of stunting and wasting. Malnourishment, as well as Zn deficiency, affects FA composition of tissues. In all lipid fractions, malnourished children have been found to have a marked decrease of polyunsaturated FA with low LA, and ARA, mainly in sterol esters (Holman et al., 1981; Koletzko, Abiodun, Laryea, & Bremer, 1986). In severely malnourished children the FA composition was low in PUFA and LCPUFA (Babirekere-Iriso et al., 2016; Decsi, Zaknun, Zaknun, Sperl, & Koletzko, 1995; Leichsenring, Siitterlin, Bäumann, Anninos, & Becker, 1995; Wolff, Margolis, Bujdoso-Wolff, Matusick, & MacLean, 1984).

It should also be noted that FA synthesis is not simply the conversion of EFA to their longer-chain metabolites (Berg et al., 2002). The complexity of metabolic processes that are involved in FA metabolism should not be overlooked. Processes such as FA desaturation and elongation, transport from sites of synthesis to target cells, and acylation and deacylation reactions all come into play (Ma et al., 1995). Furthermore, non-dietary factors such as absorption, metabolism, genetic and life style determinants are all factors that can and may affect FA compositions in tissues (Wilkins & Lee, 1998).

In conclusion, in this study, we give convincing evidence that the DGLA:LA or GLA:LA ratio may be a good biomarker of Zn status. Furthermore, water fortified with Zn (2.8mg) daily, increased the overall desaturation and elongation of FA in Beninese children.

Key Messages

- The LA:DGLA/DGLA:LA ratio is a potential Zn status biomarker
- The provision of water fortified with low concentrations of Zn has a beneficial effect on plasma Zn as well as plasma total phospholipid FA composition
- Zn influences FA metabolism and may affect desaturase and elongase activity
- Sex has an effect on the plasma total phospholipid FA composition
- The improvement in LCPUFA status appears to be directly dependent on Zn dose

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Conflict of Interest Statement

None of the researchers had a conflict of interest. None of the study funders had any involvement in the study design, analysis, or interpretation of data; the writing of the report; or the decision to submit the article for publication.

Contributor statement

T.C. involved in conceptualisation, performed data analyses and interpretation and wrote the manuscript; L.M. involved in data analysis and interpretation, revision of manuscript; J.B. conceptualised secondary study, involved in revision of manuscript; P.J.vJ supervised FA analyses, cleaned FA data se, revision of manuscript; V.G. and D.M. conceptualised and conducted parent study research; M.B.Z. initiated and conceptualised the parent study. All authors read and approved the final version of the paper.

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CHAPTER 4: CONCLUSIONS AND RECOMMENDATIONS

4.1 Introduction

The overall aim of this thesis was to investigate the associations between plasma Zn and plasma total phospholipid FA. The present study further aimed to generate some knowledge on the interactions that exist between FA and Zn in humans. More specifically we aimed to 1) investigate associations that exist between Zn and FA 2) investigate whether the LA:DGLA could be a potential Zn biomarker in humans 3) determine the effect Zn fortification on plasma total phospholipid FA composition.

This concluding chapter will provide a summary of the main findings, the limitations of the methodology that was used, and recommendations for future research. To provide an overview, the study's aims and objectives are used as headers.

4.2 Interactions between FA and Zn and effect of Zn deficiency on FA status

In Chapter Two the literature review gave a thorough theoretical background on Zn, FA and Zn and FA interactions. Both Zn and FA are essential for growth, immunity, and skin integrity, neural and visual development. In the literature review, it was noted that Zn and FA deficiencies result in similar physical and physiological impairments such as growth retardation, delayed, sexual maturation, infertility, dermal lesions, alopecia and decreased rate of wound healing. The need for a specific, reliable and sensitive Zn status biomarker was also highlighted in the review. A potential Zn status biomarker was discovered by Reed and colleagues in an *in vivo Gallus gallus* model. Evidence from this study demonstrated that the LA:DGLA ratio may be a potential Zn biomarker, as the LA:DGLA ratio was significantly elevated in the Zn-deficient group compared to the Zn-sufficient group (Reed *et al.*, 2014). In humans, researchers confirmed that the LA:DGLA ratio responded to dietary Zn manipulations as the LA:DGLA ratio was increased in people with lower dietary Zn intake (Knez *et al.*, 2016). The literature review was able to illustrate that there are interactions that are present between Zn and FA as well as the proposed mechanisms by which the two interact. The majority of the studies were conducted in Zn-deficient rats. Compared to either pair-fed rats and/or control rats, it was found that the LA composition/concentration of either plasma or RBC was increased and the ARA composition reduced in Zn-deficient rats. In studies that measured the activity of desaturase enzymes, Zn deficiency was found to reduce the activity of delta -5 and -6 desaturase enzymes. These results are, however, inconsistent and further research should be conducted to ascertain the mechanisms involved between Zn and FA. Despite Zn deficiency and inadequate FA status being a public health concern, there are limited

data on Zn and FA interactions in humans. Furthermore, of concern is the scarce information on the FA status of children in Africa.

4.3 Baseline associations of Zn status and plasma total phospholipid FA composition

This study set out to evaluate if there were associations that existed between plasma Zn and plasma total phospholipid FA composition, and whether the LA:DGLA ratio can be used as a Zn status marker. In Beninese school children at baseline plasma Zn strongly correlated with DGLA:LA and DGLA ratios. These results support what has been reported by Knez *et al.* and Reed *et al.* as well as the notion that Zn acts as a cofactor in desaturase and elongase enzymes. Furthermore, these results support the use of the DGLA:LA ratio as a biomarker, as it encompasses both delta-6 desaturase activity as well as elongase activity. Subgroup analyses found that at baseline boys had lower plasma total phospholipid compositions for LA, adrenic acid and ARA:LA ratio than girls. At baseline there was a significant difference between the FA composition of Zn-sufficient and Zn-deficient boys. Zn-sufficient boys had a higher plasma total phospholipid composition for GLA and tended to have a higher DGLA:GLA ratio than Zn-deficient boys.

4.4 Effect of Zn fortification on plasma total phospholipid FA composition

In this study we tested if increased dietary Zn in the form of Zn-fortified water actually had an effect on FA status. This study demonstrated that in 6-10-y Beninese children with a high prevalence of Zn deficiency, Zn water fortification did have an effect to some extent on the plasma total phospholipid FA composition. Contrary to results from previous research, that found an effect on the n-6 LCPUFA composition of Zn-deficient rats, we found that there was a strong intervention effect on the n-9 long-chain monounsaturated FA, nervonic acid, as its composition was increased in the Zn+filter group when compared against the filter group. Furthermore, Zn fortification showed a trend towards reducing ALA. In the Zn-deficient children, nervonic acid was also significantly higher in the intervention than in the control group. Additionally, the ALA composition was reduced in the Zn+filter group as compared to the filter group, indicating that the desaturase and elongase enzyme activity may be improved in the intervention group. There were, however, no differences in the intervention effects between boys and girls.

4.5 Proposed mechanism of enzyme activity

The proposed molecular interaction that Zn affects delta-6 and delta-5 desaturase activity as well as that of elongase enzymes, was supported by our results. The plasma total phospholipid composition of LA and ALA showed a trend to increase over time in both the Zn+filter and filter

groups but more so in the filter group. This effect became significant for LA in the filter group, indicating an impaired delta-6 desaturase enzyme activity in the control group.

Our results mostly favoured the n-9 FA, nervonic acid, indicating that the activity of elongase also to be involved. As a result we suggest that microsomal Zn levels directly affect the electron transfer system from NADP or NADPH. Thus, inhibiting the terminal desaturase reaction (Cunnane, 1988), as well as the FA elongation reaction that utilises NADPH as reductant (Coupland, 1999). The estimated desaturase as well as elongase activities were determined from the product-precursor ratios; generally we found an impaired desaturase and or elongase activity in the filter (control) group as well as in the Zn-deficient children at baseline.

