

**Zinc intake-status-health relationships and
the impact of multiple micronutrient
supplementation on cognitive function in
Peruvian pre-school children**

by

Marisol Warthon Medina

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Thesis supervisors

Professor Nicola M.Lowe, B.Sc, PhD, R.Nutr, PGCert TLHE
Professor of Nutritional Sciences, University of Central Lancashire
nmlowe@uclan.ac.uk

Dr Stephanie Dillon
Senior Lecturer in Nutrition, University of Central Lancashire
SDillon@uclan.ac.uk

Dr Stephen Atkins
Division leader for sport, exercise and nutritional sciences, University of Central
Lancashire
SATkins@uclan.ac.uk

"The measure of intelligence is the ability to change"

-Albert Einstein

"Courage is not the absence of fear but the triumph over it"

-Nelson Mandela

ABSTRACT

“Zinc intake-status-health relationships and the impact of multiple micronutrient supplementation on cognitive function in Peruvian pre-school children”.

Background: Dietary zinc recommendations vary widely across Europe. The EURRECA (European Micronutrient Recommendations Aligned) Network of Excellence was brought together to harmonise the approach to setting micronutrient recommendations. The overall aim was to produce a set of guidelines and an extensive database that may be of use to expert panels in underpinning future micronutrient recommendations, based on a series of systematic reviews and meta-analysis of published data. A secondary aim was to identify gaps in knowledge regarding micronutrient status, intake and health outcomes for future research. A third aim, coming from the result of the secondary aim, was to investigate the long term effect of supplementation on cognition.

Objectives: The objectives of this thesis were: (1) To adapt the methodology for undertaking a systematic review and meta-analysis developed by EURRECA for zinc in all population groups (infants, children, adolescents, pregnant and lactating women and adults and elderly). (2) To identify knowledge gaps in the research through the assessment of the interrelationships between zinc intake, status and health outcomes. (3) To design and implement a study to explore the intake-status-health relationships between micronutrient supplementation and cognitive function in Peruvian children.

Methods: Phase 1: The EURRECA systematic review. Database searches were conducted in MEDLINE, Embase and the Cochrane library, from inception to March 2014. For the assessment of the intake-status relationship, an intake-status regression coefficient ($\hat{\beta}$) was estimated for each individual study and the overall pooled $\hat{\beta}$ and SE $\hat{\beta}$ was calculated using random effect meta-analysis on a double log scale. The systematic review included randomized controlled trials, cohort studies, cross sectional studies and nested case-control studies in healthy children and adult populations that measured zinc intake (diet and supplements) and zinc status (serum/plasma zinc) in

association health outcomes that included cognitive function and psychomotor development.

Phase 2: Empirical study. This included the assessment of multiple micronutrients (MMN) supplementation and cognitive development in Peruvian children when compared with giving iron supplements alone. These tests were considered to reflect theoretical dimensions of working memory (Nine boxes), and inhibitory control (Day/Night stroop task). Intelligence Quotient (IQ) was measured using the Wechsler Preschool and Primary Scale of Intelligence (WPPSI); social-emotional behaviour was assessed via the Brief Infant–Toddler Social and Emotional Assessment (BITSEA) and reasoning through the theory of mind test.

Results: Meta-analysis of data reporting zinc intake and status revealed that doubling intake increased plasma zinc concentration by 9% in children, by 3% during pregnancy, by 1% during lactation and by 6% in adults and elderly. The systematic review of zinc and health outcomes highlighted the need for further research on the relationship between micronutrients and cognitive function. The empirical study was therefore designed and undertaken. The results revealed that there were no significant differences between the iron and the MMN supplemented groups for all cognitive tests, with the exception of the vocabulary WPPSI subtest in girls (mean±SD), (MMN, 6.83±2.05; iron 5.78±1.59, $p = 0.028$) and no significant differences were found for plasma zinc and haemoglobin concentrations between the two groups.

Conclusions: The systematic reviews provided novel dose-response estimates between zinc intake-status that could be used either qualitatively or quantitatively with balance studies when setting future zinc recommendations. Following an assessment of the impact of micronutrient supplementation on cognitive and social-emotional development in Peruvian preschool children, it can be concluded that MMN supplements had no long term additional effects on cognitive function compared with iron alone, however the timing of the supplement for maximal potential benefit needs to be explored further.

Key words: zinc intake-plasma zinc status-cognitive function-systematic review-meta-analyses.

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Dedication

In memory of my sister Minerva Warthon Medina, who gave me the drive to restart my life abroad and for making me believe that some dreams in life can be accomplished, and in memory of my granny Julia Cruz de Revilla who passed away in 2012 while I was doing my research in Peru.

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Marisol

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List of abbreviations

AAS:	Flame atomic absorption spectrophotometry
ANOVA:	Analysis of Variance
BITSEA:	The Brief Infant-Toddler Social and Emotional Assessment.
CANTAB:	Cambridge Automated Neuropsychological Test Battery
CERAD:	Consortium to Establish a Registry for Alzheimer's Disease; Word List Learning Test; The CERAD word List Recall Test
CNS:	Central Nervous System
CPAS-R:	Cognition-Psychomotor Assessment System-Revised
CSID:	Community Screening Instrument for Dementia; Consortium to Establish a Registry for Alzheimer's Disease
DBP:	Diastolic Blood Pressure
DS:	Digit Span
DTLA:	Detroit Tests of Learning Aptitude
DRVs:	Dietary Reference Values
EF:	Executive function
EURRECA:	EUROpean micronutrient RECommendations Aligned
FFQ:	Food Frequency Questionnaire
GDP:	Gross domestic product
24h:	24 hour
ICP-MS:	Inductively coupled plasma-mass spectrometry
ICP-AES:	Inductively coupled plasma atomic emission spectrophotometer
IIN:	Institute de Investigacion Nutricional, a nutritional research institute
IQ:	Intelligence Quotient

IU:	The Indiana University; The IU Story Recall, the IU Token test
MABC:	Movement Assessment Battery for Children
MANOVA:	Multivariate Analysis of Variance
MMSE:	Mini-mental state examination
MTS:	Matching to Sample visual search
MMN:	Multi-MicroNutrient
MUAC:	Middle Upper Arm Circumference
NaFeEDTA:	Sodium iron ethylenediaminetetra-acetate
PMSQ:	Pfeiffer's mental status questionnaire
PN:	Parenteral Nutrition
PRM:	Pattern Recognition Memory
RPM:	Raven's Progressive Matrices
Raven CPM/RCPM:	Raven's Coloured Progressive Matrices Test
RPM:	Revolutions per minute
RCTs:	Randomized Controlled Trials
RRT:	Recognition Reaction Time
SBP:	Systolic Blood Pressure
SD:	Standard Deviation
SES:	Socio-Economic Status
SMD:	Standard mean difference
SSP:	Spatial Span
SRT:	Simple Reaction Time
SWM:	Spatial Working Memory
TAZ:	Total absorption of zinc,
TDZ:	Total dietary intake of zinc,
TDP:	Total dietary phytate.

UCLAN:	University of Central Lancashire
UEA:	University of East Anglia
UNIT:	Universal Nonverbal Intelligence Test
UNC-SENC:	Community Nutrition Unit of Bilbao (Spain),
ULPGC:	Universidad de Las Palmas de Gran Canaria
UNU:	United Nations University
USA:	United States of America
VES:	Villa El Salvador
WHO:	World Health Organisation
WISC-III:	Wechsler Intelligence Scale for Children-Third edition
WPPSI:	Wechsler Preschool and Primary Scale of Intelligence
WPPSI-R:	Wechsler Preschool and Primary Scale of Intelligence Revised
WU:	Wageningen University

CHAPTER 1. –
BACKGROUND TO THE THESIS

Chapter 1 - BACKGROUND TO THE THESIS

Micronutrient deficiencies and malnutrition are worldwide public health concerns (WHO, 2006). Malnutrition is one of the most common causes of morbidity and mortality among children throughout the world and coupled with infectious disease has a powerful impact on child mortality (Pelletier et al., 1995). Micronutrients, including zinc and iron, are essential for growth and cognitive development (Rivera et al., 2003) (Grantham-McGregor and Ani, 1999).

Dietary recommendations for micronutrients differ between countries because of differences in the data and methodologies used to derive them. This has led to confusion among health professionals and consumers (Doets et al., 2008). To address this problem the EURRECA Network of Excellence (EUROpean micronutrient RECommendations Aligned) was established. The primary purpose of EURRECA was to review and undertake an alignment of methodologies for the derivation of micronutrient dietary reference values (DRVs), in the European Union (EU) populations (van't Veer et al., 2013). The overall aim was therefore to harmonise the approach to setting micronutrient recommendations, through expert opinion, systematic reviews to identify best practice and meta-analysis of published data. There were 8 prioritised micronutrients that were included in the EURRECA project: iodine, folate, iron, riboflavin, selenium, zinc and the vitamins B12 and D.

This network of scientists was funded by the European Commission Sixth Framework Programme (FP6) and coordinated by the International Life Sciences Institute in Europe (ILSI). The Network included 35 partners comprising more than 200 individual scientists from 17 European countries, drawn up not only from nutrition science but also from industry, consumer groups, national nutrition societies, and health professions, with a budget of €13.2 million spread over 5.5 years, the project started in 2007 and ended in 2012 (eurreca.org).

The author of this thesis was a member of the UCLAN team, which in turn was a key member of the EURRECA consortium. The author's role within the team was to lead the systematic review and meta-analysis of studies relating to zinc. The author played a central role in co-ordinating and undertaking the zinc systematic review and meta-analyses of data from all population groups, namely adults and elderly, pregnant and

lactating women, children and adolescents, and infants. The review focussed on the relationships between zinc intake, status and health outcomes in these population groups.

Part 1 of this thesis presents the results of this series of systematic reviews, focussing on the aspects of the review that the author was actively involved with. One important finding from the zinc systematic review work was that, for many health outcomes, there are limited high quality data and that the data that are available are too heterogenic to allow meta-analysis. In particular, previous work examining the relationship between micronutrient intake, status and cognitive function is limited. Part 2 of the thesis develops this theme further to corroborate the findings of the systematic review/meta-analysis and to add further validity, presenting an empirical field study, conducted in Peru, that examines the long term impact of micronutrient supplementation in infancy on indices of growth and cognitive function in pre-school children.

Peru was chosen as the location for this empirical study because child undernutrition is highly prevalent in low-income and middle-income countries (Black et al., 2008) and micronutrient deficiencies and anaemia are common problems in children in Peru (World, 2011, ENDES, 2010), where approximately 9% of children below 5 years of age suffer from wasting and are at risk of death or severe impairment of growth and psychological development (WHO, 1999). The empirical study assessed the long-term effect of multiple micronutrient supplement containing zinc on the cognitive and social emotional development in children. This study supported research in human nutrition in a middle-income country and scientific and educational knowledge was transferred between organizations. This study was exceptional in public health because it examined the aspect of micronutrient deficiencies that are a critical concern among the children throughout the world. This study generated new evidence which is needed in examining the impact of micronutrient supplementation on cognitive function and neuropsychological development.

1.1 - AIMS

The aims of this research were the following:

1. To undertake a series of meta-analyses following a comprehensive literature review of zinc intake, status and health outcomes.
2. To identify gaps in knowledge regarding zinc intake-status-health in infants, children, pregnant, lactating women and adults and elderly.
3. To investigate the long term impact of micronutrient supplementation during infancy on cognitive function in pre-school aged children, in Lima, Peru (informed by the outcome of aim 2).

1.2 – PERSONAL REFLECTION; THE STORY OF THIS PhD

The EURRECA network of excellence was established to address discrepancies in micronutrient recommendations across Europe through a series of systematic review and meta-analyses in priority micronutrients: iron, folate, vitamin B12, selenium, iodine, and zinc, and to work towards a framework of advice to better inform policy makers as they formulate precise recommendations. The focus was on the most vulnerable groups in Europe such as the adult and elderly, pregnant and lactating women, children and adolescents, those with low incomes and migrant populations. The UCLAN team conducted the systematic review and meta-analyses of zinc micronutrient. The author was working as part of a dynamic UCLAN and EURRECA team.

Undertaking the zinc systematic review and meta-analyses was an enormous task, contributing to the development of protocol, best practice method for zinc intake, zinc biomarkers and zinc health outcomes. These health outcomes were of a wide range for each population group, namely: growth, immune response, neurodevelopment in infants, immune function, cognitive function and psychomotor development in children, dermatitis, foetal growth, foetal malformation in foetus, preeclampsia and preterm delivery in pregnant and lactating women, cognitive function in adults and elderly, anorexia, hypogeusia, ischemic heart disease, depression, diabetes mellitus, carcinogenesis. In this thesis, only one zinc health outcome will be described, which is the cognitive function. Carrying out the search, the screening and sorting of extensive

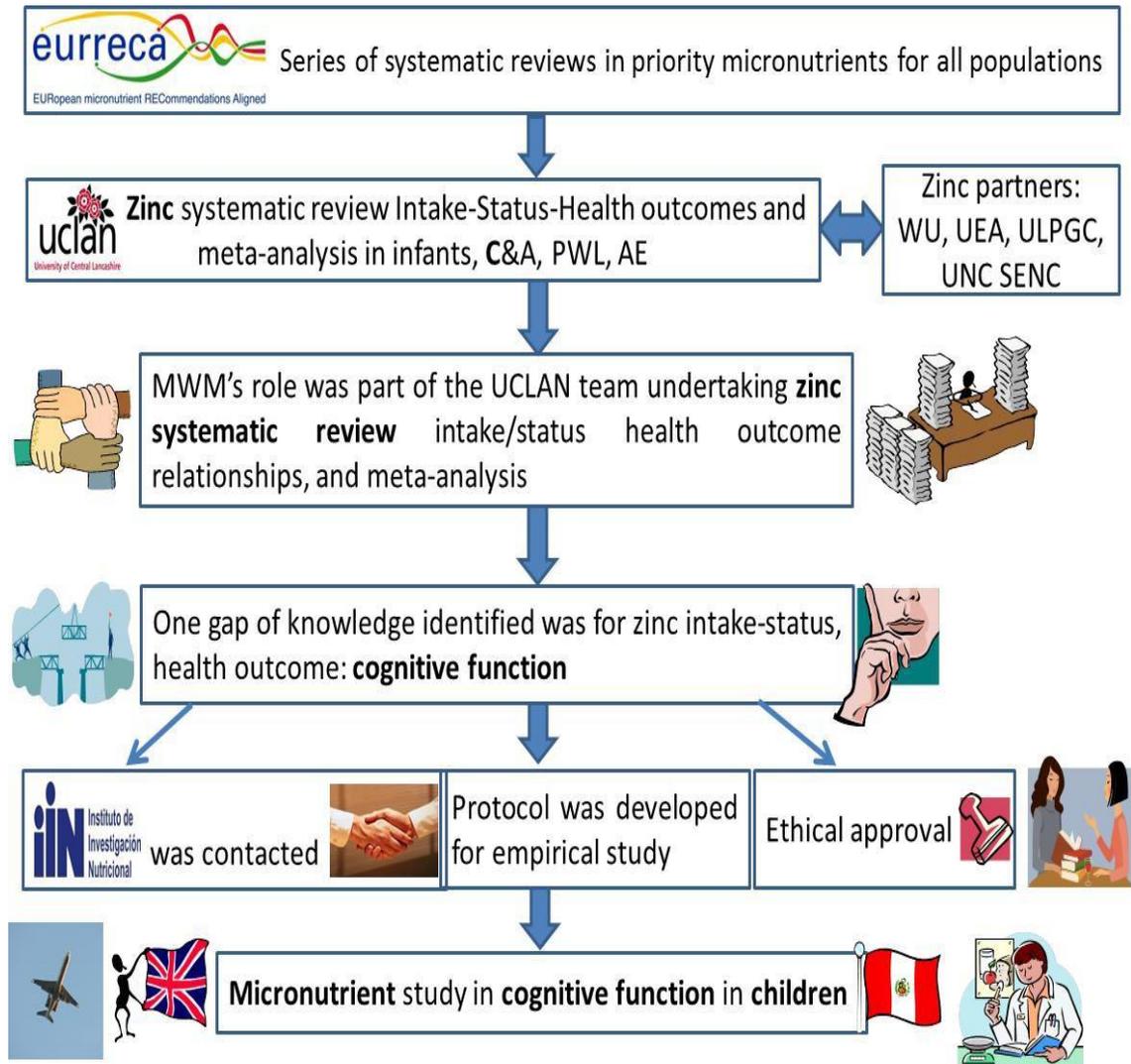
number of abstracts was quite a large task, reading the full papers was enjoyable, but the data extraction was laborious. The meta-analysis compensated for that time. Meta-analyses and meta-regression was relatively simple, fast and very interesting and this was one of the new skills acquired through training, then reports were written for the studies that were not considered for meta-analysis and this was also manageable.

Following the zinc systematic review process and, in view of the preliminary findings of the limited number of studies on the cognitive function outcome in children and adolescents, and adults and the elderly, then there was the need to explore this health outcome. This was an important identified gap of knowledge and, after some consideration and research of the possible psychological tests to evaluate cognition, so the idea of a new study was born, which led to the empirical work in the children population.

After searching for potential collaborators, an initial contact was established with the team at IIN lead by Dr Nelly Zavaleta, this in turn led to a short visit to discuss the project proposal. While in the UK, a joint study design protocol was developed, then the following visit, further review of the protocol and the preparation of an executive summary, forms, and training continued in Peru. Once the ethical approval was granted, a jubilant moment, the field work study began, and this was an incredible experience working with the professional IIN team and meeting the parents and children at the IIN clinic in Villa el Salvador, where the study took place. Gathering data, and laboratory analysis by IIN, was crucial for the subsequent data entry, data analysis and write-up of findings which were carried out at UCLAN.

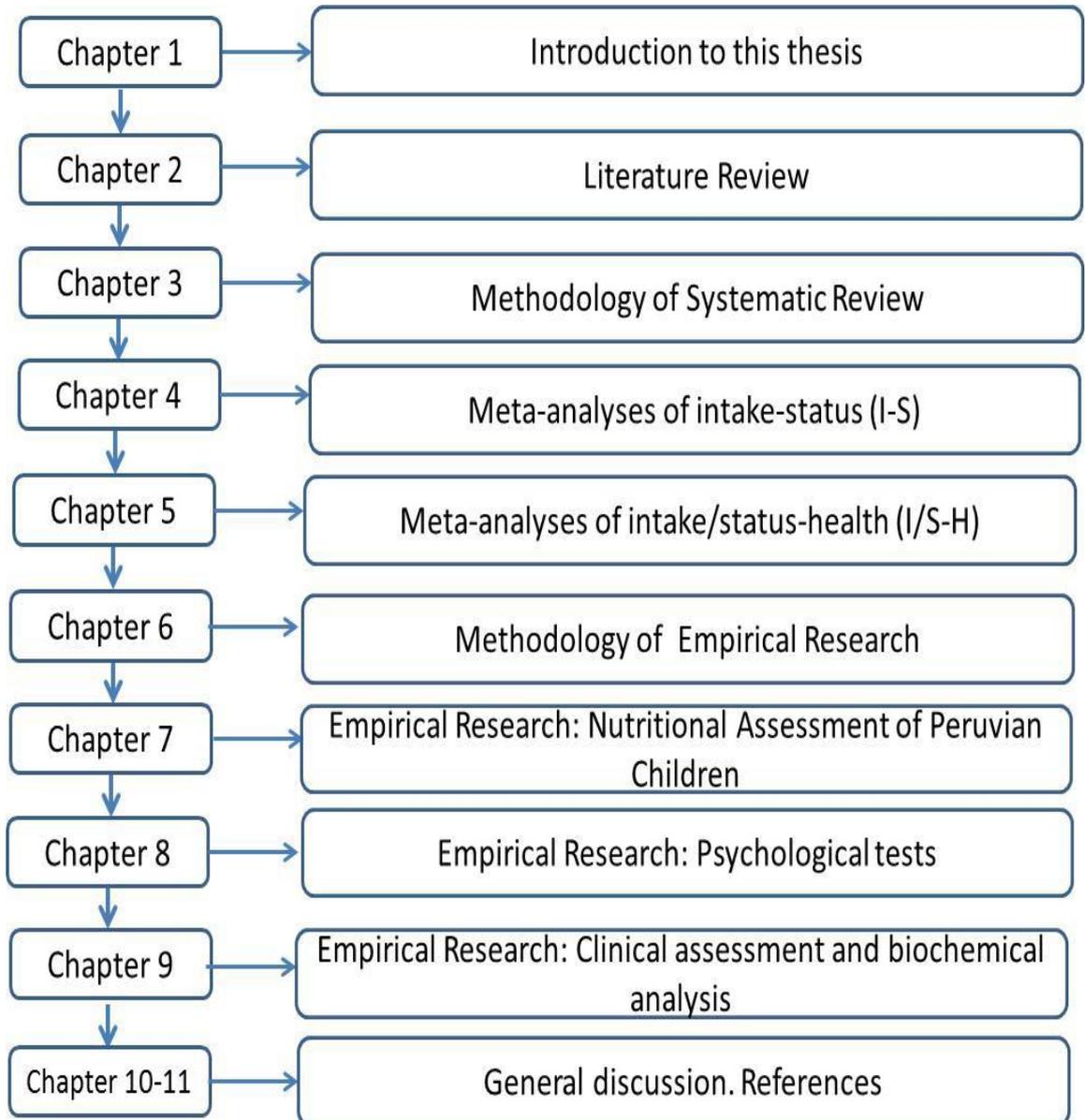
The overall completion of this PhD research, was arduous, with highs and lows, and at the same time a gratifying learning process, recognizing errors and amending them, but with the guidance of eminent scientists and all who contributed to this PhD, then the author felt fortunate to have had the opportunity to work alongside both UCLAN and IIN teams. This PhD research has concluded its journey and has landed with a desire to explore the next phase.

Figure 1.1-Flowchart summarizing the conceptual framework of this thesis



EURRECA: The European Micronutrient Recommendations Aligned, WU: Wageningen University (the Netherlands), UEA: University of East Anglia (UK), ULPGC: Universidad de Las Palmas de Gran Canaria (Spain), UCLAN: University of Central Lancashire (UK), UNC-SENC: Community Nutrition Unit of Bilbao (Spain), CA: Children and adolescents, PWL: Pregnant and lactating women, AE: Adults and elderly, IIN: Instituto de Investigación Nutricional; MWM: the author's thesis Marisol.

Figure 1.2- Flowchart demonstrating the structure of this thesis



CHAPTER 2. – LITERATURE REVIEW

Chapter 2 – LITERATURE REVIEW

2.1 GENERAL BACKGROUND OF ZINC

Zinc is a bluish-white metallic element that makes up about 0.02% of the earth's crust (Brown et al., 2001). Zinc is an essential micronutrient and is one of the most abundant elements in the human body and, unlike iron, zinc has no storage site in the human body (Lowe et al., 2003).

The human body's total zinc content has been estimated to be 30 mmol, approximately 2.5–3g, 57% of which is present in skeletal muscle (Jackson and Lowe, 1992). Other body tissues where zinc is found are skin, bone, liver, kidney and brain. Around 90% of zinc is found in muscle and bones. The highest concentration of zinc is found in the choroid of the eye, optic nerve (Watts, 1988), followed by the prostate gland and semen (IZA, 2011) and approximately 0.2 % of total body zinc circulates in plasma (mean concentration, 15 $\mu\text{mol/L}$) (Brown et al., 2001).

Zinc is important for human health and was first recognized as an essential nutrient for microorganisms more than 137 years ago, during the late 19th century. Appreciation of the essentiality of zinc was observed in plants, rats, mice, poultry and swine in studies undertaken in the 1920s to 1950s (Thureen and Hay, 2006). However, despite these observations, researchers were sceptical about the possibility of zinc deficiency in humans because zinc is an ubiquitous element. This remained so until the 1960s when evidence of human zinc deficiency began to emerge (Brown et al., 2001). Furthermore, human zinc nutrition was not considered to be a major public health issue until 1961 when some cases of dwarfism and delayed sexual maturity caused by zinc deficiency were reported in Egyptian adolescents and in rural Iran by Dr Ananda Prasad (Prasad et al., 1963). The hypothesis was that zinc deficiency was a major etiological factor in the syndrome of “adolescent nutritional dwarfism” in mid-Eastern countries. The underpinning of this hypothesis were the identified cases in Iranian men suffering from malnutrition and a syndrome characterized by iron deficiency anaemia, hepatosplenomegaly (enlargement of both the liver and the spleen), dwarfism (abnormal low height), hypogonadism (reduced hormonal secretion by the gonads: testes). The

possibility of zinc deficiency was considered as an explanation of the hypogonadism, retarded growth and changes in alkaline phosphatase (Prasad et al., 1961). The full impact of this hypothesis, and its implications regarding zinc were not fully appreciated due to the presence of multi-micronutrient deficiencies and a lack of high quality data derived from randomized controlled trials. The case reported by Prasad involved a 21 year old male patient from Shiraz, Iran who presented with dwarfism, hypogonadism, hepatosplenomegaly, rough and dry skin, mental lethargy, geophagia, iron deficiency anaemia and had an unusual diet. His intake of animal protein was negligible; he consumed only unleavened bread and 0.5 kg of clay daily. His intake of calories and protein (cereal) was adequate, and he had iron deficiency, but no other deficiency was documented (Prasad, 2003). Prasad demonstrated zinc deficiency in Iranian and Egyptian subjects whose plasma zinc concentration levels were reduced, their dietary history were similar in that their intake of protein was negligible and their diet was mainly of bread and beans (Prasad, 1991). The findings by Prasad made an outstanding contribution to the history of the recognition of zinc as a micronutrient of practical importance in human nutrition (Hambidge, 2000b). Since then, considerable research has been carried out to understand the role of zinc in human nutrition (Hambidge, 2000b) and clinical studies in children with acrodermatitis enteropathica have confirmed the critical role of zinc in growth and immune function (Brown et al., 2001).

2.2 PHYSIOLOGICAL ROLES OF ZINC

Zinc has an atomic number of 30 and an atomic weight of 65.4 (Halsted et al., 1974). The study of zinc biochemistry started in 1948 with the detection of zinc in carbonic anhydrase (Keilin and Mann, 1940, Thureen and Hay, 2006). Since then, over 300 zinc metalloenzymes have been identified, with zinc participating in a broad range of biochemical processes, including the synthesis and degradation of carbohydrates, lipids, proteins, nucleic acids, and in the metabolism of other micronutrients such as vitamin A (Vallee and Galdes, 1984, Prasad, 1995, FAO&WHO, 2002, McClain et al., 1985).

2.2.1 Structural Roles

Zinc plays a key structural role within the human body, both at the tissue and sub-cellular level. Zinc is an integral structural component of tissues including bone, hair and liver where it is found in concentrations of 0.77g, <0.01g, 0.13g respectively (Gibney, 2009). The following table shows the zinc content of major organs.

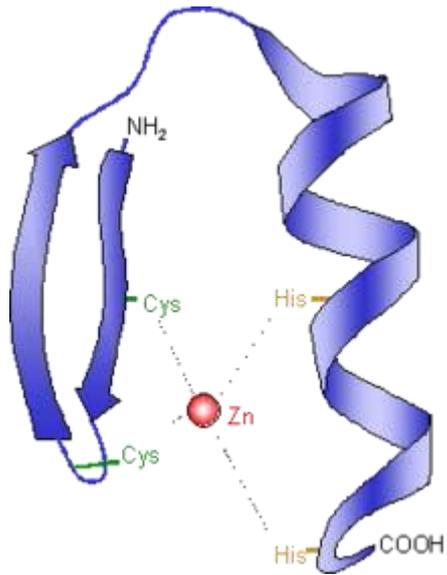
Table 2.1 - Zinc content of major organs and tissues in the adult male

Tissue	Total zinc content (g)	Percentage of body Zn (%)
Skeletal muscle	1.53	~ 57
Bone	0.77	29
Skin	0.16	6
Liver	0.13	5
Brain	0.04	1.5
Kidney	0.02	0.7
Heart	0.01	0.4
Hair	<0.01	~ 0.1
Blood (plasma)	<0.01	~ 0.1

Adapted from Gibney, M. J. 2009. Introduction To Human Nutrition, United Kingdom, Wiley-Blackwell. Modified from Mills CF, ed, Zinc in Human Biology, copyright 1998

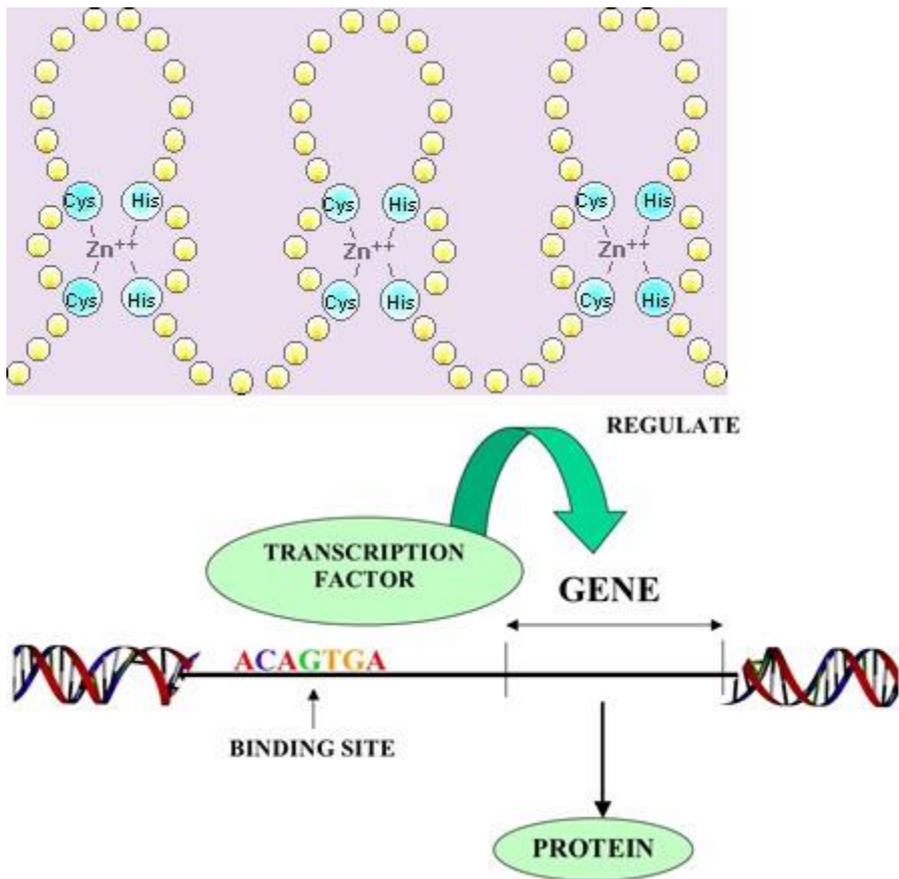
At the sub-cellular level, one of the key structural roles of zinc is with the so-called “zinc fingers”. These are protein motifs, many of which are involved in the regulation of gene expression as DNA binding transcript factors and zinc ions bind to the *his* and *cys* aminoacids of the protein motif, and stabilise the folding of the protein into a finger-like structure as illustrated in Figure 2.1 .

Figure 2.1 - Structure of zinc finger



These zinc fingers play a key role in the correct positioning and binding of transcription proteins to DNA (Thureen & Hay, 2006), which initiates the transcription process (Niaragu, 2007) as illustrated in Figure 2.2 , where the transcription factor Sp1 has a DNA-binding domain that consists of 3 zinc fingers. This transcription factor binds to the DNA thus regulating the protein production from a gene.

Figure 2.2 - “Zn fingers” binding to DNA and Transcription factor Sp1 (3 zinc fingers)



Zinc plays a structural role in proteins such as alcohol dehydrogenase, superoxide dismutase, keratin (hair), among others (Williams, 1984, Frederickson et al., 2000). The structural role for zinc is best demonstrated in the zinc-finger motif of proteins, which became a generic term for the role of zinc in many protein domains (Maret, 2013). The zinc-finger motif binds zinc or DNA to stabilize a very small functional protein domain or “finger”, then the term ‘zinc finger’ applies to a various set of protein motifs which have in common the property of binding zinc ions in order to stabilize the structure of a small protein domain (Klug, 2010, Klug and Schwabe, 1995).

The structural role of zinc involves proteins that form domains that are capable of zinc coordination, which facilitates protein folding to produce biologically active molecules (Klug and Schwabe, 1995). The majority of such proteins form a “zinc finger-like” structure and some of these proteins take part in gene regulation as deoxyribonucleic acid binding transcription factors (Institute of Medicine, 1999). “Zinc fingers”, the conformation of which depends on a zinc atom at the base of the finger, play a key role in the correct positioning and binding of these transcriptions proteins to DNA (Thureen

and Hay, 2006). Thus, the linking of zinc fingers to corresponding sites in the DNA initiates this transcription process and gene expression (Nriagu, 2007). Perhaps one of the most important discoveries is the involvement of zinc in the synthesis of ribonucleic acid (RNA) (Watts, 1988). As zinc plays a crucial role in genetic expression (Vallee and Auld, 1990), it affects the structure of chromatin, the template function of its DNA, the activity of various transcription factors and of RNA polymerases (Falchuk, 1998), then it is not surprising that zinc deficiency reduces growth because of decreased cell replication (Fairweather-Tait, 1988).

2.2.2 Functional Roles

Zinc plays a wide range of functional roles. A few of these roles are described in more detail in this section.

Zinc and Enzymatic Activities

Zinc metalloenzymes are present in all classes of enzymes. The role of zinc in these proteins can be either catalytic or non-catalytic. The chemical properties of the metal ion make it an ideal component of the active site of enzymes, with its small size and high electron affinity. Zinc may also play a structural role within the active site, stabilising the protein conformation such that the removal of zinc would render the enzyme inactive. Well studied zinc metalloenzymes defined as catalytically active metalloproteins (firm binding of metal to protein), are the ribonucleic acid (RNA) polymerases, alcohol dehydrogenase, carbonic anhydrase and alkaline phosphatase which are decreased in cases of severe zinc deficiency (Parisi and Vallee, 1969, DRI, 2014).

Zinc may be present in the enzyme, but not involved in its catalytic action or structural stabilisation of the active site, for example within alcohol dehydrogenase. In this way zinc plays a non-catalytic role and removal of these zinc ions has a minor impact on the enzymes' activity.

Zinc and Growth

Due to the role zinc plays in enzyme structure and function, this trace element is essential for protein synthesis, cell division, DNA and RNA synthesis, and therefore growth. Zinc deficiency is one of the main causes of stunting in children (Gibson et al.,

2007, Salgueiro et al., 2002) and indeed, the essentiality of zinc was first recognised by Ananda Prasad in hypogonadal dwarfs in Iran (Prasad and Oberleas, 1970, Prasad, 2009) where lack of zinc had resulted in delayed sexual development and stunted growth.

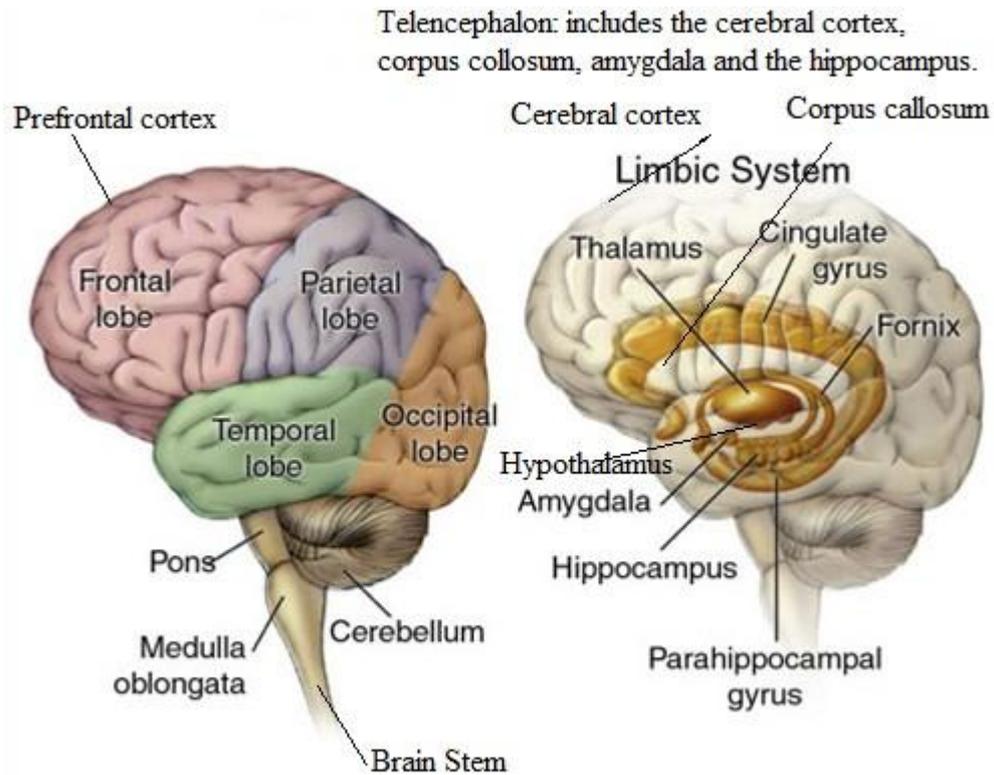
Zinc and Immune function

Zinc plays a role in both the innate and adaptive immune response. Within the innate system, defined as the first barrier of defence, zinc is required for the function of the monocytes, macrophages and granulocytes. Within the adaptive system, zinc is required for the proliferation of T cells and the production of antibodies by the B cells (Rosenkranz et al., 2011). Poor immune function and thus susceptibility to infection is one of the consequences of zinc deficiency (Prasad, 2009). Zinc act as potent mediator of host resistance to infection, however it is unclear how zinc influence the ability of the cell to engulf and kill organisms (Shankar and Prasad, 1998).

Zinc and the Central Nervous System

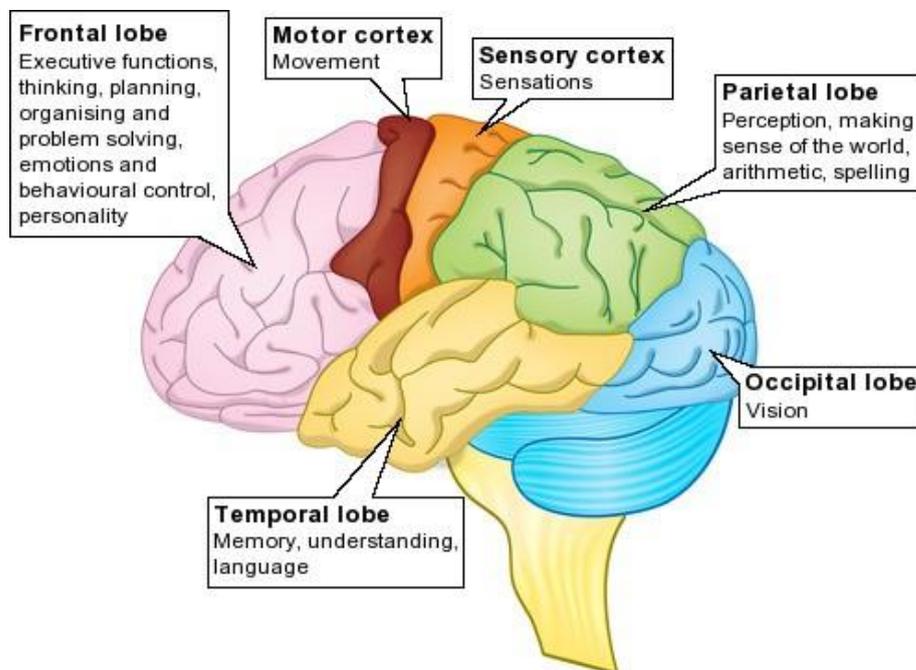
Zinc plays essential roles in the central nervous system across the lifespan from early neonatal brain development through the maintenance of brain function in adults (Gower-Winter and Levenson, 2012). Zinc plays a crucial role in the survival of cell neurons (Sensi et al., 2009) and there is evidence that zinc is involved in the process of cortical plasticity which is the brain's ability to develop and adapt to a changing environment (Pascual-Leone et al., 2011). The central nervous system consists of the brain and the spinal cord, which is contained within the bony compartments formed by the skull and spinal column (Bloom and Lazerson, 1988).

Figure 2.3 - The human brain



The Telencephalon includes the cerebral cortex (corpus callosum), hippocampus and the limbic system.

Figure 2.4 - Functional areas of the brain



Figures 2.3 and 2.4 show the structure of the brain, identifying its functional areas. The cerebrum or cortex is the largest part of the brain, where the frontal lobe is associated with reasoning, planning; the parietal lobe is associated with the movement, perception,

recognition; the occipital lobe is associated with visual processing. Temporal lobe is associated with memory and speech. The cerebellum is associated with movement's coordination and balance. The limbic system or "emotional brain" is comprised of the thalamus, hypothalamus, amygdala, and hippocampus (Schoenemann, 2006).

Zinc is found in the mossy fibres, approximately 220-300 μM (Frederickson et al., 1983), of the hippocampus (within the temporal lobe, which mediates learning and memory function), the cerebellum, which controls coordination, balance, learning and motor skills) and the prefrontal cortex, which is involved in the formation of plans, concepts and focussing attention. The highest concentration of zinc is in the telencephalon and grey matter of the cerebral cortex (Caulfield et al., 2011, Hu and Friede, 1968). Approximately 80% of the total brain zinc exists as zinc metalloproteins. The rest mainly exists in the presynaptic vesicles where it is histochemically reactive (responds to a stimulus) or chelatable (that can combine with a chemical compound to form a ring) (Frederickson, 1989, Frederickson and Bush, 2001).

Zinc ions are contained in nerve terminals (Assaf and Chung, 1984), and act as key modulator of both intracellular and intercellular neuronal signalling (Sensi et al., 2011), and plays an additional role as a neurosecretory product within the Central Nervous System. Zinc is found in particularly high concentrations in a group of neurons called "zinc-containing neurons" which are found mainly in the fore-brain, the limbic and the cerebro-cortical regions. It is thought that the zinc here may play a role in the modification of synaptic strength (Frederickson and Danscher, 1990, Frederickson et al., 2000), modulating N-methyl-D-aspartate receptor (NMDA) receptor response to the synapses, and exerting cellular effects particularly on neurotransmitter receptors (Smart et al., 2004, Vogt et al., 2000).

Zinc is an important trace element which is critically needed during brain development and for brain function. Zinc deficiency has been associated with lower learning ability, apathy, fatigue, and cognitive delay" in children (Pfeiffer and Braverman, 1982). The homeostasis of zinc in the brain is very complex (Takeda, 2001). Zinc is supplied to the brain through the brain barrier system which comprises the blood-brain barrier and the blood-cerebrospinal fluid barrier. From the total amount of zinc the brain, about 10% is present in the synaptic vesicles. The turnover of zinc in the brain is slow compared to turnover in the peripheral tissues such as the liver. But when the brain is deprived of zinc this has an adverse effect on the hippocampus, affecting learning and memory

(Takeda, 2001, Szewczyk, 2013). Previous research exploring the impact of zinc deficiency on cognitive and behavioural function in humans and animals is reviewed in section 5.4.

Other roles

Zinc is required for neurosensory functions, including cognition, taste and vision (Nriagu, 2007). The role of zinc in cognition is described in detail elsewhere (see Section 5.4). There are high concentrations of zinc present in the choroid of the eye (Samman, 2007, Mann and Truswell, 2012). A decline of zinc levels with age appear to play a role in age related macular degeneration, leading to loss of vision (Lengyel et al., 2007). Zinc has also been linked to taste acuity, where zinc activates the sensor areas for taste, smell and appetite in the brain (IZA, 2011, Henkin, 1984). Zinc status appears to influence appetite and taste preference and elemental zinc supplementation of about 25-100 mg/day has been shown to be an effective treatment for taste dysfunction (Heyneman, 1996). Finally, zinc has been shown to be important for fertility in men, protecting against prostatic hypertrophy and infection, helping to maintain sperm count, motility and normal levels of testosterone (IZA, 2011).

2.3 ZINC METABOLISM

Understanding the pathways involved in zinc metabolism has been the focus of much research over the last 20 years. Technological advances in the analysis, imaging and modelling of zinc in the body have contributed to an increased understanding of how zinc is absorbed, excreted, transported to maintain homeostasis.

2.3.1 Zinc Absorption

Zinc absorption can be simplified into three stages, firstly, zinc is chelated in the intestine, then zinc is transferred intracellularly by zinc-binding ligands and, finally, zinc is removed from the basolateral membrane of the epithelial cells to enter systemic circulation (Fairweather-Tait, 1988).

Zinc is released from food as zinc free ions that bind to endogenous ligands or exogenous materials in the lumen of the intestine, before being absorbed in the duodenum and jejunum (Cousins, 1979, Brown et al., 2004). Zinc absorption can occur by an active or passive transport (Salgueiro et al., 2000). The absorbed zinc is carried by

the portal system directly to the liver where it is taken up and released into the systemic circulation to be delivered to other tissues (Brown et al., 2004). During this process, zinc may be transported by albumin in plasma to the liver where it form a hepatic pool bound to hepatic metallothionein or may circulate to the rest of the body (Salgueiro et al., 2000).

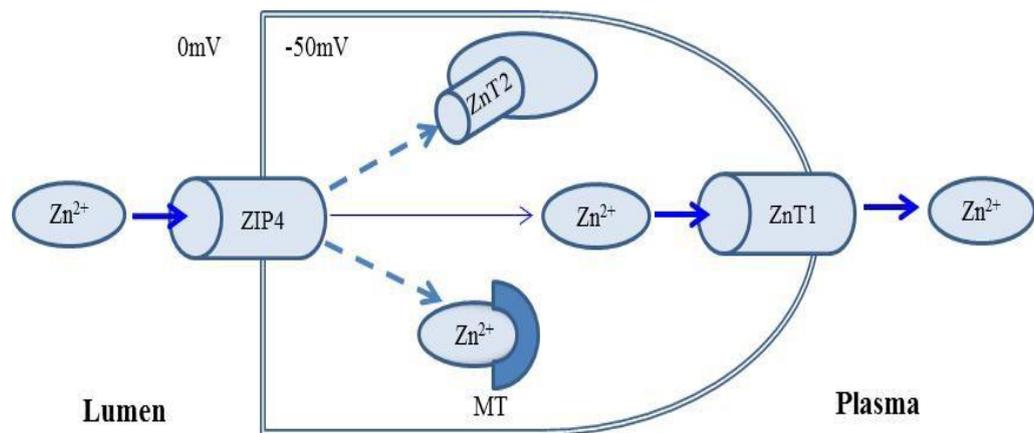
Zinc binds to protein to enter the enterocyte, and that in this process low molecular weight organic substances such as amino and hydroxy acids facilitate zinc absorption (FAO, 2001b). The ZIP (Zrt-Irt-like protein) have 8 transmembrane domains and transport zinc from the extracellular space into the cytoplasm (Eide, 2006), whereas the ZnT (solute-linked carrier) protein have 6 transmembrane domains and generate zinc efflux out of cell or into organelles or vesicles (Cousins et al., 2006, Rink, 2011a). Among the number of proteins dedicated to Zn²⁺ are 10 members of the ZnT family, 15 members of the ZIP family and 3 different forms of metallothionein (Sekler et al., 2007). There are 9 ZnT and 15 Zip transporters in human cells, ZnT decrease cellular intake by promoting zinc efflux, from cells or into intracellular vesicles, whereas Zip increase intracellular zinc availability by promoting extracellular zinc uptake (Liuzzi and Cousins, 2004). The uptake and efflux of cellular zinc is carried out by zinc transporters (e.g. ZnT3, ZnT1). Zn transporter 1 function mainly as a zinc exporter but may also play a role in zinc homeostasis by acquiring or eliminating excess of zinc (Krebs, 2000).

Zinc transports are responsible for the movement of zinc across cellular membranes (Eide, 2006) and there is general consensus that there are 2 gene families of zinc transport that are the major players: the ZnT solute-linked carrier 30 ZnT(SLC30) and the ZIP solute-linked carrier 39 ZIP (SLC39) (Cousins et al., 2006, Rink, 2011b). The first mammalian zinc transport ZnT1 was identified in 1994 (Cousins et al., 2006). Subsequently 14 zinc importers (SLC39/ZIPs), and 10 zinc exporters (SLC30/ZnTs) have been further described in mammals (Kambe, 2011).

In the intestine, ZIP4 is believed to provide the major uptake pathway for zinc from the diet. At cellular level, free zinc is bound by metallothionines (MTs) which are ubiquitous proteins characterized by low molecular weight, high metal content, no aromatic amino-acids and high cysteine content (Tapiero and Tew, 2003). MTs are small zinc-binding proteins that maintain a low cytosolic 'free' zinc concentration. ZincT2 can be accumulated in the cytoplasm, being a possible mechanism whereby a cell can store significant amounts of this metal (Falcón-Pérez and Dell'Angelica, 2007),

where it can be integrated into zinc-dependent proteins and enzymes. Zinc uptake is transporter mediated (a passive component may be evident at high zinc concentrations in the lumen), however the exact cellular mechanisms are still unclear. Ultimately, zinc leaves the enterocyte via ZincT1 and is transported in the portal circulation to the liver bound mainly to albumin (70%) and bound to α 2-macroglobulin (20–30%) (Figure 2.5) (Geissler and Powers, 2011).

Figure 2.5 - Mechanisms involved in the intestinal absorption of zinc (Geissler & Powers, 2005)



Zinc absorption is concentration dependent and is homeostatically regulated in the small intestine, primarily in the jejunum which is a site for the highest rate of absorption (Rosenberg, 1982, Lee et al., 1989). Up to a maximum of 50-60% of zinc consumed is absorbed from food, but this depends on the composition of the diet. Using stable isotopes techniques to measure zinc absorption from composite meals given to young women, Turnlund et.al., reported an average zinc absorption of 25% (Turnlund et al., 1984). Absorption of zinc from cow milks and infant formulas is around 30% and then soy protein formula, 15% (Sandstrom et al., 1983). Absorption of zinc from human milk averages 50-60%. These studies were conducted in adults, but a similar pattern has been suggested in the premature infant (Ehrenkranz et al., 1989), however studies in premature infants before 36 weeks pre-conceptual age has been contradictory (Peirce et al., 1989), this is because of the data reported on zinc absorption using traditional balance techniques in premature infants is variable and to avoid these errors, zinc stable isotopes are usually recommended to measure true zinc absorption.

2.3.2 Mechanisms of Zinc Absorption

We have seen from Figure 2.5 an overview of the mechanisms involved in absorption of zinc, with the associated transport mechanisms.

Zinc transport

As mentioned before zinc is transported in plasma bound mainly to albumin and then to α_2 macroglobulin, and oligopeptides. Thus, to deliver zinc to the cells, albumin acts as the principal zinc carrier in the plasma followed by other plasma proteins and free aminoacids (Tapiero and Tew, 2003).

Whole body level

As dietary zinc absorption occurs in the small intestine, zinc may form an intestinal pool bound to the intestinal metallothionein or may be transported by albumin in plasma, to the liver. Then in the liver, it may form a hepatic pool bound to the hepatic metallothionein or may be distributed to the rest of the body to perform a wide range of biological and biochemical functions. Zinc is excreted through faeces from pancreatic, biliar and intestinal secretions, and mucosal desquamated cells. Endogenous zinc secretions and dietary zinc that was not absorbed may also be reabsorbed at distal segments of the intestine (Salgueiro et al., 2000).

2.3.3 Factors Affecting Zinc Absorption

There are a number of dietary factors that can affect zinc absorption, including the total amount of zinc consumed, plus dietary components that can either inhibit or enhance absorption. These are summarized in Table 2.2.

Table 2.2 - Summary of the dietary components of the effects on zinc absorption in adults

Dietary component	Summary of reported effect on zinc absorption
Phytate	Inhibitor
Micronutrient	
Calcium	Conflicting reports. Possible inhibitor of zinc absorption
Folic acid	
Iron	Conflicting reports. Depends on relative levels of zinc and iron and dietary matrix
Tin and copper	Tin inhibits zinc absorption. Copper has no reported effect
Vitamin B6	Possible enhancer of zinc absorption when added to B6-deficient diets
Ascorbic acid	No reported effect on zinc absorption
Dietary matrix	
Organic versus inorganic zinc	No significant difference in absorption of zinc from organic versus inorganic sources with a meal. Presence of food inhibits absorption of zinc from inorganic sources.
Bio-fortified Crops	Bio-fortification of crops with zinc can enhance zinc absorption from the diet
Level of zinc in the diet	Fractional zinc absorption is inversely related to the total zinc content of the meal
Vegetarian matrix	Zinc absorption is adversely affected by various components of the vegetarian diet
Protein	
Oxalate	No significant effect reported
Cholesterol	Possible enhancer of zinc absorption
Tea	Small but not statistically significant adverse effect on zinc bioavailability
Maillard browning (protein cooked at high temperature)	Inhibitor of zinc absorption
Dairy	
Milk and yogurt	Addition of milk or yogurt to a plant-based diet increases bioavailability of zinc
Human milk versus cow's milk versus infant formula	Bioavailability of zinc is greater from human milk than cow's milk or infant formula

Adapted from Bel-Serrat, S., Stammers, A. L., Warthon-Medina, M., Moran, V. H., Iglesia-Altaba, I., Hermoso, M., Moreno, L. A. & Lowe, N. M. 2014. Factors that affect zinc bioavailability and losses in adult and elderly populations. *Nutr Rev*, 72, 334-352.

Dietary factors affecting zinc absorption

Zinc absorption is influenced by dietary zinc intake, not zinc status (King, 2010). Dietary zinc intake is the main determinant of zinc absorption, as zinc in the diet increases then the total amount of absorbed zinc augment and some of the absorbed zinc

is kept by the liver during the first pass (Wastney et al., 1986). The study by Sandstrom and Cederblad also demonstrated that the total zinc content of a meal was the main factor that influenced the amount of zinc absorbed. (Sandström and Cederblad, 1980). Isotopes studies have identified 2 factors, namely phytate and dietary protein that together with the total amount of zinc play a major role in zinc absorption. Zinc availability from the diet can be improved by reducing the phytate content from the diet and including sources of animal protein (FAO/WHO/UNU, 2001).

Iron

Competition between iron and zinc has been demonstrated in animals and human studies. Several studies have shown that high iron concentrations can negatively affect zinc absorption in adults when they are given in solution. However, when iron and zinc are given in a meal, this effect has not been observed (Solomons, 1986). Whittaker suggested that the total amount of ionic species affects the absorption of zinc and that a large amount of iron supplement of more than 25 mg may decrease zinc absorption (Whittaker, 1998).

Non-heme iron has been demonstrated to interact with dietary zinc in the intestine (Solomons and Jacob, 1981). When zinc and iron were taken with meals, a damping of their interaction was observed in absorption experiments, this observation is significant in dietary context, for instance, in a metabolic study of 9 young women that were fed with 22 mg of iron and 11.5 mg of zinc, there was a 0.1 mg daily absorption of zinc (Solomons, 1986). What is evident is that the risk for competitive interaction between zinc and iron is related to high doses in the form of supplement or in aqueous solution, but when zinc is present at normal levels in the food this may not affect zinc absorption (FAO/WHO/UNU, 2001).

Calcium

The reports on calcium as a possible inhibitor of zinc absorption are conflicting (Bel-Serrat et al., 2014). Spencer et al. (1984) studied the effect of calcium in adult men through metabolic studies where 3 levels of calcium intake were administered: 200, 800 and 2000 mg/d. This study showed that the increase of calcium intake from 200 to 2000 mg/d did not significantly change the urinary and faecal zinc excretion. Dawson-Hughes et al. (1986) studied the effect of calcium on zinc retention in Post-menopausal woman who were given a standard meal supplemented with Zn-65 or 500 mg of

calcium carbonate or hydroxyl apatite compared to a placebo group, this study showed that zinc retention was not affected by the use of calcium carbonate. Wood and Zheng (1997) also studied the effect of calcium supplementation on zinc absorption in 18 post-menopausal women aged 59-86 year old. The period of study was 36 days. Subjects were given diets that contained 17.6 mg Zn/d and 890 mg Ca/d, in this study net zinc absorption and zinc balance was reduced by 2 mg/day. Wood et al suggested that a high calcium intake can decrease net zinc absorption and balance and may increase zinc dietary requirements in adults. Another study by Snedeker et al. (1982) in 9 adult males who were fed with moderate and high calcium intake ranging from 780-2382 mg/d found that calcium intake had no effect on faecal and urinary losses and neither on apparent zinc retention. Plasma zinc was also not affected by dietary calcium. In another study, when a fortificant supplement mixture that contained sodium iron ethylenediaminetetra-acetate (NaFeEDTA) zinc methionine, ascorbic acid and citric acid but not calcium was given to 13 non-pregnant adult women aged 20-31 year old, this showed an improvement of zinc absorption from food products (Mendoza et al., 2004).

Phytate

Despite the general presence of zinc in commonly consumed foods, zinc deficiency occurs because of the inhibitory substances affecting zinc absorption. There was a reduction in zinc absorption in children from Iran and Egypt whose diet had high content of inhibitors such as phytate and fibre (Prasad, 2001). These phytates are found in wholegrains, cereals, and legumes bind with zinc and inhibit its absorption, consequently zinc bioavailability from grain and plant sources are lower than from animal sources. Plant foods rich in zinc are legumes, whole grains, nuts, and seeds, which are also high in phytic acid (Harland and Oberleas, 1987). Phytate is the primary dietary factor that inhibits zinc absorption. A trivariate model described by Miller et al. (2007) allows a quantitative prediction of this inhibitory effect in adults, indicating that dietary zinc and phytate explain more than 80% of the variance in the quantity of zinc absorbed as a function of the quantity of ingested zinc (Hambidge et al., 2010). Based on animal experiments, a daily phytate: zinc molar ratio of 10 or less is considered acceptable in providing adequate dietary zinc, and daily ratios above 20 may jeopardize zinc status (Oberleas and Harland, 1981).

Amirabdollahian estimated the phytate intake and the phytate-to-zinc molar ratio of the UK population, developing tables of the phytate content of foods which were extrapolated from 28 studies and adding this data to the nutrient databank of the National Diet and Nutrition Survey (NDNS). This was a retrospective analysis from the NDNS of the food and drink consumed by children and adults which was based on 4-7 days weighed dietary intake. The phytate-to-zinc molar ratio was calculated by the division of (Phytate (mg)/660)/Zinc (mg)/65.4. The database produced an estimate of the phytate content of foods consumed in the UK. Then the database of the dietary intake of phytate and the phytate-to-zinc molar ratio of the diet of the UK population can be used for estimating the average requirement of zinc (Amirabdollahian and Ash, 2010).

2.3.4 Excretion of Zinc

Zinc excretion occurs via the gut, kidney and integument (epithelial cells and sweat). Faecal zinc is composed of unabsorbed dietary zinc, plus endogenous zinc from intestinal secretions and sloughing of intestinal cells (Krebs, 2000). These endogenous intestinal losses vary from 7-45 $\mu\text{mol/day}$ (0.5-3mg/day) which depends on the amount of zinc intake (FAO&WHO, 2002). Urinary and integumental losses vary from 7-10 $\mu\text{mol/day}$ (0.5-0.7mg/day) and is less dependent on zinc intake (FAO&WHO, 2002).

2.3.5 Zinc Homeostasis

The major sites of whole-body zinc homeostasis are in the gastrointestinal tract and involve both zinc absorption and excretion of endogenous zinc in the faeces (Sian et al., 1996). When large amounts of zinc are secreted into the lumen of the intestine, this is then reabsorbed and both process of secretion and absorption are regulated and play an important role in zinc homeostasis (Hambidge and Krebs, 2001b). There is a well-developed homeostatic control for the absorption, metabolism and excretion of zinc. Zinc absorption and endogenous intestinal excretion are very important to maintain zinc homeostasis (King, 2010). Zinc from the diet and endogenous zinc are homeostatically regulated in the intestine (Salgueiro et al., 2000). But the maintenance of zinc balance depends not only on absorption of some fraction of exogenous dietary zinc but also on the efficient reabsorption of endogenous zinc (Krebs, 2000).

It is important to remember that we cannot measure zinc absorption or endogenous excretion directly in either humans or animals. Determinations are therefore mainly based on models that cannot be validated (King et al., 2000). The adjustments in gastrointestinal zinc absorption and endogenous excretion are synergistic. Changes in the endogenous excretion appear to occur quickly with variations in zinc intake of just above or below optimal intake while the absorption of zinc responds more slowly, but it has the capacity to cope with large fluctuations in intake (Roohani et al., 2013).

Zinc transporters from the ZIP and ZnT family maintain zinc homeostasis by controlling the process of zinc absorption and excretion (see section 2.3.1 Zinc Absorption) by regulating zinc intracellular and between organs (Cousins et al., 2006, Rink, 2011b, Josko, 2011, Murakami and Hirano, 2008).

The factors that affect zinc homeostasis was studied during acute zinc depletion on zinc homeostasis in men. This study looked at the homeostatic response using a compartmental model. 12 men were placed in a metabolic ward in San Francisco for 57 days. At baseline these men had an adequate zinc intake which was assessed by a food frequency questionnaire and a diet history. This study comprised 2 metabolic studies, for a period of 16 days men were supplemented with 12.2 mg/d of zinc, and during the period of zinc depletion, at day 41, subjects were given 0.23 mg/d of zinc. Zinc stable isotopes were administered on days 6 and 7 of the initial period and then at day 35 of the depletion period. This study showed that a decrease in plasma zinc concentrations occurred after 5 weeks of acute zinc depletion because there was a decrease in the rate of zinc release from the slow turnover of body zinc pool (King et al., 2001).

2.4 DIETARY REQUIREMENTS

2.4.1 What are the requirements / how much zinc is needed from the diet?

In the UK, the Dietary Reference Values (DRVs) are used as a reference for dietary recommendations. The amount of zinc the body needs is 5.5-9.5 mg/day for men and 4-4.7 mg/day for women (NHS, 2012). In the US, the Dietary Reference Intakes (DRI) are used. DRI were developed by the Institute of Medicine's Food and Nutrition Board(FNB), and are a general term for a group of reference values that vary by age and gender. These set of references are: The recommended dietary allowance (RDA), Adequate intake (AI), Tolerable upper intake level (UL). The RDA is the level of intake that is necessary to meet the nutrient requirements of 97-98% of healthy individuals (see

Table 2.3); the AI is the level necessary to meet nutritional adequacy, the UL is the maximum level of intake that is unlikely to cause adverse health effects (NIH, 2013).

Table 2.3 – US Recommended Dietary Allowances (RDAs) for Zinc by age and gender

Age	Male	Female	Pregnancy	Lactation
0–6 months	2 mg*	2 mg*		
7–12 months	3 mg	3 mg		
1–3 years	3 mg	3 mg		
4–8 years	5 mg	5 mg		
9–13 years	8 mg	8 mg		
14–18 years	11 mg	9 mg	12 mg	13 mg
19+ years	11 mg	8 mg	11 mg	12 mg

* Adequate Intake (AI)

Source: National Institute of Health, 2013

In the UK, the adequacy of micronutrient intake is assessed by comparing intake with age/sex specific DRVs for each vitamin and mineral. Mean intake is compared with the Reference Nutrient Intake (RNI) (Bates et al., 1997) and the proportion with intakes below the Lower Reference Nutrient Intake (LRNI) (Hill et al., 2009) is assessed. The RNI for a mineral is the amount of the nutrient that is sufficient for about 97% of people in the group (IOM, 2003). If the average intake of the group is at the RNI, then the risk of deficiency in the group is judged to be very small. However, if the average intake is lower than the RNI, then it is possible that some of the group will have an intake below their requirement (NDNS, 2012). The RNIs and LRNIs set for zinc are shown in Table 2.4, and average daily zinc intake in the UK is shown in Table 2.5 (NDNS, 2008).

Table 2.4 – UK Reference Nutrient Intake (RNIs) and Lower Reference Nutrient Intakes (LRNIs) for zinc, by sex and age (NDNS, 2008)

Mineral		Age group (years)								
		1-3	4-6	7-10	11-14	15-18	19-50	51-64	65-74	75+
Males										
Zinc (mg/d)	RNI	5	6.5	7	9	9.5	9.5	9.5	9.5	9.5
	LRNI	3	4	4	5.3	5.5	5.5	5.5	5.5	5.5
Females										
Zinc (mg/d)	RNI	5	6.5	7	9	7	7	7	7	7
	LRNI	3	4	4	5.3	4	4	4	4	4

Table 2.5 – UK average daily intake of zinc from food sources only, by age and sex, aged 1.5-64y (NDNS, 2008)

Zinc intake	Sex and age group (years)											
	Boys			Men	Girls			Women	Total			
	4-10	11-18	Total boys	19-64	4-10	11-18	Total girls	19-64	1.5-3	4-10	11-18	19-64
Zinc mg												
Mean	6.8	8.7	7.9	10.1	6.2	6.9	6.6	7.8	5.2	6.5	7.8	8.9
Median	6.4	8.4	7.2	9.8	6	6.9	6.4	7.8	5.1	6.2	7.7	8.8
SD	2.2	2.7	2.7	3.3	1.7	1.8	1.8	2.5	1.3	2	2.5	3.1
Upper 2.5 percentile	12.8	15.8	14.4	18.2	10.1	9.8	9.9	13.3	8	11	13.5	15.3
Lower 2.5 percentile	3.6	5.1	3.9	4.6	3.4	3.1	3.3	3.6	2.9	3.4	4.2	4

Recommendations

The aim of micronutrient recommendations is to provide guidelines for the nutrient composition of diets as a basis of good health and quality of life for populations. They are based on judgments and knowledge of micronutrient requirements in a particular population.

Table 2.6 shows the recommended daily intake of zinc (mg/d) in the different population groups (IZA, 2011).

Table 2.6 - Recommended daily intake in infants, children, women and men (IZA, 2011)

Population	Recommended daily intakes
Infants	5 mg
Children	10 mg
Women	12 mg
Pregnant women	15 mg
Lactating women	16 mg
Men	15 mg

- Recommended daily intakes (IZA, 2011).

Source: International Zinc association, 2011

Table 2.7 shows the different concepts that were used in Europe, USA and the United Nations University (UNU). The most commonly given values are for the estimated average requirements (EAR) of groups, and for the 2 SD above the EAR, which covers most of the population (97.5%), assuming that there is a normal distribution of individual requirements (Ashwell et al., 2008). There are different terminologies for the same nutrition concept as it is shown in Table 2.8. The common terminology of micronutrient recommendations varies across the world as most countries set their own nutrient recommendations, which results in a large variation of nutritional recommendations. This is in part due to the different approaches taken to establish micronutrient recommendations which, in turn, add to the confusion on a general consensus of micronutrient recommendations (Dhonukshe-Rutten et al., 2010). Table 2.9 shows the variability of recommended intakes of zinc for adults (over 18 years old) from European countries, USA/Canada and Australia/New Zealand. The recommended intakes for males, females (pre and post-menopausal woman), the Average Nutrient Requirement (ANR) 50th percentile and coefficient of variation percentage; the methods for estimating the ANR/Adequate Intake (AI) daily losses through faeces, urine, sweat, menstrual and the percentage of bioavailability are shown in Table 2.9 (Doets et al., 2012).

Table 2.7 - A selection of concepts and acronyms used for micronutrient recommendations across the world

Source	Mean	Mean + 2SD	Mean-2 SD	Definition used in absence of information on distribution of requirements	Upper limit of intake	General term to encompass all values
EC Scientific Committee on Food (1993, 2000/01/02/03)	Average Requirement (AR)	Population Reference Intake (PRI)	Lowest Threshold Intake (LTI)	Acceptable ranges	Tolerable Upper Levels (UL)	
UK Department of Health (1991)	Estimated Average Requirement (EAR)	Reference Nutrient Intake (RNI)	Lowest Reference Nutrient Intake (LRNI)	Safe Intake	Safe upper levels	Dietary Reference Values (DRV)
Nordic Council of Ministers (2004)	AR	Recommended Intake (RI)			Upper limit of intake	
DACH (2000)	AR	Recommended Nutrient Intake (RNI)		Estimated value		Reference values
Health Council of the Netherlands (2000)	AR	Recommended Dietary Allowance (RDA)		Adequate Intake (AI)	Tolerable upper intake level (UL)	Dietary Reference Intakes (DRI)
US institute of Medicine (1997)	EAR	RDA		AI	Tolerable upper intake level (UL)	DRI
United Nations of University (2007)	Average Nutrient Requirement (ANR)				Upper nutrient level (UNL)	Nutrient Intake Values (NIV)
Ashwell, M., J. P. Lambert, et al. (2008). "How we will produce the evidence-based EURRECA toolkit to support nutrition and food policy." European Journal of Nutrition 47: 2-16.						

Table 2.8 - Common terminology proposed by UNU and currently used terminology

UNU term	UNU definition	Terminology used by European countries / organizations and key non-European countries for equivalent concepts
NIV	Nutrient intake value encompasses the set of recommendations	Dietary reference intakes (US) Reference values for nutrient intake (DACH ^a) Dietary reference values (UK, France)
ANR	The Average Nutrient Requirement is the average or median requirement estimated from a statistical distribution of required intakes for a specific criterion (such as biomarker or health indicator) and for a particular age and sex-specific group.	Estimated Average requirement
INLx	The individual Nutrient levels is the recommended nutrient level for all healthy individuals in a specific sub-population	Recommended nutrient intake (DACH, UK, WHO) Population reference intake (France, EC) Recommended average (Latvia) Recommended daily allowance (The Netherlands, US) Recommended intake (Nordics) =all equal to INL 97.5
<i>Other general terms and definitions</i>		
AI ^b	The Adequate intake is defined as the observed or experimentally derived intake in a defined population group that appears to sustain health. It is used when there are insufficient data to establish a statistical distribution of individual requirements and, therefore, an ANR and INLx.	Estimated value for adequate intake EC, US) (DACH)Adequate intake (France, Netherlands, Safe intake (France, Netherlands, EC, US) Acceptable Intake (WHO)
Acceptable range	The acceptable range is a range of safe intake values and is given where insufficient information is available	Acceptable range (EC) Estimated value for adequate intake (DACH) Adequate area of intake (Netherlands) Safe intake (UK)

^aDACH stands for the German-speaking countries: Germany, Austria and Switzerland.

^bFrom a scientific point of view, this term is not advocated, as it is a default approach that should be used only if too little information is available for the ANR and/or INLx.

^cUNU, United Nations University

Dhonukshe-Rutten, R. A. M., L. Timotijevic, et al. (2010). "European micronutrient recommendations aligned: a general framework developed by EURRECA." *European Journal of Clinical Nutrition* 64: S2-S10.

Table 2.9 - Recommended zinc intakes for adults (Doets et al., 2012)

Recommended intakes of zinc for adults (age≥18years) and underlying approaches																	
Zinc	Year	Recommended intake (mg/d)			Approach	ANR (mg)/ 50th pct CV (%)				Method for estimating ANR/AI: daily losses				Total losses (mg/d)			Bioav (%)
		m	f pre	f post		m	f pre	f post	m+f	Faeces	Urine	Sweat	Menstrual	m	f pre	f post	
GB	1991	9.5	7	2.5	ANR + 2SD	7.3	5.5		15					2.2	1.6		30
EC	1993	9.5	7	2.6	ANR + 2SD	7.5	5.5		15					2.2	1.6		30
DACH	2000	10	7	3	ANR + 2SD	n.a	n.a		10-15					2.2	1.6		30
FR	2001	9	7	2	AI	x	x		x					n.a	n.a		30
NL	1992	10	9	1.2	ANR + 2SD	7.6	6.8		20	0.3-0.5	0.3-0.7		0.27	1.3-1.9	1.1-1.7		25
NNR	2004	9	7	0.9	ANR + 2SD	6.4	5.7		15	1.4	Urine + Sweat + Semen: m: 1.27, f: 1.0			1.4	1		30
WHO/FAO	2004	7	4.9	2.2	ANR + 2SD	4.7	3.2		25					2.67	2.4		40
US/CA	2001	11	8	3.1	ANR + 2SD	9.4	6.8		10	m: 2.57, f: 2.3	0.63	0.54	0.10	3.84	3.3		m:41, f: 48
AU/NZ	2005	14	8	6.6	ANR + 2SD	12	6.5		10	m: 1.54, f: 1.06	0.63	0.54	0.10	2.81	1.96		m:24, f:31

m, males; f, females; pre, premenopausal; post, postmenopausal; pct, percentile, ANR, Average Nutrient Requirements; AI, Adequate Intake; bioav, bioavailability; GB, United Kingdom; EC, European Community, DACH, Germany-Austria-Switzerland; FR, France; NL, Netherlands; NNR, Norway-Sweden-Finland-Denmark-Iceland; US/CA, USA/Canada; AU/NZ, Australia/New Zealand; n.a., not available in report; x, not available because the intake-based approach was used; n, negligible.

§For men and women aged ≥75 years, the recommended Zn intake was 8.0 mg

Doets, E. L., A. E. J. M. Cavelaars, et al. (2012). "Explaining the variability in recommended intakes of folate, vitamin B-12, iron and zinc for adults and elderly people." Public Health Nutrition

2.4.2 Methods used to derive dietary requirements (Factorial approach)

Since the mid-1990s, the expert committees from the World Health Organization / Food and Agriculture Organization / International Atomic Energy Association (WHO/FAO/IAEA) together with the Food and Nutrition Board (FNB) of the United States Institute of Medicine (IOM) developed the estimates of human requirements using a factorial method, which is the amount of zinc needed for tissue growth, maintenance, metabolism and endogenous losses (FAO, 2001b, Brown et al., 2004).

The type of data used to estimate zinc physiological requirement for each age group will be described below.

Adult men

The estimation of mean urinary zinc excretion by adult men of 0.63 mg/day was based on 17 studies whose subjects had a zinc intake from 4-25 mg/day which was a range at which urinary zinc concentrations are not affected by the amount of ingested zinc. This estimation is reliable for 3 reasons: 1) It was reviewed from a large number of studies; 2) Included only studies in which zinc intakes fell within a range in which urinary zinc excretion is constant and likely to include the true physiological requirement; and 3) provided lots of information on the analysis.

Adult women

The estimation of mean urinary excretion by the FNB/IOM for adult women was 0.44 mg/day. Exogenous zinc losses were 1.06 mg/d. This estimation resulted from the examination of 10 studies. There is little information in endogenous zinc losses in menstrual fluid, the average menstrual losses were estimated to be 0.1 mg/day which is negligible and consequently ignored in the estimation of zinc requirements.

Children (Infants 0-6 m)

There is little information on zinc homeostasis and on physiological requirements for absorbed zinc in infants (under 6 months old). Studies on breast fed infants in the United States found no differences in growth between those who received zinc supplements or placebo from 4-6 months old. This suggested that their zinc intake from breast milk together with any additional zinc from pre-existing stores was adequate for the maintenance of normal growth. Infants from 4-9 months of immigrant population had increased rates of growth when given 5 mg/day of zinc for 3 months, but these

infants were not entirely breast-fed, so it is believed that foods that had lower zinc density were displacing breast milk or these foods interfered with zinc absorption from breast milk. Because of few studies in infants physiological requirements were not estimated, but the Adequate Intakes (AIs) were described by the FNB/IOM, which proposed an AI of 2 mg/day for infants aged 0-5 months old. On the contrary the WHO committee estimated zinc requirements in infants by extrapolating data for adults in relation to metabolic rate and incorporating the amount of zinc from tissue, this was estimated to be 0.7-1.3 mg/day for infants aged 0-5 months old. There is less information on zinc requirement in infants of low birth weight, therefore, there is a need for further research on zinc physiological requirements in infants particularly those with low birth weight (IZINCG, 2011).