4.6 Limitations of the research project

1. The major limitation of this study was the lack of dietary FA intake data, especially with the baseline (cross-sectional) investigation. Dietary intake is known to correlate with FA plasma composition (Hodge *et al.*, 2007) and should be put into consideration when investigating the effect of Zn supplementation/ fortification on essential FA metabolism. As a result we were also unable to explore dietary factors that are associated with FA status.
2. Plasma as a FA biomarker is not as sensitive to, and as reflective of, long term dietary interventions as the other blood or tissue pools such as RBC or adipose tissue. Plasma FA composition reflects the last 1–2 weeks of diet, while red blood cell FA reflect the last 1–2 months and adipose tissue FA, the previous years (Katan *et al.*, 1997; Arab, 2003).
3. The use of gas chromatography with a flame ionisation detector (GCFID) resulted in us not being able to assess some FA, as detection of status markers such as n-6 DPA and mead acid was not possible. Chromatographic-mass spectral (GC-MS) assays of FA methyl esters (FAME) is more sensitive (Ren *et al.*, 2013). It is recognised that GC-MS is more selective than GCFID making it more suitable for determination of complex biological samples such as blood (Dodds *et al.*, 2005).
4. The maintenance of plasma Zn at baseline values in the parent study throughout its duration might have resulted in a small intervention effect in this sub-study. The Zn status of Beninese children was maintained at baseline concentrations throughout the intervention (Galetti *et al.*, 2015).
5. The study might not have been powered enough to demonstrate the effect of the intervention as well as Zn deficiency on the rate limiting delta-6 desaturation step that is involved in the biosynthesis of LA to GLA as well as the elongase step that is involved in the synthesis of GLA to DGLA. The subjects in the Zn-deficient groups in the Zn+filter and filter groups for GLA, and the ratio of GLA:LA and DGLA:GLA ratio had below 30 subjects that had their FA composition analysed.

4.7 Future research

As observed by Reed *et al.* the physiology of membrane FA accumulation in relation to Zn homeostasis has been the subject of little investigation in literature, even though Zn is thought to play a crucial role as a cofactor for desaturase enzymes (Reed *et al.*, 2014). In view of results obtained from this study, the following prospective studies could address gaps found in literature

1. Most of the studies that found an effect were supplementation studies that utilised higher Zn concentrations (>10mg/kg) than those used in the Zn+filter group. Therefore, future work should intervene with higher concentrations of Zn in the water as this might be able to cause a more pronounced effect on plasma Zn and thereby FA.
2. More studies are required to elucidate the mechanisms by which Zn and FA interact. Because hepatic desaturase activity cannot be determined in humans due to ethical and practical reasons and can only be measured indirectly through product to precursor ratios, we propose that to determine fully the sensitivity of FA to Zn interventions, a kinetic study could be conducted to understand best the underlying mechanisms that occur during desaturase and elongase activity in humans. The enzyme activity can be measured by use of a radiochemical method that utilises reverse-phase high performance liquid chromatography (HPLC). Additionally, instead of using FA total composition to determine the content of blood we suggest that the absolute concentrations of the FA should be used, as it may be more sensitive.
3. To understand the manner in which Zn affects FA composition fully, factors that affect the conversion of EFA into their long chain derivatives such as genetic factors should be assessed as they are thought to affect FA composition. African children from rural Limpopo, South Africa who had the lowest intake of omega-3 and omega-6 FA presented the highest RBC DHA and ARA percentages (Ford *et al.*, 2016). For this reason, the difference in capacity between different populations to synthesise LCPUFA and the supposed genetic advantage that people from an African descent should also be assessed, specifically investigating the FA desaturase (FADS) and ELOVL2 genes.
4. We also recommend that future studies should use 24 h recalls as well as quantified food frequency questionnaires to assess habitual dietary intake. Additionally, to assess the FA composition of individuals we propose the use of RBC as they are known to be a more sensitive FA biomarker than plasma.

Interactions with other micronutrients have to be taken into account when food fortification or supplementation programmes are initiated, especially when directed to population groups with

high micronutrient requirements (Sandström, 2001). This is because the prevalence of malnutrition (protein–energy malnutrition and micronutrient) in developing countries is high (Müller & Krawinkel, 2005). Although there is evidence that African populations are genetically advantaged as far as desaturation and elongation of FA are concerned, other deficiencies such as Zn, may be detrimental to this advantage (Ford *et al.*, 2016). An increase in Zn concentration might be able to increase the effectiveness of a Zn intervention on Zn and FA status but might be detrimental to the micronutrient status of individuals. Higher dosages of Zn may cause negative nutrient-nutrient interactions. A high Zn dosage causes reduced copper and iron concentrations in the body (Sandström, 2001). Therefore, single supplementation strategies may not be effective in determining the interaction and/or the mechanism present. Therefore, it will be beneficial for future research to intervene with FA and Zn, alone and in combination. This will have the advantage that it can reduce Zn deficiency prevalence; as well improve Zn and LCPUFA status, while being able to assess interactions with each other.

4.8 Public health relevance

Nutritional deficiencies rarely occur as single moieties. These deficiencies can also influence other nutrients such as the case of Zn deficiency which influences FA composition by impairing desaturation and elongation. From this study nutritional strategies will be influenced by gaining knowledge on the impact of fortification of single nutrients on other micronutrient systems, such as FA composition.

4.9 Strengths of study

Little has been reported on the FA status of children in developing countries in Africa. This study was able to provide data on the plasma total phospholipid FA composition of Beninese children. The strength of this study also comes from the fact that, to our knowledge no other study has looked at the associations between Zn status and FA in humans using continuous data. Furthermore, the effect of Zn fortification on FA composition in humans to our knowledge has not been explored before. Most of the studies assessing the presence of an interaction between Zn and FA were conducted in animals under controlled conditions. In this study group allocation was randomised, and at baseline plasma Zn did not differ between the groups. Factors known to affect the quality of our results such as baseline FA composition as well as BMI and sex were taken into account during analysis. This study also provided evidence that consumption of Zn-fortified water at low Zn dosages is an effective strategy to maintaining higher longer chain FA compositions compared to controls. The results also provide additional supporting evidence that plasma total phospholipids can respond to Zn fortification.

4.10 Recommendations

The following recommendations are proposed based on our main findings, in order to develop an understanding of interactions between Zn and FA composition, and how to use this knowledge in improving micronutrient status and thereby also public health.

1. Although large national nutrition surveys have been conducted among African children, there is paucity of data concerning the FA status of children in particular. Therefore, more research in African populations should be conducted.
2. As the mechanisms that are involved in FA and Zn synthesis are not fully understood, additional research should be carried out to improve the understanding of these relationships.
3. Providing children with water fortified with low bioavailable Zn has a beneficial effect on plasma Zn concentrations (Galetti, 2014) as well as plasma total phospholipid FA composition, which may be an effective and economic strategy over the long term.

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ANNEXURE 1: ETHICAL APPROVAL FROM THE NORTH-WEST UNIVERSITY



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Institutional Research Ethics Regulatory Committee

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ETHICS APPROVAL CERTIFICATE OF PROJECT

Based on approval by **Health Research Ethics Committee (HREC)**, the North-West University Institutional Research Ethics Regulatory Committee (NWU-IRERC) hereby approves your project as indicated below. This implies that the NWU-IRERC grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

Project title: EFFECTS OF ZINC FORTIFICATION ON THE PLASMA FATTY ACID COMPOSITION OF BENINESE SCHOOL CHILDREN: A RANDOMIZED, DOUBLE-BLIND CONTROLLED TRIAL.																													
Project Leader: Ms L Malan																													
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Institution			Project Number					Year		Status																			
Approval date: 2015-08-11	Expiry date: 2016-12-31																												
Category: N/A																													

Special conditions of the approval (if any): None

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- The project leader (principle investigator) must report in the prescribed format to the NWU-IRERC:
 - annually (or as otherwise requested) on the progress of the project,
 - without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.
- The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-IRERC. Would there be deviations from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.
- The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-IRERC and new approval received before or on the expiry date.
- In the interest of ethical responsibility the NWU-IRERC retains the right to:
 - request access to any information or data at any time during the course or after completion of the project;
 - withdraw or postpone approval if:
 - any unethical principles or practices of the project are revealed or suspected,
 - it becomes apparent that any relevant information was withheld from the NWU-IRERC or that information has been false or misrepresented,
 - the required annual report and reporting of adverse events was not done timely and accurately,
 - new institutional rules, national legislation or international conventions deem it necessary.

The IRERC would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the IRERC for any further enquiries or requests for assistance.