Children (6m-18y)

The method used by the FNB/IOM to estimate zinc physiological requirements for children aged 6m-18year old was the factorial approach. Exogenous zinc losses was estimated to be 0.014 mg/kg/day, faecal excretion of endogenous zinc was estimated to be 0.050 mg/kg/day for infants aged 6-11 months old and 0.034 mg/kg/day for older children. To these amount of endogenous losses, the amount of zinc needed for growth was added and this amount was estimated to be 0.020 mg/g of gained tissue. To estimate zinc physiological requirements for children age 14-18 year old, an extra 0.1 mg/day was added to account for zinc losses in semen. The WHO committee estimated zinc physiological requirements in childhood by extrapolating the data used to estimate zinc endogenous losses in adults. The international zinc group (IZiNCG) follow the factorial approach used by the FNB/IOM, but for endogenous zinc losses follow the estimation carried out by the IZiNCG, and use the National Center for Health Statistics, Centers for Disease Control and prevention, World Health Organization (NCHS/CDC/WHO) body weight reference standard. Endogenous zinc losses are calculated as 0.064 mg/kg/day for infants aged 6-11 months old, and 0.034 mg/kg/day for children over 1 year old, estimation of intestinal losses are 0.05 mg/kg/day for infants 6-11 months old or 0.02 mg/kg/day for children over 1 year old. For instance, children aged 6-11 months old with a body reference weight of 9 kg and expected weight gain of 13 g/day will need 0.576 mg/day (i.e. 9kg x 0.064 mg/kg) to replace endogenous losses and 0.260 mg/day (13 g/day x 0.020 mg/day) for tissue, this result in

a total amount of zinc required of 0.836 mg/day. Similar calculations was performed for children aged 1-3, 4-8, 9-13 and 14-18 year old (IZINCG, 2011).

Pregnancy

Additional zinc requirements are needed during pregnancy for foetal and maternal tissue, this was estimated to be 0.16 mg/day during the first trimester of pregnancy, 0.39 mg/day during the second trimester and 0.63 mg/day during the third trimester by the FNB/IOM, whereas the WHO estimated the additional requirement to be 0.1 mg/day during the first trimester, 0.3 mg/day during the second trimester and 0.7 mg/day during the third trimester. The IZiNCG proposed the figure of 0.7 mg/day, which covers the additional need during the third trimester of pregnancy, however this figure overestimates zinc average requirements for the first and second trimester pregnancy. Both the FNB/IOM and WHO committees based zinc requirements during pregnancy on the data from Swanson and King (Swanson and King, 1987).

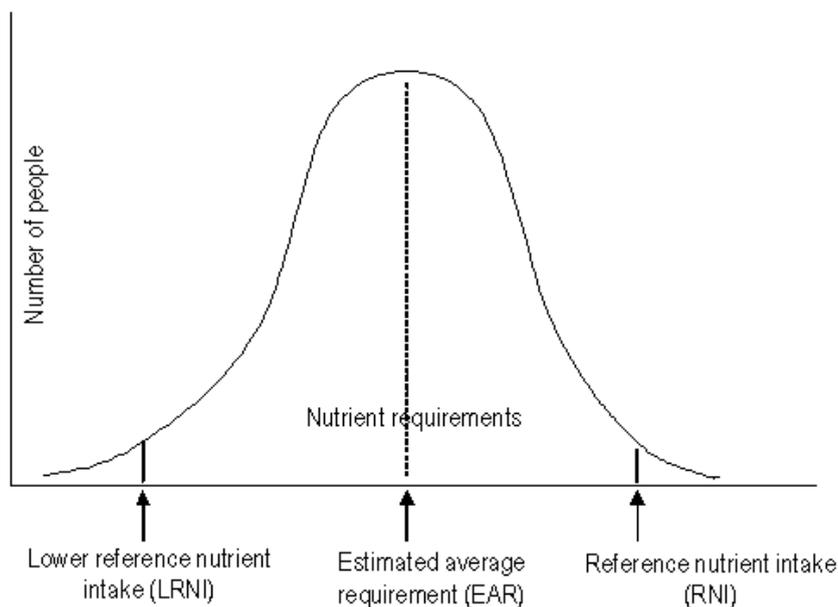
Lactation

The calculation of zinc requirements for women during lactation by the FNB/IOM, was as follows: the average volume of milk transferred from the mother to the infant was multiplied by the amount of zinc concentration in human milk at various post-partum periods. Milk volumes were measured during the first year post-partum in US women (0.78 L/d), then the analysis of 12 studies provided information on zinc concentration of human milk at 4 weeks (2.75 mg/L), at 8 weeks (2 mg/L), at 12 weeks (1.5 mg/L) and at 24 weeks (1.2 mg/L). The combination of these 2 data sets produced a single measurement of 1.35 mg/day which is the average additional amount of absorbed zinc needed during lactation. On the other hand the WHO committee estimated zinc concentrations in human milk using only 3 studies, then the WHO estimated that additional 1.4 mg zinc/day is needed from 0-3 months post-partum, an additional 0.8 mg/day for 3-6 months, and 0.5 mg/day after that. It was clear that additional needs are increased during lactation particularly during the first months of breastfeeding, then on average 1 mg of additional zinc is needed during lactation.

To calculate the Estimated Average Requirement (EAR), the factorial approach has been used, which is illustrated in Figure 2.6. The Estimated Average Requirement (EAR) is the daily intake value that is estimated to meet the requirement, as specified by the index of adequacy, in half of apparently healthy population by group and age. The

EAR serves as a basis for setting the Recommended Dietary Allowance (RDA). There are variations in nutrient absorption and metabolism because of individual biological variation. Some people have a low requirement for a nutrient, meaning lower than average and some people have a higher requirement for a nutrient, meaning higher than average. If the requirement of a nutrient X for a determined life stage and age group were plotted (e.g. 19-30 year old women who are neither pregnant nor lactating) as a normal or Gaussian distribution, this might result and create a bell-shaped curve. This indicated that there is an assumption that nutrient requirements are normally distributed, however this is not always the case. It may be that the nutrient requirements of a particular group may be unknown, or that the data may be insufficient, or in the case of infants, it would unethical to conduct studies on them to determine their nutrient requirements. Overall, the EAR are sometimes based on scarce data or data which is drawn from studies with limitations in their study design. The nutrient requirement level that is 2 standard deviation above the EAR is generally selected as the Reference Nutrient Intake (Lee and Nieman, 2013).

Figure 2.6 - Estimated Average Requirement (EAR)



Source: Dietary Reference Values: A Guide, Department of Health, London 1991.

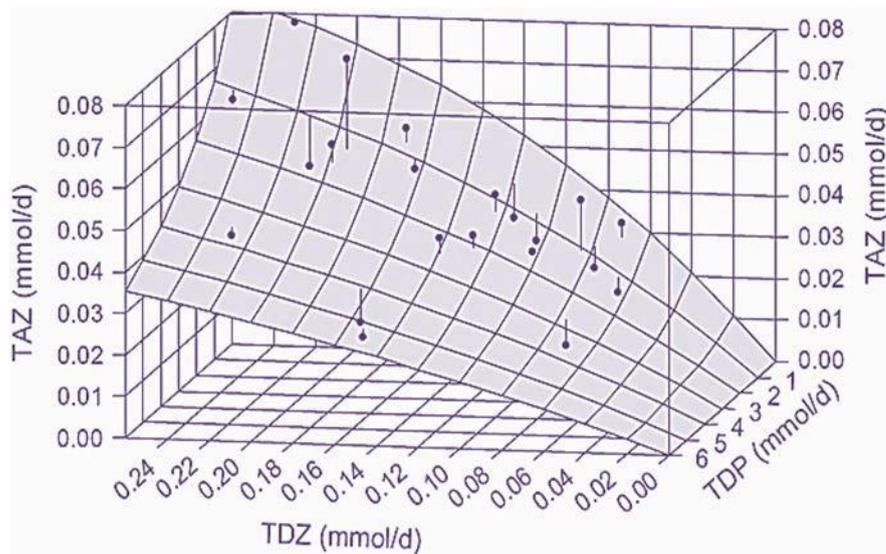
Regarding the sources of zinc losses these are: integumental (ranging from 0.24-0.67 mg/d of zinc), endogenous (zinc that is secreted into the intestine form within the body and excreted via the faeces; ranges from 1.3-2.62 mg/day of zinc), urinary (ranging from 0.2-6.5 mg/d day with a dietary zinc intake ranging from 1.43 to over 22 mg./d of

zinc), faecal (In adults, ranging from 0.69 mg/d on a diet containing 1.43 mg/d of zinc, to 22.4 mg/d on a diet containing 23.26 mg/d of zinc, seminal (0.63 mg per ejaculation on a diet containing 15.7 mg Zn/day), and menstrual losses ($5\mu\cdot$ /d for women taking oral contraception and $6\mu\text{g}/\text{d}$ for women with normal menstrual cycle) (Bel-Serrat et al., 2014).

To determine zinc losses, a number of methods have been used including metabolic balance studies, turnover time of radio labelled endogenous zinc pools and measurements of total endogenous zinc loss, however, none of these methods have been shown to be an ideal measurement, but they all indicate that in a western diet, people eating 10-12 mg/zinc, the amount of endogenous loss will amount to 2-3mg/day (Geissler and Powers, 2011). Unlike for iron, no specific body store is known for zinc, and consequently any metabolic requirement of zinc has to be met by food intake and/or supplementation (Nriagu, 2007). Furthermore, although zinc is present in all body tissues and fluids (total body zinc content accounts approximately 30 mmol (2 g)), which is distributed in skeletal muscle (approximately 60 % of the total body content), and bone mass (approximately 30%), with a zinc concentration of 1.5–3 $\mu\text{mol}/\text{g}$ (100-200 $\mu\text{g}/\text{g}$), “the body has no zinc stores in the conventional sense” (FAO, 2001b).

Miller developed a mathematical model based on total absorption of zinc (TAZ) as a function of total dietary intake of zinc (TDZ) and total dietary phytate (TDP). This mathematical model is important to comprehend zinc absorption considering that phytate inhibits zinc absorption by binding with zinc in the gut, forming an unabsorbable complex (Miller et al., 2013), and this model can be used to predict zinc requirements. TAZ was measured using established isotope techniques, TDZ was determined by chemical analysis of diets in the majority of studies, and TDP was calculated from diet composition. This model of zinc absorption was developed from a basic conception of the relevant intestinal biochemistry and fit it to selected existing data, 21 mean data were used from 4 to 21 adult subjects, and no data in children was included for this model. The goodness of fit R^2 of 0.82 was very supportive of this model, indicating that 82% of the variance on total absorption of zinc (TAZ) was explained by this mathematical model. The data points are represented by round symbols, the vertical “stems” on the data symbols indicate the distance, up or down, from each datum to the surface Figure 2.7 (Miller et al., 2007). Also see section 2.3.3 Factors Affecting Zinc Absorption.

Figure 2.7 - Mathematical model of zinc absorption in humans as a function of dietary zinc and phytate, a three-dimensional plot of the data (Miller et al., 2007)



TAZ: Total absorption of zinc, TDZ: Total dietary intake of zinc, TDP: Total dietary phytate.

Factorial approach

Estimates of human requirements depends on the factorial approach (Hambidge and Krebs, 2001a). The factorial approach relies on measurements of various factors that include requirements for growth, pregnancy and lactation and faecal and urinary losses that determine requirements to maintain plasma levels or body stores which results in normal tissue and body function and prevents adverse health outcomes. Then the factorial approach consists of adding the requirement for growth to the requirement for replacement of the inevitable losses in urine, faeces and from the skin (Fomon, 1991). The reference values derived from this approach also depend on a bioavailability factor, in order to convert the physiological requirement into a dietary intake value.

Matthys et al. (2011) indicates that the factorial approach is based on data of micronutrient losses and maintenance absorption/bioavailability that includes measurements of physiological outcomes, such as body stores. These measurements can be affected by the many factors for instance, homeostatic mechanisms, the micronutrient intake, body status, and demography.

Estimated average requirements for micronutrients are important to derive Dietary Reference Values.. Zinc average requirements are derived using the factorial approach in which physiological needs for tissue growth, endogenous losses and maintenance are estimated, and the total converted to a dietary requirement by taking into account the

total absorption from the diet, for example, multiplying the requirement by a bioavailability factor which in turn can be calculated using algorithms, or estimates from absorption studies (Fairweather-Tait and Collings, 2010). To date, a useful algorithm to establish dietary zinc requirements based on the presence of other nutrients and food components have not yet been established, therefore more information is needed to develop an algorithm that can predict zinc bioavailability (Hunt, 1996).

2.4.3 Food sources

Dietary Sources

Zinc is found in higher concentrations in animal source foods, particularly beef, pork, chicken, fish, shellfish and in lower concentrations in eggs and dairy products. Relatively higher content of zinc is found in nuts, seeds, legumes and whole grain cereals, and lower content of zinc is found in tubers, refined cereals, fruits and vegetables (IZINCG, 2011), see Figure 2.8.

Figure 2.8 - Food sources of zinc



Adapted from Insel, et al. (2013). *Discovering Nutrition*, Jones Barlett.

According to the National Diet and Nutrition Survey (NDNS) (2000/01), a continuous cross-sectional survey designed to assess the diet, nutrient intake and nutritional status of the general population aged over 18 months old living in private households in the UK (Whitton et al., 2011), showed that the mean average zinc intake from food sources is 10.1 and 7.8 mg/d for men and women respectively with a Reference Nutrient Intake (RNIs) of 9.5 mg/d for men and 7.0 mg/d for women (NDNS, 2008). Figure 2.9 and Table 2.10 show that rich sources of zinc are meat (especially red meat rather than white meat), poultry, fish, seafood, shellfish (oysters), whole-grain and dairy products. The majority of zinc is provided by animal tissue as high levels of zinc are found in muscle, up to 50mg/Kg (Jackson, 1989, Plum et al., 2010). By contrast zinc content in fat is only 5 mg/Kg.

Figure 2.9 - Main dietary sources of zinc micronutrients in the UK diet (MAFF 1994a, 1997) (Geissler and Powers, 2011)

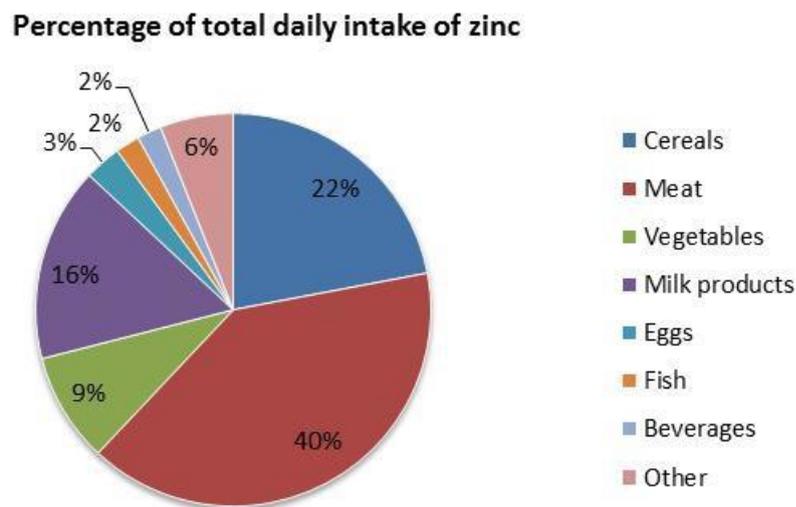


Table 2.10 - Sources of zinc and their average content (mg/100g) (IZA, 2011)

Sources of zinc	Average content (mg/100g)
Oysters	25
Meat (especially red meat)	5.2
Nuts	3
Poultry	1.5
Eggs	1.3
Milk products	1.2
Cereals	1
Bread	1
Fish	0.8
Sugars & preserves	0.6
Canned vegetables	0.4
Green vegetables	0.4
Potatoes	0.3
Fresh fruits	0.09

Evaluation of zinc status

The rationale for serum or plasma zinc remaining as the best available biomarker of the risk of zinc deficiency in populations is because serum or plasma zinc reflects dietary zinc intake and responds constantly to the supplementation of zinc (de Benoist et al., 2007). Although plasma or serum zinc concentration is the most widely used measure of zinc status, the reliability of this index has been criticized since change in plasma zinc is not linked to whole-body zinc, plasma zinc is insensitive to the changes in zinc diet (King, 1990) and plasma zinc does not reflect actual zinc absorption, this may be because an increase in plasma zinc may not be seen until an amount of zinc is absorbed, which depends on the amount of zinc intake and the type of food (Casey et al., 1981). Zinc concentrations, e.g. of plasma, blood cells, hair, and urinary zinc, decline in severe zinc deficiency but other conditions, not related to zinc status, can also affect all these indices, namely, infection, fever, stress and pregnancy (WHO, 1996).

It is known that zinc concentrations in muscle and liver tissues is 50 times greater than in plasma, therefore differences in zinc intake or zinc delivery from the peripheral areas can have a deep effect on plasma zinc concentrations and this is a reason of why plasma zinc concentrations is not a reliable index of total body zinc content in individuals (Brown et al., 2001).

Searching for a reliable indicator for zinc is problematic because the effective regulation of zinc homeostasis buffers the functional response to dietary deficiency and excess (Lowe et al., 2009). Nowadays, there is no consensus on the appropriate biochemical indicators of zinc status and neither much information on the zinc status of populations worldwide (Hess et al., 2007).

2.5 ZINC DEFICIENCY AND ZINC TOXICITY

2.5.1 Consequences of zinc deficiency

Zinc deficiency is a major public health problem worldwide (Prasad, 2000), and has a big effect in both humans (Sandstead, 2013) and in animals. In the case of humans, cases of severe zinc deficiency have been shown to affect several organ systems such as the central nervous system, immune system, gastrointestinal, and reproductive systems (Hambidge, 2000b), the zinc content in human organs and tissues has been determined in healthy and ill subjects (Eggleton, 1940).

In the study of animals, zinc deficiency has been studied in rats (Follis et al., 1941). It was found that pregnant zinc deficient rats caused foetal malformation. Weanling rats with severe zinc deficiency had retarded growth, abnormal hair and dermatitis (Hurley, 1969). In animal models, it has been shown that zinc deficiency is especially devastating during foetal and early postnatal development, causing increased oxidative stress and resulting in damage that can contribute to teratogenicity (Uriu-Adams and Keen, 2010). Looking at the effect of nutritional zinc deficiency on zinc concentration in various regions of the brain, only in the cerebellum was there a significantly decreased zinc concentration, whereas the hippocampus and cortex did not show this. In rats, severe zinc deficiency did not affect lateral distribution of zinc in the hippocampus (Wallwork et al., 1983). However, zinc deprivation in pregnant rats showed neuronal degeneration and a reduction in the brain size (McKenzie et al., 1975, Prohaska et al.,

1974). Moreover, moderate zinc restriction reduced motor activity (Golub et al., 1985) and performance of visual attention tasks (Golub et al., 1996). Animals that were zinc deficient presented cognitive deficits, for example monkeys that were zinc deprived had difficulty in remembering what they learnt and in learning new problems (Black, 2003a), and when monkeys were fed with a zinc deficient diet they had poor visual-attention performance task and poor short term memory (Golub et al., 1994a). It has also been shown that the neuronal function in the hippocampal mossy fibres have been affected in pigs suffering with chronic zinc deficiency (Hesketh et al., 1985, Hesse, 1979).

Zinc deficiency has been recognized as an important and widespread risk to human health and it is common throughout the developing world (Prasad, 2009). Despite the fact that zinc has been indicated as an important factor in children's activity, attention, and development, the mechanisms underlying this relationship are still unclear (Black, 1998), but these are the specific cognitive deficits associated with zinc deficiency. Although infants, children, pregnant women and elderly are considered risk groups, zinc deficiency may affect the whole population (Salgueiro et al., 2000). The critical consequences of zinc deficiency are: growth retardation and male hypogonadism as those clinical signs reported in Iran and Egypt, have been observed in many other countries (Prasad, 2009); impaired immune function, rough skin, poor appetite, delayed wound healing, susceptibility to infections such as diarrhoea and pneumonia and consequently increased mortality rate, negative effects in pregnancy outcome and neuro-behavioural development and mental lethargy (IZA, 2008, Prasad, 2009).

Risk for zinc deficiency occurs mainly during the foetal period due to maternal zinc deficiency and often accompanied by protein malnutrition, since protein is a major source of dietary zinc (Georgieff, 2010). Moderate zinc deficiency in infants and children is not only associated with reduced growth and development, but also with impaired immunity and increased morbidity and mortality from infectious diseases (Osendarp et al., 2003). There is little evidence of the possible effect of zinc deficiency on behaviour, one example being the study of Guatemalan children aged 12-24 months by (Sazawal et al., 1996). This study found that supplementation of zinc together with vitamins increased activity levels in children which was measured by the children's activity rating scale of Puhl et al. (1990).

An early zinc deficiency will lead to a diminished cognitive function, behavioural problems, mood changes, reduced memory, problems with spatial learning and neuronal degeneration (IZA, 2004). Explanations for altered cognition and other mild and severe zinc deficiencies clinical outcomes mentioned above have not been categorically established. Zinc deficiency also complicates many diseases including alcoholic liver disease, sickle cell anaemia, protein calorie malnutrition, intestinal diseases such as Crohn's disease, celiac disease and short bowel syndrome (McClain et al., 1985).

The inclusion of zinc in interventions to prevent micronutrient deficiencies in young children will have an impact on child morbidity and mortality (Penny, 2013). Prenatal zinc nutrition has been shown to be protective against diarrhoea morbidity in infant offspring through 12 months of age (Iannotti et al., 2008) and, if diarrhoea occurs, then zinc supplementation is recommended, especially in developing countries (WHO/UNICEF, 2004) and this has been shown to be beneficial in reducing the incidence of persistent diarrhoea by approximately 25% (Patel et al., 2010) and to enhance serum zinc concentration (Baqui et al., 2006).

Zinc deficiency is likely to be prevalent especially in countries where cereal proteins are mainly consumed by the population (Prasad, 2009) and because of poor dietary zinc, chronic infections are also known to diminish zinc availability (Georgieff, 2010). Two general dietary patterns are likely to be the major factors in zinc deficiency; first, non-fermented cereal and legume-based diets are high in phytic acid consequently inhibiting zinc absorption; second, diets based on starchy roots and tubers have lower zinc content and when these diets are also combined with lower meat intake then zinc absorption would be also insufficient (IZA, 2008). However, the actual prevalence of zinc deficiency amongst populations cannot be assessed because of a lack of definite criteria for zinc requirements (Abrams and Wong, 2003). Children from many low-income countries are at risk of illness and death from infectious diseases (Black, 2003b) the global burden of disease is very large and could be reduced by improving the available zinc in the diet (Penny et al., 1999).

Mild chronic zinc deficiency

Mild zinc deficiency occurs specially in developing countries and evidence exist of the uncertainties associated with mild zinc deficiency (Hambidge, 1989, Hambidge, 2000a). In the cases of mild chronic zinc deficiency, the study by Gibson et al. (1989) in Ontario

boys aged 5-7 years showed that zinc deficiency affected growth development. Prasad also indicated that characteristics of moderate zinc deficiency were growth retardation and delayed puberty in adolescents, rough skin, reduced appetite and delayed wound healing, and typical characteristics of mild zinc deficiency are reduced number of sperm in the semen, mild weight loss, and excess of ammonia in the blood (Prasad, 1985). Prasad et al also studied the effect of mild zinc deficiency in 4 male volunteers who had a restricted zinc diet for several weeks under metabolic conditions. As a result of this dietary zinc restriction the following changes occurred: There was a reduction in plasma zinc concentrations, erythrocyte, leukocytes and urine, changes in alkaline phosphatase and ribonuclease were also observed and the participants of this study also lost weight because of dietary zinc restriction (Prasad et al., 1978, Maywald and Rink, 2014). Similar the review by Solomons (1998) indicated that a restriction in zinc diet was linked to a reduction in cellular production and hormonal signalling of cell mediated immunity (lymphocytes, granulocytes and platelets), where plasma zinc concentrations were also diminished. For the treatment of mild zinc deficiency, a standard recommendation is 1-2 mg/kg/day of zinc. 1 mg of the zinc ion (Zn^{++}) corresponds to 4.5 mg of zinc sulphate ($ZnSO_4$) (Thureen and Hay, 2006).

Acrodermatitis enteropathica

Inherited zinc deficiency, or acrodermatitis enteropathica (AE), occurs worldwide with an estimated incidence of 1 per 500,000 children (Maverakis et al., 2007). AE is a relatively rare genetic disorder that has been mapped to human chromosome 8, and where the zinc transporter ZIP4 has been attributed to be responsible for this disorder due to a diminished efficiency in transport (Rink, 2011b). AE is typically seen in infants that have congenital zinc transporter defect in the intestine or can be acquired AE which sometimes is the result of an intravenous nutritional programme that are prepared without the adequate amount of zinc and occur when zinc status levels are depleted. Moreover, AE is an inborn, autosomal recessive, genetic disorder, characterized by decrease in serum zinc levels, alopecia, vesicular, pustular and acral dermatitis (affecting a limb or other extremities), eczematoid skin lesions of the mouth, face, hands, feet and groin, growth retardation, mental apathy, behavioural and neurological disturbances, diarrhoea and malabsorption. AE is primarily a disease of zinc malabsorption or the inability to absorb zinc adequately (Rosenberg, 1982, Prasad, 2009, Antala and Dempski, 2012, Macdonald et al., 2012, Schmitt et al., 2009, Aggett,

1983, Weismann et al., 1979). This defect in zinc absorption can be corrected with zinc supplementation or higher zinc intake. The skin lesions and immune defects can be overcome by oral zinc supplementation, whereas the neurological defect may not be corrected. This syndrome received its name because diarrhoea was a cardinal feature (Moynahan, 1974). The clinical signs of AE are listed in Table 2.11

Table 2.11 - Clinical manifestations of Acrodermatitis Enteropathica

Symptom	Frequency (%)
Dermatitis	84
Diarrhoea	54
Alopecia	48
Growth retardation (stunting)	46
Weight loss (wasting)	43
Mood changes	39
Birth defects	31
Recurrent infections	30
Nail deformation	25
Miscarriage	23
Blepharoconjunctivitis (inflamed eyelids and conjunctiva)	22
Death	20
Anorexia/hypogeusia (reduced ability to taste)	15
Photophobia (abnormal visual intolerance of light)	14
Pale skin	8
Neurological defects	3

Adapted from Van Wouwe (1989). Clinical and laboratory diagnosis of acrodermatitis enteropathica. *European Journal of Pediatrics*, 149, 2-8.

Oral zinc supplementation has been successful in treating patients with AE, improving skin lesions of patients who can survive and lead a normal life (Moynahan, 1974). Thus, permanent zinc therapy is necessary for AE to achieve and complete a total clinical remission. The administration of zinc sulphate intravenously or orally in doses of 40-220 mg/d has been shown to reverse the signs of AE such as skin lesions, diarrhoea and mental depression (Gordon and White, 1978, Neldner and Hambidge, 1975). Improvements were also shown when patients were given zinc sulphate, e.g. a girl aged 11 months old who had AE was treated with 5 mg/kg/day of zinc sulphate, an 8 year old girl with AE was given 220 mg/d of zinc via oral for 3 days, a 16 year old boy was also given 220 mg/day of zinc sulphate, and in all cases treatments were successful (Álvarez et al., 2007, Larralde et al., 1995, Robertson and Sotos, 1975, Gartside and Allen, 1975),

Treatment of zinc deficiency with parenteral nutrition

A range of nutrients including trace elements are added to parenteral fluids intravenously to prevent the appearance of deficiency syndromes. Cases of zinc deficiency has been reported in patients on parenteral nutrition (PN) without added zinc, for instance skin lesions were observed after 6 months of providing this type of PN (Fessler, 2005). In children, the syndrome of acrodermatitis enteropathica has been observed in patients on total parenteral nutrition (TPN) who do not receive zinc. Balance studies have estimated that zinc requirements in TPN are 3 mg/d in patients without gastrointestinal losses and an average of 12 mg/d in patients that have diarrhoea and fistula (abnormal passage) losses (Leung, 1995, Jeejeebhoy, 2009). Clinical findings of zinc deficiency in an infant receiving TPN were low plasma zinc levels, skin lesions as opposed to the advantages of receiving TPN which are weight gain, serum proteins and increase of albumin (Principi et al., 1979). The decline of plasma zinc levels is common during TPN using fluid that do not include supplementation with trace metals and this is a reason why supplementation with zinc is recommended during TPN (Solomons et al., 1976). The administration of zinc chloride or zinc sulphate intravenously has been shown to rapidly improve the signs and symptoms of zinc deficiency, such that skin lesions disappeared within 8 days post therapy (Younoszai, 1983).

2.5.2 Consequences of zinc toxicity

Zinc is relatively non-toxic (Murphy, 1970) if taken orally, and rather non-toxic compared to other trace metals such as manganese and iron (Mocchegiani et al., 2005). The intake of zinc in drinking water needs to exceed 15 mg/L (highly unusual in potable water) for it to possibly produce nausea, vomiting and diarrhoea. Ingestion of zinc and zinc-containing compounds can have an effect on the gastrointestinal system, haematological, respiratory, cardiovascular and neurological systems. Intake of zinc in the range of 100–300 mg/d is likely to induce chronic toxicity, which is common among people who are self-medicated and are using zinc-containing supplements (Nriagu, 2007). In general, ingestion of large amounts of zinc (or over 2 grams of zinc sulphate) can produce symptoms of zinc toxicity such as nausea, vomiting, abdominal pain, lack of energy and fatigue and, in the absence of these symptoms, the use of zinc supplements seem to interfere with the utilization of other nutrients, mainly copper, and

impair immune function (Fosmire, 1990, Brown et al., 2001, Prasad, 1976). Zinc toxicity has been studied in the brain; zinc can act as a neurotoxin (Choi et al., 1988), where high intracellular zinc levels may induce cell death, normally homeostatic mechanisms would prevent the accumulation of zinc in the brain to reach toxic concentrations as a result of increased oral zinc. Cases of zinc in toxicity has been reported, for instance the case of a boy who showed lethargy and focal neurological deficits 3 days after he ingested 12 g of metallic zinc (Murphy, 1970). Intakes of zinc of 150-450 mg/d of zinc were associated with reduced copper levels, affected iron function, impaired immune function and decreased high density lipoproteins (Hamilton et al., 2000, Fraga, 2005, Chandra, 1984). Overall, a severe impact on human health by intoxication is a rare event, but at cellular level, zinc may have an impact on cell survival and be a key regulator of apoptosis and neuronal death following brain injury (Plum et al., 2010).

2.6 SUMMARY

Zinc is an essential micronutrient that plays multiple roles, and a deficiency of zinc has been associated with several clinical manifestations, such as growth retardation, predisposal to infections, delay of wound healing, neurodevelopment, alopecia, hypogonadism in males, decreased immunity, inter alia (Florescu et al., 2009, Saper and Rash, 2009). Then to prevent zinc deficiency, an adequate diet is very important, particularly during brain growth and development, which has a significant implication on cognitive function (Benton, 2010). However, micronutrient recommendations vary widely throughout Europe, despite that people's physiological needs differ little (Doets et al., 2008) and, to address this variation, the systematic review undertaken for zinc exploring the relationship between zinc intake-status and health outcomes will be described in chapters 4 and 5.

Regarding cognition, the systematic review for the intake/status-health, will be described more in chapter 5. Healthy motor and cognitive development in children may be promoted by providing multi-micronutrients supplements to mothers during pregnancy and lactation, mainly to those undernourished or anaemic may promote (Prado et al., 2012). Evidence from observational studies suggests that micronutrients may play an important role in the cognitive development of children, but results are inconclusive for intervention trials of single micronutrients. There is evidence that malnutrition can hinder cognitive development (Nyaradi et al., 2013), in particular many aspects of brain development and function (Morgane et al., 1993).

A review of the literature on the roles of zinc and its proposed associations with cognition was carried out by Black (2009) who stated that, since 1980, at least 9 studies have assessed the potential associations between zinc supplementation and cognitive development in human infants and children but at present, the body of evidence is insufficient to warrant recommending routine zinc supplementation to enhance cognitive performance among children. In the United States and internationally, there are few available data on zinc status or deficiency rates in children and further study is necessary to assess the efficacy of zinc supplementation, alone or in combination with other nutrients, for improving cognitive outcomes (Black et al., 2009).

A series of zinc intervention trials have been conducted in developing countries, however the results on the effectiveness of improved zinc status on cognitive function have been ambiguous. The limited evidence from human studies of the associated zinc deficiency to children's cognitive skills and motor function suggests a relationship for the most vulnerable children, though there is a lack of any clear consensus. This highlights the need for further research into the timing of zinc supplementation, particularly during the early years which are a sensitive time period, which influence development; the aspects of zinc deficiency and its occurrence with other micronutrient deficiencies (Black, 2003a, Cusick and Georgieff, 2012, Wachs et al., 2013). Likewise, no evidence exists on the long-term effect of interventions, supplements or foods rich in zinc on growth and cognitive development.

Despite the literature on zinc being limited in humans and animals, there are suggestions that zinc has a greater influence during the early periods of life (Benton, 2010). Studies in very low birth weight infants showed that formula plus zinc supplements improved motor scores (Friel et al., 1993). The first study reporting improvement of cognitive function after the infancy period is the study by Penland et al., in children aged 5-7 year old, where zinc plus micronutrients had the greatest effect on neuropsychological functions (Penland et al., 1997).

The role of zinc in cognitive function has been demonstrated in animal trials which have shown a positive effect of zinc supplementation on functions such as learning, knowledge retention, attention, play and functional activity. There are scant human studies, e.g results of studies on zinc deficiency or zinc supplementation in humans are less consistent than in animal studies (Brown et al., 2001), and most of the evidence linking zinc supplementation to cognitive development is inconclusive but there is some indication that zinc supplementation may have an effect in motor development in the most vulnerable infants. Thus, further research is required to explore the role of zinc and micronutrient supplementation in areas of child development such as motor and cognitive function and psychomotor development (IZA, 2008).

Therefore, the aims and objectives of this of this study were:

2.6.1 Aims:

1. To undertake a series of meta-analyses following a comprehensive literature review of zinc intake, status and health outcomes.

2. To identify gaps in knowledge regarding zinc intake-status-health in infants, children, pregnant, lactating women and adults and elderly.
3. To investigate the long term impact of micronutrient supplementation containing zinc during infancy on cognitive function in pre-school aged children, in Lima, Peru (informed by the outcome of aim 2).

2.6.2 Objectives:

In order to achieve the aims of this research, the following objectives were attained:

1: The methodology for undertaking a systematic review and meta-analysis was developed by the EURRECA consortium experts (Does et al, 2008). The Embase, Medline and Cochrane databases will be searched according to predefined search protocols, the papers sorted and the data extracted onto pro-forma. The systematic review assessed the zinc intake-status-health relationship: Intake-status (I-S), Intake-health (I-H) and Status-Health (S-H). Meta-analysis of data was undertaken where possible and forest plots generated using STATA/IC 11.0.

2: Gaps in knowledge assessing zinc intake-status-health relationships in adults and elderly, pregnant and lactating women, infants and children were identified mainly in the I-S/H association per population, for the following health outcomes: dermatitis, cognitive function and psychomotor development in children, foetal malformation, preeclampsia, preterm delivery in pregnant and lactating women; cognitive function, dermatitis, anorexia, hypogeusia, ischemic heart disease, depression in adults and elderly. The number of included studies per health outcome was limited, and the studies were heterogeneous to perform a meta-analysis.

The link between the systematic review and the empirical research lies in that, in view of the undertaken systematic review and health outcomes, there were a limited number of studies looking at the health outcome cognitive function, which was considered a priority health outcome for zinc. Therefore, a protocol was developed to explore this health outcome: cognitive function in a clinical setting in Peruvian children to evaluate if there was long term impact of multiple micronutrients in cognitive outcomes.

3: A study was designed to investigate the effect of micronutrient supplementation during infancy on cognitive function in pre-school children, and assess the long-term effect on cognitive, social-emotional, growth, anaemia and micronutrient status.

Specific sub-objectives for objective 3:

1. Measure cognitive and socio-emotional development in response to supplementation with iron vs. multi-micronutrient powder with zinc in a subsample of 200 children from the cohort study.
2. Measure growth in response to supplementation with iron vs. multi-micronutrient powder with zinc in a subsample of 200 children from the cohort study.
3. Measure anaemia and zinc status in response to supplementation with iron vs. multi-micronutrient powder with zinc in a subsample of 200 children from the cohort study.

The rationale for this study was to provide evidence of the long-term effect of micronutrient supplementation on cognitive, social-emotional, growth, anaemia and micronutrient status. The justification of the empirical study lies in the significance of nutrition research in shantytown regions of Peru and their contribution towards aiding their inhabitants for a better healthy life, raising the awareness and education in nutrition, contributing significantly to public health nutrition for the world.

Hypothesis of the empirical study:

Multi-micronutrients with zinc given during infancy at 6-17 months will result in long term benefits in children's cognitive function, growth, and micronutrient status measured at 3-4 years old.

CHAPTER 3. –

METHODOLOGY OF THE SYSTEMATIC REVIEW OF ZINC INTAKE-STATUS-HEALTH (I-S-H) RELATIONSHIPS

Chapter 3 – METHODOLOGY OF THE SYSTEMATIC REVIEW OF ZINC INTAKE-STATUS-HEALTH(I-S-H) RELATIONSHIPS

Section 3.1 provides an overview of the systematic review and meta-analysis protocols that were used to establish a current database of high quality published data relating to zinc intake-health-status interactions. This work was undertaken as part of a European Commission funded Network of Excellence, entitled European Micronutrient Recommendations Aligned (EURRECA). Section 3.2 gives the rationale behind the systematic review process, this is followed by section 3.3 that describes the research questions that the reviews were designed to address. Section 3.4 gives an overview of the methodological approach used, followed by section 3.5 which concludes with a validity assessment for the systematic review.