Yours sincerely

Linda du Plessis
Digitally signed by Linda du Plessis
 DN: cn=Linda du Plessis, o=NWU,
 ou=Vaal Triangle Campus,
 email=linda.duplessis@nwu.ac.za,
 c=ZA
 Date: 2015.08.11 12:57:34 +0200

Prof Linda du Plessis
 Chair NWU Institutional Research Ethics Regulatory Committee (IRERC)

ANNEXURE 2: MOU BETWEEN NWU AND MRC



COLLABORATION AGREEMENT

ENTERED INTO BY AND BETWEEN:

1. PARTIES

The **NORTH-WEST UNIVERSITY**, a public higher education institution and a juristic person established in terms of the Higher Education Act No. 101 of 1997 represented by Prof Frederik van Niekerk, duly authorised thereto by a Schedule of Authority by the Council.

From: Office of the Institutional Registrar, Building C1, 53 Borchard Street, Potchefstroom, 2522

(hereinafter "NWU")

and

SOUTH AFRICAN MEDICAL RESEARCH COUNCIL

herein represented by Prof Glenda Elisabeth Gray, in her capacity as President of the Council, duly authorized thereto.

From: Office of the President, SAMRC, Francie van Zyl Drive, PAROW VALLEY, 7505

(hereinafter "the Collaborator")





PREAMBLE

- A. The Parties want to collaborate with the aim of furthering education, applied scientific and technological research, capacity building and community involvement in the two institutions;
- B. The Parties recognize the mutually beneficial interdependencies of collaboration established to render a service, to educate and train students, and to undertake research, and commit themselves to the continuation and strengthening of this collaboration;
- C. The Parties wish to combine their respective expertise and resources to establish an association of excellence and, as a joint effort, to make available accredited and professional training and development programs, research and consultation capacity and related activities; and
- D. This Agreement between the Parties is intended specifically for the collaboration between the Parties pertaining to the total phospholipid fatty acid analysis to be performed by the Non-Communicable Disease Research Unit (NCDRU) of the collaborator, in accordance with the attached Project Schedule, marked "A" and any extension thereof as may be agreed upon in writing.

NOW THEREFORE the parties wish to record in writing the agreement they have concluded:

1. INTERPRETATION / DEFINITIONS

In this Agreement, unless inconsistent with or otherwise indicated by the context –

- 1.1. **"Agreement"** means the collaboration agreement contained in this document and any written amendments made thereto from time to time, and will including any schedules attached hereto;
- 1.2. **"Background Intellectual Property"** means Material, Know-How and Intellectual Property Rights that are made available for the purposes of a Project by either Party, but that can be demonstrated by such Party not to have arisen in the course or as a result of the conduct of a Project under this Agreement, including those as may be specified in Schedule A hereto;
- 1.3. **"Commercialization"** means commercial exploitation of the Research Products and/or the Intellectual Property Rights pertaining to the Research Products, for monetary or non-monetary consideration, including through the lease, assignment, sale or licensing thereof, and "Commercialize" shall have a corresponding meaning;
- 1.4. **"Confidential Information"** means any information of a confidential nature, which has been, or may be obtained directly or indirectly by one Party hereto (the "Receiving Party") from the other Party hereto (the "Disclosing Party"), whether in writing or in electronic format, or pursuant to discussions held between the Parties, or which is obtained by the Receiving Party through examination, testing, visual inspection or analyses, including, without limitation all information pertaining to a Disclosing Party's Background Intellectual Property and the Research Products, including the properties, characteristics, content and composition thereof and the logic, coherence and methods of use or implementation thereof, as well as all materials, technologies, inventions, information relating to a Disclosing Party's past, present

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- and future research and development or to a Disclosing Party's trade secrets, business activities, opportunities, products, services, clients, or suppliers, or to a Disclosing Party's Know-How, as well as the terms and conditions of this Agreement, any information identified as confidential, and any other material which contain or otherwise reflect, or are generated or derived from any such information as is specified in this definition;
- 1.5. **"Contract Period"** means a period specified as such in Schedule A hereto commencing on the Start Date;
 - 1.6. **"Effective Date"** means the date specified in Schedule A of this Agreement and failing such specification, the date of signature of this Agreement by the Party signing last;
 - 1.7. **"Improvement"** means any derivative version of or improvement, enhancement, alteration or modification to or of Background Intellectual Property wholly or partly developed pursuant to or in the course of the Project;
 - 1.8. **"Intellectual Property"** means any and all intellectual property rights, including without limitation, patents, plant breeders' rights, design rights, trade marks (whether registered or otherwise), copyright, performers' rights and rights to claim something as a Party's Confidential Information, including all similar and related rights in other jurisdictions, that grant similar rights as the foregoing, and the right to apply for registration of any of the foregoing;
 - 1.9. **"IPR Act"** means the Intellectual Property Rights from Publicly Financed Research and Development Act, Act 51 of 2008;
 - 1.10. **"Know-How"** means any and all concepts, ideas, methods, methodologies, procedures, processes, formulae, techniques, models (including, without limitation, function, process, system and data models), templates, designs, utilities and routines, and logic, coherence, properties, composition and methods of operation and manipulation of materials, systems, structures or processes, which is not generally known or readily ascertainable and which a Party has created, acquired or otherwise has rights in;
 - 1.11. **"Material"** means any material or substance (including of natural, synthetic, biological or chemical nature) including in particular as made available by one Party to the other Party pursuant to this Agreement or arises in the course of a Project;
 - 1.12. **"NIPMO"** means the National Intellectual Property Management Office, a function managed within the Department of Science and Technology or any of its instruments or entities, as established in terms of the IPR Act;
 - 1.13. **"the Parties"** mean the Collaborator and North-West University;
 - 1.14. **"Personnel"** means any employee, contractor, representative or student of an institution;
 - 1.15. **"the Project(s)"** means the collaborative research work / services to be undertaken by the Parties as described in the Schedules hereto;
 - 1.16. **"Researchers"** means the members of the Personnel of each Party responsible for conducting the research under a Project, including as may be identified in the Schedules hereto;
 - 1.17. **"Research Facilities"** means the locations at which each Project will be performed by the Parties as described in the relevant Schedule hereto;

- 1.18. **"Research Product"** means any new process or product arising within the scope of a Project and any derivative forms thereof, including without limitation, any Improvements and modifications of the Background Intellectual Property, reports, results, data, software, documentation, processes, inventions, techniques, methods, new genetic sequences and any new Material so arising;
- 1.19. Where any term is defined within the context of any particular clause in this agreement, the term so defined, unless it is clear from the clause in question that the term so defined has limited application to the relevant clause, shall bear the meaning ascribed to it for all purposes in terms of this agreement, notwithstanding that the term has not been defined in this interpretation clause.
- 1.20. Any reference to writing shall include any modes of reproducing any words in a legible and non-transitory form, including e-mail that shall be deemed to have been received on the date of e-mail confirmation by the other party in person.
- 1.21. The terms of this agreement having been negotiated, the *contra proferentem* rule shall not be applied in the interpretation of this agreement.
- 1.22. Unless specifically otherwise provided, any consent, approval or agreement to be provided by a Party in terms of this Agreement may not be unreasonably withheld, delayed or conditioned.

2. STRUCTURE

This Agreement between the Parties is intended to lead to specific collaborative research work / services between the Parties in the research field and Project defined in Schedule A hereto.

3. DURATION

- 3.1. This agreement will commence on the Start Date and endure for the Contract Period, subject to provisions that provide for its earlier termination, and provided further that the clauses relating to ownership of intellectual property, confidentiality, disclaimers and liability and arbitration will survive any termination of this Agreement. All rights that have already accrued as at the date of termination will remain unaffected.

4. ROLES AND RESPONSIBILITIES OF THE PARTIES

With respect to the Project the Parties shall jointly have the following specific functions listed under "Specific Research Responsibilities" in the Schedule as well as "General Research Responsibilities" listed hereunder:

4.1 "Specific Research Responsibilities"

The primary responsibilities in respect of the execution of the Project (if anything more than already contained in the general responsibilities as set out hereunder) are agreed to in Schedule A.

4.2 "General Research Responsibilities"

The institutions will jointly as part of the execution of the Project in a pre-agreed mutual manner:

- Improve mechanisms for collaborative training and development programs, research and consultation capacity and related activities;
- Develop a system that would enable students to participate in training and development programs, research and consultation capacity and related activities.
- Develop a system that would enable the associated staff and students of the parties to work within each other's research facilities on a continuing or intermittent basis;
- Work jointly and in partnership to identify fields of research that could further the interests of the Parties, as well as the broader community in order to meet the aims and objectives of this Agreement. This will include the identification of research agendas and the obtaining of funding;
- Create mechanisms for sharing resources, data and environmental assessment information, integrated geographic information systems, *etcetera*. Maintain joint support on specific resource applications of information management systems;
- Jointly foster support for national programs and international obligations;
- Work jointly and in partnership to investigate the feasibility of presenting the publication of papers or conference proceedings.
- The Parties shall commence the performance of their work under a Project after signature of the Schedule, and shall all reasonable efforts to perform such work substantially in accordance with the terms and conditions of such Schedule.
- Either Party may terminate the research conducted by it pursuant to a Schedule by giving notice thereto in the event of one or more of its personnel involved in the Project become unavailable to continue the research.
- The Parties shall do all acts and sign all documents as may be required from time to time in order to implement and carry out the terms and conditions of this agreement.
- The Parties shall immediately disclose to one another in writing all new information in their possession or under their care relating to the work being conducted, provided that such new information must have been developed during the course of the work relating to the relevant Project.
- If necessary, during the term of a Project, representatives of NWU will meet with representatives of the Collaborator at times and places mutually agreed upon to discuss the progress and results, as well as ongoing plans, or changes therein, of the work to be performed.

5. FINANCES

It is recorded that, save for the specific fees and payments agreed in the Schedule, there shall be no additional consideration payable by either Party for the performance of work by the other Party under the Project referred to in clause 4 and each Party shall be responsible for procuring its own funding and paying its own costs incurred in respect of such Projects.

6. SUPPORT

6.1. The Parties undertake at all times to do all such things, to perform all such acts and to take all such steps and to procure the doing of all such things, the performance of all such actions and the taking of all such steps as may be open to them and necessary for or incidental to the putting into effect or maintenance of the terms, conditions and import of this agreement and furthermore to act in good faith towards each other and to co-operate with each other to the fullest extent.

6.2. The Parties recognise that it is impracticable to provide for every contingency which may arise during the period of this agreement, and shall use their best endeavours to agree upon means of alleviating any hardship or detriment which may be caused to the parties by circumstances at present unforeseen.

7. AGENCY

7.1. No Party shall present itself as the representative or agent of the other Party for any purpose nor shall it have the power of authority to commit the other Party to any obligation, unless it receives the other Party's prior written consent.

7.2. Nothing in this agreement shall be interpreted as establishing a partnership or joint venture between the Parties.

8. NON-EXCLUSIVITY

Nothing contained in this agreement shall be construed as binding the Parties to any form of exclusivity. Both Parties shall be entitled to conduct business independent of each other and to pursue any work on its own where market requirements so dictate, unless otherwise agreed upon in writing and signed by the duly authorised representatives of the Parties.

9. PUBLICITY

Neither Party will use any trade mark nor name of the other Party or of the other Party's Personnel for advertising or marketing purposes or make any press release or other publication regarding the Work without the prior written approval of the other Party.

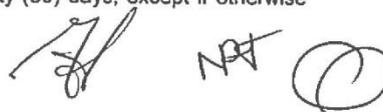
10. PUBLICATIONS

10.1. In the event that a Parties' institutional policies requires that results obtained by either Party must be publishable, then in such an event each Party agrees that the other Parties' researchers shall be permitted to present and to publish the methods and results of the Project at symposia and professional meetings, in journals, theses or dissertations, or otherwise of their own choosing, subject to the provisions of this Article. With respect to said publications, the Parties shall agree upon appropriate co-authorship in accordance with academic customs.

10.2. Each Party shall submit to the other Party all intentions to publish or to otherwise disclose the methods and results of the Project. The receiving Party shall report in writing within thirty (30) days if such Party is of the opinion that its commercial interest is likely to be prejudiced by the aforementioned publication or disclosure. In case of this prejudice, each Party may request to delay the publication or disclosure and may request to omit Confidential Information of such Party and/or to delay the publication or disclosure. Such delay shall sufficiently be motivated in writing and shall not exceed thirty (30) days, except if otherwise agreed between the Parties.

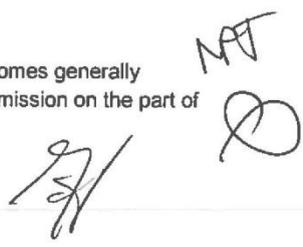
11. CONFIDENTIALITY

11.1 The Parties recognize that in their dealings with one another, governed by the terms of this



Agreement, they may each receive Confidential Information relating to the other Party.

- 11.2 Each Party agrees that it shall take all reasonable steps to protect Confidential Information against any unauthorized or improper access or use.
- 11.3 Neither Party shall grant access to any Confidential Information received from the other Party to any of its personnel save to the extent that access to such Confidential Information is required for the performance of a Party's obligations in terms of this Agreement.
- 11.4 Each Party shall ensure that such of its members of staff, consultants, advisors or contractors who have been granted access to the Confidential Information for execution of the Project, enter into personal confidentiality agreements prior to access being granted to the Disclosing Party's Confidential Information.
- 11.5 Neither Party shall grant access to any Confidential Information received from the Disclosing Party to any third party without the prior written consent of the Disclosing Party, unless the Receiving Party is legally obliged to make disclosure of such Confidential Information without obtaining the prior written consent of the Disclosing Party. The Disclosing Party will nevertheless be notified of such disclosures.
- 11.6 The Parties acknowledge the provisions of sections 65 and 71 of the Promotion of Access to Information Act 2000 (Act 2 of 2000) ("the PAIA Act"), and Section 4 (prohibition of disclosure of certain information) of the Protection of Information Act, 1982 (Act 84 of 1982) ("the PIA Act").
- 11.7 If a third party requests access to any Confidential Information which is subject to any one of the provisions of this Agreement, regard shall be had to the obligation of the Party of whom the request is made in terms of the PAIA Act, to:
 - 11.7.1 promptly notify the Disclosing Party of the request; and to exercise the mandatory right of refusal to grant access to the Confidential Information, the disclosure of which would constitute a breach of the duty of confidence owed by the Parties to one another in terms of this Agreement or in terms of the PAIA Act.
 - 11.8 In the event of one Party visiting any of the establishments of the other Party, the visiting Party undertakes that any further information relating to the Project which may come to the visiting Party's knowledge as a result of any such visit, inclusive of the form, materials and design of the various elements of any product, item and equipment which may be seen at such establishments, the methods of operation thereof and the various applications thereof, shall be kept strictly confidential and that any such information will not be divulged to any third party and will not be made use of in any way by the visiting Party without the other Party's prior written consent.
- 11.2. The above undertakings shall not apply to information which:
 - 11.2.1. can be established by documentation was, at the time of disclosure, published or otherwise generally available to the public, otherwise than through any act or omission on the part of the Receiving Party;
 - 11.2.2. after disclosure by the Disclosing Party is published or becomes generally available to the public, otherwise than through any act or omission on the part of the receiving Party;



- 11.2.3. the Receiving Party can establish by documentation was in its possession at the time of disclosure and which was not acquired directly or indirectly from the Disclosing Party;
- 11.2.4. has been rightfully acquired from others who did not obtain it under pledge of secrecy to the Disclosing Party;
- 11.2.5. has been independently developed by the Receiving Party without the use of information from the Disclosing Party;
- 11.2.6. has been approved for release by a written authorisation of the Disclosing Party.
- 11.3. The terms of this Agreement shall be deemed to apply also to the servants and agents of the Receiving Party who shall require its said servants and agents to observe the foregoing obligations.
- 11.4. The Parties' obligation of confidentiality or that of any person acting under the direction or control of the respective Parties shall endure for a period of 5 (Five) years after expiration or prior termination of this Agreement, subject thereto that this period can be extended or shortened by a written and signed agreement between the Parties.