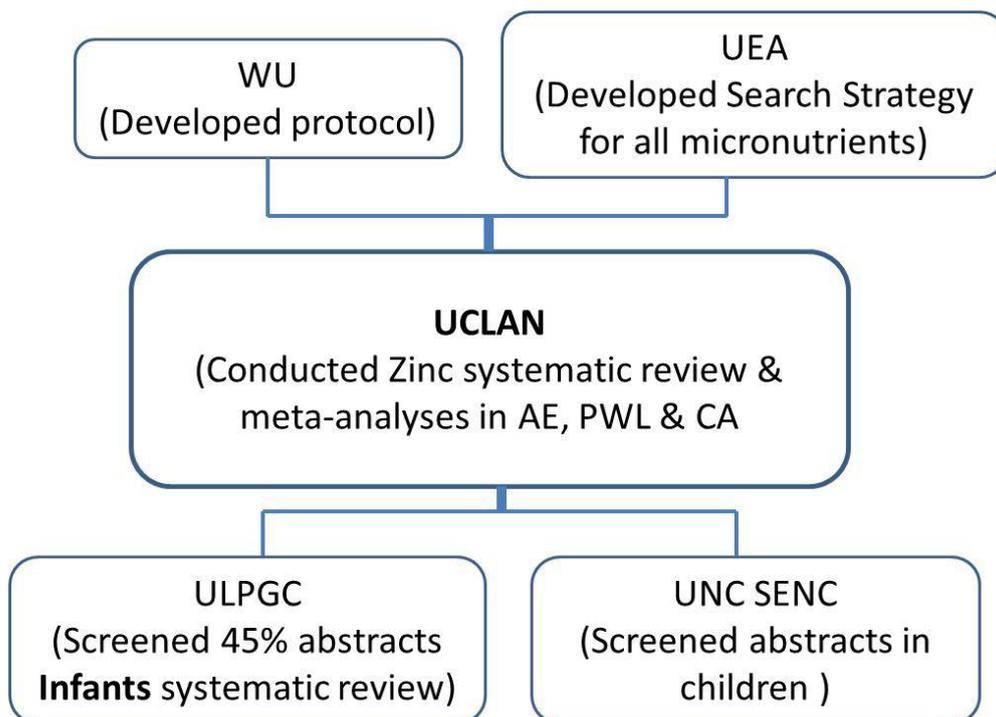
3.1 THE EURRECA APPROACH

A brief overview of EURRECA was provided in chapter 1. The specific global objectives of this Network of Excellence were defined as follows:-

- To provide an aligned set of standards with robust scientific basis for establishing micronutrient requirements and for devising micronutrient recommendations.
- To focus on the needs of specific groups: infants, children and adolescents, adults, pregnant and lactating women, elderly, as well as people with low income and immigrants.
- To evaluate the impact of socio-economic status, ethnic origin, inter-individual variability and vulnerability due to genetics, environmental factors and epigenetic phenomena.

UCLAN led the research activities relating to Zinc in collaboration with partners from Wageningen University (WU), University of East Anglia (UEA), Universidad de Las Palmas de Gran Canaria (Spain) (ULPGC), and Community Nutrition Unit of Bilbao (Spain) (UNC SENC). The relationships between these partners and their specific roles are illustrated in Figure 3.1.

Figure 3.1 - EURRECA Zinc Partners



WU: Wageningen University (the Netherlands), UEA: University of East Anglia (UK), ULPGC: Universidad de Las Palmas de Gran Canaria (Spain), UCLAN: University of Central Lancashire (UK), UNC-SENC: Community Nutrition Unit of Bilbao (Spain), AE: Adults and elderly, PWL: Pregnant and lactating women, CA: Children and adolescents.

3.2 PURPOSE OF THE ZINC SYSTEMATIC REVIEW

The zinc systematic review was undertaken because the prevalence of inadequate zinc intake is high among all populations, and the public health concern is considerable. In addition, recent evidence demonstrated a large heterogeneity among current micronutrient recommendations on zinc intake across Europe (Doets et al., 2008).

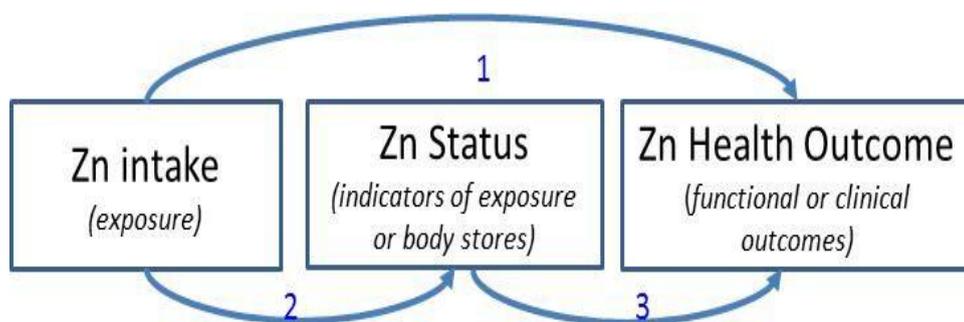
Zinc intake requirements have been mainly based on balanced studies focusing on the prevention of zinc deficiency. However, one of the aims of the EURRECA project was to define zinc requirements of general European populations in an evidence-based manner by performing a systematic literature review and meta-analysis. The zinc review considered the relationships between zinc intake, zinc status and health outcomes (I-S-H) for all populations, specifically infant, children and adolescents, pregnant and lactating women and the adults and elderly.

These are defined as follows:

- Infants (0-12 months),
- Children and adolescents (1-18 years),
- Adults (19-64 years),
- Elderly (65+ years),
- Pregnant women and
- Lactating women

Figure 3.2 illustrates the relationship between dietary zinc intake and health outcome (I-H), dietary zinc and status markers (I-S), and zinc status markers and health outcome (S-H).

Figure 3.2 - Intake-Status-Health relationships



3.3 RESEARCH QUESTIONS

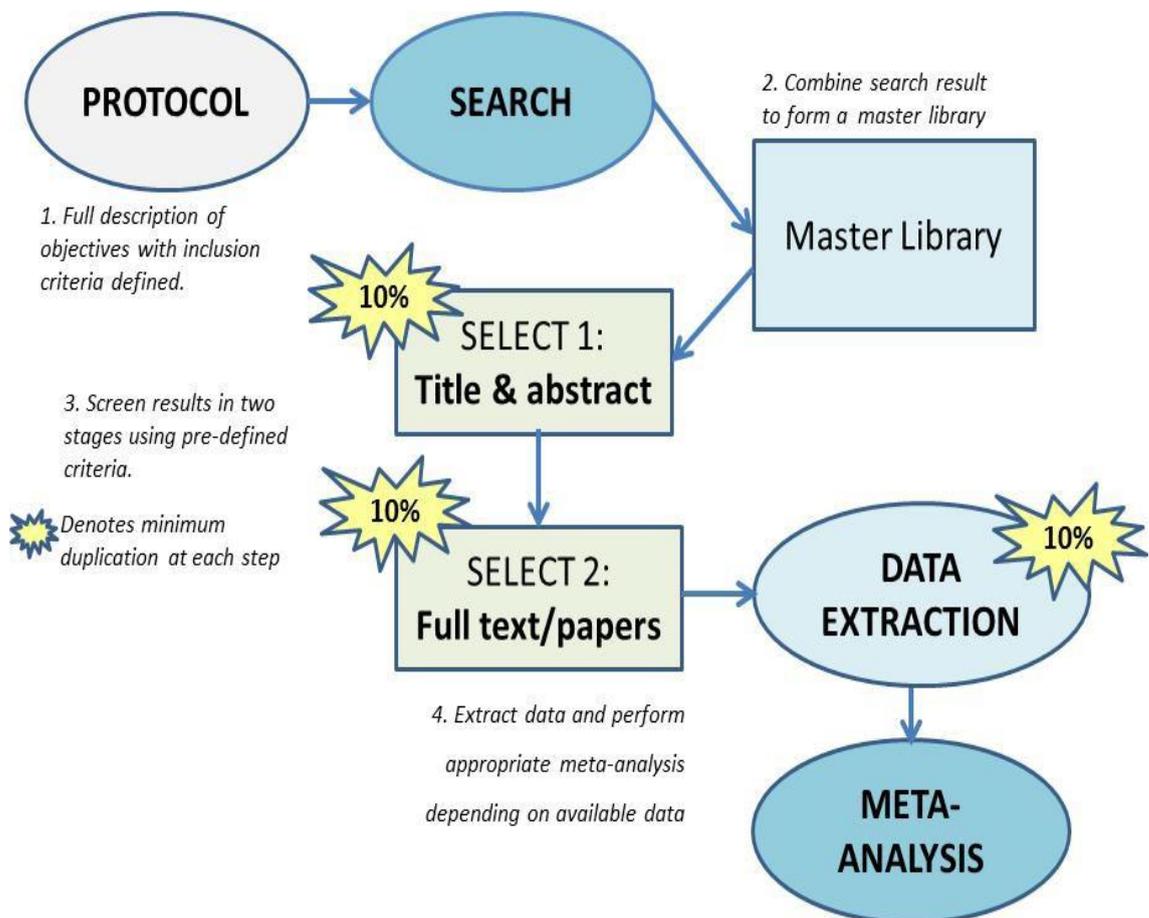
The zinc micronutrient review was divided into three main questions:

1. What is the effect of zinc intake on functional or clinical outcomes? (Intake – Health). What factors affect this relationship?
2. What is the effect of zinc intake on indicators of exposure or body stores? (Intake – Status). What factors affect this relationship?
3. What is the effect of indicators of exposure or body stores on functional or clinical outcomes? (Status - Health). What factors affect this relationship?

3.4 OVERVIEW OF THE METHODOLOGICAL APPROACH

The systematic review started with a question, then a protocol was developed, and the following steps were taken: searching for studies in 3 major databases, title and abstracts were screened, full text papers were assessed using an in/out form, then included studies were data extracted into a database, comparable studies were grouped for meta-analysis and meta-regression, finally reports summarizing the evidence were written and submitted for publication. This process is illustrated in Figure 3.3.

Figure 3.3 - Systematic Review Process Overview



3.4.1 Step 1: Search Protocol Development and Undertake Search

In order to answer the research questions, the search protocol for zinc was developed by both WU and UEA EURRECA partners. A protocol is a record document that outlines the research question that the review intend to address, detailing the inclusion and exclusion criteria and describes of how the authors will undertake the review process and plan to answer the research question (Cochrane, 2014). The protocol included the selection of papers based on best practice guidelines for the assessment of zinc intake

and zinc status. These best practice guidelines were developed from the identification of the most robust and appropriate methods from the literature undertaken by members of the EURRECA consortium (Serra-Majem et al., 2009, Dhonukshe-Rutten et al., 2013, EURRECA, 2013). These guidelines are outlined in the following subsections.

Best Practice: Measurement of Zinc Intake

These were identified as 24-hour recall, food records/ diary, validated food frequency questionnaire (FFQ)/ diet history. A minimum of 3 days was considered acceptable for the 24-hour recall and food record/diary. FFQs were only acceptable if validated against a food diary method. The review of the methodology used in validation studies showed that the most frequently used method to ascertain dietary intake was the FFQ, whereas dietary records and 24-hour recalls were the most used reference methods. Only papers using these methods were included in the systematic reviews (Serra-Majem, 2009).

Best Practice: Measurement of Zinc Status

Best practice guidelines for biomarkers of zinc status were created based on an eminence-based (expert opinion) approach, derived as an outcome of a EURRECA workshop at which key biomarkers for zinc were assessed with respect to their specificity, sensitivity and reliability in responding to changes in zinc intake (Fairweather-Tait, 2008, Lowe et al., 2009).

Biomarkers considered included: serum/plasma zinc concentration, erythrocyte zinc concentration, urinary zinc excretion, monocyte metallothionein mRNA, exchangeable zinc pool size, lymphocyte metallothionein mRNA (MT-2A), ectopurine5' nucleotidase activity, lymphocyte gene expression, hair zinc concentration.

These biomarkers were selected by using a triage technique (Fairweather-Tait, 2011), and were given a rating system of excellent, good, limited use and not useful. The serum/plasma zinc was considered "good" therefore for this systematic review serum/plasma zinc was the biomarker of choice. However, there are a number of important caveats that are recognised in the published outcome of this workshop and expert consultation. It is recognised that a specific, sensitive and reliable biomarker was not identified for zinc, however serum/plasma zinc concentration is widely used and the best biomarker available at the current time, providing the confounding factors are understood and taken into consideration when interpreting the data (Matthys et al., 2011).

Measures of Health Outcome

Important health outcomes associated with inadequate zinc intake were identified using an eminence approach that was developed using a pilot search carried out by EURRECA team members UEA and WU. The health outcomes chosen were those most relevant to the population group (based on public health reports and the scientific literature, i.e. current evidence of a relationship and the number of preliminary search hits from online databases, i.e. Embase, Cochrane and Medline) and not recently and thoroughly covered by a similar review. An overview of the measures of zinc intake, biomarkers of status and selected health outcomes studied for zinc micronutrients which were reviewed within the framework of EURRECA for different life-stage groups are shown in Table 3.1. It was acknowledged that there was insufficient time and resource available to address all the health outcomes for each population group, therefore the outcomes were prioritised based on the volume of published literature on each. Prioritised health outcomes are shown in **bold** in this table.

Table 3.1 Overview of intake, status and health measures for zinc micronutrient

Micronutrient	Intake Measures	Status Measures ¹	Health Outcomes ²			
			Infants	Children and Adolescents	Pregnant and lactating women	Adult and Elderly:
Zinc	Intake from natural food sources, fortified foods and supplements	<ul style="list-style-type: none"> • Serum/plasma zinc** • Urinary zinc* • Hair zinc* 	1.Growth 2.Immune response to vaccination 3.Neurodevelopment	1.Growth 2.Immune function 3.Cognitive functions and Psychomotor development 4.Dermatitis	<u>Fetus:</u> 1. Fetal growth 2. Fetus malformation <u>Mother:</u> 1. Preeclampsia 2. Preterm delivery	1. Immune function 2. Cognitive function 3. Dermatitis 4. Anorexia 5. Hypogeusia 6. Ischemic heart disease 7. Depression 8. Diabetes Mellitus 9. Carcinogenesis

¹Status measures graded according to eminence-based review (Fairweather-Tait, 2011) (*** excellent, ** good, * limited use)

²Health outcomes in bold were prioritised, the others were covered if the number of relevant papers for the priority outcomes was small

3.4.2 Database Search

To ensure comprehensive retrieval of relevant articles, a multi-database search was conducted. The following databases were searched: Medline, Embase (both on Ovid) and the Cochrane Library CENTRAL database until the date of the search in February 2009. A further updated search was conducted in February 2010 and March 2013. All searches were undertaken from inception for randomized controlled trials and observational studies, using text terms with appropriate truncation and relevant indexing terms. The date of the search was registered in the reference database or log book/file.

A general search strategy was devised which included terms for

[study designs in humans] AND [intake or status] AND [micronutrient]

and was then tailored to zinc micronutrient. In other words, the search terms for intake, status and health outcome measurements were those corresponding to zinc, e.g. dietary zinc intake from food and supplements, biomarkers for zinc status (serum/plasma zinc, urine zinc and hair zinc status), health problems related to zinc intake in all populations.

Indexing and text terms were used for each search strategy on individual databases used (Ovid EMBASE, Ovid Medline and Cochrane Central). The protocol for data-analysis and reporting for Zinc is available at <http://www.eurreca.org/everyone/8568/7/0/32>.

General Inclusion/Exclusion Criteria for the systematic reviews

The standard criteria assessment, developed by the scientific network, indicated that studies included in the systematic reviews needed to fulfil the criteria shown in Table 3.2

Table 3.2 - Inclusion/Exclusion Criteria for the systematic reviews

Criteria	Intervention Studies	Observational Studies
Population characteristics (age, health at baseline)	Age: ≥ 18 years old	Age: ≥ 18 years old
	Apparently healthy participants or appropriate health at baseline	Apparently healthy participants or appropriate health at baseline
	Infants	Age: 0-12 months
	Children and Adolescents	Age: 1-<18 years
	Pregnant and lactating women	
Study design	Randomised controlled trial	Prospective cohort
	<i>(exclude non-randomised, group randomised, uncontrolled trials)</i>	Nested case-control
		Cross-sectional (intake-status only)
Intervention	Supplements, fortified foods or natural diet intakes vs a placebo or untreated group	N/A
	<i>(exclude studies using multiple micronutrient supplements and if effect of micronutrient cannot be separated from another aspect of the intervention e.g. advice or physical activity)</i>	
Duration	Minimum duration for zinc, B12 and folate is 2 weeks. Ideally, for iron ≥ 4 months (depending on available data)	Suitable duration for health outcome
	<i>(exclude single meal intakes and isotope tracer studies)</i>	N/A for cross-sectional data
Outcomes	Must report at least one of the following relationships:	Must report at least one of the following relationships:
	<i>Intake-Status</i>	<i>Intake-Status</i>
	<i>Status-Health</i>	<i>Status-Health</i>
	<i>Intake-Health</i>	<i>Intake-Health</i>
Intake measures	Report of intake from supplements, fortified foods and natural food	· Validated FFQ/ Dietary history
		· 24h recall/food records/ diary measures for at least 3 days
		· 24h recall/ food records/ diary measure <3days adjusted for intra-individual variability
Status measurements	Micronutrient specific	Micronutrient specific
Health outcomes	Micronutrient specific	Micronutrient specific
Baseline information	Baseline data must be present for all reported outcomes	Baseline data must be present for all reported outcomes

N/A: Not applicable

The protocol used for the Medline database is shown in Table 3.3. This was adapted for the electronic searches on Embase and Cochrane database which are shown in Table 3.4 and Table 3.5. The author will focus on the adult elderly population group as this was its main role while undertaking the zinc systematic review.

Table 3.3 - Medline search strategy

N °	Search term	Results	Search type
1	randomized controlled trial.pt.	280821	Advanced
2	controlled clinical trial.pt.	79998	Advanced
3	randomized.ab.	196604	Advanced
4	placebo.ab.	117891	Advanced
5	clinical trials as topic.sh.	146242	Advanced
6	randomly.ab.	145491	Advanced
7	trial.ab.	203467	Advanced
8	randomised.ab.	38423	Advanced
9	6 or 3 or 7 or 2 or 8 or 1 or 4 or 5	734511	Advanced
10	(animals not (human and animals)).sh.	4482479	Advanced
11	9 not 10	642665	Advanced
12	(cohort* or "case control*" or cross-sectional* or "cross sectional" or case-control* or prospective or "systematic review*").mp.	768885	Advanced
13	exp meta-analysis/ or exp multicenter study/ or follow-up studies/ or prospective studies/ or intervention studies/ or epidemiologic studies/ or case-control studies/ or exp cohort studies/ or longitudinal studies/ or cross-sectional studies/	1013635	Advanced
14	13 or 12	1203767	Advanced
15	14 not 10	1154385	Advanced

16	11 or 15	1599094	Advanced
17	((zinc or zn or zinc sulphate or zinc gluconate or zinc acetate or methionine or zinc isotope*) adj3 (intake* or diet* or supplement* or deplet* or status or serum or plasma or leukocyte or concentration* or expos* or fortif* or urine or hair)).ti,ab.	16681	Advanced
18	Nutritional Support/ or Dietary Supplements/ or nutritional requirements/ or Breast feeding/ or exp infant food/ or bottle feeding/ or infant formula/	63098	Advanced
19	exp Nutritional Status/ or exp Deficiency Diseases/ or supplementation/ or diet supplementation/ or dietary intake/ or exp diet restriction/ or exp mineral intake/ or Diet/ or Food, Fortified/ or nutrition assessment/ or Nutritive Value/	176014	Advanced
20	(intake* or diet* or supplement* or deplet* or status or serum or plasma or leukocyte or concentration* or expos* or fortif* or urine or hair).ti,ab.	3166092	Advanced
21	18 or 19 or 20	3263114	Advanced
22	zinc/	41027	Advanced
23	22 and 21	20745	Advanced
24	23 or 17	26943	Advanced

Table 3.4 - EMBASE search protocol

N °	Search term	Results	Search type
1	random\$.ti,ab.	424441	Advanced
2	factorial\$.ti,ab.	8992	Advanced
3	(crossover\$ or cross over\$ or cross-over\$).ti,ab.	41414	Advanced
4	placebo\$.ti,ab.	116034	Advanced

5	(doubl\$ adj blind\$.ti,ab.	88626	Advanced
6	(singl\$ adj blind\$.ti,ab.	7916	Advanced
7	assign\$.ti,ab.	116276	Advanced
8	allocat\$.ti,ab.	37030	Advanced
9	volunteer\$.ti,ab.	103810	Advanced
10	crossover procedure.sh.	22365	Advanced
11	double-blind procedure.sh.	75974	Advanced
12	randomized controlled trial.sh.	180619	Advanced
13	single blind procedure.sh.	9006	Advanced
14	or/1-13	704875	Advanced
15	animal/ or nonhuman/ or animal experiment/	3598371	Advanced
16	human/	6816877	Advanced
17	16 and 15	584651	Advanced
18	15 not 17	3013720	Advanced
19	14 not 18	613713	Advanced
20	(cohort* or "case control*" or cross-sectional* or "cross sectional" or case-control* or prospective or "systematic review*").mp.	510309	Advanced
21	epidemiologic studies/ or case-control studies/ or cohort studies/ or longitudinal studies/ or follow - up studies/ or prospective studies/ or cross-sectional studies/ or clinical trials as topic/ or intervention studies/ or pilot projects/ or Meta Analysis/	1041831	Advanced
22	21 or 19 or 20	1585753	Advanced
23	22 not 18	1560501	Advanced
24	((zinc or zn or zinc sulphate or zinc gluconate or zinc acetate or methionine or zinc isotope*) adj3 (intake* or diet* or supplement* or deplet* or	22167	Advanced

	status or serum or plasma or leukocyte or concentration* or expos* or fortif* or urine or hair)).mp.		
25	*zinc/	20164	Advanced
26	zinc blood level/	2541	Advanced
27	zinc deficiency/	3312	Advanced
28	supplementation/ or diet supplementation/ or dietary intake/ or exp diet restriction/ or exp mineral intake/ or Infant nutrition/ or artificial milk/ or breast milk/ or bottle feeding/ or breast feeding/ or lactation/	138878	Advanced
29	exp nutritional status/ or nutritional deficiency/ or exp zinc deficiency/ or trace metal blood level/ or exp zinc blood level/	27625	Advanced
30	(intake* or diet* or supplement* or deplet* or status or serum or plasma or leukocyte or concentration* or expos* or fortif* or urine or hair).mp.	2919410	Advanced
31	28 or 30 or 29	2941543	Advanced
32	25 and 31	12965	Advanced
33	27 or 32 or 24 or 26	27863	Advanced
34	33 and 23	3360	Advanced

Table 3.5 - Cochrane search protocol

N °	Search term	Results
1	(zinc or zn or methionine or "zinc sulphate" or "zinc acetate" or "zinc gluconate" or "zinc isotope*")	3179
2	(MeSH descriptor Zinc explode all trees)	19
3	(#1 OR #2)	3179
4	(deplet* or status* or expo* or plasma or serum or leukocyte or concentration* or fortif* or urine or hair)	196565

5	(supplement* or diet* or "diet* restriction*" or "mineral intake*" or intake* or "infant nutrition" or "artificial milk" or "breast milk" or "bottle feeding" or "breast feeding" or "lactating")	56330
6	(#4 OR #5)	223056
#7	(#3 AND #6)	2152

3.4.3 Step 2: Combine Search Result to Form a Master Library

The results of the searches were combined in EndNote databases, duplicates were then searched and removed and libraries were screened on the basis of title and abstract, with any reference clearly not meeting the review criteria excluded. The duplicate checks were performed first and any discrepancies discussed before the remainder of the references were screened. Any potentially relevant references were then located as full texts to be screened in more detail. For this process a paper-based in/out form was devised by the University of East Anglia (UEA), which used the inclusion/exclusion criteria in Table 3.2 to assess each reference. Only those papers meeting all the criteria on the form were included in the next step which was data extraction.

After merging all the searches in database and removing duplicates, the total search yielded 7154 studies. Additional studies were added from reviewed articles.

3.4.4 Step 3: Screening and Sorting Abstracts

The screening/sorting process involved building the endnote libraries and importing references from the searched database, screening the references on the basis of title and abstract, and sorted by relationships of intake-status (I-S), intake-health (I-H) and intake-status-health (I-S-H), and population clusters. Step 3 was a stepwise process: a) Screening and sorting on basis of title and abstract, b) Selection on basis of full papers.

Screening and selecting the abstracts that met the inclusion criteria was undertaken by the UCLAN team (adult and elderly populations, pregnant/lactating women), the ULPGC team (infant population), and UNC-SENC (children/adolescent populations), with UCLAN taking over the database for children/adolescents later. Reasons for the excluded abstracts were registered in the Endnote library and separate libraries were created per population group.

Full-texts of potentially relevant articles were collected and assessed using in/out forms (See Appendix A1 and A2), this form used the inclusion/exclusion criteria described above and acted as an assessment form for each reference.

Abstracts for which the full article was unavailable were not included, and articles were considered in a range of languages spoken by the EURRECA network partners including English, Dutch, French, German, Hungarian, Italian, Norwegian, Polish, Spanish, Greek and Serbian. A minimum of 10% of full-texts were independently assessed by two reviewers.

Full-texts were grouped per type of Intake-Status-Health relationship and population group: young and adult and elderly (>18yr). All papers identified as young were further classified into infant (<1yr), child and adolescent (\geq 1-18 yrs) and pregnant and lactating groups.

Screening title and abstracts were carried out for the intake-status-health zinc relationships for each population group. In this section, the results of the screening and selection process involved in step 3, are illustrated in Figure 3.4, Figure 3.5, and Figure 3.6 for different population groups.

The flow diagrams show the systematic review process, from searching databases, sorting abstracts, requesting full text papers, excluding studies not meeting criteria, and the final included papers with those considered for meta-analyses and those which were not comparable for meta-analysis but relevant for a narrative review.

The reasons for excluding randomized controlled trials were:

- Studies did not provide both the mean and standard deviation of zinc biomarker (both required for the calculation of beta and standard error (SE) beta)
- Control/placebo data at all of the time points corresponding to treatment groups were not reported.
- Study population not healthy

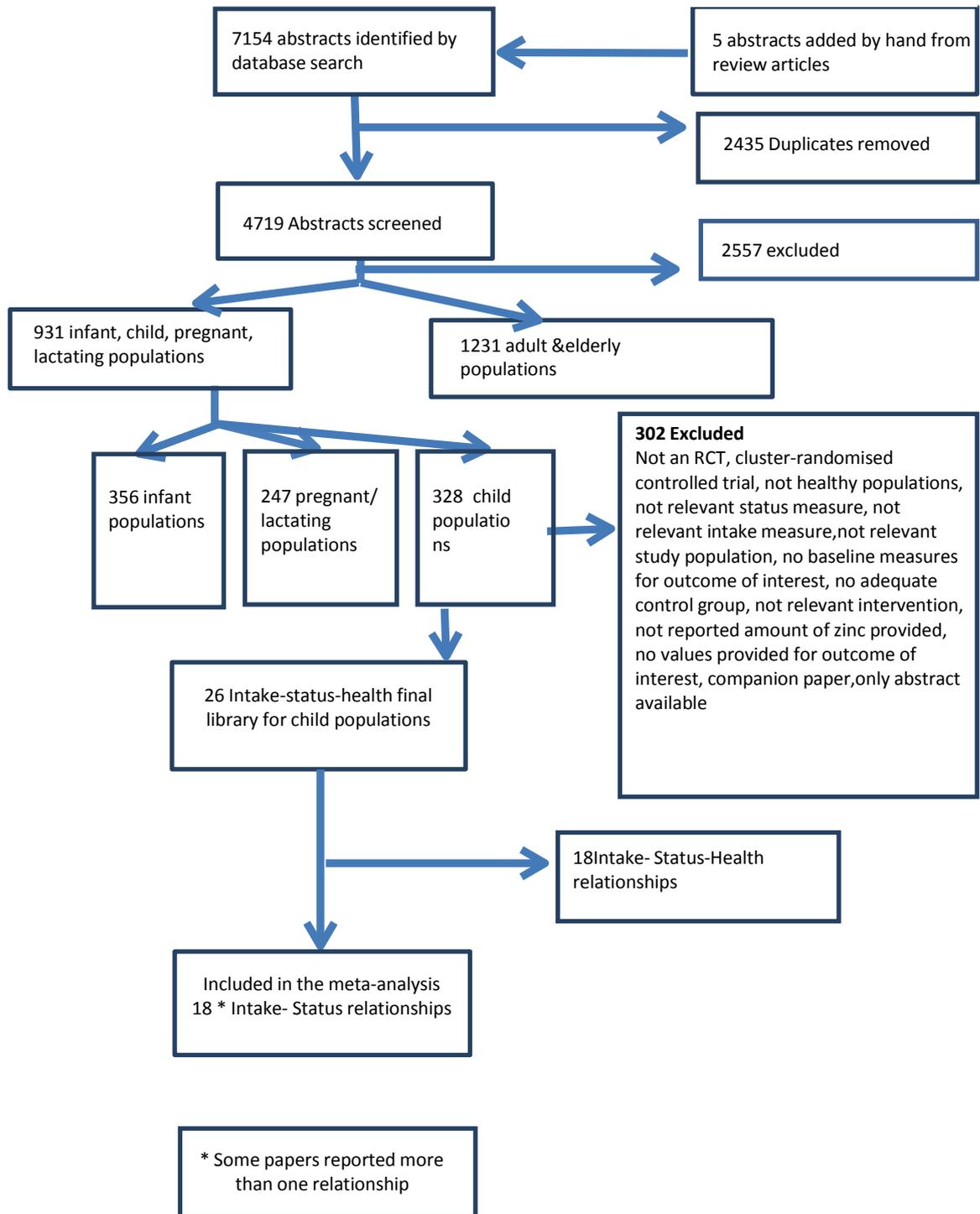
The most common reasons for excluding observational studies were:

- Studies did not provide standard deviation (SD) of intake then beta values could not be calculated without interquartile range (IQR), range or confidence interval for the zinc intake.
- Studies did not provide SD of plasma/serum zinc

- Studies did not provide the correlation (r) value of association of the zinc intake and plasma serum zinc.
- Studies did not provide the association type (Regression coefficient; Pearson correlation coefficient; Spearman rank correlation coefficient).

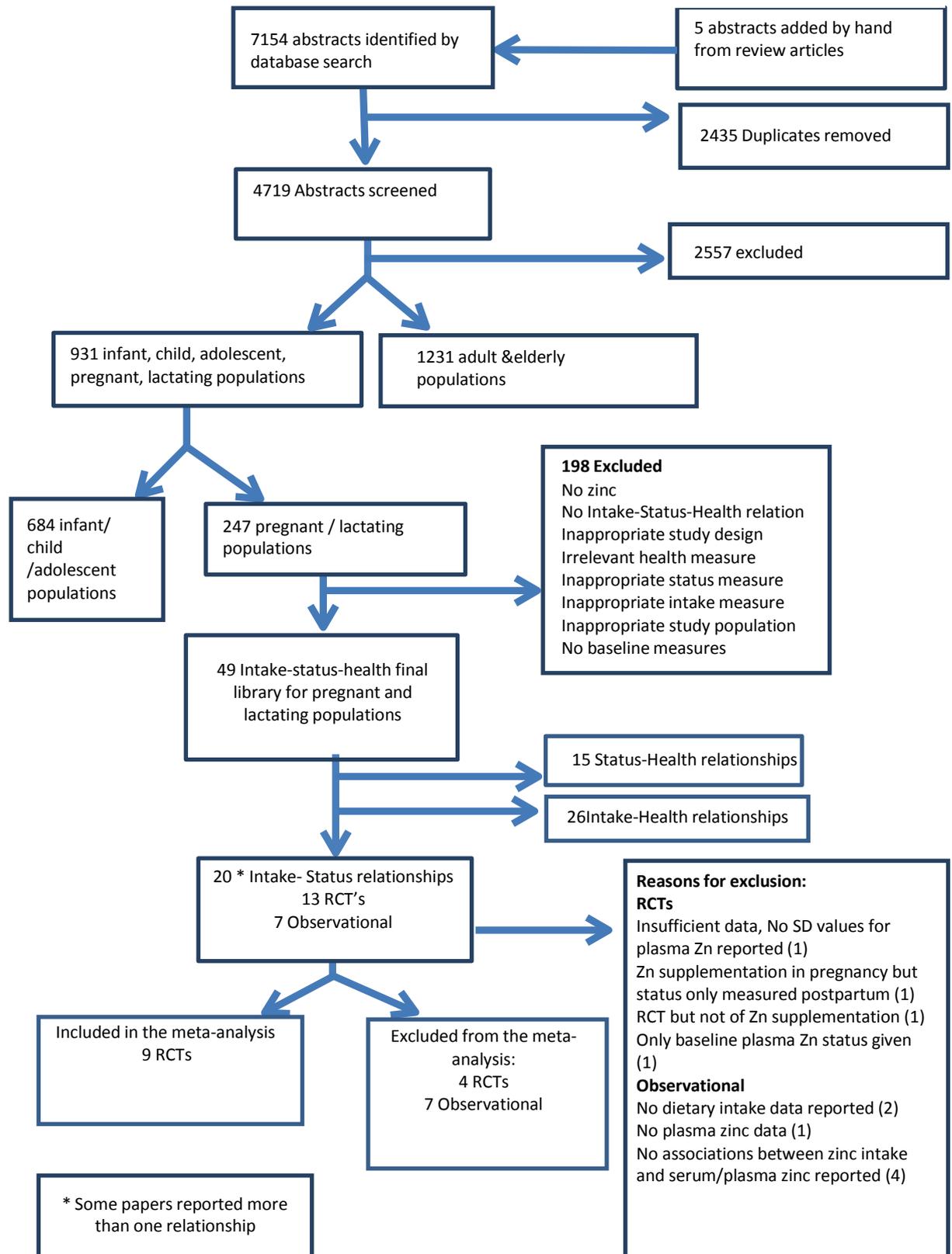
In Figure 3.4, after duplicates were removed, of 7154 articles the number of abstracts that were screened remained 4719; these were divided in two main clusters, the young (infant, pregnant and lactating women, and the old group (adult and elderly). After this, the full text of the 328 studies were assessed using the in/out form (inclusion/exclusion criteria) by two independent reviewers from the UCLAN team and disagreements were settled through discussion. Of the 26 studies included in the final library and after assessment for opportunities for meta-analysis, a final number of 18 studies were included in the meta-analysis for zinc intake-status relationships in children.

Figure 3.4 - Children, Adolescent process for systematic review (Intake-Status-Health)



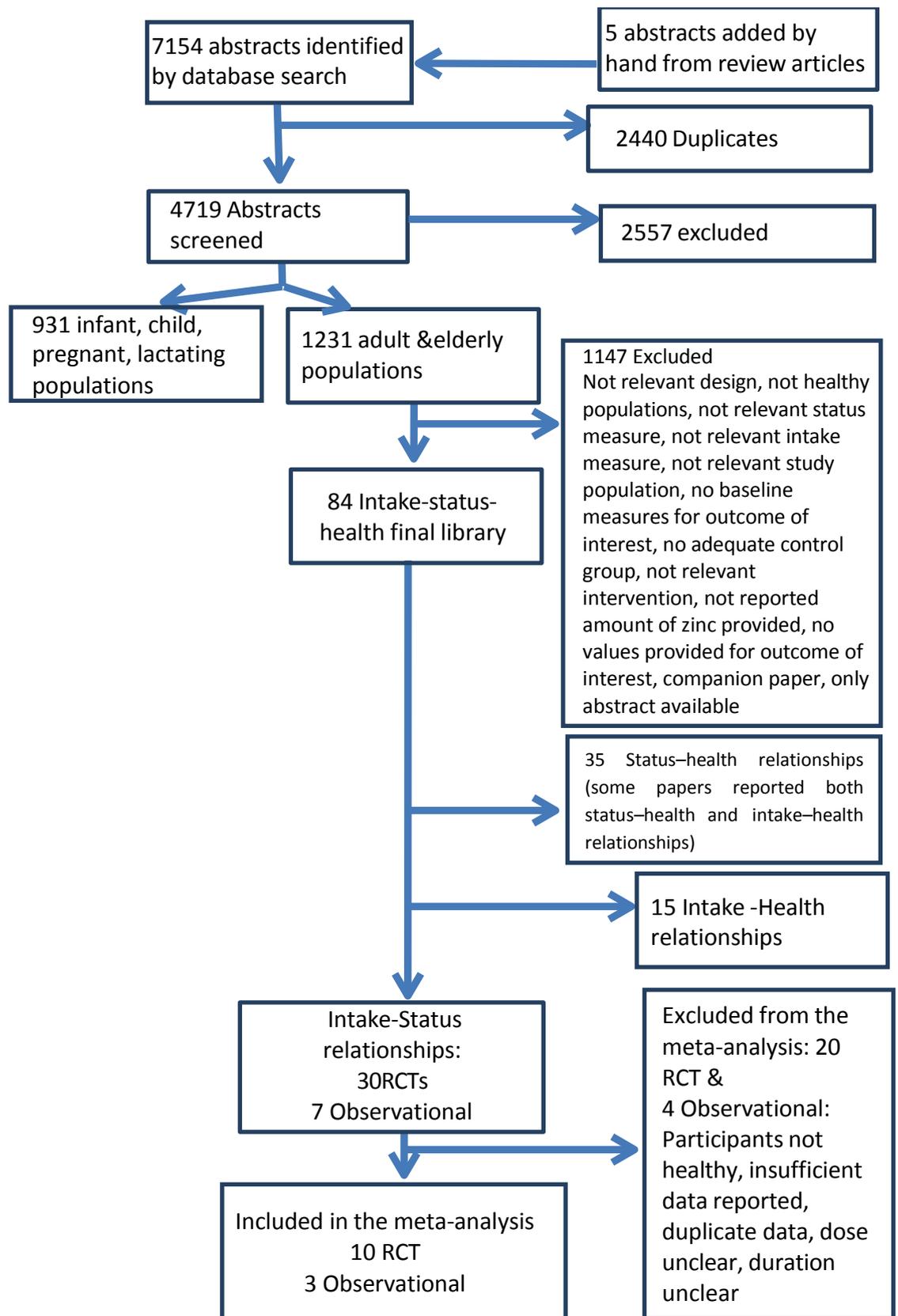
In Figure 3.5 the flow diagram on pregnant and lactating women, shows that of 7159 studies, 4719 abstracts were screened. After excluding 2557 abstracts, 931 studies were included in the infant, child and adolescent, pregnant and lactating population group. Of these, 247 studies were included in the pregnant and lactating group. Data was extracted for 49 studies. 13 randomized controlled trials (RCTs) and 7 observational studies were included for the intake-status relationship. Finally 9 RCTs were finally included for meta-analysis.