12. INTELLECTUAL PROPERTY (IP)

12.1. Background IP

- 12.1.1. The Parties may make certain Background Intellectual Property available in order to facilitate and promote the research work being conducted under a Project, including as may be stipulated in the relevant Schedule. Each Party agrees to treat such Background Intellectual Property of the other Party as the other Party's Confidential Information pursuant to Clause Error! Reference source not found. hereof.
- 12.1.2. Each Party ("the Licensor") hereby grants the other Party ("the Licensee") a non-exclusive non-transferable license to use the Background Intellectual Property made available by the Licensor to the Licensee solely for the purposes of performing the research work under the relevant Project and evaluating the Research Products arising from such Project. The Licensee may also be permitted by the Licensor to use and/or sub-license such Background Intellectual Property for other research and educational purposes and/or in connection with the Commercialisation of the Research Products, provided it has obtained the Licensor's prior written consent thereto. The Licensor agrees to negotiate the terms of such consent in good faith upon request by the Licensee.
- 12.1.3. No Licensee shall, and shall not permit, whether directly or indirectly, any third party, except as expressly permitted in writing by the Licensor to distribute, use, rent, lease, share, sell, assign, sub-license or otherwise transfer any part of the Licensor's Background Intellectual Property or Licensee's rights to use the Background Intellectual Property.
- 12.1.4. Licensee acknowledges and agrees that notwithstanding delivery of any Background intellectual Property to it by the Licensor, all Background Intellectual Property will remain and constitute the sole and exclusive property of the Licensor that first provided it. Such Licensor shall retain full right, title and interest in and to all of its Background Intellectual Property (including all Intellectual Property Rights therein) and, save for the limited licences granted in Clause 12.1.2 hereof, the Licensee shall not obtain any rights in respect of any of the Background Intellectual Property

pursuant to this Agreement. The Licensee undertakes that it shall not in any manner whatsoever represent that it is the owner or has any interest, other than as licensee in terms hereof, in the Licensor's Background Intellectual Property.

12.2. Research Products

- 12.2.1. Each Party shall upon request provide the other Party with a written progress report in respect of the research conducted by it under a Project, detailing all the Research Products developed. Upon request by either Party, the other Party shall provide it with samples of the Research Products developed by it and with such information pertaining thereto as may be reasonably requested.
- 12.2.2. Intellectual Property Rights resulting directly from, or developed in the course of the Project, shall vest in the Party responsible for the creation thereof and such Party shall, subject to clauses 12.2.3 and 12.2.4, be solely entitled to file an application for the registration of a patent, utility model, design or any similar Intellectual Property Right in respect thereof.
- 12.2.3. If a Party wholly or partly creates any Improvement to the Background Intellectual Property of the other Party, such Party shall promptly inform the other Party of such Improvement. No Party may register or apply for the registration of any Intellectual Property Right (including without limitation under patent, utility model or design) with respect to any such Improvement without the prior written consent of the other Party.
- 12.2.4. Joint ownership of Project Intellectual Property shall be established in the case of joint creation or inventorship of Research Products. All Intellectual Property Rights that may vest in such Research Products shall vest in the Parties jointly and equally unless otherwise agreed in writing. The Parties shall negotiate in good faith and use reasonable efforts to agree in writing on the proportion of ownership to be allocated to each Party taking into consideration each Party's contribution to the creation of the relevant Research Products. Neither Party will cede, assign or transfer any of its rights, title or interest pertaining to such Research Products without the consent of the other Party. Subject to 12.2.3, each Party will be entitled to take such steps as may be necessary to maintain, enforce and protect the Intellectual Property Rights in such jointly owned Research Products and may prosecute, maintain and defend such Intellectual Property Rights, including by applying for the registration of such Intellectual Property Rights and enforcing such Intellectual Property Rights once registered, provided that it gives the other Party reasonable advance notice of any such actions and reasonable opportunity to protect its own rights and interests pertaining to such Research Products. The other Party will provide such assistance in this regard as may reasonably be required, including by procuring that its Personnel who have contributed to the Research Products fulfill all required formalities, provide suitable Material for deposit and execute all required documents for the filing, prosecution, maintenance and defense of the relevant Intellectual Property Rights.
- 12.2.5. Each Party shall be entitled to use and permit the use of any of the Research Products for academic research and other non-profit or scholarly purposes which are undertaken at an academic, non-profit or governmental institution anywhere in the world. Neither Party may use or exploit for any other purposes (including for Commercialization purposes) any Research Product that is wholly or partly owned by the other Party or any derivative form thereof, nor permit, enable or purport to authorize any third party to do so, unless authorized in advance in writing by the other Party. Neither Party will unreasonably withhold its authorization in this regard.

13. COMMERCIALISATION

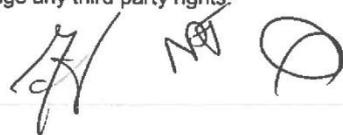
- 13.1. The Parties will notify each other in writing of any Commercialization opportunity for the Intellectual Property Rights in the Research Products. Upon request by either Party, the Parties will negotiate in good faith regarding their respective contributions to such Commercialization and sharing of revenue arising from such Commercialization. The collaborator shall be given the right of first refusal to receive a license to commercialize all jointly owned Research Products on behalf of the Parties and shall be entitled to recover its costs incurred in this regard prior to the distribution of revenue shares.
- 13.2. Unless otherwise agreed in writing, the Parties shall report to each other quarterly, on the revenue received in connection with the Commercialisation of the Research Products during the preceding year and pay over to the other Party its share of the revenue that may be due.
- 13.3. Each Party shall ensure that complete and suitably detailed written records of the data pertaining to Commercialisation are kept and, upon request by the other Party shall provide to the other Party and/or its auditors (including internal audit staff and external auditors) access to such records at all reasonable times to verify the accuracy of the revenue reports provided and revenue shares paid.

14. MATERIAL

- 14.1. Each Party is hereby granted a non-transferable, non-exclusive right to use the Materials (and the information pertaining to such Materials that is made available to it by the other Party) solely in the pursuit of the Project for which it is provided. Save as aforesaid all rights are reserved and neither Party shall use or permit the use of the Material provided by the other Party for any other purpose, save with the other Party's prior written consent.
- 14.2. Each Party acknowledges and agrees that notwithstanding delivery of Material to it by the other Party, all Material will remain and constitute the sole and exclusive property (including all Intellectual Property Rights therein) of the Party from which it originates.
- 14.3. Upon request by a Party or termination of a Project, each Party will dispose of the Material of the other Party made available for such Project as reasonably directed by the other Party and shall certify such disposal upon request.
- 14.4. Each Party shall comply with applicable Law in handling the Material, including as may be applicable to the import and export, transport, use, storage and disposal of the Material.
- 14.5. Each Party acknowledges that the Material provided to it may have defects or hazardous properties and are provided without warranty of any kind, whether express or implied. Use of Material will be at the sole and exclusive risk of the user.

15. DISCLAIMERS

- 15.1. The Parties make no representations and provides no warranties of any kind, either expressed or implied by Law, with respect to the execution of a Project, Background Intellectual Property or Research Products. In particular, each Party hereby excludes and disclaims any express or implied warranty of reasonable quality, merchantability or fitness for a particular purpose, that the Background Intellectual Property and Research Products provided by it will be free from defects (latent or otherwise), or that use of the Background Intellectual Property and Research Products will not infringe any third party rights.

Handwritten initials and a circle, likely representing a signature or mark.

15.2. Use of the Background Intellectual Property and Research Products will be at the sole and exclusive risk of the Party using it, and each Party hereby indemnifies and agrees to hold the other Party and its Personnel harmless against any and all Losses that may arise in connection with such use of the other Party's Background Intellectual Property and Research Products.

15.3. In no event shall a Party or its Personnel be liable to the other Party for any punitive, indirect, incidental, extrinsic, special or consequential loss (whether foreseeable or unforeseeable) of any kind that may arise in connection with this Agreement, whether based on contract, delict, statute or otherwise, save to the extent that the limitation of liability contained herein is not permitted by applicable Law.

16. SEVERABILITY

Should any provision of this agreement in any manner whatsoever contravene any law of the Republic of South Africa, such provision shall be deemed to be severable and shall not affect any other provisions which are not in contravention of any law.

17. CESSION

Neither party shall be entitled without the prior written consent of the other party to cede, delegate, or otherwise transfer any of its rights or obligations in terms of this agreement which consent shall not be withheld unreasonably.

18. APPLICABLE LAW

This agreement is governed by the laws of and is subject to the jurisdiction of the Republic of South Africa, as constituted from time to time.

19. NOTICES AND DOMICILIA

19.1. The parties choose as their *domicilia citandi et executandi* ("Domicilium") for all purposes under this Agreement the physical addresses set out on the first page of this Agreement.

19.2. Any notice given in terms of this agreement shall be in writing and shall –

19.2.1. if delivered by hand be deemed to have been duly received by the addressee on the date of delivery;

19.2.2. if posted by prepaid registered post be deemed to have been received by the addressee on the 8th (eighth) day following the date of such posting;

19.2.3. if transmitted by facsimile be deemed to have been received by the addressee on the day following the date of dispatch, unless the contrary is proved.