Figure 3.5 - Pregnant and lactating women flow diagram (Intake-Status)



In Figure 3.6 the flow diagram for the adult and elderly population shows that a total of 7154 title and abstracts were screened after electronic searches, and 5 additional abstracts were added from expert recommendations or review article reference lists. Of these 1231 appeared potentially relevant to the present review of adult and elderly populations, and the full text paper was obtained. 1147 of these were subsequently excluded. 84 were retained, 30 of which were included in the I-S category for this population. 25 RCTs and 5 observational studies were included for the intake-status-health relationship. Of these, 10 RCTs and 3 observational studies were included in the meta-analyses.

Figure 3.6 - Adult and Elderly flow diagram (Intake-Status)



3.4.5 Step 4: Data Extraction and Meta-Analysis

Once papers were identified as relevant to the research questions, data was extracted for each individual paper, to characterize studies and to facilitate meta-analysis. Extracted data was entered into a database (in Access) specifically designed by the WU team. Information pertaining to bibliographic details, study characteristics, quality and validity and any data presented for the review associations (intake-status-health) were extracted and entered into the electronic form. A minimum of 10% duplication was again carried out and data were entered in a standard format.

Besides the key variables of interest: zinc intake, status and health outcomes and measures of the relationship, data extracted included information that concerned the quality of the study such as study design, response rates/drop-outs rates, confounders examined, study population size, study duration, methods of intake measurement, methods of status measure, association studied (intake-status, status-health, intake-health), etc. The reported serum/plasma zinc concentrations of all papers were converted to $\mu\text{mol/L}$, where applicable, using the following formula: $1\mu\text{g /dL} \times 0.153 = \mu\text{mol/L}$ (Ctdslab, 2012). Companion papers which are papers that report on the same study and the same data were also added to the database.

Potential zinc confounders and effect modifiers for the relation between intake-status, intake and status, and intake and health in general were: age, gender, country of the study, ethnicity, social class/living conditions/income, smoking, physical activity, body mass index, total energy intake and, intake of other macro and micronutrients.

When data extraction was completed, then it was decided whether studies would be used for meta-analysis or for the narrative part of the systematic literature review. This was the result of the zinc systematic review assessment and synthesis of the research evidence from individual studies based on the EURRECA protocol. A meta-analysis was then executed for selected RCTs and observational studies

3.5 VALIDITY ASSESSMENT

For publication, validity assessment was undertaken based on the Cochrane Guidelines (Higgins, 2011). Assessment of internal validity was focussed on the quality of the study, the risk of bias, and the methodology (RCT, cohort, case control). Risk of bias for each paper was assessed using criteria specified for RCT's, Cohort and nested case control studies, and cross sectional studies. Each study had a set of indicators of internal validity. The internal validity focuses on the quality of the study and provides information on the risk of bias. Criteria of judgement for the RCT validity were adequacy of the generation, allocation, blinding, dropout, funder and lack of other potential threats to validity. Criteria for the judgement of observational studies were: confounders, intake or status, drop out, funders were dealt adequately and lack of other potential threats to validity. Studies were graded low, medium or high risk of bias independently in duplicate by 2 reviewers. Where disagreements occurred, they were resolved through discussion, including a third team member to arbitrate where necessary. Qualities of studies were assessed for RCTs and observational studies.

CHAPTER 4. –

RESULTS OF THE META-ANALYSIS OF ZINC INTAKE-STATUS (I-S) RELATIONSHIPS

Chapter 4 – RESULTS OF THE META-ANALYSIS OF ZINC INTAKE-STATUS (I-S) RELATIONSHIPS

In this chapter, the results of the systematic review and meta-analysis of intake-status relationships in all populations groups are presented. As described in chapter 3, step 4 of the systematic review process involved extracting data for each of the identified published papers. Extracted data included population characteristics, study design, zinc dietary intake and dose of supplement, as well as mean concentration of zinc in plasma/serum.

Meta-analysis is the quantitative analysis of a collection of study results (Berlin et al., 1993). The specific questions to be addressed by this meta-analysis review were

- 1) What is the effect of zinc intake on plasma/serum zinc concentration?
- 2) What factors affect this relationship?

4.1 STATISTICAL APPROACH

The dose-response relationship between zinc intake and plasma zinc was transformed to a regression coefficient ($\hat{\beta}$) and the standard error ($SE(\hat{\beta})$) of this regression coefficient. To calculate the regression coefficient ($\hat{\beta}$) per study for the RCTs the following equation was used:

$$\hat{\beta} = \frac{\log(\text{outcome}_{\text{int}}) - \log(\text{outcome}_{\text{cont}})}{\log(\text{Intake}_{\text{int}}) - \log(\text{Intake}_{\text{cont}})}$$

Where:

- outcome_{int} is the mean of the status or health variable in the intervention group,
 - outcome_{cont} is the mean of the status or health variable in the control group,
 - intake_{int} is the mean micronutrient intake in the intervention group,
 - intake_{cont} is the mean micronutrient intake in the control group, and
- the log transformation is a *natural* logarithmic transformation (with base $e (=2,718\dots)$)

To calculate the standard error of the $\hat{\beta}$ per study ($SE(\hat{\beta})$), the following equation was used:

$$SE(\beta) = \frac{((SD_{int}/outcome_{int})(N_{int} - 1)) + ((SD_{cont}/outcome_{cont})(N_{cont} - 1))}{(N_{total} - 2) * (1/N_{cont} + 1/N_{int})}$$

where:

- SD_{int} is the standard deviation (SD) of the outcome in the intervention group,
- SD_{cont} is the standard deviation (SD) of the outcome in the control group,
- N_{int} is the number of subjects in the intervention group,
- N_{cont} is the number of subjects in the control group, and
- N_{total} is the total number of subjects (i.e., $N_{total} = N_{int} + N_{cont}$).

To calculate the regression coefficient ($\hat{\beta}$) per study for the observational studies, the estimated ($\hat{\beta}$) and its standard error from each study was estimated using the linear regression model $Y = \alpha + \beta X + \gamma Z$, where Y represents the continuous outcome of interest, X represents the exposure and Z represents a vector of covariates

The statistical transformations to obtain $\hat{\beta}$'s and $SE(\hat{\beta})$'s were performed using GenStat version 13-SP2 (VSN International Ltd., <http://www.vsn.co.uk/>), developed by WU, (Souverein et al., 2012), and the meta-analysis for zinc was performed using STATA version 11.0 (College Station, TX), with statistical significance defined as $P < 0.05$. $\hat{\beta}$ and standard error ($SE(\hat{\beta})$) were used to estimate an intake-status regression coefficient for each individual study, using the mean serum/plasma zinc concentrations, based on the assumption of a linear relationship on the $\log_e - \log_e$ scale (natural logarithm of intake v. natural logarithm of status). Because a base- e logarithmic transformation on the zinc intake and serum/plasma zinc concentration was applied, the interpretation of these results are as follows: the overall $\hat{\beta}$ represents the difference in the \log_e transformed predicted value of serum/plasma zinc status for each one-unit difference in the \log_e transformed value in zinc intake (Moran et al., 2012b). The I-squared (I^2) measured the heterogeneity between studies. An overall pooled $\hat{\beta}$ and $SE(\hat{\beta})$ was calculated using random effects meta-analysis, which estimates the variance between studies and incorporates the heterogeneity of effects in the overall analysis (DerSimonian and Laird, 1986), this heterogeneity may be reduced if considering covariate information. For this zinc systematic review, covariates assessed were: age, gender, dose, duration of study. Separate meta-analyses were performed for each population group, however the author was most closely involved with that of the adult and elderly group. Therefore, this meta-

analysis will be presented in the most detail but the same procedures were followed for all population groups.

4.2 ADULTS AND ELDERLY: META-ANALYSIS OF ZINC INTAKE-STATUS RELATIONSHIP

From the systematic search (Steps 1-3) as outlined in chapter 3, and as shown in Figure 3.6 of section 3.4.4, the following papers were identified and selected for meta-analysis: **10 RCTs** (Abdulla and Svensson, 1979, Bogden et al., 1988, Boukaiba et al., 1993, Feillet-Coudray et al., 2005, Feillet-Coudray et al., 2006, Hininger-Favier et al., 2007, Prasad et al., 2007, Preziosi et al., 1998, Sakagami et al., 2009, Sullivan et al., 1998) **3 observational studies** (Chandyo et al., 2009, Gibson et al., 2001, Sanchez et al., 2009).

A description of the RCTs and observational studies is summarized in Table 4.1 and Table 4.2 respectively.

Table 4.1 - RCTs reporting the effect of dietary zinc intake on serum/plasma zinc status in adults.

Study, year, country	Sex, age	Treatment group	Micronutrient type	Duration	Status marker reported & analytic method
Abdulla & Svensson (1979)	Mean age 25 years SD, age range and sex not reported	Study 1 Placebo (n 5) 135.3 mg Zn/d (n 7) Study 2 Placebo (n 8) 45 mg Zn/d (n 7)	Zinc sulfate	12 weeks	Plasma Zn, AAS
Bogden <i>et al.</i> (1988), USA	Males and females aged 60 – 89 years	Placebo (n 36) 15 mg Zn/d (n 36) 100 mg Zn/d (n 31)	Zinc acetate	3 months	Plasma Zn, AAS
Boukaiba <i>et al.</i> (1993), France	Males and females aged 73 – 106 years	BMI ≤ 21 kg/m ² Placebo (n 21) 20 mg Zn/d (n 21) BMI ≥ 24 kg/m ² Placebo (n 23) 20 mg Zn/d (n 23)	Zinc gluconate	8 weeks	Serum Zn, AAS
Preziosi <i>et al.</i> (1998), France	Males and females aged 35 – 60 years	Placebo (n 200) Multi-micronutrient supplement (20 mg Zn/d) (n 201)	Zinc gluconate	3 and 6 months	Serum Zn, AAS
Sullivan <i>et al.</i> (1998), USA	Males aged 19 – 35 years	Placebo (n 13) 50 mg Zn/d (n 13)	Zinc gluconate	15 d	Plasma Zn, AAS
Feillet-Coudray <i>et al.</i> (2005), France	Males aged 58 – 68 years	Placebo (n 16) 15 mg Zn/d (n 16) 30 mg Zn/d (n 16)	Zinc gluconate	6 months	Plasma Zn, ICP-MS
Feillet-Coudray <i>et al.</i> (2006), France	Females aged 55 – 70 years	Placebo (n 16) 15 mg Zn/d (n 16) 30 mg Zn/d (n 15)	Zinc gluconate	6 months	Serum Zn, ICP-MS
Hininger-Favier <i>et al.</i> (2007), France, UK, Italy	Males and females aged 55 – 85 years	Age 55 – 70 years Placebo (n 63) 15 mg Zn/d (n 16) 30 mg Zn/d (n 16) Age > 70 years Placebo (n 16) 15 mg Zn/d (n 66) 30 mg Zn/d (n 66)	Zinc gluconate	6 months	Serum Zn, AAS
Prasad <i>et al.</i> (2007), USA	Males and females aged 55 – 87 years	Placebo (n 25) 45 mg Zn/d (n 24)	Zinc gluconate	12 months	Plasma Zn, AAS
Sakagami <i>et al.</i> (2009), Japan	Males and females aged 21 – 77 years	Placebo (n 28) 17 mg Zn/d (n 27) 34 mg Zn/d (n 26) 68 mg Zn/d (n 28)	Zinc carnosine	12 weeks	Serum Zn, AAS

AAS, atomic absorption spectroscopy; ICP-MS, inductively coupled plasma MS.

Sample size for the RCTs included ranged from 5 to 201, the total number of participants was 1285. The majority of intervention studies provided zinc in the form of zinc gluconate, where the range of zinc dose was from 15 to 135.3 mg/day and the range of dietary zinc intake was from 5.4 to 10.8 mg/d.

Table 4.2 - Observational studies reporting the association between dietary zinc intake and serum/plasma zinc status in adults.

Study, year, country	Subjects and ages	Mean Zn intake (mg/d)	Mean plasma/serum Zn ($\mu\text{mol/l}$)	Zn intake (source)	Zn intake (assessment)	Zn status biomarker and analytical method
Gibson <i>et al.</i> (2001), New Zealand	330 females aged 18–40 years	10.44 (SD 3.51)	12.00 (SD 1.36)	Diet	FFQ and 24 h recall	Serum Zn, AAS
Chandyo <i>et al.</i> (2009), Nepal	500 females aged 13–35 years	8.6 (SD 3.3)	8.5 (SD 2.4)	Diet	FFQ and 24 h recall (2 d)	Plasma Zn, ICP-AES
Sánchez <i>et al.</i> (2009), Spain	170 males aged 25–60 years	12.24 (SD 7.16)	17.48 (SD 6.68)	Diet	24 h recall (2 d)	Plasma Zn, AAS
	184 females aged 25–60 years	9.07 (SD 4.40)	16.32 (SD 6.21)			

AAS, atomic absorption spectroscopy; ICP-MS, inductively coupled plasma MS.

In the observational studies, the total number of participants of the included studies was 1184, the dietary zinc intake was measured by FFQ and 24 h recall, and the range of zinc intake was from 8.6 to 12.2 mg/d.

Figure 4.1 – Forest plot depicting the β values calculated from the Intake-Status relationships from RCT's and observational studies in the adult and elderly population group

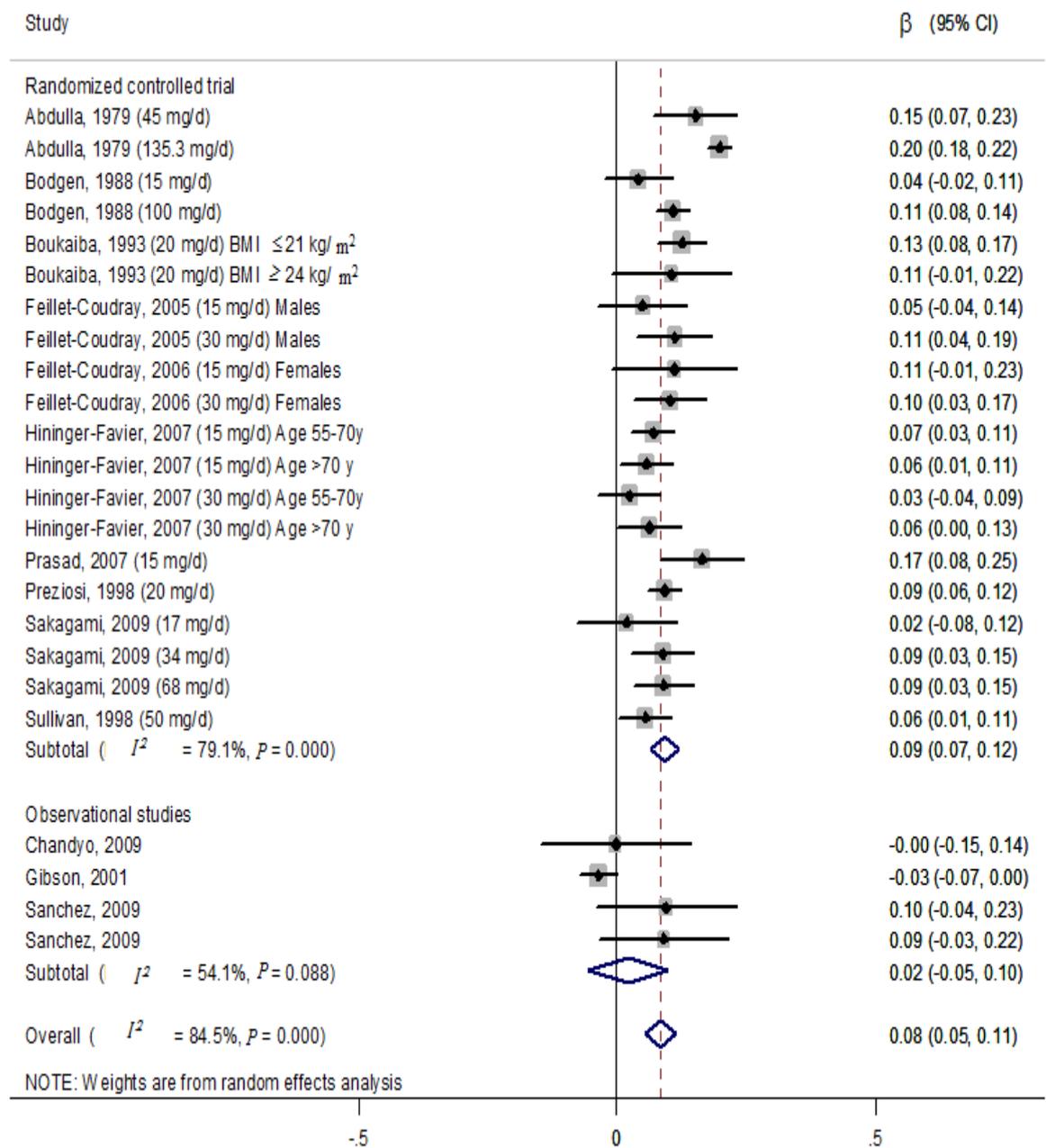


Figure 4.1 shows the random effect meta-analyses of RCTs and observational studies. The β for each individual study is plotted, which is represented as the centre of the grey squares. The horizontal line which runs through the grey squares represent the 95% confidence intervals. The area of the square is proportional to the study's weight in the meta-analysis. The overall β and its 95% confidence interval from the meta-analysis is plotted as a diamond and added to the bottom of the plot. The centre of the diamond

represents the overall β , and its lateral tips represent the 95% confidence interval. The overall pooled $\hat{\beta}$ for the studies in adults and elderly was 0.08 (95%CI: 0.05–0.11; $P < 0.0001$; $I^2 = 84.5\%$). This indicated that zinc supplementation was positively associated with the increase of serum/plasma zinc concentrations. Meta-regression was also conducted to evaluate sources of heterogeneity; co-variables assessed were age, gender, dose and duration of the study. From this analysis, zinc dose was a significant determinant of the overall $\hat{\beta}$. To calculate $\hat{\beta}$ or $SE(\hat{\beta})$ the following data was necessary, the mean values of the status or health variable in both intervention and control group, the SD's of the status or health variable in both groups, the number of subjects on whom the status or health variable was measured in both groups. Since a base- e logarithmic transformation was applied: $2^{\hat{\beta}}$, where the overall $\hat{\beta}$ of the randomised controlled trials and observational studies evaluating the pooled effect of dietary zinc on plasma zinc status in adults was 0.08, then this resulted in ($2^{0.08} = 1.06$), ($1.06 - 1 = 0.06$, multiplied by $100 = 6$), which is 6%, then interpretation of this result is as follows: for every doubling in zinc intake, the difference in zinc serum/plasma concentration is 6%. This means that a person with an intake of 14 mg/d of zinc will have a concentration of serum/plasma zinc that is 6% higher than a person consuming 7 mg/d of zinc. Zinc intake-status association was significant for the RCTs but not significant for the observational studies ($P = 0.09$).

Table 4.3 summarizes the internal validity of the RCTs. 5 out of 10 assessed studies were considered to have high risk of bias due to insufficient information on sequence generation, blinding, dropouts, funding bodies, compliance and unclear intake data.

Table 4.3 – Assessment of validity of included randomised controlled trials reporting zinc intake and serum/plasma zinc status in adults.

Study	Adequate sequence generation	Adequate blinding	Drop-outs adequate and outcome data complete	Funder Adequate	Compliance Check and results	Dose Check and results	Dietary intake data reported and results	Status reproducibility reported	Similarity of most and least exposed groups at baseline	Lack of other potential threats to validity	Overall risk of bias
Abdulla & Svensson (1979)	No	No	Unclear	No	Unclear	Unclear	NR	No	Yes	No	High
Bodgen <i>et al.</i> (1993)	Yes	Yes	Yes	Yes	NR	Yes	Yes	No	Yes	Yes	Low
Boukaiba <i>et al.</i> (1993)	Unclear	Yes	Yes	Unclear	Yes	NR	Yes	NR	Yes	Yes	High
Preziosi <i>et al.</i> (1998)	Yes	Yes	Yes	Unclear	Yes	Yes	NR	Yes	Yes	Yes	High
Sullivan <i>et al.</i> (1998)	Unclear	Unclear	Yes	Yes	Yes	NR	NR	No	Yes	Yes	High
Feillet-Coudray <i>et al.</i> (2005)	Unclear	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Low
Feillet-Coudray <i>et al.</i> (2006)	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
Hininger-Favier <i>et al.</i> (2007)	Unclear	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
Prasad <i>et al.</i> (2007).	Yes	Yes	Yes	Yes	Yes	Yes	NR	Yes	Yes	Yes	Low
Sakagami <i>et al.</i> (2009)	Unclear	Yes	Yes	Unclear	NR	Yes	NR	Yes	Yes	Unclear	High

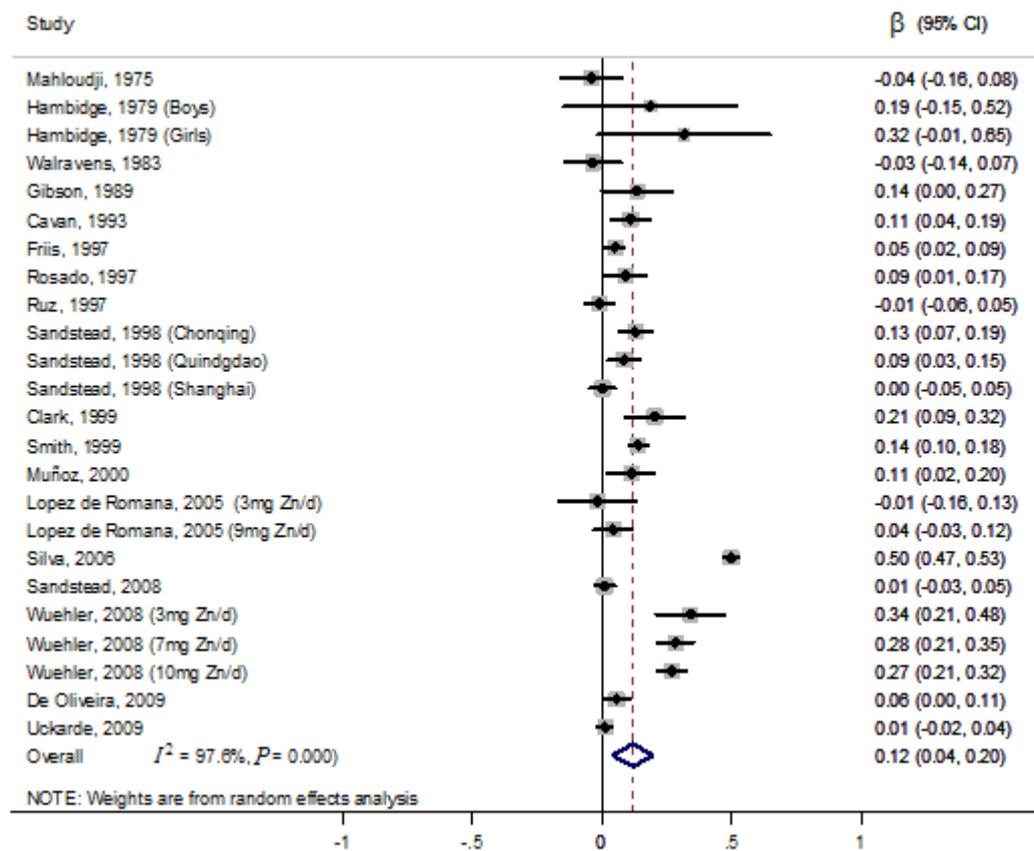
NR, not reported

4.3 CHILDREN: META-ANALYSIS OF ZINC INTAKE-STATUS RELATIONSHIP

For this review only studies that were conducted in healthy children (1-17 year old), that reported zinc intake either as a supplement or as part of a fortified meal, plasma/serum zinc concentration, and those who reported sufficient data to enable estimation of $\hat{\beta}$ and $SE(\hat{\beta})$, were included. From the results systematic search (Steps 1-3) as outlined in chapter 3, and as shown in Figure 3.4 of section 3.4.4, meta-analysis was possible for 18 studies.

These 18 studies were conducted in Latin America ($n=9$), North America ($n=4$), Asia ($n=3$), and in Africa ($n=1$) and Europe ($n=1$) (Mahloudji et al., 1975, Hambidge et al., 1979, Walravens et al., 1983, Gibson et al., 1989, Cavan et al., 1993, Friis et al., 1997, Rosado et al., 1997, Ruz et al., 1997, Sandstead et al., 1998, Clark et al., 1999, Smith et al., 1999, Munoz et al., 2000, Lopez de Romana et al., 2005, Silva et al., 2006, Sandstead et al., 2008, Wuehler et al., 2008, de Oliveira et al., 2009, Uçkarde et al., 2009).

Figure 4.2 - Forest plot depicting the β values calculated from the intake-status relationships from RCT's in Children



RCT's: Randomized controlled trials.

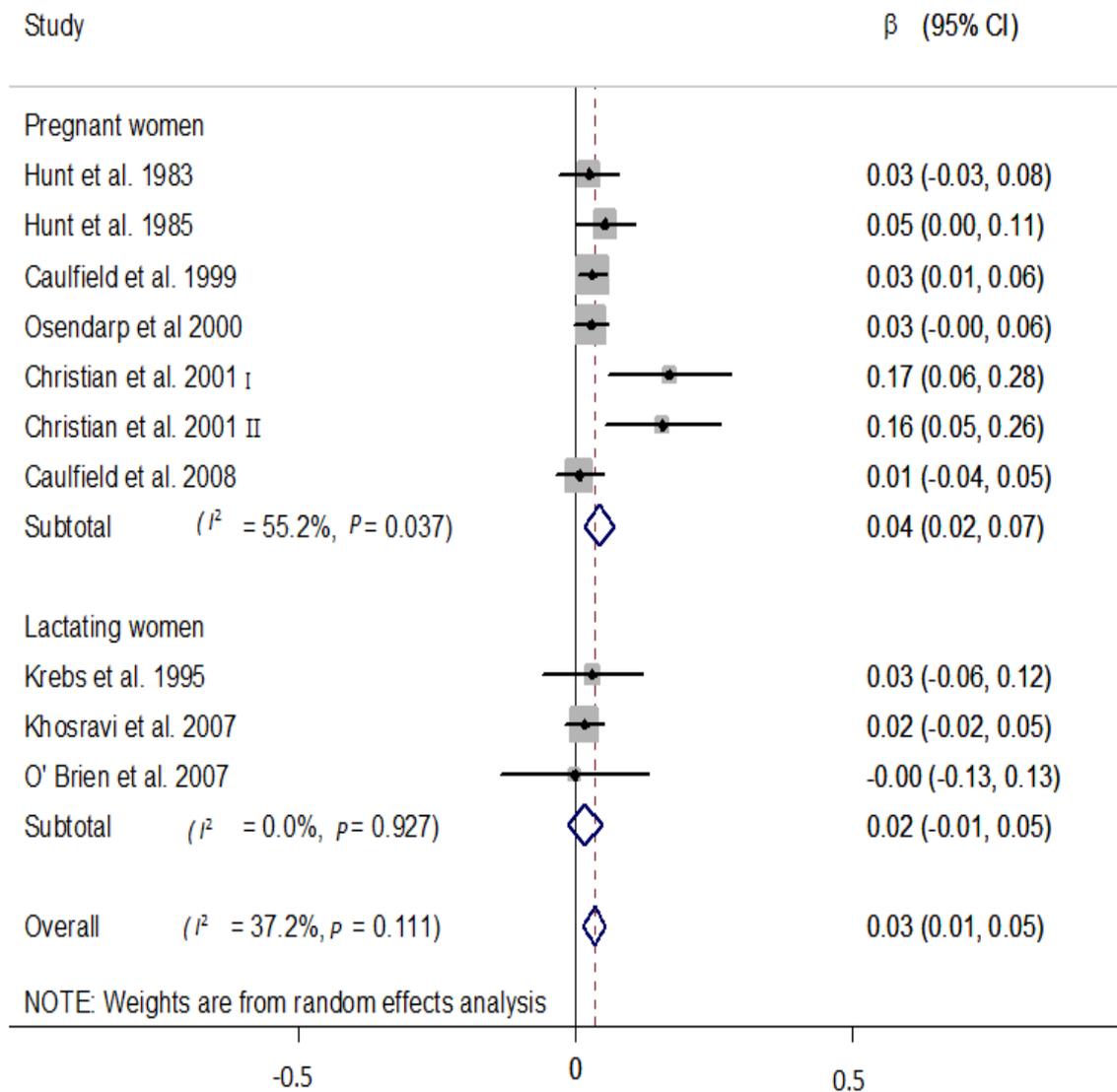
The overall meta-analysis of 18 studies performed in children yielded an overall β of 0.12 (95% CI 0.04, 0.20); I^2 97.6%, $p < 0.0001$. This means that the overall effect or regression coefficient from 18 studies in children was 0.12. Therefore, an overall $\hat{\beta}$ of 0.12 means that for every doubling in zinc intake, the difference in zinc serum or plasma concentration is $2^{\hat{\beta}}$ ($2^{0.12} = 1.09$), or 9%. This indicates that, for example, an

individual consuming 14 mg/day of zinc has a plasma zinc concentration that is 9% higher than an individual consuming 7 mg/day of zinc.

4.4 PREGNANT AND LACTATING WOMEN: META-ANALYSIS OF ZINC INTAKE-STATUS RELATIONSHIP

For this review from the results systematic search (Steps 1-3) as outlined in chapter 3, and as shown in Figure 3.5 of section 3.4.4, 6 RCTs were included that assessed zinc intake-plasma/serum zinc in pregnant women and (Hunt et al., 1983, Caulfield et al., 1999, Osendarp et al., 2000, Hunt et al., 1985, Christian et al., 2001, Caulfield et al., 2008) and 3 RCTs in lactating women (Krebs et al., 1995, Khosravi et al., 2007, O'Brien et al., 2007). One study, Christian (2001), included two different zinc treatments and two controls and this was treated as two independent estimates in the meta-analysis.

Figure 4.3 - Forest plot depicting the β values calculated from the intake-status relationships from RCT's in pregnant and lactating women



The overall pooled $\hat{\beta}$ for the studies in pregnancy was 0.04 (95%CI: 0.02–0.07; $p = 0.04$; $I^2 = 55.2\%$) and for lactating women studies was 0.02 (95% CI: -0.01, 0.05; $p = 0.93$; $I^2 = 0\%$). This meta-analysis provided an estimate of the dose-response relationship for zinc intake-status in pregnant and lactating women, and revealed that during pregnancy, doubling the zinc intake; increases zinc concentration by 3%, and during lactation this increased by 1%.

4.5 SUMMARY OF THE RESULTS OF THE INTAKE-STATUS META-ANALYSES IN ALL POPULATION GROUPS

Table 4.4 - Summary of the meta-analysis of intake-status relationship in all populations

Population group	Overall Beta	95% CI's	I ²	p
Infants‡	0.09	0.06,0.12	95%	P<0.0001
Children	0.12	0.04, 0.20	97.6%	p<0.0001
Pregnant women	0.04	0.02, 0.07	55%	p=0.037
Lactating women	0.02	-0.01,0.05	0%	p=0.927
Adults and Elderly (RCTs)	0.09	0.07,0.12	79.1%	P<0.0001

‡ Infants review was undertaken by ULPGC, final result is shown in the summary table.

These results mean that the systematic review on the randomized controlled trials on the zinc intake-status association (effect $\hat{\beta}$) for infants, children, pregnant women and adults and elderly was significant ($p<0.05$), doubling the zinc intake, increased plasma zinc levels by 6% in adults, by 3% in pregnant women, by 1% in lactating women and by 9% in children, with a degree of heterogeneity of studies between 55%-97.6% which suggests the need for standard methods in reporting results e.g the median or the standard error or the interquartile range or the 95% confidence interval, the range, the mean difference of the outcome was reported or no estimate of variability was reported. The reason for presenting analysis results primarily from intervention studies is because the number of studies from observational studies was limited and because randomized controlled trials are considered of higher quality studies in the hierarchy of evidence of study designs. Well conducted randomized controlled trials remain “the gold standard for evidence of efficacy” (Barton, 2000).

The meta-analysis approach was conducted to provide an estimate of the dose-response relationship between zinc intake and plasma zinc status which is indicated by the $\hat{\beta}$ value.

These systematic reviews addressed 2 main questions, firstly to evaluate the effect of zinc intake on indicators of exposure or body stores (i.e. plasma/serum zinc status) (I-S)

and secondly to look at the factors that affected this relationship. The effect of dietary zinc on plasma zinc status was determined for each individual study designated as regression coefficient $\hat{\beta}$ and a calculated overall pooled $\hat{\beta}$ on a double log scale showed that the effect was significant increasing plasma zinc concentrations between 1 to 9%. However the effect may have been altered by co-variables of duration of the study, age, gender and dose administered.

These meta-analyses were carried out within the context of the EURRECA project as a means to provide complementary evidence that may be useful to underpin dietary zinc reference values. To derive micronutrient recommendations, a dose response approach can be used (Dhonushe-Rutten et al., 2013), then these reviews were part of the dose-response approach which involved at least two of the 3 components: dietary intake (I), micronutrient status (S) and health outcome (H) and assessed the effect of zinc micronutrient intake on indicator of zinc status: plasma.

The main result and zinc guidance report can also be found at <http://www.eurreca.org/everyone/8567/7/0/32>. The zinc systematic review for infants, children, pregnant and lactating women, adults and elderly has been published elsewhere (Nissensohn et al., 2013, Moran et al., 2012a, Lowe et al., 2012, Moran et al., 2012b).

CHAPTER 5. –

RESULTS OF THE META-ANALYSIS OF ZINC INTAKE/STATUS-HEALTH (I/S- H) AND NARRATIVE REVIEW OF ZINC AND COGNITIVE FUNCTION

Chapter 5 – RESULTS OF THE META-ANALYSIS OF ZINC INTAKE/STATUS-HEALTH (I/S-H) RELATIONSHIPS AND NARRATIVE REVIEW OF ZINC AND COGNITIVE FUNCTION.

In this chapter, the results of the systematic review and meta-analysis of intake/status-health relationships in all populations groups are presented. The chapter concludes with a short narrative review of the literature relating to the role of zinc in one of the key health outcomes, cognitive function.

The purpose of this review was to specifically address the following questions:

- 1.) What is the effect of zinc intake on indicators of functional or clinical outcomes (Intake-Health) and what are the factors that affect this relationship?
- 2.) What is the effect of indicators of exposure or body stores (i.e. plasma/serum zinc status) on indicators of functional outcome (Status – Health), and what are the factors that affect this relationship?

Health outcomes were selected using an eminence approach developed by EURRECA team members UEA and WU (as described in chapter 3, section 3.4.1 about the measures of health outcome). The result of the systematic search for studies addressing zinc intake plasma zinc status and health relationships yielded over 1000 articles that were obtained in full text for eligibility evaluation. The main results for the others zinc health outcomes will be shown, then, the rest of this chapter will focus on a meta-analysis of the data available for zinc intake/status and indices of cognitive function.

5.1 MAIN RESULTS OF INTAKE/STATUS-HEALTH SYSTEMATIC REVIEW IN ALL POPULATION GROUPS

Table 5.1 provides a summary of the systematic review of zinc intake/status health relationships, dividing the studies identified into those that were eligible for meta-analysis, and those that were available for narrative review based on the criteria described in chapter 3 section 3.4.2 and its inclusion/exclusion criteria.

Meta-analysis was possible in 2 health outcomes: Growth, and cognitive function. For the purpose of this thesis only the cognitive health outcome will be described.

With reference to growth, the zinc systematic review looked at the effect of zinc supplementation in children's growth aged 1-8 year old (Intake-Health association). It found that of the 9 studies that were eligible for meta-analysis, there was no significant effect of zinc supplementation of between 2 weeks to 12 months duration on weight gain, height for age (HAZ), weight for age (WAZ), length for age (LAZ), weight for height (WHZ) and weight for length (WLZ) scores in children aged 1-8 years. The results of this review were that there was no modifying effect of zinc dose, supplement duration, age or gender on zinc supplementation and growth outcomes in meta-regression analyses (Unpublished data).

Table 5.1- Health outcomes identified from the systematic review in all population groups

Intake-Status-Health outcomes	N° of studies	
	Meta-analysis	Narrative review
<i>Infants</i>		
Intake-Status	13	
Growth	24	
Immune response to vaccination		0
Neurodevelopment	5	
<i>Children, Adolescents</i>		
Intake-Status	18	
Growth	9	
Immune function		3
Cognitive function, psychomotor development	6	7
Dermatitis		0
<i>Pregnant, Lactating women</i>		
Intake-Status	9	
Fetus		
Foetal growth		1
Foetus malformation		3
Birthweight		14
Mother		
Preeclampsia		4
Preterm delivery, gestational age at birth		9
Cognitive function		1
<i>Adults, Elderly</i>		
Intake-Status	13	
Immune function		9
Cognitive function	0	4
Dermatitis		0
Anorexia		0
Hypogeusia		0
Ischemic heart disease, cardiovascular disease		12
Depression		1
Diabetes mellitus		6
Carcinogenesis		7

Many of the studies identified by the systematic review process contained data that were not combinable for meta-analysis due to the heterogeneity of the methodological approaches for the outcome measurements and diversity of study design. However, a few studies were suitable for meta-analysis for the cognitive function health outcome. This was further enhanced by an updated search which was carried out for this health outcome to include papers published between March 2013 and March 2014.

5.2 META-ANALYSIS OF ZINC INTAKE/STATUS AND COGNITION HEALTH OUTCOME

The aim of the zinc systematic review and meta-analysis on cognition was to assess the impact of zinc intake/status on indices of cognitive function.