19.2.4. if sent by e-mail deemed to have been received on the date of e-mail confirmation by the other party in person.

19.3. Notwithstanding anything to the contrary contained or implied in this agreement, a written notice or communication actually received by one of the parties from another including by way of facsimile transmission shall be adequate written notice or communication to such party.

20. BREACH AND CANCELLATION

20.1. In the event, subject to clause 18 of this agreement, of any of the parties ("the defaulting party") committing a material breach of any of the terms of this Agreement and failing to remedy such breach within a period of 10 (ten) days after receipt of a written notice from the

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other party ("the aggrieved party") calling upon the defaulting party so to remedy, then the aggrieved party shall be entitled, at its sole discretion and without prejudice to any of its other rights in law, either to claim specific performance of the terms of this agreement or to terminate this Agreement and/or any of the Schedules forthwith and without further notice, claim and recover damages from the defaulting party.

- 20.2. This Agreement may be terminated by a Party if the other Party takes steps to place itself or is placed in liquidation, whether voluntarily or compulsory, or in judicial management, in either case whether provisionally or finally, or takes steps to deregister itself or is deregistered.
- 20.3. Either Party may terminate this Agreement by giving 90 (ninety) days written notice thereto if the research agreed to be provided under the existing Schedules are completed or terminated.
- 20.4. Either Party may terminate any of the Schedules by giving 60 (sixty) days written notice to the other Party.

21. ADJUDICATION OF DISPUTES AND ARBITRATION

- 21.1 The Parties shall first of all solve any dispute between them by way of mediation and either Party to this Agreement may request that such dispute be referred to mediation by a single mediator. If the Parties agree to mediation then a mediator shall be selected by agreement between the Parties or, failing such agreement, be nominated by a mutually respected neutral party. The written opinion expressed by the mediator should be accepted by both Parties hereto unless and until otherwise ordered in arbitration proceedings referred to below. The mediator shall formulate his costs in respect of the mediation and these shall be borne equally by the Parties hereto.
- 21.2 Should the Parties fail to resolve any dispute in the manner stipulated in clause 21.1 above (other than a dispute in respect of which urgent relief may be obtained from a court of competent jurisdiction) that dispute shall be referred to and be determined by arbitration in terms of this clause, provided that a party to the dispute has demanded the arbitration by written notice to the other parties.
- 21.3 The arbitration shall be held at Potchefstroom, South Africa in terms of the Arbitration Act, No 42 of 1965, it being the intention that the arbitration shall be held and completed within 21 (twenty one) days after it was demanded.

22. WHOLE AGREEMENT

This agreement constitutes the whole agreement between the parties as to the subject matter hereof and no agreements, representations or warranties between the parties other than those set out herein are binding on the parties.

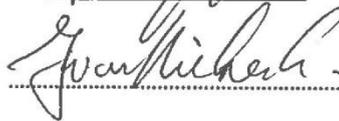
23. VARIATION

No addition to or variation, consensual cancellation or novation of this agreement and no waiver of any right arising from this agreement or its breach or termination shall be of any force or effect unless reduced to writing and signed by all the parties or their duly authorized representatives.

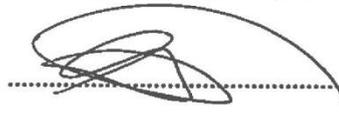
24. RELAXATION

No latitude, extension of time or other indulgence which may be given or allowed by any party to the other parties in respect of the performance of any obligation hereunder or the enforcement of any right arising from this agreement and no single or partial exercise of any right by any party shall under any circumstances be construed to be an implied consent by such party or operate as a waiver or a novation of, or otherwise affect any of that party's rights in terms of or arising from this agreement or estop such party from enforcing, at any time and without notice, strict and punctual compliance with each and every provision or term hereof.

Signed at Potchefstroom on this 16th day of September 2016.


.....(Signature of NWU)

Signed at Cape Town on this 12th day of Aug 2016.


.....(Signature for SAMRC)



ANNEXURES 3: ETHICAL APPROVAL FROM ETH ZURICH (SWISS GERMAN AND ENGLISH)



Eidgenössische Technische Hochschule Zürich
Swiss Federal Institute of Technology Zurich

Herr
Prof. Dr. Michael Bruce Zimmermann
LFV D 20
Schmelzbergstrasse 7
8092 Zürich

Zürich, 31.10.2012
EK 2012-N-47

Sehr geehrter Herr Zimmermann Ihr Gesuch
Vizepräsident für Forschung und Wirtschaftsbeziehungen

ETH Zürich, HG F 57
Rämistrasse 101
CH-8092 Zürich, Schweiz

Prof. Dr. Roland Siegwart
Tel.: +41 44 632 20 39 Fax: +41 44 632 15 92
roland.siegwart@sl.ethz.ch

Efficacy study of a water filter used as distribution system of zinc for primary schools children in municipality of Natitingou, North of Benin (24.09.2012)
wurde online durch die folgenden Mitglieder der Ethikkommission beurteilt:

Name	Institut	am Beschluss beteiligt		
		ja	nein (Grund)	
			abwesend	im Ausstand
Prof. Dr. Lutz Wingert, Präsident	Professur für Philosophie	X		
Dr. Ines Egli	Lebensm.wiss., Ern., Ges.	X		
Dr. Roberto La Marca	Psychologisches Institut	X		
Prof. Dr. Matthias Mahlmann	Rechtswissenschaftliches Institut UZH	X		
Dr. Marino Menozzi	Innovations- und Technologiemanagement	X		
Prof. Dr. Kurt Murer	Inst. f. Bewegungswiss. und Sport	X		
Dr. Kai-Uwe Schmitt	Inst. f. Biomedizinische Technik	X		

Aufgrund der Empfehlung der Ethikkommission der ETH Zürich ist die Schulleitung zu folgendem Beschluss gekommen:
Bewilligung

Bewilligung mit Vorbehalt (schriftliche Mitteilung an Ethikkommission ausreichend)
Rückweisung zur Überarbeitung mit Auflage
Schriftliche Mitteilung an Ethikkommission ausreichend
Neubegutachtung durch Ethikkommission notwendig
Negativ (mit Begründung und Erläuterung für Neubeurteilung)
Nicht-Eintreten (mit Begründung)
1 / 3

Vorbehalte

Stellungnahmen der Mitglieder der Ethikkommission zu Ihrem Gesuch:

1) Abstract

2) Projekt

- a) The investigators argue that a control group receiving unfiltered water is required for this study design. They mention that the microbiological quality of the unfiltered water will be monitored. Monitoring however, does not ensure the quality and investigators should refer to the cut-offs or standards which they will apply to ensure the water quality. A data safety monitoring board should be suggested for this study that would independently evaluate the data (e.g. diarrhea rates) during the study and could intervene in case problems.
- b) The control group should be better described in the protocol in chapter 2.3.1.
- c) In der Studie sollen die Kinder gefiltertes bzw. ungefiltertes Wasser trinken. Ist das die Regel vor Ort? Trinken die Kinder i.d.R. Wasser vom Schulbrunnen? Es sollte sichergestellt werden, dass kein Kind schlechteres Wasser bzw. Getränke zu sich nimmt, als es sonst trinken würde (wenn es die Studie nicht gebe). Dieser Punkt sollte v.a. bei der am schlechtesten gestellten Kontrollgruppe sichergestellt werden.
- d) Die Antragsteller sollten ein Cut-off-Kriterium festlegen, unter welchen Umständen die Studie abgebrochen wird bzw. unter welchen Vorkommnissen ihres Erachtens eine relevante Verschlechterung des Gesundheitszustandes der teilnehmenden Kindern vorliegt, welche ein Studienabbruch rechtfertigen würden.

3) Zu erwartende Risiken und entsprechende Vorsichtsmassnahmen

- a) The information about the average zinc intake should be clarified (see p. 14: "3.2 Zinc toxicity"). According to the WHO guideline the intake by adults should not exceed 45 mg/day. What is the estimated daily zinc intake by a six to ten year old child? (Laymen need help to read table 3 on page 14.)

4) Projektleiter/-in

-

5) Probanden/-innen

- a) As regards the blood analysis: "HIV/AIDS will be not diagnosed." (p. 14; also Annex I, p. II.) Does this imply that the analysis is not apt to diagnose HIV? If it were apt, what would be the policy by the research team? (E.g. the team would abstain from further data analysis or from enabling others to do the analysis etc.)
- b) Fiche de recrutement: Why are so detailed questions about personal data asked (schooling, religion, ethnic background, profession) - what is the connection to the study?