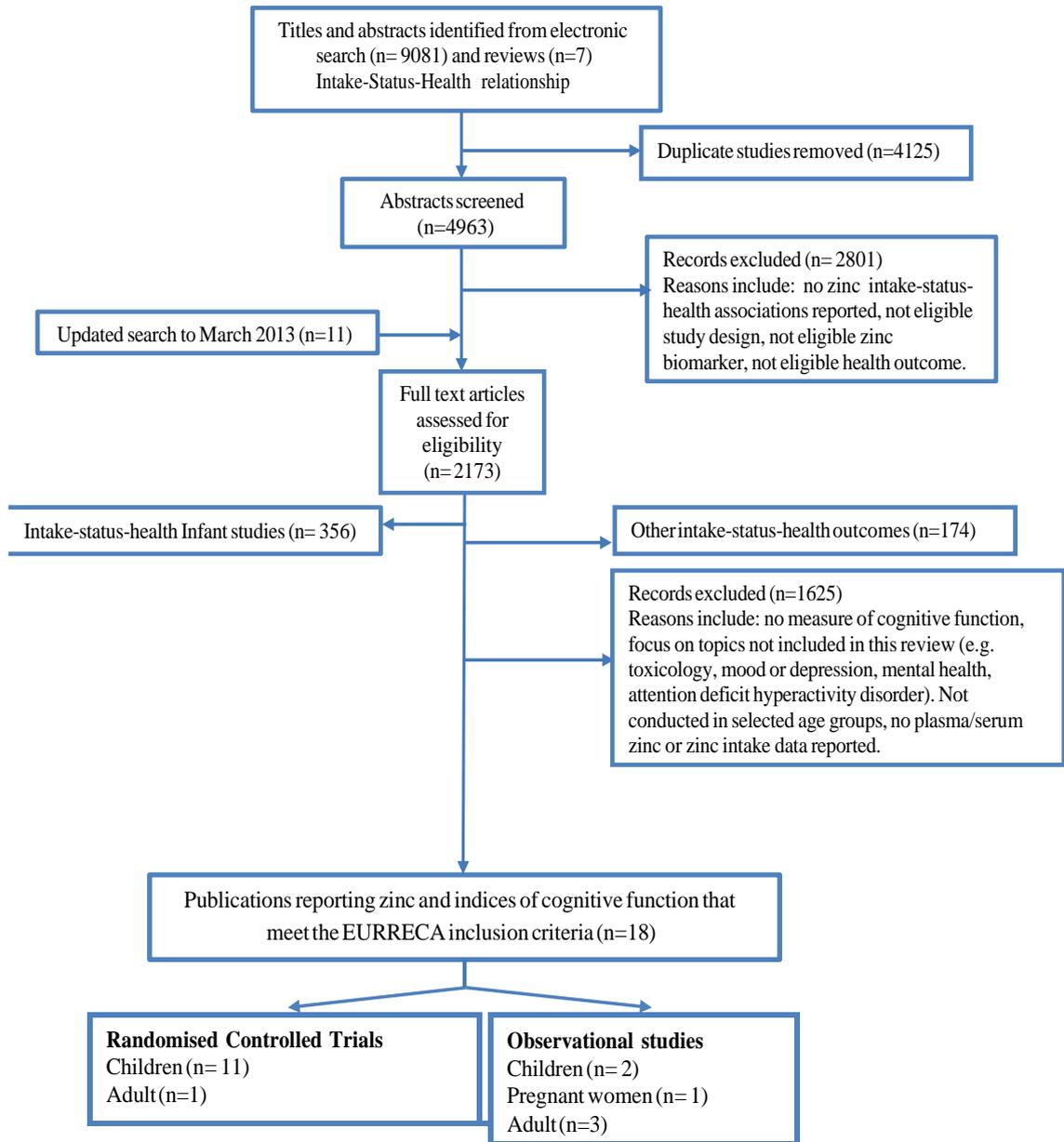
5.2.1 Search Strategy (STEP 1)

The same search strategy described in chapter 3 was conducted for the cognitive function health outcome in the 3 main databases: Embase, Medline, and Cochrane. The updated search (up to March 2013) yielded 1927 articles, which was added to the initial search of 7154 articles.

5.2.2 Screening Process for Cognitive Studies (STEPS 2-3)

The number of abstracts identified through the main databases was 9081 articles, plus 7 reviews. After duplicate abstracts were removed (4125), the number of abstracts that were screened and sorted was 4963. Afterwards, 2173 full text papers were assessed, including the 11 studies from the updated search. After excluding studies that did not report zinc intake/plasma zinc status or cognitive health outcome, the total numbers of included studies that examined the association between zinc and indices of cognitive function was 18. This is shown in Figure 5.1.

Figure 5.1 - Study selection process for the systematic review.



Cognitive aspects or categorization of cognitive function tests are shown in Table 5.2. The relevance of this table was to identify the areas of cognition that were assessed by each study included in this review, to find the same tests used or the same cognitive outcome to facilitate comparisons between the cognitive studies.

Table 5.2 - Cognitive aspects

Aspects of cognitive function	Cognitive Test	Specific functions assessed by test
Motor skills	• CPAS-R	Assesses cognitive abilities, interests, and dispositions by questionnaires.
	• Gross motor scale	Development of gross motor function
	• McCarthy Scales of Children's Abilities	Measures mental and motor abilities
	• MABC, finger tapping	Assessment of motor coordination, motor speed and dexterity
	• Grooved pegboard, dominant, non-dominant hands	Manipulative dexterity
	• Concept formation	Draw a person
Executive Function: Memory	• CANTAB :PRM	Visual memory
	• Spatial Span, SWM	Working memory
	• Visual and auditory sequential memory	Visual or auditory memory span
	• Short term memory	Storage of information for a limited period
	• SRT, RRT, Visual memory	Cognitive speed for reaction tasks
	• Buschke Recall	Short and long-term storage, retention of the total recall, Memory for geometric forms,
	• Heaton Recall	Concentration and memory,
	• Blessed items,	Visuomotor tracking and attention,
	• Trails, part B,	The subject names as many animals as possible in 1 minute
	• Category Fluency	
	• Stroop test	Inhibitory control
Attention	• Reaction time, MTS	Measures attention
	• Attention span scores	Length of time to concentrate
	• MMSE,	Screen dementia, measures orientation, registration, attention, calculation, memory, language skills.
Language	• Bear story, number concepts	Language and narrative development
Global cognitive function	• CSID, CERAD, The IU Story Recall Test, Animal Fluency Test, The IU Token test.	CSID, screening tool for dementia, CERAD word list recall. Animal Fluency Test, measure of executive function, The IU Token test, measure of language comprehension and working memory
	• PMSQ	Cognitive capacity
Intelligence	• Verbal, Non-verbal ability, general conceptual ability IQ	Differential ability scales, IQ is the ratio of tested mental age to chronological age and is expressed as a quotient multiplied by 100.
	• Binet-Kamath scale	Determine the level of intellectual and cognitive functioning
	• UNIT	The UNIT provides a comprehensive assessment of nonverbal intelligence.
	• WISC III	Measures intellectual functioning
	• RAVEN CPM	Test of non-verbal intelligence
	• WPPSI	Assess cognitive and intellectual abilities
	• DTLA	Measures general and specific mental abilities

Abbreviations: CANTAB, Cambridge Automated Neuropsychological Test Battery; PRM, Pattern Recognition Memory; SWM, Spatial Working Memory; MTS, Matching to Sample visual search; WISC-III, Wechsler Intelligence Scale for Children-Third edition; RAVEN CPM/RCPM, Raven's Coloured Progressive Matrices Test; IQ, Intelligent Quotient; CPAS, Cognitive Psychometric Assessment; CPAS-R, Cognition-Psychomotor Assessment System-Revised; SRT, Simple Reaction Time; RRT, Recognition Reaction Time; RPM, Raven's Progressive Matrices; UNIT, Universal Nonverbal Intelligence Test; MABC, Movement Assessment Battery for Children; DS, Digit Span; WPPSI, Wechsler Preschool and Primary Scale of Intelligence; DTLA, Detroit Tests of Learning Aptitude; MMSE, Mini-mental state examination; PMSQ, Pfeiffer's mental status questionnaire; CSID, Community Screening Instrument for Dementia; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; Word List Learning Test; The CERAD word List Recall Test; IU, The Indiana University; The IU Story Recall, the IU Token test.

5.2.3 Data Extraction and Meta-Analysis of Cognitive Outcome (STEP 4)

The zinc systematic review in cognition yielded a total of 18 studies that reported zinc intake or plasma/serum zinc and its association with cognitive function and, of these, 12 were RCTs and 6 were observational studies.

Characteristics of identified studies assessing zinc intake/status and cognitive function and the main results for each included study are shown in Table 5.3 and Table 5.4 for the intervention and observational studies, respectively.

Table 5.3 - Randomized controlled trials (n=12) reporting the effect of dietary zinc intake/serum or plasma zinc status on cognitive function in adults and children.

Study, year, country	Sex, age	Treatment groups	Micronutrient type	Duration	Outcome measure	Main results
Adults						
Maylor (Maylor et al., 2006) 2006 UK France Italy	188 males & females aged 55-70y. 199males &females aged 71-87y.	Placebo (n=63) 15 mg Zn/d (n=60) 30 mg Zn/d (n=65) Placebo (n=67) 15 mg Zn/d (n=66) 30 mg Zn/d (n=66)	Zinc gluconate	6 months	CANTAB consisting of: -Visual memory by PRM, -Working memory by SSP and SWM, -Attention by reaction time and MTS.	Significant improvement for SWM errors with 15 and 30 mg/d at 3 months (p=0.030). Significant detrimental effect of 15 mg/d for MTS latency (p=0.015).
Children						
Gibson (Gibson et al., 1989) 1989 Canada	60 males aged 5-7y.	Placebo (hair zinc concentration > 1.68 µmol/L) (n=42) Zinc group (hair zinc concentration < 1.68 µmol/L) 10mg Zn/d (n=14)	Zinc sulphate	12 months	Attention span assessed using 4 subtests from the DTLA: -sentence imitation, -word sequences, -oral directions, -and design reproduction.	No significant effect of zinc supplementation on attention span scores.
Cavan (Cavan et al., 1993) 1993 Guatemala	162 males & females aged 81.5 ±7.0 m.	Placebo (n= 79) 10 mg Zn /d (n= 75)	Zinc as amino acid chelate	25 weeks	Total cognition score assessed using 3 subtests from the DTLA: -Letter sequences, -Oral directions, -Design reproduction	No significant effect of zinc supplementation on cognition measures.
Penland (Penland et al., 1997) 1997 China	372 males & females aged 6-9y. Number of children in each treatment group not	20 mg zinc (Z) 20 mg zinc with micronutrient (ZM) Micronutrients alone (M)	Zinc alone (Z) Zinc with micronutrients (ZM) Micronutrients alone (M).	10 weeks	CPAS-R consisting of 6 subtests: -Continuous performance -Design matching -Delayed design matching -Concept formation -Finger tapping -Visual motor tracking	Z and/or ZM significantly improved performance on all subtests compared to M (p<0.05).

stated						
Sandstead (Sandstead et al., 1998) China	740 males & females aged 6-9y 'subjects divided equally between treatments'	20 mg zinc alone (Z) 20 mg zinc with Micronutrients (ZM) Micronutrients alone	Zinc alone (Z) Zinc with micronutrients (ZM) Micronutrients alone (M).	10 weeks	CPAS-R consisting of 6 subtests: -Continuous performance -Design matching -Delayed design matching -Concept formation -Finger tapping -Visual motor tracking	Significant effect of ZM on continuous performance, visual motor tracking and concept formation compared to M or Z (P<0.01).
Tamura (Tamura et al., 2003) UK	355 males & females aged 5.3 ±0.3 y.	Placebo (n=182) Zinc group (n=173)	Zinc sulphate	21 weeks Given prenatally at ~19 weeks gestation	-Differential Ability Scales (non-verbal, verbal, general conceptual ability: IQ), -Visual Sequential Memory, -Auditory Sequential Memory -Knox Cube -Gross Motor Scale -Grooved Pegboard	No significant effect of zinc supplementation on any cognition measure.
Gewa (Gewa et al., 2009) Kenya	554 males & females aged 7.6 ±1.3 y.	Control (n=130) Vegetarian supplement (n=147) Milk supplement (n=144) Meat supplement (n=133)	No additional Zn 1.35-1.68 mg Zn 1.46-1.66 mg Zn 2.38-2.89mg Zn	24 months	-RCPM -Verbal meaning test, -Arithmetic test, -Digit span-forward test -Digit span-backward test	Available Zn intake was associated with significantly higher gains in digit span test scores over time (p<0.05). A child with a daily high intake of available zinc gained 0.73 more points in the DS-total test. No significant differences were found for RCPM, verbal meaning score and arithmetic score.
Tupe (Tupe and Chipplonkar, 2009) India	180 females aged 10-16y.	Control (n=60) Diet supplementation (n=60). 20 Zn mg/d (n=60).	No additional Zn 1.6-2.6 mg Zn 20 mg ayurvedic zinc.	10 weeks	-SRT -RRT -Memory -RPM	SRT (p<0.05) and RRT (p<0.05) decreased significantly in the Zn and diet supplemented groups compared to baseline. Memory (p<0.05) and RPM (p<0.05) scores significantly increased in Zn and diet supplemented groups compared to baseline and control.

Caulfield (Caulfield et al., 2010) 2010 Peru	205 males & females aged 4-5y.	Control (iron & folic acid only) (n=96) Iron, folic acid + 25 mg Zn (n=85)	Zinc sulphate	26-30 weeks given prenatally starting 10-14 weeks gestation	-WPPSI -Language development -Number concepts -Concept formation	No significant effect of zinc supplementation for any outcome measure.
Christian (Christian et al., 2010) 2010 Nepal	676 males & females aged 7-9y.	Control (vitamin A only) (n=177) Iron + folic acid (n=103) Iron, folic acid + 30 mg zinc (n=178) Multiple micronutrients (n = 218)	Zinc sulphate	From early pregnancy to 3 months postpartum)	-UNIT (6 subtests: symbolic memory, cube design, spatial memory, analogic reasoning, object memory, mazes) -Go/no-go task -Stroop test -Backward Digit Span -MABC -Finger-tapping test	No significant effect of zinc supplementation for any outcome measure.
Murray-Kolb (Murray-Kolb et al., 2012) 2012 Nepal	688 males & females aged 7-9 y.	Placebo (n=176) Iron and folic acid (n=169) 10 mg Zn (n=144) Iron, folic acid + 10 mg Zn (n=199)	Zinc	12-35 months	-UNIT -Go/no-go task, -Stroop test, -Backward Digit Span test; -MABC -Finger-tapping test.	Unadjusted analyses revealed a significant overall difference across tests for Zn supplementation compared to no zinc (p=0.04). No significant effect of Zn supplementation was found for individual tests.
Pongcharoen (Pongcharoen et al., 2012) 2012 Thailand	560 males & females, aged 9.3 ±0.3y.	Placebo (n=139) 10 mg Zn (n= 139) Iron (n=147) Iron & 10 mg Zn (n=135)	Zinc sulphate	6 months Infants supplemented aged 4-6 months	-WISC III (6 verbal subtests: information, similarities, arithmetic, vocabulary, comprehension, digit span; 6 performance subtests: picture completion, coding, picture arrangement, block design, object assembly, symbol search), -RCPM.	No significant effect of Zn supplementation was found for any test.

Table 5.4 - Observational studies (n=6) reporting the effect of dietary zinc intake/ serum or plasma zinc status on cognitive function

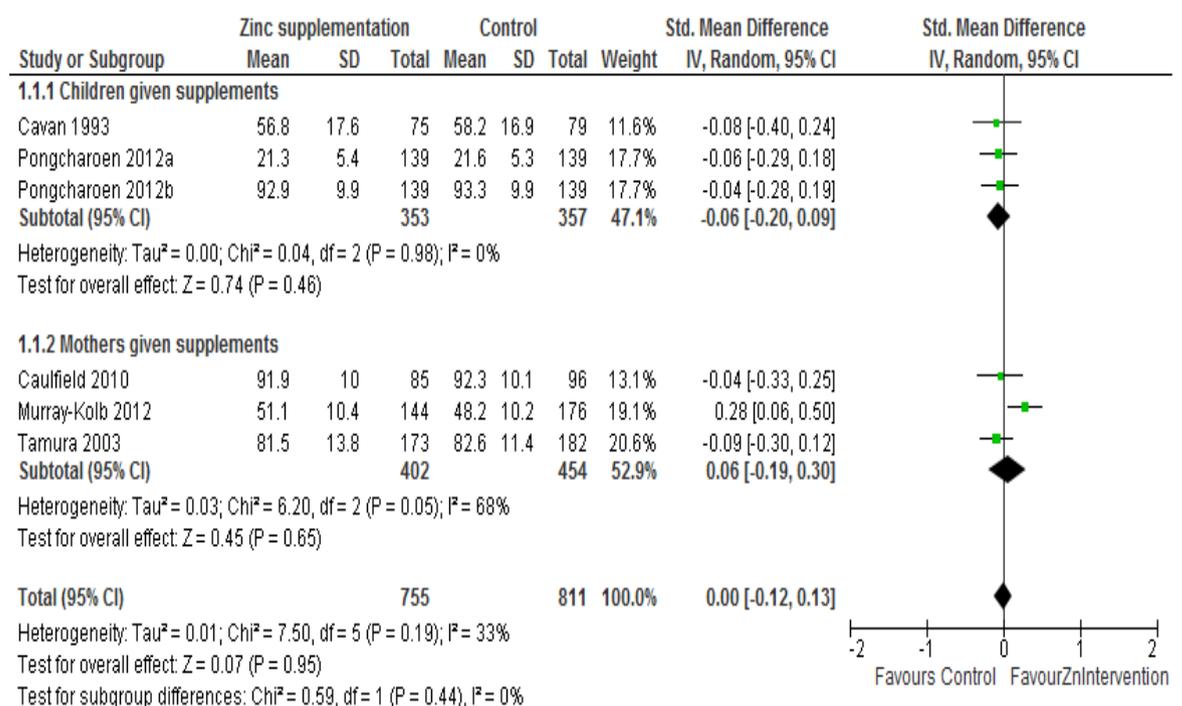
Study, year, country	Sex, age	Study design	Duration	Zinc intake/status marker reported and dietary/ analytical method	Outcome measure	Main results
Adults						
Ortega (Ortega et al., 1997) 1997 Spain	260 males & females aged 65-90y.	Cross-sectional	N/A	7 day weighed food record & FFQ.	-MMSE -PMSQ	MMSE results improved with increasing intakes of zinc (r=0.1349, p<0.05).
Gao (Gao et al., 2008) 2008 China	188 males & females, aged ≥ 65y.	Cross-sectional	N/A	Plasma zinc [ICP-MS]	Composite cognition score based on 6 tests: -CSID, -IU Story Recall Test, -Animal Fluency Test, -CERAD Word List Learning -CERAD Word List Recall and -The IU Token test.	Plasma zinc was not significantly associated with the composite cognitive score.
Lam (Lam et al., 2008) 2008 USA	1451 males & females aged 60-94y.	Cross-sectional	N/A	Plasma zinc [ICP-AES]	Cognitive function scores of a battery of 12 tests: -Buschke Total Recall, -Buschke Long-Term Recall, -Buschke Short-Term Recall, -Heaton immediate Recall, -Heaton Delayed Recall, -Heaton Copying, -Mini-Mental State Exam (MMSE), -Serial 7's, -"World" Backward, -Blessed items, -Trails, part B, -Category Fluency.	-In men, plasma Zn concentrations were not significantly associated with cognitive function scores. -In women, lower plasma zinc concentrations were related to poorer performance on tests of concentration (p=0.008).
Pregnant Women						
Stoecker (Stoecker et al., 2009) 2009 Ethiopia	99 females >24 wks gestation aged 27.7 ±4.7y.	Cross-sectional	N/A	Plasma zinc [AAS]	RAVEN CPM.	RAVEN CPM (A) score was correlated with plasma zinc (r=0.27, p<0.008).

Children						
Hubbs-Tait (Hubbs-Tait et al., 2007) USA	42 males & females aged 3-5y.	Cross-sectional	N/A	Plasma zinc [AAS]	McCarthy Scales of Children's Abilities which included verbal and perceptual score. -5 subtests of the verbal scale, -6-7 subtests of the perceptual performance scale.	Hierarchical regression analyses revealed that zinc explained significant unique variance in McCarthy scales of children's abilities verbal score (p=0.01).
Umamaheswari (Umamaheswari et al., 2011) India	100 males and females aged 6-11y.	Interventional 5mg Zn/d (in the form of syrup).	3 months	Serum zinc [AAS]	-Binet-Kamath scale -Digit forward -Sentence repetition -Story recall -Picture recall -Benton visual retention test -Cattell's retentivity test	Verbal (p=0.05), non-verbal memory (p<0.01) and IQ (p=0.05) were significantly improved after supplementation in 9-11y old children. In 6-8y old children, only verbal memory was significantly improved after zinc supplementation (p<0.01).

Abbreviations: DTLA Detroit Tests of Learning Aptitude; CANTAB, Cambridge Automated Neuropsychological Test Battery; SSP, Spatial Span; PRM, Pattern Recognition Memory; SWM, Spatial Working Memory; MTS, matching to sample visual search; MMSE, Mini-mental state examination; PMSQ, Pfeiffer's mental status questionnaire; WISC-III, Wechsler Intelligence Scale for Children-Third edition; WPPSI, Wechsler Preschool and Primary Scale of Intelligence; RAVEN CPM, Raven's Coloured Progressive Matrices Test; RPM, Raven's Progressive Matrices; IQ, Intelligent Quotient; CPAS, Cognitive Psychometric Assessment; CPAS-R, Cognition-Psychomotor Assessment System-Revised; SRT, simple reaction time; RRT, Recognition Reaction Time; UNIT, Universal Nonverbal Intelligence Test; MABC, Movement Assessment Battery for Children; DS, Digit Span; CSID, Community Screening Instrument for Dementia; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; IU, the Indiana University Story Recall; FFQ food frequency questionnaire; AAS, Atomic Absorption Spectroscopy; ICP-MS, Inductively coupled plasma-mass spectrometry; ICP-AES, Inductively coupled plasma atomic emission spectrometer.

The purpose of the zinc systematic review on cognition health outcome was to address the effect of zinc intake/serum or plasma zinc on cognitive function in children and adults and elderly population. The common identified cognitive outcomes were: intelligence, executive function and motor skills, and that is the reason the meta-analysis was conducted to evaluate these indices of cognitive function. Then final included studies were grouped according to main cognitive outcomes assessed in each study and divided in two groups: children who were given zinc supplementation, or mothers who received zinc supplements. After examining if studies reported mean and SD data on comparable cognitive measurements, standard mean differences analysis on intelligence, executive function and motor outcomes was conducted using Review Manager (RevMan) version 5.2. Forest plots of the meta-analyses are shown in Figure 5.2, Figure 5.3 and Figure 5.4.

Figure 5.2 - The effect of zinc supplementation on intelligence in children.



Abbreviations:

^aPongcharoen, 2012 [22] RCPM (Raven test)

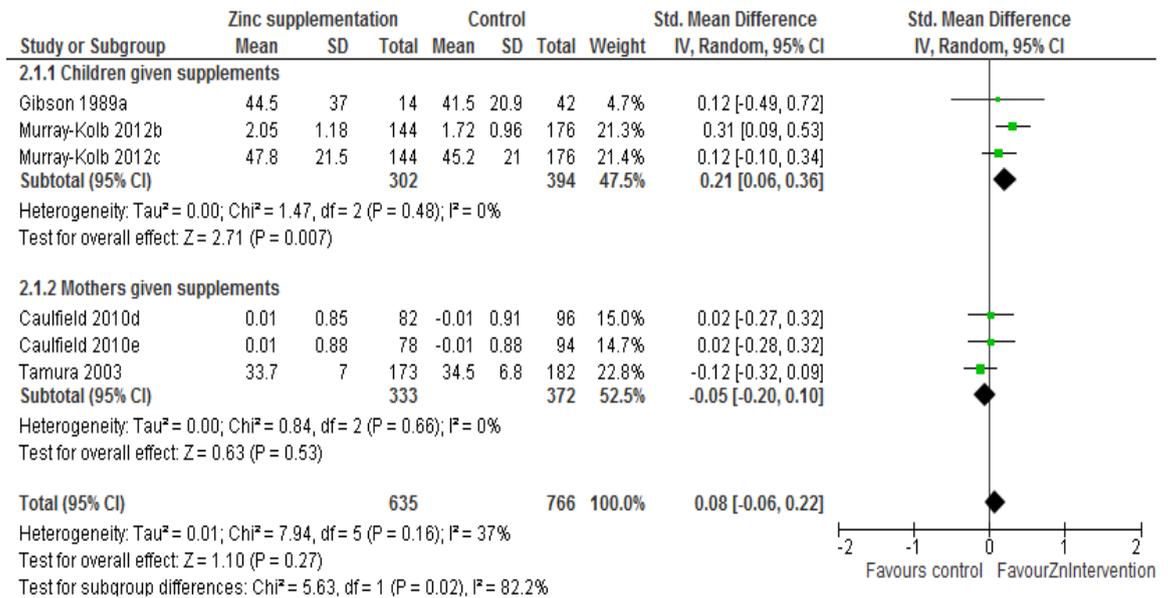
^bPongcharoen, 2012 [22] WISC test (full scale)

RCPM (Raven's Colour Progressive Matrices)

WISC (Wechsler Intelligence Scale for Children)

The Intelligence outcome analysis yielded an overall β of < 0.001 (95% CI -0.12, 0.13; I^2 33%), $P=0.95$.

Figure 5.3 - The effect of zinc supplementation on executive function in children.



^aGibson, 1989 [28] Cognitive score median converted to mean value

^bMurray-Kolb, 2012 [21] Backward digit span

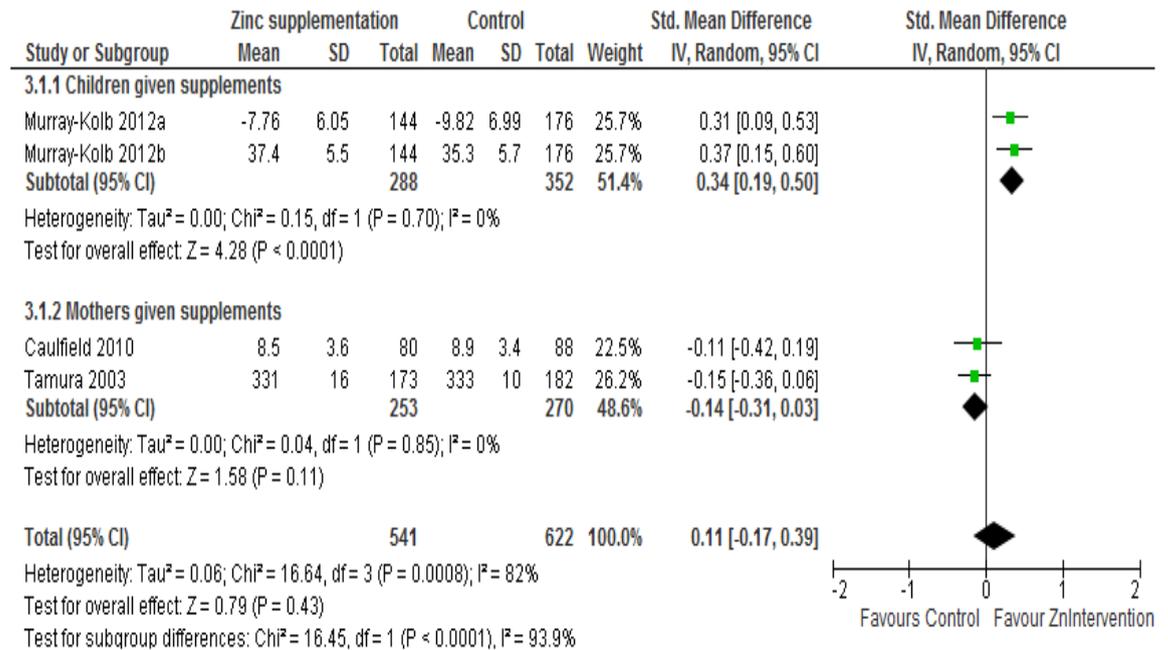
^cMurray-Kolb, 2012 [21] Go no go test %

^dCaulfield, 2010 [25] Language development

^eCaulfield, 2010 [25] Counting game

The executive function outcome analysis yielded an overall β of 0.08 (95% CI -0.06, 0.22; I^2 37%), $P=0.27$.

Figure 5.4 - The effect of zinc supplementation on motor outcome in children.



Abbreviations:

^aMurray-Kolb, 2012 [21] MABC test where lower score indicates a higher motor skill, therefore means have been converted to a negative score for meta-analysis.

^bMurray-Kolb, 2012 [21] Tapping

The motor outcome analysis yielded an overall β of 0.11 (95% CI -0.17, 0.39; I^2 82%), $P=0.43$. These results revealed that there was no significant overall effect of zinc supplementation on intelligence ($P=0.95$), executive function ($P=0.27$), and motor development ($P=0.43$).

5.2.4 Internal Validity Assessment

In order to assess the quality of the included studies, an internal validity assessment was executed to determine the overall risk of bias of each study (See Table 5.5).

Table 5.5 - Assessment of validity of included randomised controlled trials reporting zinc intake/serum or plasma zinc status on cognitive function in adults and children

Study	Adequate sequence generation	Allocation concealment adequate	Adequate Blinding	Dropouts adequate and outcome data complete	Funding adequate	Lack of other potential threats to validity	Overall risk of bias
Maylor, (2006)	Unclear	Unclear	Yes	Yes	Yes	Unclear	High
Tamura (2003)	Yes	Unclear	Yes	Unclear	Yes	Unclear	High
Penland (1997)	Unclear	Unclear	Yes	Unclear	Yes	Unclear	High
Tupe (2009)	Unclear	Unclear	Yes	Unclear	Yes	Unclear	High
Murray-Kolb (2012)	Unclear	Yes	Yes	Yes	Yes	Yes	Moderate
Pongcharoen (2012)	Yes	Yes	Yes	Yes	Yes	Yes	Low
Gewa (2009)	Unclear	Unclear	No	Yes	Yes	Unclear	High
Christian (2010)	Unclear	Yes	Yes	Yes	Yes	Yes	Moderate
Caulfield (2010)	Yes	Yes	Yes	Yes	No	Yes	High
Sandstead (1998)	Unclear	Unclear	Yes	Unclear	Yes	No	High
Gibson (1989)	Unclear	Yes	Yes	Yes	No	Yes	High
Cavan (1993)	Unclear	Unclear	Yes	Unclear	Yes	No	High

Table 5.6 - Meta-analysis summary for cognitive function

Cognitive outcome	Overall β	95% CI's	I^2	<i>P</i>
Intelligence	<0.001	-0.12,0.13	33%	<i>P</i> =0.95
Executive function	0.08	-0.06,0.22	37%	<i>P</i> =0.27
Motor skills	0.11	-0.17, 0.39	82%	<i>P</i> = 0.43

Table 5.6 presents the overall effect β value that gives estimates of zinc intake for each cognitive outcome assessed and the degree of heterogeneity (I^2) of studies, which was higher in the group of studies that evaluated motor outcome ($I^2=82\%$). There was no significant effect on zinc supplementation on cognitive measurements.

5.3 SUMMARY OF META-ANALYSES OF THE INTAKE/STATUS-HEALTH RELATIONSHIPS

The aim of this systematic review was to look at the relationship between zinc intake and/or zinc status (plasma/serum zinc concentration) and indices of cognitive function in vulnerable populations children and adolescents, adult and elderly and pregnant women. This was because cognitive function was a prioritized health outcome for zinc intake and its effect on cognition. A conclusion of this systematic review was that the number of studies in cognition were limited in determining zinc intake-status/health. Therefore, further studies were necessary to fully explore this association. The empirical study described in part 2 of this thesis investigated the cognition health outcome with the difference that zinc was not the only trace mineral of study. In the empirical study multiple micronutrients were evaluated to assess the long term effect on cognitive function from infancy to preschool age.

The inclusion/exclusion criteria for the systematic review resulted in only a small number of studies being included in the meta-analyses in cognition studies in the children (n=6) group. Within this meta-analysis, three studies were excluded from the meta-analysis because the authors only reported percentage change in measurements (Tupe and Chiplonkar, 2009), lacked control group (Penland et al., 1997, Sandstead et al., 1998), or reported differences of test score gains (Gewa et al., 2009) rather than the mean and SD data for both intervention and placebo group to enable analytical

comparison. Results from the meta-analyses of the impact of zinc supplementation on cognitive domains in children indicated that supplements given prenatally did not have a long term impact on offspring during childhood but supplements given directly to children may have a positive impact on executive function and motor skills.

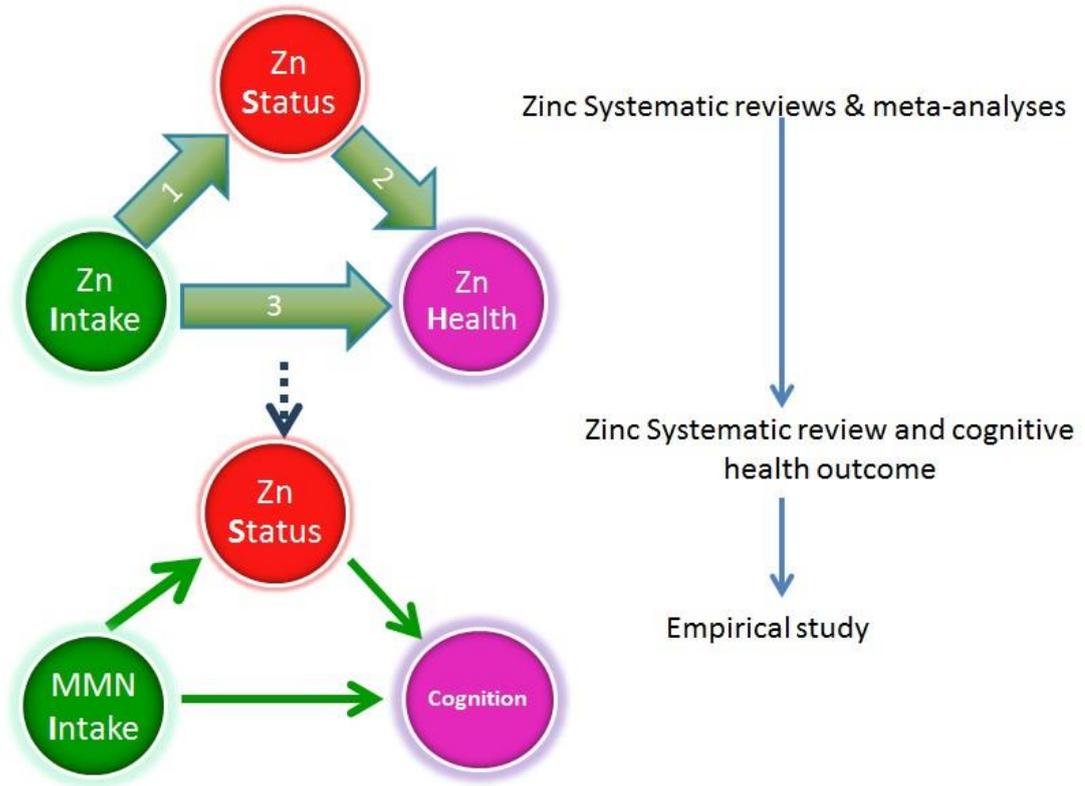
Despite the small number of studies that were eligible for the meta-analysis, it could be argued that the usefulness of this meta-analysis lies in the analyses per cognitive domain and in the categorization of prenatal supplementation and supplements given to children that add an insight into the effect of zinc supplementation in both situations. This systematic review showed that the evidence regarding the effect of zinc intake or status on cognitive function is lacking and inconclusive. Although the meta-analysis of studies conducted in children showed no effect of zinc supplementation on any of the identified cognitive domains (intelligence, executive function, motor outcome), there were some small indicators of improvements in cognition (Penland et al., 1997, Sandstead et al., 1998, Gewa et al., 2009, Tupe and Chiplonkar, 2009, Ortega et al., 1997, Stoecker et al., 2009, Hubbs-Tait et al., 2007, Umamaheswari et al., 2011, Maylor et al., 2006), following zinc supplementation when all studies across all population groups are considered as a whole. It is difficult to determine a specific effect of zinc intake or status on indices of cognition, partly because of the methodological challenges of assessing long-term cognition effects, but also because the identification of “at risk populations” (identified vulnerable population exposed to zinc deficiency) seems to be a key factor in disentangling the impact of supplementation on cognitive outcomes (Schmitt, 2010a).

In terms of the relationships between zinc intake, status and health outcomes, this review process highlighted a number of gaps in knowledge. These findings from the EURRECA systematic review relate to the first objective of this study which was to identify research gaps from intake-status-health zinc relationships in populations groups. Cognition was a health outcome of interest and further research is needed in this area. The studies not included in the meta-analyses have been reported in a narrative review at ‘eurreca.org/everyone’.

The results of the relationship between zinc intake-status and health outcomes was the starting point for the interest cognitive function in children. There were a limited number of studies identified by the systematic review process and thus highlighted the

gap of knowledge in this area. This led to the design of the empirical study that was undertaken as part of this doctoral research, described in Chapter 6, and results presented in Chapter 7, Chapter 8 and Chapter 9. The purpose of this empirical research was to evaluate the long term impact of multi-micronutrient supplementation on cognition in young children (see Figure 5.5).

Figure 5.5 - Link between the zinc systematic review and the empirical study



5.4 NARRATIVE REVIEW OF ZINC AND COGNITIVE FUNCTION

This literature review is placed in this section because it was part of the systematic review in zinc intake/status and cognitive function. This review provides a summary of published research relating to cognitive function in animals and humans with a particular focus on the consequences of malnutrition. This review was undertaken in order to bring together knowledge regarding the effect of zinc supplementation on cognitive function in developing countries and to understand the state of malnutrition in children of the country setting where the empirical study took place.

Nutrition provides an essential role in cognitive development and function. This role includes the modulation of long-term cognitive processes such as neurodevelopment and neurodegeneration (Schmitt, 2010b). Evidence exists that deficiency of some micronutrients influences cognition in children (Benton, 2010). One of these micronutrients is zinc which is found in high levels in the brain where it plays important structural and functional roles (Black, 1998). However, 10% of total brain zinc is located in the lumen of glutamate containing synaptic vesicles which may be released on excitation and play a role in modulation of synaptic signalling (Palmiter et al., 1996). Both the structural components of the brain and the energy it uses to function come from the diet. The possibility that a child's behaviour and cognition may be influenced by what is eaten while the brain is initially developing has therefore attracted attention, for instance in nutritional deficiencies, such as an early shortage of iodine, zinc or iron, permanently decrease cognitive ability.

Other nutrients that play a role in brain function include docosahexaenoic acid (DHA, 22:6 n-3 fatty acid) that improves mental development in infants; folate which prevents neural tube defect in infants; and selenium has been shown to affect activities of brain enzymes in animals, which are necessary for brain development and function (Wasantwisut, 1997). By having roles as coenzymes or as a constituent of important molecules, micronutrients influence general metabolism. Several trials have found that micronutrient supplementation improved both behaviour and cognition, however the details are not as yet clear (Benton, 2011).

Before the decision was taken of the types of cognitive tests to be used in the empirical study, a literature review of the cognitive tests was undertaken and is presented below.

5.4.1 Cognition and the Developing Brain

Cognition comes from the Latin word, co“gnoscere” meaning “to come to know”(MedNet, 2011), it is a complex process of encoding, selection, maintenance, storing, retrieving, transforming and manipulating information that is obtained through the senses (Ashman and Conway, 1997), consequently the assessment of cognitive development will involve evaluation of perception, thought, attention, memory, language and activity (Bhatnagar and Taneja, 2001).

Cognitive development

Cognitive development is the development of intelligence, and problem solving skills. The cognitive processes involve changes in the child’s thinking, intelligence, and language whereas socio-emotional processes involve changes in the child’s relationships with other people. Gross motor skills involve large-muscle activities, e.g. running and playing. Fine motor skills involve finely tuned movements e.g. finger dexterity required for writing and drawing.

The human brain undergoes remarkable structural and functional changes between 24 and 40 weeks after conception, progressing in the last trimester of pregnancy to become a complex brain that resembles morphologically to an adult brain (Georgieff, 2007).

The period of most rapid brain growth occurs during gestation through the first 2 years of life (Tran et al., 2010). Thus, the prenatal period to eight years of age is the period of most intensive brain development during the lifespan, and at the age of 2 the brain is 80% the weight of the adult brain (Anderson, 2010, Lenroot and Giedd, 2006) and at 5 year old, the brain reach a size of about 90% of its adult size (Dekaban and Sadowsky, 1978). Brain development continues beyond foetal life (DeLong, 1993), starts days post conception and continues through adolescence and beyond (Shonkoff and Phillips, 2000) hence the vital role of nutritional adequacy for normal brain development and function. Nutrition plays an important role during the preschool years (1 to 5 years of age), which is a period of rapid and dramatic post-natal brain development, and the main period of cognitive development, namely working memory, attention and inhibitory control (Rosales et al., 2009).

Cognitive development during the child lifecycle

Cognitive development is thought to change as the child goes through different stages of life

- Sensorimotor (0-2yrs): sensory perceptions and motor activity (reflexes, reactions, coordination),
- Preoperational (2-6yrs): basic language use (Intuitive, symbol use and construction),
- Concrete operational (7-11yrs): mental operations and logic (categories, concrete logical systems),
- Formal Operational (12+yr): abstract concepts (systematic testing of possibilities, application of systems reasoning).

These are only approximate age guidelines of Piaget's theory of cognitive development and it is the universal sequence of the stages and not the age, which is the most important aspect. This psychological stages can serve as an organizing framework for the study of cognitive development (Feldman, 2004).

Aging and the brain

With age the brain loses size, weight and volume. The cerebellum loses weight in proportion to the loss in the cerebrum. This atrophy or decrease in size is a result of loss of neurons which are replaced by fibrous astrocytes which are star-shaped cell of a type of neuroglia. The most significant change is the decline in the firing rate of some neurons, the information-processing abilities slow down and this accounts for lower IQ scores reached by old people (Bloom and Lazerson, 1988). Thus, the importance of adequate zinc supply for brain function for the prevention of neurological diseases (Takeda, 2001).

5.4.2 Physiology and Neurobiology of Zinc

The structural and functional roles of zinc have been described in chapter 2. In this section, a short summary of the physiology and neurobiology of zinc will be provided.

Zinc is an essential micronutrient (Sandstead, 2000) and has a catalytic (rate of chemical reaction), co-catalytic and a structural role. About 85% of whole body zinc is in muscle

and bone and 11% is in the skin and the liver, with the remaining 4% found in tissues (Tapiero and Tew, 2003). Zinc concentrations remain constant through adult life, measurements of zinc in the adults was reported to be on average 13.3 (SD 0.3) $\mu\text{g/g}$ (wet weight) $n=175$; and in infants 8.2 (SD 0.8) $\mu\text{g/g}$ (wet weight) $n=31$ (Markesbery et al., 1984). Zinc is essential for brain function (Atsushi and Haruna, 2014). Hence, zinc homeostasis in the brain is vital and is regulated by the brain barrier system: the blood-brain and blood-cerebrospinal fluid barriers (Takeda, 2001). To maintain this zinc homeostasis the brain capillary endothelial cells respond to changes in zinc status by increasing zinc uptake when zinc concentrations are low and decreasing zinc uptake when zinc concentrations are high (Lehmann et al., 2002).

Physiological roles of zinc become manifest during zinc deficiency. Zinc depletion has been demonstrated in kinetic studies (chemical reactions) where changes in tissues concentrations occur when plasma zinc levels decrease (King et al., 2001).

To the present, the importance of zinc in neurobiology is very well acknowledged (Dreosti, 1989). Zinc deficiency increases the risk for neurological disorders, affecting neurogenesis and increasing neuronal death which may lead to learning and memory deficits. Finally, zinc homeostasis disorders may be a risk factor for neurological problems such as Alzheimer's disease and depression (Szewczyk, 2013).

5.4.3 Animal Studies

Relatively few studies exist between zinc and cognition and most animals studies have shown that zinc deficiency affects cognitive development by alterations in attention, activity, and other features of neuropsychological behaviour and motor development. However, it is not always possible to extrapolate findings from animals studies to human studies.

To understand this association in zinc and cognition, it is important to determine the period which is most affected by zinc deficiency and from animals studies, it appears that the most sensitive periods are during brain growth and pre-adolescent spurt growth (Bhatnagar and Taneja, 2001).

A study in young monkeys showed that zinc deficiency was linked with reduced activity, poor memory and attention (Golub et al., 1994b). Similarly zinc supplementation greatly delayed hippocampal-dependent memory deficits in mice and supported that zinc supplementation may prevent cognitive deficits (Corona et al., 2010). Opposite results were found in rats, in which enhanced zinc consumption causes

memory deficits and increased brain levels of zinc (Flinn et al., 2005). At a physiological level, evidence exists that zinc is involved in the developmental regulation of neurotrophins and N-methyl-D-aspartate receptors (NMDA), controlling use of glutamate as a neurotransmitter in the central nervous system (CNS), particularly in the hippocampus, the region of the brain involved in learning and memory (Levenson, 2006). Furthermore, short-term memory can be affected by dietary zinc restrictions, this was shown in male rats which were sensitive to zinc when fed as adequate zinc (20 mg/kg of zinc) or fed as zinc-deficient (2 mg/kg) (Keller et al., 2001).

Another study, in offspring rats showed that zinc deficiency during the last trimester of pregnancy and during lactation impaired spatial learning and memory and also had a negative effect on motor activity. Pregnant rats after mating were divided into three groups. The control group was fed a standard diet, a zinc deficient (ZnD) group fed a diet deficient in zinc (0.5-1.5 ppm), and a zinc supplement (ZnS) group fed a standard diet and enhanced zinc in the drinking water (10 ppm). Rat's offspring in these groups were tested for spatial learning and memory at post natal day (PND) 56 in Morris Water Maze (MWM) and motor activity in open field at PND 66. The Escape Latency (EL) and Travelled Distance (TD) in the ZnD group were increased but Percentage of Time Spent (PTS) in the target quadrant was decreased compared to the control group (Boroujeni et al., 2009).

Cognitive performance was also assessed in young adult rats, where male rats were fed for 17 days on either adequate zinc (24.4 µg Zn/g) or low zinc (5.3 µg Zn/g) and were tested on visual, auditory, association, and discrimination learning using a skinner box. The results of these tests found no differences for the visual discrimination performance test between zinc deficient rats and the control group. However, the zinc deficient rats performed better than the control group during the last auditory discrimination task which involved transferring a learned food-relevant cue. (Massaro et al., 1982).

5.4.4 Human Studies

To date, human studies examining zinc and cognitive function show inconsistent results. Zinc supplementation has been shown to have a positive impact on various functions such as learning, knowledge retention, attention and functional activity among school children aged 7 year old. Data for this study was from the Child Nutrition Project (CNP) in Kenya, a 2 year feeding intervention study (1998-2000) using animal food sources.

The CNP used several cognitive tests such as the Raven's Coloured Progressive Matrices (RCPM) which are considered to be good non-verbal indicators of general intelligence (Wicherts et al., 2010); the verbal meaning test to measure expressive language and verbal skills; the arithmetic test adapted from the Wechsler Intelligence Scales for children to assess numeric and reasoning skills; and the digit span (DS) forward and backward test to assess memory and concentration. The result of this study was that available zinc intake was associated with higher gains in DS-total test scores over time (Gewa et al., 2009).

Zinc supplementation resulted in significantly greater activity levels in children, shown in 93 Indian children aged 12 to 23 months where the supplemented group (n=48) received 10 mg of Zinc gluconate along with selected vitamins for more than 120 days. 5 activity levels were recorded according to the children activity rating score (CARS) representing the extent of movement such as low movement (stationery 1, stationery 2, slow movement) and high movement (moderate, fast movement). The zinc supplemented children were observed to spend on average some 72% more time in performing activities in the high movement group than the control group (8.1% vs 4.7%). This study suggested further examinations on the implications of improved zinc status on activity, cognitive and locomotor development (Sazawal et al., 1996).

Regarding cognitive tests used in infants, the Bayley Scales of Infant Development have been widely used in infants research, this mental scale assess various sensory-perceptual abilities, memory, learning, problem solving ability, communication and verbal skills (Niccols and Latchman, 2002). When the Bayley motor test was applied to Egyptian infants from 1 to 42 months of age, at 6 months, examining the mental, motor development and behaviour of the infants, poor attention was found (Kirksey et al., 1994).

Evidence from observational studies of the benefits of including animal source foods (ASF) in the diets of children in developing countries reveals that higher intake of ASF in those countries was associated with better growth, status of some micronutrients, cognitive performance, motor development and activity. Only three randomized trials supplemented children with milk and compared outcomes with a non-intervention control group. Both height and weight growth were improved, although in Kenya height was increased only in younger school children who were stunted at baseline. Meat supplements were evaluated only in two randomized controlled trials, in Kenya and

Guatemala (mean baseline age 8 years and 1 year, respectively); meat improved cognitive function and activity in Kenya; milk was less effective than meat for improving cognitive function and physical activity, possibly due to its lower content of iron, zinc, or riboflavin. Meat and especially cow's milk are excellent sources of vitamin B12 which is a micronutrient commonly deficient in populations which consume low amounts of ASF. Other micronutrients such as iron have been added to cow's milk and resulted in improved nutritional outcomes for children (Allen and Dror, 2011).

Cognitive functions are more vulnerable to missing breakfast in poorly nourished children. Simeon and Grantham-McGregor found that severely malnourished, stunted and wasted children were adversely affected in a number of cognitive functions when they missed breakfast. This contrasted with children who were neither stunted nor wasted and were not adversely affected. Therefore missing breakfast could be a serious contributor to poor school achievement in undernourished children who are more likely than adequately nourished children to benefit in school performance (Simeon and Grantham-McGregor, 1989).

Food fortification has been recognized as an effective approach to improve a population's micronutrient status (Hess and Brown, 2009) and impaired cognitive development in children can be prevented through home-fortification. Reduction of diarrhoea and improvement of haemoglobin concentrations was associated with the reduction in child mortality and increased intelligent quotient (IQ) scores, thus, home-fortification seemed to improve clinical outcomes at a reasonable cost (Sharieff et al., 2008). Zinc fortification have been investigated by (Penland et al., 2003) in young adolescents (65 girls and 47 boys) who consumed 4 oz fruit juice which contained 0, 10 or 20 mg of zinc gluconate for a period of 10 weeks. Psychomotor cognitive outcome was measured through manual dexterity and eye-hand coordination, cognitive measurements were attention, perception, memory and reasoning, and the findings of this study were that zinc fortification improved overall memory.

Micronutrient deficiencies which are linked with impaired growth and cognitive function (Nga et al., 2011) may also be complicated with parasite infestation. A randomized, double-blind, placebo-controlled 2 x 2 factorial trial was conducted to assess the efficacy of multi-micronutrient fortified biscuits (FB) with or without deworming on growth, cognitive function, and parasite load in 510 Vietnamese

schoolchildren aged, 6-8 years old. Schoolchildren were randomly allocated to receive albendazole or placebo at baseline and 4 months of multi-micronutrient (FB) or non-fortified biscuits. Children receiving FB for 4 months scored higher on two cognitive tests: Raven's Colored Progressive Matrices and the Digit Span Forward test whereas children receiving albendazole plus FB had the lowest parasite load after four months. A school-based fortification program might help schoolchildren but a high prevalence of parasite infestation may reduce its efficacy.

Prenatal micro nutrient supplementation has been positively associated with general intellectual ability and motor functioning. Christian et al. (2010) followed up 676 Nepalese children aged 7 to 9 years, whose mothers randomly received iron/folic acid, iron/folic acid/zinc or multiple micronutrients containing iron/folic acid/zinc plus 11 other micronutrients, all with vitamin A compared to a control group of vitamin A alone. In this study, in order to assess the intellectual and motor function, the following tests were used: the universal nonverbal intelligence test (UNIT), the movement assessment battery for children (MABC) and the finger tapping test.

In another study, maximum motor development scores were found to be higher in zinc supplemented group of infants of low birth weight ($1,117 \pm 287$ g), (98 ± 10) than the placebo (98 ± 8). The infants supplemented group received a regular term formula plus zinc supplements (4.4 mg/L; final content, 11 mg/L) for a period of 6 months and the placebo group received the same formula and placebo (final content, 6.7 mg/L), for this examination, the Griffiths developmental assessment was used (Friel et al., 1993).

Zinc supplementation during infancy did not lead to long-term cognitive improvement in 9-y-old children (Pongcharoen et al., 2011). Pongcharoen and colleagues investigated the long-term effects of iron or zinc supplementation or both nutrients on cognitive performance, 560 Thai children were followed up after 8 years, and the randomized control trial involved 4 groups receiving daily iron, zinc, iron plus zinc, or a placebo at 4–6 mo. of age for 6 months. Cognitive performance was assessed by using the Wechsler Intelligence Scale for Children–Third Edition (Thai version), the Raven's Coloured Progressive Matrices (CPM), and school performance tests.

Caulfield used the Spanish adaptation of the Wechsler preschool and primary scale of intelligence to test verbal and performance abilities of 205 children aged 4.5 year old and found that prenatal zinc supplementation did not influence child development (Caulfield et al., 2010). In addition, a study by Gibson et al. (1989) reported that in

older children there is less evidence to suggest that zinc supplementation has a beneficial impact on cognition, this has been shown in a 12 month study of 60 Canadian boys aged 5-7 y, where attention span scores did not respond after 10 mg of zinc sulphate.

Eilander et al. (2010) reviewed the effect of 3 or more micronutrients compared with placebo on cognition in healthy children aged 0-18 and showed that multiple micronutrient supplementations may be associated with a marginal increase in fluid intelligence (the ability to reason and think logically) and academic performance in healthy schoolchildren but not with crystallized intelligence (the ability to utilise skills and knowledge). The cognitive tests used in these randomized controlled trials were grouped into several cognitive domains (e.g., fluid and crystallized intelligence). Pooled effect size were 0.14 SD (95% CI: -0.02, 0.29; P = 0.083) for fluid intelligence and -0.03 SD (95% CI: -0.21, 0.15; P = 0.74) for crystallized intelligence. Eilander et al., recommends more research before public health recommendations can be given.

Overall, the results from zinc intervention trials on the effectiveness of improved zinc status on cognitive function have been ambiguous and remain unclear.

Below is an introduction of the link between malnutrition and cognition and the scale of undernourishment in Latin American countries and specifically the country of Peru, setting for the empirical study which will be described in chapters 6 to 9.

5.4.5 Malnutrition and Cognition

Malnutrition is a worldwide problem that affects millions of unborn and young children mainly during the vulnerable stages of brain development. (Morgane et al., 2002).

Substantial evidence exists that severe malnutrition in early childhood can lead to poor cognitive function and a causal relationship exist between undernutrition and delayed child development (Pelletier, 1995). This link between malnutrition and impairment of mental function in children has been reviewed by (Connolly and Kvalsvig, 1993), and this indicates that although there is good evidence linking malnutrition and adverse cognitive outcome, there are no grounds that establish that an improved diet will also improve measured intelligent quotient (IQ). However a review by Grantham-McGregor and Baker-Henningham (2005) suggests that a well-balanced diet appears to be necessary for optimal development in children and considerable evidence exists that the first 2 years of life are the most sensitive to under nutrition.

Malnutrition during early brain development has been shown to reduce both protein synthesis and DNA synthesis. This results in a stunted brain which contains fewer cells of normal size (Winick and Rosso, 1974). Among the earliest functions to be affected are those associated with the brain enzymes involved in cognition and behaviour (Scrimshaw, 1998). Furthermore, the review by Morgane states that malnutrition exerts its effects during development, not only during the brain growth spurt period, but also during early organizational processes such as neurogenesis, cell migration, and differentiation (Morgane et al., 1993).

The demand for zinc is higher especially in the stages of rapid growth, such as infancy. As malnutrition interacts with short-term food deprivation on children's cognitive function, it is likely, that other nutrient deficiencies or infections may have an effect on cognition (Grantham-McGregor, 1995).

Other micronutrients involved in cognitive function are iron, B12 and studies have shown that children who are zinc deficient are also likely to be iron and B12 deficient as the main source for these micronutrients is animal protein (Grantham-McGregor and Ani, 1999). In supplementation studies it may be difficult to interpret the effect of a single micronutrient supplementation, when children are deficient in multiple micronutrients (Black, 2003a).

Nutritional deficiencies can have a detrimental effect on mental development (Simeon and Grantham-McGregor, 1990). Iron and zinc micronutrient deficiencies are recognized public health problems globally (Hambidge, 2003). It is prevalent in children worldwide and very common in most developing countries, and while zinc deficiency may be prevalent, data is lacking (Wasantwisut et al., 2006, Rossander-Hulten et al., 1991). Iron and zinc deficiencies are common and may occur together because iron and zinc are mostly bioavailable from many of the same food sources, and zinc and iron absorption are also inhibited by many of the same dietary substances. Furthermore, mild to moderate deficiencies impair neuropsychological function (Sandstead and Smith, 1996). For instance, if iron deficiency occurs during infancy and is moderate to severe, then there is likely to be permanent neurological damage that cannot later be corrected by iron supplementation (Scrimshaw, 1998). Zinc deficiency occurs in combination with other micronutrient deficiencies but the mechanisms that link zinc deficiency with cognitive development are unclear. Zinc deficiency may influence children's development by altering the ability of the child to respond or

interact (Black, 1998). Black (1998) suggest a path model in which neuropsychological functioning (e.g. attention), activity, and motor development facilitate the relation between zinc deficiency and cognitive development and where age acts a potential moderator, depending on age, on the relation between zinc deficiency and cognitive development.

Regarding malnutrition in school-aged children, this is a public health issue, especially in developing countries and countries in transition. The nutritional status of school-aged children impacts their health, cognition, and subsequently their educational achievement. Other factors also associated with long-term deficits in cognition and school achievement are small-for-gestational-age birth weight, iodine deficiency, and protein–energy malnutrition (PEM) (Grantham-McGregor et al., 2000).

School settings are an opportunity to provide health and nutrition services to disadvantaged children. Yet, school-aged children are not commonly included in health and nutrition surveys. An up-to-date overview of their nutritional status across the world is not available (Best et al., 2010). (Best et al. (2010) reviewed the literature from 2002 to 2009 on the nutritional status of children).

In order to understand the role of nutrition in the brain and behavioural development of toddlers and preschool children, evidence from 125 studies in preschool children versus 232 infant and 303 school age children studies has been reviewed by (Rosales et al., 2009). The preschool years (i.e. 1-5 years of age) is a time of rapid and dramatic postnatal brain development and of fundamental acquisition of cognitive development, such as working memory, attention and inhibitory control. It is also a time of transition from a direct maternal mediation/selection of diet-based nutrition to food selection that is more based on self-selection and self-gratification. However, there have been fewer published studies in preschool children than in infants or school-aged children that examined the role of nutrition in brain/mental development. One reason may be because of age-related variability, in terms of individual differences in temperament, linguistic ability, and patterns of neural activity that may affect the assessment of neural and cognitive development in pre-school children.

The scale of undernourishment in Latin America and malnutrition in Peru

Malnutrition is one of the most widespread health problems in South America, particularly for infants below 5 year old (Dutra de Oliveira et al., 1981, Escudero, 1978). Latin America and the Caribbean is home to some 6% of the developing world's undernourished people and to 11% of its total population (FAO, 2006). Food insecurity deteriorated in Peru during the 1970s and, especially, the 1980s. The prevalence of undernourishment doubled from 21% in 1969–71 to 42% in 1990–92. In the 1990s, the trend was finally reversed. Between 1990–92 and 2001–03, the number of undernourished fell from 9.3 million to 3.3 million people, and the prevalence of undernourishment fell from 42% to 12% of the population. The improved food security can be attributed, among other things, to the reduction of inflation. Between 1990–92 and 2001–03, real per capita Gross Domestic Product (GDP), market value of goods, grew by 2.1 % per year, despite the difficulty caused by world financial market turmoil in the late 1990s. A key factor behind the success was the strong agricultural growth. Peru introduced reforms in the agriculture sector, including legislation on land transactions and entitlements, which led to improvements in access to credit. Agricultural value added per worker increased by 4% annually between 1990–92 and 2001–03. Nevertheless, significant levels of undernourishment and poverty remain. The prevalence of undernourishment (the population below minimum level of dietary energy consumption) was reported at 16 in 2008 and the percentage of the population whose food intake is insufficient to meet dietary energy requirements was below 2.5% (Index, 2008).

Undernourishment exists when caloric intake is below the minimum dietary energy requirement, which is the amount of energy needed for light activity and a minimum acceptable weight for attained height, and it varies by country and from year to year depending on the gender and age structure of the population. According to the Food and Agriculture Organization (FAO), there is food insecurity in Peru, which occurs when people do not have adequate physical, social or economic access to food (FAO, 2009) (Table 5.7).

Table 5.7 - Prevalence of undernourishment (FAO, 2009).

Country	Total population 2004-2006 (millions)	Number of people undernourished 2004-2006 (millions)	Proportion of undernourished in total population (%)
Peru	23.7	3.6	13

When the intake of animal sourced foods is low the bioavailability of minerals (iron, zinc, calcium), vitamins (vitamin A, vitamin B12) is also low (Gibson et al., 2003, Neumann et al., 2004). In Peru, as in many other lower-income countries, the diets are composed primarily of cereals and legumes (Gibson, 1994), which are relatively good sources of zinc, but even if net zinc intake appears adequate by general recommendations, zinc status is usually compromised and thus the importance to recognize the dietary factors affecting zinc absorption, such as phytate, which is present in staple foods like cereals, corn and rice (Lonnerdal, 2000). Dietary strategies to prevent zinc deficiency can be implemented at household level such as increasing intakes of food with high content of bioavailable zinc e.g. fish, nuts as snacks (Gibson et al., 1998), other dietary strategies that included dietary diversification and modification in a community-based trial reduced the prevalence of inadequate intakes of protein, and micronutrients such as calcium, zinc and vitamin B-12, but not iron in young Malawian children (Gibson et al., 2003).

The challenge for the future is to maintain the pace of improvements in poverty and hunger reduction and broaden the gains to poorer regions of the country (FAO, 2009). According to (FAO, 2012) statistics, Peru has a total population of 29.4 million inhabitants with 3.0 million undernourished people and with a prevalence of undernourishment of 11%. The principal issue in Peru is still chronic malnutrition which was reduced by 5% between 2005 and 2010 from 22.9 % to 17.9% (Mejia, 2011).

Figure 5.6 - Country of Peru in South America (WHO, 2013)



Aguiar Christine (2007) analysed malnutrition and policies in Peru to reduce chronic malnutrition and indicated that causes of malnutrition could be direct, such as insecurity and poverty, or indirect, namely lack of access to markets, transportation, education; water and sanitation inadequacy and a weak government. To reduce the problem of chronic malnutrition in Peru, Rogers (2002) proposed a national strategy that addressed the causes of stunting, that is, growth retardation, which surprisingly did not focus either on poverty or food security strategies, but indicated that because supplementary food strategies in Peru were ineffective in the reduction of chronic malnutrition, strategies should aim first to allow accessibility to food, health, water and sanitation and continue working towards long term goal of reduction of poverty. New figures from the Peruvian National Statistical Office (INEI) are encouraging and show that malnutrition rates in Peru have decreased from 40.1% in 2005 to 31.3% in 2010, which is success in the fight against malnutrition in Peru (Mejia, 2011).

5.5 CONCLUSIONS FROM THE SYSTEMATIC REVIEW

The systematic review of the literature reporting studies investigating the relationship between zinc intake and plasma/serum zinc concentration (I-S, dose response), described in Chapter 4, revealed a high volume of strong evidence of I-S association, obtained from randomised controlled trials, however there were limited number of studies assessing the intake-status relationship from the observational studies; similarly

there was a limited number of studies assessing the Intake/Status-Health outcome (I/S-H) relationship for each of the population groups, described in Chapter 5. In addition, the heterogeneity of the studies made it difficult to find comparable studies to conduct a meta-analysis in most of the health outcomes assessed. The findings from the large systematic review in (I-S) and (I/S-H) association for zinc, indicated that more data was required to explore the health outcomes. For one health outcome, cognitive function, there was sufficient data available and homogeneity between studies to conduct a limited meta-analysis that showed that no significant overall effect of zinc supplementation on cognitive function domains was observed. For example, the pooled standard mean difference of intelligence was <0.001 (95% CI -0.12, 0.13) $p=0.95$, executive function, 0.08 (95% CI, -0.06, 0.22) $p=0.26$ and motor skills, 0.11 (95% CI -0.17, 0.39) $p=0.43$.

While there were limited number of studies available assessing the interrelationships between zinc intake-status/health cognition, and the finding from this review that although no overall significant effect of zinc supplementation was observed on cognitive domains, we also had a sub-finding that when supplements were given directly to children this may have a positive impact on executive function, 0.21 (95% CI 0.06, 0.36) $p=0.006$, and motor skills, 0.34 (95% CI 0.19, 0.50) $p<0.0001$, however this result should be taken with caution as the 2 datasets were from the same study. There was a need to explore the cognition health outcome and the effect of zinc supplementation on cognition in children.

These findings were used to design the empirical part of this PhD and drove the exploration of the effect of multi-micronutrients supplementation including zinc in pre-school children, which will be described in Chapter 6.

CHAPTER 6. –

DESIGN AND METHODOLOGY OF EMPIRICAL RESEARCH

Chapter 6 – DESIGN AND METHODOLOGY OF EMPIRICAL RESEARCH

This chapter begins with a review of previous studies that have examined the impact of changes in dietary zinc on cognitive function and behaviour in humans and animals (section 6.1), leading to the hypothesis of the empirical research. The context of the study, that took place in Peru, is presented in section 6.2. The design of the study is presented in sections 6.3 and 6.4 respectively. Section 6.5 describes the detail of the methods selected for the empirical research, and the reasoning behind the choices made.

6.1 REVIEW OF PREVIOUS RESEARCH AND RATIONALE FOR EMPIRICAL STUDY

Micronutrient deficiencies and anaemia are common problems in children in Peru (World, 2011, ENDES, 2010). Malnutrition is one of the most common causes of morbidity and mortality among children worldwide (WHO, 1999). The sub optimal zinc status has been demonstrated in Peru, where over 40% of children are deficient in zinc (IZA, 2010). Dietary patterns of Peruvians are characterized by inadequate intake of foods of animal origin, which are important sources of protein, zinc and micronutrients (Rogers, 2002).

Zinc is an essential micronutrient found at high levels in the brain, particularly in the hippocampus which is located in the temporal lobe of the brain, and which has the function of strengthening the information of the short and long term memory. It is known that the human brain undergoes remarkable structural and functional changes between 24 and 40 weeks after conception, progressing to start the third quarter of a smooth bilobed structure with few twists or complex paths for a term brain, which morphologically resembles the adult brain (Pomeroy, 2004). During brain development, zinc is important for the structure and DNA synthesis and release of neurotransmitters, and its deficiency alters the autonomic nervous system regulation and development of the hippocampus, and cerebellum (Georgieff, 2007).

A study by Caulfield et al (2011) showed that prenatal zinc supplementation improved foetal autonomic regulation in a zinc deficient population in Peru. In this study, 165

infants born to mothers supplemented during pregnancy, with iron and folic acid with or without zinc were evaluated at 54 months (80% of the original sample). Data was collected from the electrocardiogram (ECG) of children at rest and at the same time they underwent a battery of cognitive tests following a standardized protocol. Children whose mothers were supplemented with zinc had more control of the autonomic nervous system and vagal tone. This indicates that supplementation of pregnant women with zinc deficiency has long term beneficial effects for neuronal development associated with autonomic regulation (Caulfield et al., 2011).

Zinc plays important structural and functional roles in health and physical growth and emotional development of children, because it is involved in the synthesis and release of neurotransmitters, development and functions of the central nervous system (Salgueiro et al., 2004) also plays a key role in cell integrity, cell differentiation, gene expression, immune development and numerous biological functions (Black, 2003). Consequently, zinc deficiency will affect growth and development (Bhatnagar and Natchu, 2004)

Evidence of the role of zinc in neurological development and function of the central nervous system in child development, behaviour and mental health have been reviewed by Black et al (2011). The authors conclude that although observational studies have suggested an association between zinc deficiency and poor cognitive functioning and mental health, evidence from randomized controlled trials conducted during pregnancy, lactation and infancy has shown little effect. Zinc supplementation has beneficial effects for health in children with zinc deficiency and therefore maintenance of the appropriate level of zinc is an important goal for the health of children (Black, 2011).

In the cognitive development, Bhatnagar pointed out that zinc deficiency may affect cognitive development by altering attention, activity, neuropsychological behaviour and motor development. The exact mechanisms are unclear but it appears that zinc is essential for neurogenesis, neuronal migration, synaptogenesis and its deficiency can interfere with neurotransmission and subsequent neuropsychological behaviour. (Bhatnagar and Taneja, 2001).

The effect of zinc on physical growth has been demonstrated in several randomized trials, such as a study of 90 children aged 2-5 years old with size below 25 percentile of the growth chart Centre Health Statistics of the United States, showed that daily supplementation of 5 mg of zinc for 6 months improves physical growth in terms of increased height and weight gain in children with inadequate linear growth, especially

in men (Mozaffari-Khosravi et al., 2009). The results of a meta-analysis of 33 randomized controlled intervention trials conducted by Brown et al (2002) to evaluate the effect of zinc supplementation on physical growth and serum zinc concentrations of pre pubertal children, showed that zinc supplementation had positive responses in height, weight gain and a large increase in serum zinc in children (Brown et al., 2002).

Additionally an RCT meta-analysis of zinc supplementation demonstrated a positive effect on diarrhoea (Brown et al., 2002, Brown et al., 2009) and pneumonia (Bhutta et al., 1999). For the aforementioned reasons, worldwide, it is estimated that over 450,000 deaths per year (4.4% of all deaths) among children six months and five years of age are attributable to zinc deficiency which is an important problem in public health (Walker et al., 2009). Therefore, improved zinc nutrition is vital for children's growth, cognitive function and development.

Anaemia, micronutrient deficiencies and stunting remain common problems in Peru (Lopez de Romana et al., 2005). Anaemia is a highly prevalent problem in Peruvian children under 5 years old, likewise consumer surveys have shown that most children do not consume the recommendations of iron, zinc, folate, vitamin A. The powdered supplements known as "sprinkles" contain multiple micronutrients which are deficient in the diet of the child.

The sprinkles have been shown to be as effective as ferrous sulfate in the control of anaemia (Zlotkin et al., 2005) and have greater acceptability, because it can be mixed with food, without altering the taste or colour (Zlotkin et al., 2001). It is necessary to give supplements to reduce other micronutrient deficiencies. There is little information on long-term benefits of these interventions. The Ministry of Health has begun, in several regions, supplementation with iron, zinc, vitamin A, vitamin C and folic acid (micronutrients in powder), then the original study was conducted (ISRCTN, 2013) to evaluate the effect of micronutrients on anaemia and zinc deficiency.

The rationale for the micronutrient supplementation was the following: in Peru it is considered normal to give iron to children under 3 years old (MINSa, 2012). Also, when the prevalence of anaemia is less than 40% the recommended dosage is 12.5 mg iron and 50 µg folic acid daily on babies with a normal weight, for a period of 6 to 12 months (Stoltzfus et al., 1998). Studies have shown the benefits of multiple micronutrients in improving anaemia (López de Romaña et al., 2005, Smuts et al., 2005,

Untoro et al., 2005), improving haemoglobin levels, and reducing anaemia though the effects were small compared with iron alone (Ramakrishnan et al., 2011). Specifically this study aimed to evaluate the long-term effect of multiple micronutrients (including zinc) on growth and cognitive function and biochemical parameters of zinc nutritional status, anaemia, in pre-school children aged 3-4 years old. The period of supplementation of these children is considered a window of opportunity for brain development, and long-term study could provide a valuable tool in public health policy.

The purpose of this study was to investigate the long-term effect multiple micronutrient powders ("sprinkles") in the cognitive, social and child growth in populations at risk of zinc deficiency. Cognitive function was an identified health outcome from the zinc systematic review and meta-analyses in all population groups and so was identified as being of particular interest for further exploration..

Hypothesis: Children given multiple micronutrient supplements during infancy will have higher cognitive and behaviour function scores than those given iron alone.

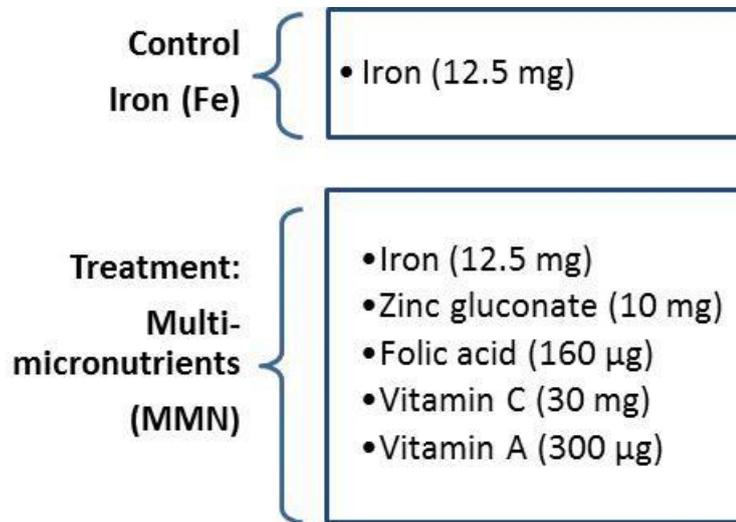
Expected outcome: We expected to see a positive effect of multiple micronutrient supplements on cognitive function tests in intelligence, inhibition, working memory and behaviour compared with infants given iron alone .

6.2 CONTEXT OF THE EMPIRICAL RESEARCH

This empirical study was a follow up study to an original RCT conducted by a team from Instituto de Investigación Nutricional (IIN) in 2010, led by Prof Nelly Zavaleta . The original study had a sample of 902 infants from Villa El Salvador (VES), in Lima, aged 6-17 months. It was a double blind randomized clinical trial (RCT) (ISRCTN, 2013) conducted by UNICEF (United Nations Children's Fund) and IIN (Instituto de Investigación Nutricional), designed to evaluate the effect of powdered multiple micronutrients on anaemia and zinc deficiency. Infants were randomized to receive a) daily powdered supplements (sprinkles) of 12.5 mg iron, (control). vs. b) multiple micronutrients which contained: 12.5 mg iron, 10 mg zinc gluconate, 160 µg folic acid, 30 mg vitamin C, and 300 µg vitamin A (Figure 6.1). Sprinkles were single-dose sachets (Schauer and Zlotkin, 2003) which contained the above micronutrients in

powder form, that were easily sprinkled onto foods prepared in the household (Sharieff et al., 2006).

Figure 6.1 – Composition of supplements provided to infants in the original study



After 6 months of supplementation, anaemia was reduced in both groups and zinc status (based on plasma zinc concentration) was improved in the infants given multiple micronutrients, but not for iron alone (Zavaleta N et al, personal communication).

The empirical study was conducted in the same district of VES in collaboration with IIN and in coordination with the Micro-red de Salud VES-LPP and UNICEF. VES is one of the 43 districts of Lima, the capital of Peru and the largest city in the country. It is a low income residential district, on the southern outskirts of Lima, that began as a shanty town in 1971 (Riofrio, 1972). Villa El Salvador (VES) is located 25 kilometres south of Lima, on the central coast. VES has an area of 3.546 hectares or 35.460 square kilometres and it is approximately located at latitude 12 ° 12' 34" South and longitude 76 ° 56' 08" west, and at an altitude of 175 meters above sea level (Figure 6.2). VES has the following boundaries:

- North: District of Villa Maria del Triunfo.
- South: Lurín District.
- East: District of Pachacamac.
- West: District Chorrillos and Pacific Ocean

With reference to demography, VES has a population of approximately 381,790 citizens, and with a population density of 10 767 citizens/km².

It is subtropical arid, hot in summer and wet in winter, the average temperature is 18° C and 19° C. The climate of VES varies because of the following factors:

- The Peruvian Current, which maintains a low temperature by cold water upwelling that restrict water evaporation, thereby controlling atmospheric stability without producing torrential rains.
- The Andes, which prevents humid air passing from the Atlantic to the Pacific causing heavy rainfall in the jungle and low rainfall on the coast.
- The South Pacific anticyclone. Cold air mass comes from the south to the coast.

Figure 6.2 - Map of the district of Villa El Salvador in Lima, Perú



There are the following zones within VES:

- Residential Zone. Represents 56% of the total area of the district and includes 9 sectors and peripheral settlements. At present, with the exception of the urbanization of Pachacamac and human settlements, the urban modular structure is composed of sector, group, block and Lot.
- Industrial Zone. Includes the Industrial Park which consists of productive business groups of handicrafts, leather, tailoring, carpentry, foundry, mechanics and food.
- Agribusiness Zone. In this zone there are livestock projects and forage crops.