6) Informationsblatt für Probanden/-innen

- a) Der Hersteller des Filters, der die Studie finanziert sollte namentlich im Infoblatt erwähnt werden.
 - b) Financing of project by filter producer is mentioned in information sheet (french) but not in the project description.
 - c) Clarify what is the research question. In the third paragraph on page 1 is the statement that water with zinc will increase health. In the fourth paragraph it is said that this statement should be proofed. Is this the research question? Please clarify that this is the research question so participants do not get false impression about benefits of study.
 - d) P. III, second paragraph: participation gratuite - this creates potentially misleading impression that one normally even pays to become participant of such a study
- 2 / 3

7) Einverständniserklärung

-

8) Generell

- a) Under ethical considerations (chapter 6) the evaluation of the protocol by the local committee in Benin is mentioned. The comments and issues raised by this committee should be made available to the ETH committee.
- b) If the hypothesis should be confirmed (= the LSF filter were an efficient system of zinc distribution, p.8), it would be nice to ask engineers to look for a cheaper version of the device to make the system available for the ordinary people.

Wir machen Sie darauf aufmerksam, dass gegenüber der Ethikkommission der ETH Zürich in folgenden Situationen eine Meldepflicht besteht:

- a) Unverzüglich bei Auftreten von unerwarteten Ereignissen, welche die Sicherheit der Versuchspersonen und/oder die Weiterführung des Versuches beeinflussen können
- b) Bei Änderungen am Forschungsprotokoll und bei Versuchspersonen
- c) Bei Abbruch der Studie

Freundliche Grüsse

Prof. R. Siegwart
Vizepräsident für Forschung &
Ethikkommission
Wirtschaftsbeziehungen

Prof. L. Wingert
Vorsitzender der

cc: Departementsvorsteher HEST

Letter 1: Provisional clearance:

Prof Zimmermann

Your study,

Efficacy study of a water filter used as distribution system of zinc for primary schools children in municipality of Natitingou, North of Benin (24.09.2012), was judged online by the following members of the Ethics Committee:

		Decision		
		Yes	No(basic)	
			absent	excluded
Prof. Dr. Lutz Wingert, president	Professor in Philosophy	X		
Dr. Ines Egli,	Institute of Food Nutrition and Health	X		
Dr. Roberto La Marca	Psychological institute	X		
Prof. Dr. Matthias Mahlmann	Institute of Law	X		
Dr. Marino Menozzi	Innovations- and Technology management	X		
Prof. Dr. Kurt Murer	Swiss Institute for movement and Sport	X		
Dr. Kai-Uwe Schmitt	Institute for Biomedical Engineering	X		

Following the recommendation of the Ethics Committee of the ETH Zurich, the Executive Board came to the following decision:

Approval
 Approval with reservation (written notification to the Ethics Committee is sufficient)

- Rejection for revision with support
- Written notice to the Ethics Committee sufficiently
- Full resubmission required by the Ethics Committee
- Negative (with justification and explanation for Reassessment)
- Non-occurrence (with justification)

Reservations:

Opinions of the members of the Ethics Committee to your request

1) Abstract

-

2) Project

a) The investigators argue that a control group receiving unfiltered water is required for this study design. They mention that the microbiological quality of the unfiltered water will be monitored. Monitoring however, does not ensure the quality and investigators should refer to the cut-offs or standards which they will apply to ensure the water quality. A data safety monitoring board should be suggested for this study that would independently evaluate the data (e.g. diarrhea rates) during the study and could intervene in case problems.

b) The control group should be better described in the protocol in chapter 2.3.1.

c) In the study, the children should drink filtered or unfiltered water. Is that the rule as usual? Do the children usually drink water from the school well? It should be ensured that no child shall drink poorer water as usual. This point should be ensured in the control group, who is the worst-off.

d) Applicants should specify a cut-off criterion, under which circumstances the trial is terminated or under which events it considers that a relevant deterioration in the health of participating children, which would justify a study discontinuation.

3) Expected risks and appropriate precautions

a) The information about the average zinc intake should be clarified (see p. 14: "3.2 Zinc toxicity"). According to the WHO guideline the intake by adults should not exceed 45 mg/day. What is the estimated daily zinc intake by a six to ten year old child? (Laymen need help to read table 3 on page 14.)

4) Project Manager

-

5) Subjects

a) As regards the blood analysis: "HIV/AIDS will be not diagnosed." (p. 14; also Annex I, p. II.) Does this imply that the analysis is not apt to diagnose HIV? If it were apt, what would be the policy by the research team? (E.g. the team would abstain from further data analysis or from enabling others to do the analysis etc.)

b) Recruitment Sheet: Why are so detailed questions about personal data asked (schooling, religion, ethnic background, profession) - what is the connection to the study?

6) Information sheet for volunteers

a) The manufacturer of the filter, which funded the study should be mentioned by name in the info sheet.

b) Financing of project by filter producer is mentioned in information sheet (french) but not in the project description.

c) Clarify what is the research question. In the third paragraph on page 1 is the statement that water with zinc will increase health. In the fourth paragraph it is said that this statement should be proofed. Is this the research question? Please clarify that this is the research question so participants do not get false impression about benefits of study.

d) P. III, second paragraph: participation gratuite - this creates potentially misleading impression that one normally even pays to become participant of such a study

7) Declaration of Consent

-

8) General

a) Under ethical considerations (chapter 6) the evaluation of the protocol by the local committee in Benin is mentioned. The comments and issues raised by this committee should be made available to the ETH committee.

b) If the hypothesis should be confirmed (= the LSF filter were an efficient system of zinc distribution, p.8), it would be nice to ask engineers to look for a cheaper version of the device to make the system available for the ordinary people.

We remind you that in relation to the ethics committee of the ETH Zurich a reporting requirement in the following situations:

a) Immediately upon the occurrence of unexpected events that may affect the safety of the subjects and / or continuation of the experiment

b) Any modification to the research protocol and in subjects

c) For termination of the study

Best Regards
Prof. R. Siegwart
Vice President for Research &
Economic Relations

Prof. L. Wingert
Chairman of the Ethics Committee

Galetti Valeria

From: Iturrizaga Raffael (F&W)
Sent: Thursday, November 22, 2012 11:09 AM
To: Zimmermann Michael Bruce
Cc: Moretti Diego; Galetti Valeria
Subject: Vollständige Bewilligung zum Gesuch "Efficacy study of a water filter used as distribution system of zinc for primary schools children in municipality of Natitingou, North of Benin" (EK 2012-N-47)

Sehr geehrter Herr Zimmermann

Die Vorbehalte, die im Bescheid vom 31.10.2012 zu Ihrem Gesuch "Efficacy study of a water filter used as distribution system of zinc for primary schools children in municipality of Natitingou, North of Benin" (EK 2012-N-47) aufgeführt wurden, sind durch die Antworten und Anpassungen in den von Ihnen eingereichten Dokumenten vom 20.11.2012 ausgeräumt. In dieser Form ist das Gesuch von der Ethikkommission vollständig bewilligt.

Mit freundlichen Grüßen
Raffael Iturrizaga

ETH Zürich | Dr. Raffael Iturrizaga | Stab Wissenschaftskoordination | HG E 34.3 | Rämistr. 101
| CH-8092 Zürich | Phone: +41 44 632 2354 | Fax: +41 44 632 1184 | iturrizaga@sl.ethz.ch |
www.forschung.ethz.ch | Anwesend im Büro: Di - Do

Galetti Valeria

From: Iturrizaga Raffael (F&W)
Sent: Thursday, November 22, 2012 11:09 AM
To: Zimmermann Michael Bruce
Cc: Moretti Diego; Galetti Valeria
Subject: Vollständige Bewilligung zum Gesuch "Efficacy study of a water filter used as distribution system of zinc for primary schools children in municipality of Natitingou, North of Benin" (EK 2012-N-47)

Dear Mr. Zimmermann

The reservations that the decision of 31.10.2012 to your request "Efficacy study of a water filter used as distribution system of zinc for children in primary schools municipality of Natitingou, North of Benin" (EK 2012-N-47) were listed, are eliminated by the responses and adaptations in the documents you submitted on 20.11.2012. In this form, the application of the ethics committee is fully approved.

With best regard

Raffael Iturrizaga

ETH Zürich | Dr. Raffael Iturrizaga | Stab Wissenschaftskoordination | HG E 34.3 | Rämistr. 101
| CH-8092 Zürich | Phone: +41 44 632 2354 | Fax: +41 44 632 1184 | iturrizaga@sl.ethz.ch |
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ANNEXURE 4: ETHICAL CLEARANCE FROM BENIN (FRENCH AND ENGLISH)

République du Bénin
MINISTÈRE DE LA SANTÉ
Direction de la Formation et de la Recherche
COMITE NATIONAL D'ETHIQUE POUR LA RECHERCHE EN SANTE

 N° 181 /MS/DC/SGM/DFR/CNERS/SA COTONOU, le 13 décembre 2012

La présidente

A

Monsieur MITCHIKPE Evariste
et collaborateurs
COTONOU

OBJET : Avis éthique favorable n° 029 du 19 octobre 2012

Monsieur,

Le Comité National d'Ethique pour la Recherche en Santé (CNERS) du Bénin, a l'honneur de vous adresser l'avis éthique concernant votre protocole de recherche :

- dont le titre actuel est « Etude d'efficacité de l'eau comme véhicule de fortification en zinc pour l'amélioration du statut en zinc chez les enfants âgés de 6 à 10 ans inscrits à l'école primaire de Kotopounga dans la commune de Natitingou, au Nord Ouest du Bénin »,
- étudié le 19 octobre 2012, par ses membres dont les noms suivent :

1) AHOSSINO Clément	2) FOURN Elisabeth
3) OGUYEMI HOUNTO Aurore	4) AYEMONNA HOUNGAN Claire
5) PADONOU Mousbaye	6) ZOMONTO Gbodja
7) QUENUM Cosme	8) GANGBO Flore

ont délibéré :

- **sur la base des documents soumis par les chercheurs**

a) Protocole de recherche
b) Résumé
c) Chronogramme
d) Budget et Financement
e) Annexe I : Equipe de recherche
f) Annexe II: curriculum vitae de l'équipe de recherche

 - Michel Bruce ZIMMERMANN
 - HOUNHOUIGAN Djidjoho Joseph



- MITCHIKPE Comlan Evariste Simon
- Diego MORETTI
- Valeria GALETTI

- g) Annexe III : Fiche d'information aux participants
- h) Annexe IV : Fiche de consentement éclairé
- i) Annexe V : Fiche de recrutement et de suivi
- j) Annexe VI : Enquête nutritionnelle
- k) Annexe VII : Protocole de prélèvement de sang humain
- l) Annexe VIII : Preuve de financement
- m) Annexe IX : Littérature scientifique sur le filtre LSF
- n) Annexe IX : Brochure de présentation sur le filtre LSF
- o) Annexe X : Proposition d'assurance responsabilité civil pour étude biomédicale
- p) Annexe XI : Approbation du Comité d'éthique de l'ETH Zurich
- q) Annexe XII : Autorisation du directeur de l'école et de l'APE
- r) Annexe XIII : Diagramme de l'étude pour explication aux enfants

- **et sur la base de l'avis scientifique fourni par le docteur HOUNSA Assomption, expert sollicité par le CNERS**

Après avoir validé globalement les aspects scientifiques étudiés par les experts sollicités et évalué les aspects éthiques centrés sur : les bénéfices attendus pour la santé des participants à la recherche et ainsi que sur la qualité des informations transmises en vue de l'obtention d'un consentement libre et éclairé, **le CNERS a émis un Avis éthique favorable n°028 du 19 octobre 2012.**

Le présent avis éthique favorable est accordé **sous réserve de l'autorisation administrative délivrée par le Ministre de la Santé.**

Par ailleurs, le CNERS vous demande de :

1) l'informer de :

- toute nouvelle information/ modification, qui surviendraient à une date ultérieure à cette approbation-ci et qui impliqueraient des changements :
 - dans le choix des participants à la recherche,
 - dans la manière d'obtenir leur consentement,
 - dans les risques encourus ou
- tout événement indésirable survenant dans le cadre du déroulement de cette recherche.

Le CNERS doit, en effet, dans ces cas, ré-évaluer et donner une nouvelle approbation avant l'entrée en vigueur desdites modifications.

Page 2 sur 3

- 2) utiliser les documents qu'il a validés ;
- 3) faire signer au participant à la recherche, le formulaire de consentement, en deux exemplaires, dont l'un lui sera remis et l'autre, conservé dans vos dossiers ;
- 4) lui adresser un rapport **au terme de la validité de cet avis**

Tout en vous remerciant pour la confiance à lui, accordée, le CNERS vous prie de recevoir, Monsieur, ses salutations les meilleures.



La présidente

Professeur agrégé Flore GANGBO

Republic of Benin
MINISTRY OF HEALTH Directorate of
Education and Health
NATIONAL ETHICS COMMITTEE FOR HEALTH RESEARCH

N°181/MS /DC/SGM/DFR/CNERS/SA

COTONOU, December 13,2012

The president

to

Mr MITCHIKPE Evariste
and Partners
COTONOU

Re : Ethics Approval Notice No 029 Of October 19 2012

Sir,

The National Ethics Committee for Health Research (Comite National d’Ethique pour la Recherche en Santé: CNERS) of Benin, is honored to give you a favorable ethics notice for your research:

Which current title is **“etude d’efficacite de l’eau comme vehicule de fortification en zinc pour l’amélioration du statut en zinc chez les enfants ages de 6 a 10 ans inscrits a l’ecole primaire de kotopounga dans la commune de Natitingou au Nord oust du Benin”, « the study of effectiveness of water as a vehicle for fortification in zinc for improving the zinc status of children aged 6 to 10 years, attending kotopounga primary school, in Natitingou canton/township, in the north west of Benin”**

Evaluated on October 19th, 2012 by the committee members whose names follow:

- | | |
|----------------------------|---------------------|
| 1) AHOSSINOU Clement | 5) PADONOU Mousbaye |
| 2) FOURN Elisabeth | 6) ZOMONTO Gbojda |
| 3) OGOUYEMI HOUNTO Aurore | 7) QUENUM Cosme |
| 4) AYEMONNA HOUNGAN Claire | 8) GANGBO Flore |

Have deliberated

Based on the documents submitted by the researchers

- a) Research protocol
- b) Summary
- c) Timetable
- d) Budget and funding
- e) Annex I : research team
- f) Annex II: resumes of research team members

Michel Bruce ZIMMERMANN
HOUNHOUIGAN Djidjoho Joseph
MITCHIKPE Comlan Evarist Simon

Diego MORETTI

Valeria GALETI

- g) Annex III: Information sheet for participants
- h) Annex IV: Consent form
- i) Annex V: Recruitment and follow up form
- j) Annex VI: Dietary survey
- k) Annex VII: Blood sample drawing protocol
- l) Annex VIII: Funding proof
- m) Annex IX: Scientific literature on the LSF Filter (LifeStraw®Family, LSF)
- n) Annex IX: Brochure on the LSF Filter (LifeStraw®Family, LSF)
- o) Annex X: Civil liability insurance for biomedical research proposal
- p) Annex XI: Ethical clearance of the ETH Zurich
- q) Annex XII: Authorization of the school director and the Pupils' Parents Association (Association des Parents d'élevés, APE)
- r) Annex XIII: study framework for explaining to children

And based on the scientific review of Doctor HOUNSA Assomption, expert commissioned/appointed by the CNERS

After validation of scientific aspects studied by the commissioned experts and evaluation of ethical aspects focusing on : the expected health benefits for the participants of the study and the quality of information provided for obtaining informed consent, the **CNERS has granted ethical approval No 028 of October 19, 2012.**

This ethical approval is **subject to the administrative authorization delivered by the Ministry of Health.**

Futhermore, the CNERS requests:

1) To inform about :

Any new information/ amendment that would occur at later date than this approval and would imply amendments

- In the choice of participants
- In the way to obtain their consent
- In the risks to participants

Any other adverse event that would happen in the course of the research

The CNERS has, indeed, in such a case, to reevaluate and give a new approval before the implementation of those amendments.

- 2) To use the validated documents**
- 3) **To request the participant to sign two copies of the consent form**, one will be given to the participant, the other kept in your files
- 4) To report at term of validity of this approval**

The CNERS thanks you for the trust granted

The president

Associate professor Flore GANGBO