- Beach zone. This includes 5.5 kilometres of beach: Venice, Windward and Conchan. It's a future economic reserve targeting tourism and recreation.

VES is divided by 9 territorial zones which in turn are formed by 10 sectors (see Figure 6.3). This division applies for a better distribution of the participatory budget

Territory I: Includes Sectors 1 and 8

Territory II: Includes the Sector 2

Territory III: Includes the Sector 3

Territory IV: Includes Private Pachacamac

Territory V: Area beaches, agricultural and Housing Association Sector 5

Territory VI: Sector 6 and Housing Association

Territory VII: Sectors 7, 9 and 10 and Human Settlements Cerro Lomo de Corvina

Territory VIII: Human Settlements Metropolitan Park

Territory IX: Industrial Park (VES council, 2011)

Figure 6.4 - Clinical setting: “Maternal and Child Center saint Joseph”



6.3 EMPIRICAL RESEARCH DESIGN OVERVIEW

The study protocol was developed at UCLAN in collaboration with IIN and approved by the IIN ethics committee in Lima. An executive summary was also approved by the Micro-red de Salud VES-LPP. Additionally, approval was also granted by the UCLAN ethics committee.

Figure 6.5 provides an overview of the methods used for the empirical field research which was undertaken to investigate the long term impact of micronutrient supplementation on cognitive and social-emotional development in Peruvian pre-school children.

Figure 6.5 - Overview of the empirical research design

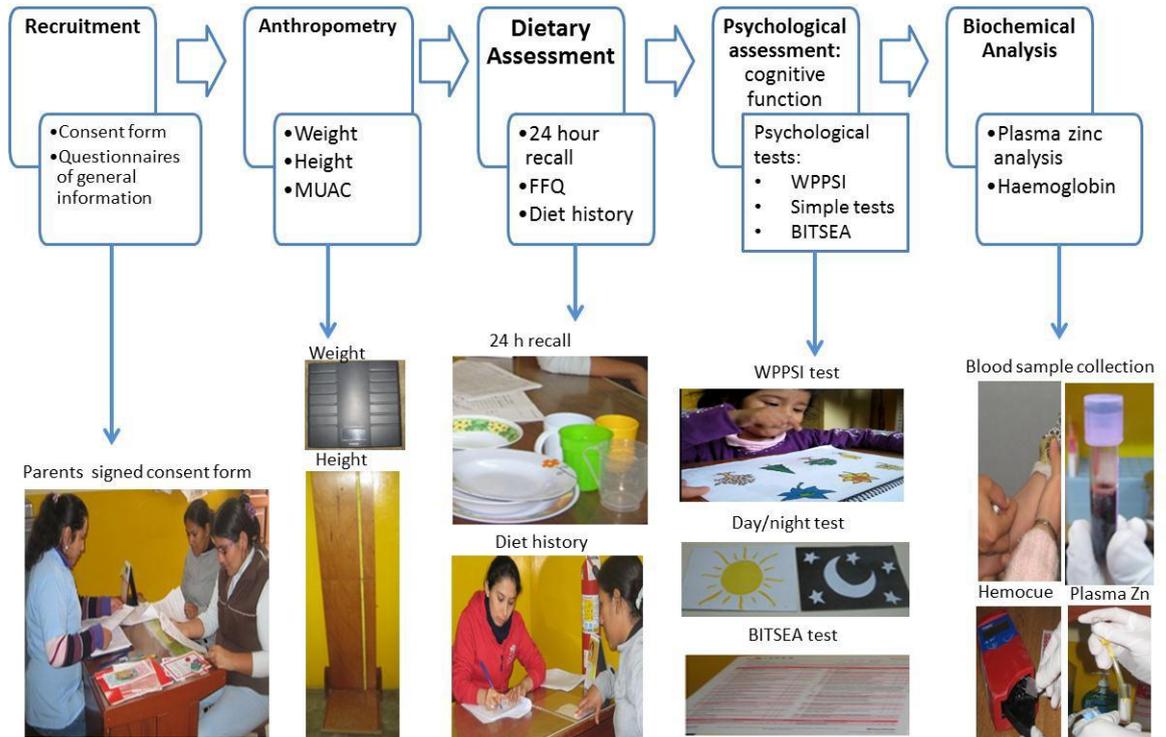
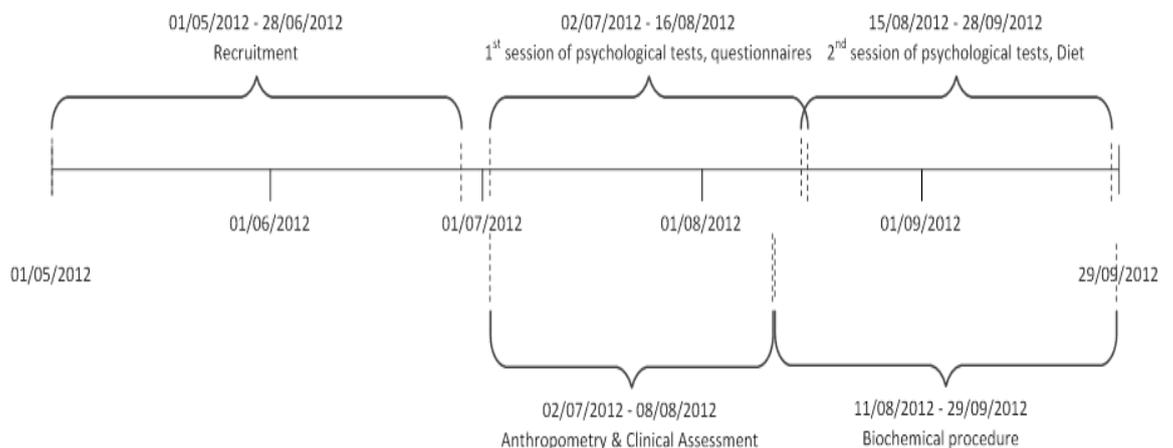


Figure 6.6 below shows the timeline for our study, with an initial period for recruitment of the children for the study, followed by the required psychological, clinical and biochemical tests. The study was performed between May and September 2012, and the number of personnel involved was 5 in total (psychologist, paediatrician, nurse, health worker, and the author (nutritionist)).

Figure 6.6 - Timeline of the empirical research



6.4 PARTICIPANT RECRUITMENT

The original study had a population of 902 children (aged between 36 and 48 months at the time of our study) and, given that our study was self-funded so had limited resource, to calculate the sample size for our study we assumed a power of 80%, a level (β) of 0.8 and a confidence interval of 95%, resulting in the number of participants required to be 166 children in total, with 10% added for the possible loss to follow up the tests, which resulted in a sample size of $n = 184$ children in total (CDC, 2011, Jones et al., 2003).

The criteria for inclusion/exclusion of children for our study were as follows:

Inclusion criteria: Children between 36-47 months of age, who participated in the study of multiple micronutrient supplementation with zinc, (IIN-270). One or both parents will sign the informed consent form.

Exclusion criteria: Children who did not participate in the study of multiple micronutrient supplementation with zinc, (IIN-270). Parents who did not accept the participation of their child. Children who are not able to complete the test because of severe or chronic disease that prevents them from participating.

In this study, 2 children were excluded at the beginning of the study because one presented poor eyesight and the other had mental retardation.

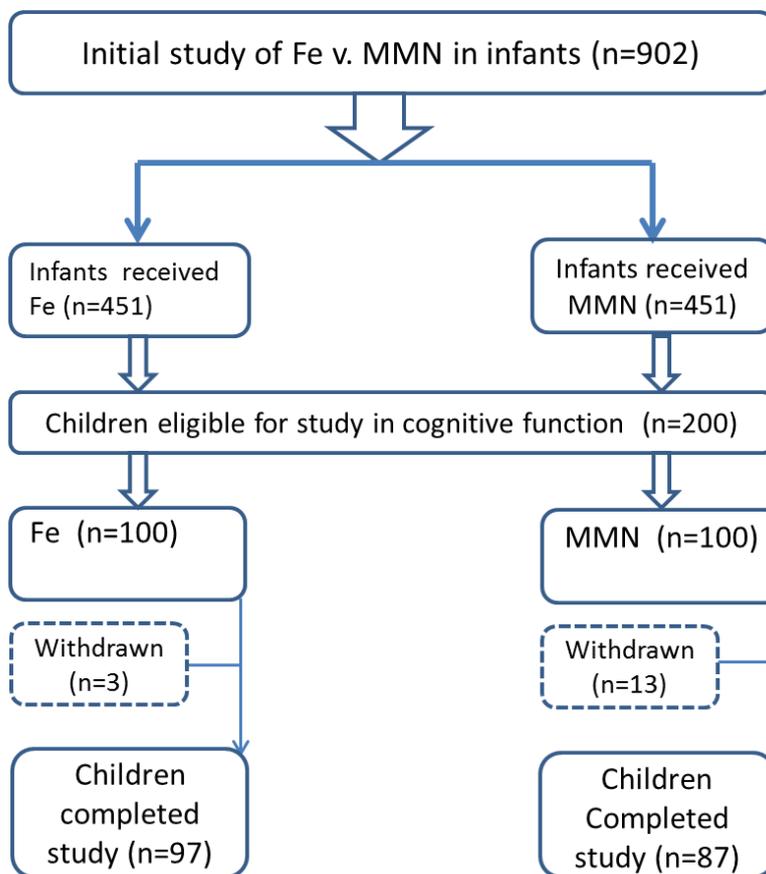
Figure 6.7 - Participant recruitment and enrolment



The final participant numbers are shown in Figure 6.8.

In preparation for the recruitment process, the following forms were prepared: pamphlet to invite parents, study and video consent form, poster for the clinic, flipchart to explain study involvement to parents, registration form to enrol children at the clinic, forms to collect general data, medical history, questionnaires of stress and depression in mothers, forms to assess diet through 24-hour recall, food and frequency questionnaire, diet history, cross check form, protocol for simple tests, summary of recommendations for psychology and nutrition to give to parents at their last appointment (All forms can be found in the Appendices B12-B18, B20-B30).

Figure 6.8 - Study participants



A computerized random number generator selection was performed to recruit mothers living in Villa El Salvador in sectors 1-10 whose children participated in the initial study (ISRCTN, 2013), which was coordinated by IIN, the Health network of the district of VES and UNICEF. Families were visited by 2 health workers to invite them to participate in the pre-school study, appointments were arranged for 2 visits and the pamphlet of information of the study was given on that day. These sectors were selected

for this study because a previous food consumption study indicated that people in this district were at risk of anaemia and micronutrient deficiency due to a low consumption of zinc-rich foods. Studies indicate a history of chronic malnutrition and anaemia in Peru (Zavaleta et al., 2005, López de Romaña et al., 2005), and the percentage of anaemia in Peruvian children in the region of Lima has been reported to be 23.6 % (INEI, 2013). This study was conducted during the winter season in Peru, and children who participated in this study following the invitation process accounted for approximately 20% of the initial sample; they were aged between 36-48 months old, specifically a sample of $n = 184$ Peruvian children (97 control and 87 study group).

The 2 groups of children were evaluated for anthropometry (growth), clinical measurements (anaemia, morbidity), psychological tests (cognitive function and socio-emotional development), nutritional assessment (zinc intake) and biochemical analysis (zinc status). The 2 groups were identified as “Iron (Fe)” and “multi-micronutrients (MMN)” group, from the initial study. Group allocation remained blinded to the researcher throughout the data collection and group analysis (the protocol can be found in Appendix B30).

6.5 STUDY METHODOLOGY

In this section we describe the methodology employed in the empirical study, and the reasons for the choices made.

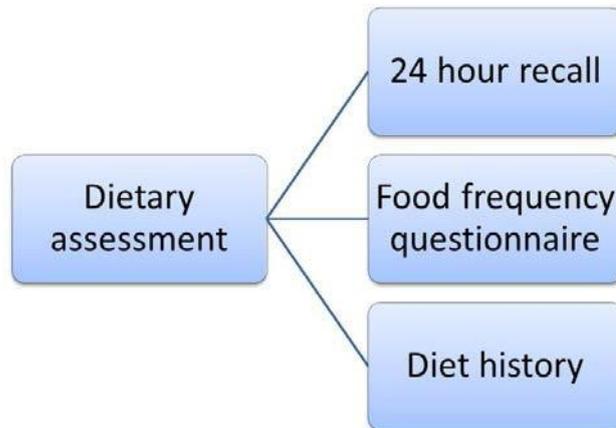
6.5.1 Anthropometry

Anthropometric measurements (WHO, 1995) are widely used to measure the nutritional status of individuals or populations and can help to identify problems of under nutrition or over nutrition (Kennedy et al., 2006). Weight-for-age, weight-for-height, and weight-for-age Z-scores below -2.00 SD of the WHO growth reference (WHO, 2006b, de Onis et al., 2007) are usually used to define stunting, wasting and underweight, respectively. For example, stunting and thinness are defined as height under 2 standard deviations (SD) or less than the 3rd centile of the WHO growth standards, respectively (Secker et al., 2010). Body weight is one of the most important measurements in nutritional assessment (Lee and Nieman, 2013). Measurement of mid-upper arm circumference (MUAC) is commonly used to identify children at risk of malnutrition. For these reasons anthropometric measurements of growth (weight, height, and middle upper arm circumference) were undertaken to monitor child health compared with International child growth standards (WHO, 2014) and to assess the long term impact of multiple micronutrient supplementation in child development. Further details of the anthropometric methods are provided in chapter 7, section 7.1.1.

6.5.2 Dietary Assessment

Children's nutrition was determined by the 24-hour recall method, food frequency questionnaire (FFQ) and diet history. The objective of this methodology was to provide a general habitual intake of Peruvian's children diet, to capture children's diet of common local foods with special emphasis on zinc rich foods and the frequency of foods consumption per child. The parent or the guardian was interviewed for this purpose as they can be reliable reporters of their children's diet; hence the ability of parents to accurately recall their children's food intake was vital (Livingstone and Robson, 2000). The 24 hour recall and FFQ questionnaire captured habitual intake by asking questions about specific list of food items for each food group. The diet history dietary method was used to obtain information of child's food consumption on a weekday and on a weekend.

Figure 6.9 - Methods of dietary assessment in pre-school children



The FFQ and diet history were applied to the full sample, whereas the 24 hour recall was applied to a group of 40 children. Only 40 children were evaluated for the 24-hour recall because this test is time consuming to perform (Gibson and Ferguson, 2008) and 40 children was sufficient to obtain the main characteristics from their diet. Because the 24 hour recall was applied to a small sample of children, the majority of the children's diet was assessed using the food frequency questionnaire. One advantage of the 24-hour recall is that literacy of the respondent is not required when this tool is administered by the interviewer. The weaknesses of this dietary assessment method are how the portion sizes are estimated, the potential bias in recording of what is considered good or bad foods, that it relies on memory (Wrieden, 2003) and that there are inherent errors (Beaton et al., 1979).

The FFQ gave us the dietary patterns and the frequency of the food consumption of local foods, and this important tool has been shown to be useful in epidemiological studies in assessing pre-schoolers dietary intakes over extended periods (Treiber et al., 1990a). FFQ is the most commonly used dietary assessment technique used in nutritional epidemiology, and is designed to assess habitual food and nutrient intake. FFQ may be useful in epidemiological studies of preschool children's intakes over extended periods, and in the assessment of daily variations in macronutrient intakes (Treiber et al., 1990b). The major advantages of the food frequency method are cost and representativeness, and for instance, the advantage of a short FFQ is that it will produce lower demands on the interviewer and/or respondent (Samman et al., 2009), however the FFQ is not without problems, the order of the listing is arbitrary and may influence responses (Barrett-Connor, 1991), also FFQ tend to overestimate total energy intake (Serdula et al., 2001).

For comparison of nutrient values, the Peruvian food composition database (INS, 2009), the energy requirements for the Peruvian population prepared by the Ministry of Health (MS, 2012), the joint report by the FAO/WHO/UNU (2001) for energy and protein requirement, and the UK DRVs (HN, 2011, SACN, 2013) were referenced and used. The 24 hour recall analysis was performed using the dietary analysis programme WinDiets version 2008. Further details of the dietary assessment methods are provided in chapter 7 section 7.2.1.

6.5.3 Cognitive Tests

Psychological tests were determined after reviewing the literature on psychological tests in both young and old children. Discussion meetings in Peru and the UK with the psychologists at IIN and at UCLAN took place in order to decide which psychological tests to employ for the assessment in cognition in Peruvian pre-school children.

Cognitive Tests literature review

The Cognitive Abilities Test (CAT)

CAT is the most common test used in the UK, for numerical, non-verbal and verbal abilities for children aged 7- 17 years old (CAT, 2011).

WPPSI

The Wechsler Preschool and Primary scale of intelligence (WPPSI) was developed in 1967 by Dr David Wechsler, (Kaufman, 2000). Wechsler believed that Intelligence Quotient (IQ) tests offered a way to peer into an individual's personality. His major contribution to the field of cognitive assessment was the inclusion of the verbal and the executive/performance scales. Children are assessed for a variety of reasons, and thus the Wechsler Preschool & Primary Scale of Intelligence Revised (WPPSI-R) may be applied in different situations. Although there are various measurements of children's intelligence, the Wechsler test remains by far the most popular (Daniel, 1997).

WPPSI-R

The WPPSI-R is a measure of cognitive function of children ranging from ages of 2 year old and 11 months to 7 year old and 3 months of age, and is administered by a licensed psychologist as part of a neuro-psychological evaluation. The WPPSI-R comprises the verbal and executive/performance scales, with each scale comprised of 5

subtests plus one optional subtest. The verbal scale includes information, comprehension, arithmetic, vocabulary, similarities and the optional test: sentence. The executive/performance scale comprises the following tests: object assembly, block design, mazes, picture completion, geometric design and the optional test animal pegs. (Kaufman, 2000). The verbal comprehension and the perceptual organization of the WPPSI-R test are shown below.

Table 6.1 – WPPSI-R

Test	Factor	Subtest
WPPSI-R	Verbal comprehension(VC)	Information
		Similarities
		Vocabulary
		Comprehension
		Arithmetic
		Sentences
		Perceptual organization (PO)
	Object Assembly	
	Picture Completion	
	Block Design	
	Mazes	
	Animal pegs	
	Geometric designs	

Verbal scale

The information subtest, the child must either point to a picture or verbally answer brief oral questions about commonplace objects and events; in the similarities subtest the child chooses which pictured objects share a common feature, or child completes a sentence that contains a verbal analogy; in the vocabulary subtest the child names pictured items and provides verbal definitions of words; in the comprehension subtest, the child verbally responds to questions about consequences of events; in the arithmetic subtest the child demonstrates the ability to count and solve more complex quantitative problems; and in the sentences subtest, the child repeats verbatim a sentence that is read aloud.

Executive/Performance scale

For the object assembly subtest, the child is required to fit puzzle pieces together to form a meaningful whole; in the picture completion, the child identifies what is missing from pictures of common objects; on the block design subtest, the child reproduce patterns from flat red-and white colour cubes; during the mazes subtest, the child solves paper-and pencil mazes of increasing difficulty; for the animal peg, the child places pegs of correct colours in the holes below a series of pictured animals; and for the geometric design subtest, in the first part of the task, the child must look at the design and point to a matching design from an array of four, in the second part, the child copies a drawing of a geometric figure (Kaufman, 2000).

WISC-IV

The Wechsler Intelligence Scale IV (WISC-IV) is designed for children aged 6 –16 year-old, it is administered between 65 and 80 minutes, and has 4 main indexes; verbal comprehension index (VCI) measures verbal concept formation, perceptual reasoning index (PRI) measures non-verbal and fluid reasoning, working memory index (WMI) assesses children's ability to memorize new information, concentrate and manipulate this information to produce some result, processing speed index (PSI) assess the ability to focus attention and quickly scan (Kaur, 2011).

Other tests

Other tests are the Universal Nonverbal intelligence test (UNIT) for intellectual functioning and the Movement Assessment Battery for Children (MABC) for motor functioning, described in the Nepalese study of 676 children aged 7-9 years old (Christian et al., 2010).

Simple tests: Day/Night

The Stroop colour test is a widely used test that was created by Ridley Stroop in 1935 (Gerstadt et al., 1994), designed to evaluate cognitive flexibility and processing speed, as well as execute functions. This test requires holding two rules in mind and exercising inhibitory control. The subject must say “night” when shown a white-sun card and say “day” when shown a black-moon card (Diamond et al., 1997).

Simple tests: 9 Boxes

The 9-box test is a memory task where the subject tries to open all boxes without repeating a choice. The subject must be able to remember the colour or shape of the box (Diamond et al., 1997).

Simple Tests: Theory of mind

This test is designed to evaluate the understanding of the change of location (Perner and Lang, 1999). “The false-belief task” involves the unexpected transfer of an object, so that the protagonist then has a false belief about the location of that object.

Theory of mind or “mentalizing” is the cluster of abilities which are necessary to understand the mental processes of others. The location of peak activations during tasks, where subjects think about their own or others' mental states, are the medial prefrontal region of the brain, particularly the paracingulate cortex (Walter et al., 2004), the superior temporal sulci and the temporal poles bilaterally (Shallice, 2001, Gallagher and Frith, 2003).

BITSEA TEST

The Brief Infant-Toddler Social and Emotional Assessment (BITSEA) test was designed as a brief, time-efficient screening tool and comprises 42 problem and competence items from the ITSEA. The BITSEA is intended as a first-stage screen for identifying children who, according to parental reports, exhibit elevated levels of problem behaviours or low levels of competence, and may benefit from additional follow-up assessment to determine whether clinically significant difficulties are present. The BITSEA comprises a 31-item Problem Scale assessing problems such as aggression, defiance, over activity, negative emotionality, anxiety, and withdrawal, and an 11-item Competence scale measures empathy, prosocial behaviour, and compliance (Briggs-Gowan and Carter, 2007).

BITSEA is a valid tool used as a screening method for identifying social-emotional and behavioural problems and/or delays or deficits in social-emotional competence, including activity, anxiety, and emotionality, in children aged from 12 months to 35 months. BITSEA responses range from 0–2, the response options being: 0 = Not true/rarely, 1 = Somewhat true/Sometimes and 2 = Very True/Often (Haapsamo et al.,

2009). BITSEA is a shorter measure of ITSEA derived from the full scale of 195 items, consists of four domains: externalizing, internalizing, dysregulation, and competencies; and three indices: maladaptive, atypical behaviour, and social relatedness. The questionnaire or interview is completed by a parent or adult caregiver, the items are rated on a three-point scale: “not true/rarely,” “somewhat true/sometimes,” or “very true/often (Sosna and Mastergeorge, 2005). The BITSEA version have a parent form and a child care provider form, that can be completed at home or in a clinic, BITSEA is intended to identify children who may need further, more comprehensive evaluation (McCabe and Altamura, 2011).

Research questions for psychological tests

The psychological tests chosen for this study focussed around answering whether the type of supplement received during infancy have an impact on the following parameters in the pre-school children:

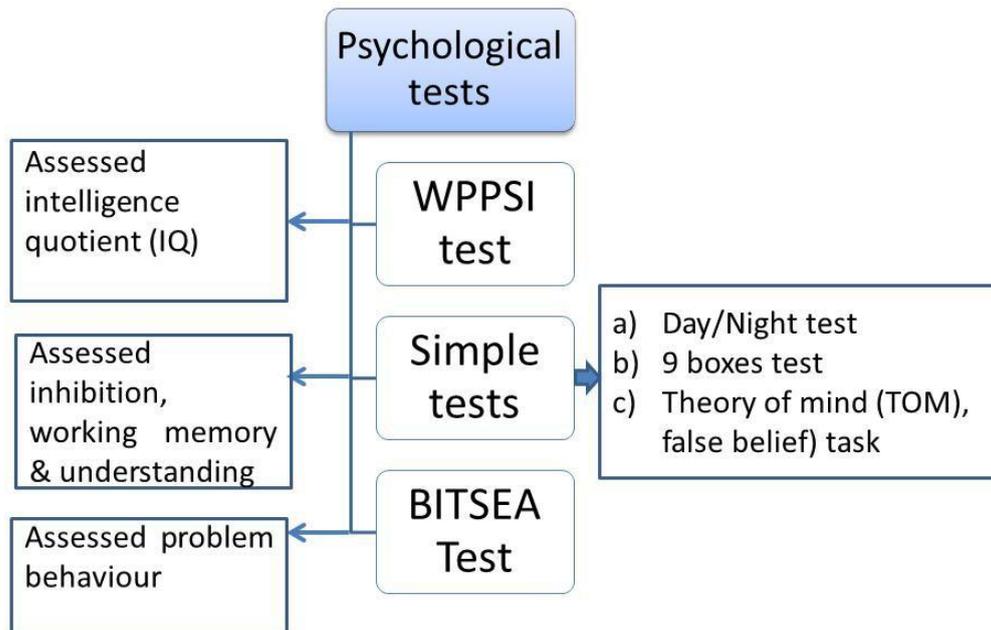
- 1) inhibition
- 2) working memory
- 3) theory of mind development
- 4) IQ
- 5) behaviour

After reviewing the type of test suitable for the age group and population, tests were agreed with psychologists in both teams: IIN and UCLAN.

Chosen psychological tests

The psychological tests chosen to address these questions are shown in the following figure.

Figure 6.10 - Psychological Tests performed



In general, cognitive tests chosen were considered appropriate to the target population; some of them have been used in previous studies in Peru.

WPPSI had been previously used in Peruvian children to measure children's IQ and this has been validated (Caulfield et al., 2011, Caulfield et al., 2010, Colombo et al., 2014), Additionally a Spanish version of the WPPSI test was available at the IIN nutritional research institute, consequently it was selected for use in our study.

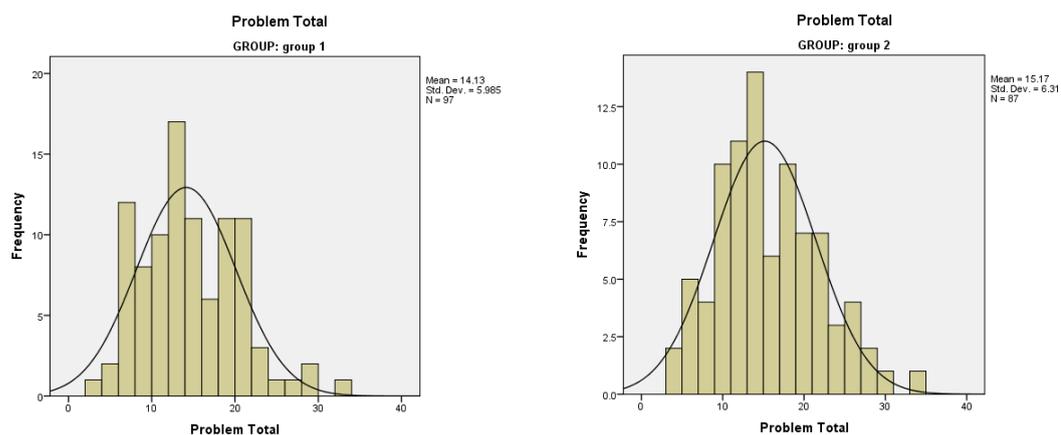
The day/night test was applied before in a pilot study by the IIN. The tests of working memory, theory of mind and BITSEA were conducted in a new pilot study (n=13) at the Villa El Salvador Clinic in June-July 2012, prior to taking the test measurement on the children of this study. Therefore, validity (how well a test measures what it intends to measure), and reliability (the consistency of a measurement) of the psychological tests were tested prior to the current study (Carmines and Zeller, 1979).

A series of simple psychological tests (day/night, 9 boxes, theory of mind) were chosen to measure how the children used their cognitive skills. The following dimensions of cognitive functioning were measured: inhibitory control (day/night)(Gerstadt et al., 1994), working memory (9 boxes), attention. The Day-Night Stroop test was selected to measure inhibitory control and was adapted from Gerstadt et al. (1994), Simpson and Riggs (2005) and Carlson (2005). The 9 boxes test was adapted from Diamond et al.

(1997) and (Wiebe et al., 2011), and was a memory task where the children had to open the boxes without repeating a choice. Diamond et al. (1997) used 3 and 6 boxes, so in our study, we decided that 3 boxes would initially be used as a trial and this would be followed by the 9 boxes test. Given its association with cognitive skills (Hughes, 2011), the theory of mind, that has been validated for use with 17-month old infants (Southgate et al., 2010), was also included. This test would be adapted from (Perner and Lang, 1999) and the false belief task was selected for the development of theory of mind. These tests were also selected because they are simple to conduct and of short duration.

To measure socio-emotional development the Brief Infant Toddler Social Emotional Assessment (BITSEA) test was administered (Briggs-Gowan and Carter, 2006). Figure 6.11 serves as normative data, showing the histogram of problem total scores for the Iron group (group 1) and the MMN group (group 2).

Figure 6.11 - Histograms of problem total scores



The Brief Infant-Toddler Social and Emotional Assessment (BITSEA) test was selected as a screening tool to determine whether a child required a more in-depth assessment of the social-emotional domain. The measure generates a total problem score and a total competence score that each can be compared to cut off points. The reason this questionnaire was selected was because some children fell under the percentile ranking of under 36 months old and the forms were also available in Spanish. (Carter et al., 2003a). Briggs-Gowan et al. (2004) indicate that BITSEA screens for social-emotional/behavioural problems and delays in social-emotional competence. Additionally, BITSEA has been used successfully in mothers, it has been translated into Spanish, and it has been piloted with Hispanic populations as a reliable and valid brief

screener for infant-toddler social-emotional and behavioural problems and delays in competence (Briggs-Gowan et al., 2004).

In terms of overlap between cognitive tests, this was as follows. The subtests from WPPSI: picture completion and animal peg measured attention which was also measured by the day/night test. The WPPSI subtest: similarities measured logical thinking which was also measured by the Theory of mind (false belief task), the WPPSI subtest: sentences measured short term memory which was also measured by the 9 boxes tests working memory.

Table 6.2 shows a summary of the selected tests. Further details of the cognitive methods are provided in chapter 8 section 8.1.

Table 6.2 - Psychological Measurements

Measurements	Test	Duration of test	Reference
<i>In children</i>			
Intelligence quotient/executive function	Wechsler preschool and primary scale of intelligence (WPPSI)	75 min	Wechsler, D. (1967)
Inhibitory control	Day/night Stroop test	5 min	Gerstadt et al., (1994)
Working memory	9 boxes	4-5 min	Wiebe et al., (2011), Diamond and Taylor (1996)
Understanding the representational mind	Theory of mind (false belief task)	3-5 min	Perner, J. & Lang, B. (1999). Perner, J. (1991)
<i>Questionnaire to parents</i>			
Child socio-emotional development	BITSEA	10 min	Briggs-Gowan, Margaret J., et al.(2004)

6.5.4 Overview of the Clinical Assessment and Biochemical Analysis

A clinical assessment of each child was performed in order to monitor the overall health of the children as part of the broader context of this research project. The clinical assessment of a child involves the comparison of measurements from a child with children with similar age. The benchmark used as a reference was the WHO child

growth standards. Referral to a specialist was executed if there were concerns about the child's development (Bellman et al., 2013).

Biochemical analysis was conducted to assess plasma zinc concentrations and haemoglobin levels. The importance of monitoring plasma zinc status in children is to identify population at risk of zinc deficiency and a biochemical indicator of zinc status may be used as a quantitative means of assessing zinc status (de Benoist et al., 2007). A systematic review in 46 supplementation and depletion studies which were mostly in healthy adults (67%) and elderly (9%), few in pregnant and lactating women and children, identified biomarkers of zinc status. This systematic review found that plasma seem to be a good biomarker of zinc status (Lowe et al., 2009).

Regarding clinical diagnosis, the paediatrician worked through a diagnostic protocol, which included the mothers obstetric history, neonatal diagnoses, congenital malformations, cardiovascular, digestive system, haematology, fluid and electrolyte, infections, neurological, ophthalmological, osteo-articular, skin and mucous, respiratory, genito-urinary system, others, adequate health, assessment codes, hospitals. This assessment was used to classify children into the groups presented in , healthy children (measured value in normal range).

1. Anaemia diagnoses was based on the reference of 11.0 g/dL (WHO, 2011)
2. Bronchitis, which is an inflammation of the bronchi (airways), includes the following symptoms cough, pain, fever; bronchitis could be acute, chronic or of a recurrent course, it is caused by a virus, bacteria or allergens (Peiser, 2012). Bronchitis diagnoses were based on the basis of symptoms and child physical examination.

Rhinitis diagnoses were based on the symptoms of sneezing, or a runny or blocked nose when the child did not have a cold or flu at physical examination. Paediatrician used a disposable wooden medical spatula to examine the mouth. The eyes, nose and mouth breathing were also observed. Rhinitis is evident in pre-school children, and diagnosis may be difficult at this age (Peroni, 2003).

HemoCue® is a reliable portable quantitative method widely used for determining Hb concentrations and consists of a portable, battery-operated photometer and a supply of treated disposable cuvettes in which blood is collected. The principle of the HemoCue

technique is based on an optical measuring cuvet of small volume (10 pL) and short light path. Dry reagents are deposited inside the cuvet cavity, then the blood sample and the reagent are mixed spontaneously. The HemoCue then calculates the concentration of Hb in the sample and displays the result (von Schenck et al., 1986, Nkrumah et al., 2011).

Further details of the clinical and biochemical assessment methods are provided in chapter 9 section 9.1.

CHAPTER 7. –

**EMPIRICAL RESEARCH: RESULTS OF
THE NUTRITIONAL ASSESSMENT OF
PERUVIAN CHILDREN**

Chapter 7 - EMPIRICAL RESEARCH: RESULTS OF THE NUTRITIONAL ASSESSMENT OF PERUVIAN CHILDREN

In order to assess physical growth and development, and ascertain whether the children demonstrated poor growth, anthropometric measurements were undertaken. Anthropometric indices are necessary to identify malnourished children (Chen et al., 1980). For instance, weight gain is used as a measure for identifying young children who are failing to thrive (Raynor and Rudolf, 2000). These data are presented in section 7.1, along with data from the original 2010 study which has enabled a longitudinal evaluation of the mean anthropometric characteristics of the participants at an earlier age when they received the micronutrient supplementation to the present time.

The results of the dietary assessment of the children is presented in Section 7.2. Assessment of dietary intake among preschool-aged children is important for nutrition monitoring and for epidemiologic research (Serdula et al., 2001). This study used three methods for dietary analysis: a 24-hour dietary recall (for a sub group), a food frequency questionnaire (FFQ) and diet history (one weekday and a weekend day to gain an overall picture of habitual intake (Livingstone et al., 1992).

The chapter concludes in section 7.3 with a brief discussion of the anthropometric and dietary analyses.

7.1 ANTHROPOMETRY

7.1.1 Methods for Anthropometry

Weight was measured to the nearest 0.05 kg on a digital display scale, which was calibrated with test weights before each weighing session. Weight was expressed in kilograms of body weight. For children unable to stand alone, the difference between the mother's weight and that of the mother and the child, on the digital scale, was recorded. Children wore a minimum of clothing and no shoes.

Height was measured using a stadiometer (conventional wooden height board) to the nearest to the nearest 0.1 centimetre (cm). A right angle headboard was used for reading the measurement and rested on crown of child's head in the Frankfort plane. The child

wore minimal clothing and no shoes, stood with heels together, arms to the side, legs straight, shoulders relaxed and back of the head was against the vertical surface of the stadiometer (Lee and Nieman, 2013), in the majority of cases when the child cooperated.

Measurements of mid-upper arm circumference (MUAC) were conducted in children and measured to the nearest centimetre using a plastic measuring tape in centimetres (cm). The middle of the left upper arm was measured.

Statistical analysis

Independent sample t-test was used to compare groups, minimum, maximum range, mean, standard deviation, and median for main descriptive variables were determined. Comparison of measurements by gender and group was made using the multivariate analysis of variance (MANOVA). The multivariate analysis of variance (MANOVA) was also conducted to assess the indices of SES. Analysis of Variance (ANOVA) was performed to compare the numbers of boys/girls per group.

Figure 7.1 - Anthropometry materials: Portable scale and stadiometer



7.1.2 Anthropometry Data From Original 2010 Study

Descriptive data from the original study are shown in the Table 7.1. These data are relevant for our study as they provide longitudinal information about the children from infancy to the present time.

Table 7.1- Baseline participant characteristics from the original study

Variable	Fe (n=76)			MMN (n=61)		
	$\bar{x} \pm SD$	Min	Max	$\bar{x} \pm SD$	Min	Max
Age (months)	7.53±4.27	1	30	7.56±3.78	6	30
Boys	7.60±4.22	1	30	6.73±1.79	6	30
Girls	7.42±4.39	6	30	7.74±2.97	6	18
Weight (Kg)	8.58±1.36	5.2	13.9	8.60±1.33	6.6	15
Boys	8.81±1.37	5.2	14	8.60±1.05	6.6	15
Girls	8.29±1.32	6.4	13.1	8.37±1.05	6.6	10.8
Length (cm)	68.32±5.16	56	93	67.80±3.49	61	76
Boys	69.09±5.33	56	93	67.47±3.33	61	76
Girls	67.31±4.85	62	89	68.21±3.69	62	75

Total N=137 available data, Min: Minimum, Max: Maximum.

The MANOVA by gender and group showed no significant gender * group effect differences between the Fe and the MMN group, for age ($p=0.340$), weight ($p=0.505$), and height ($p=0.110$) measurements, Wilks' $\lambda=0.968$, $F = 1.050$, $df = (1,132)$, $p = 0.384$.

Table 7.2 - Percentiles from original study

Variable	Fe	MMN
	Percentile	
Weight x Age	10th - 25th	10th - 25th
Length x Age	5th - 10th	10th - 25th
Weight x Length	50th - 75th	75th - 90th

Percentiles were estimated from measurements of weight and length, these were compared with the percentiles of the WHO growth standards based on the LMS method (smooth (L) curve, trends in the mean (M) and coefficient of variation (S)) (WHO, 2006b, Cole, 1990) The WHO growth standard charts are intended to reflect normal child growth in adequate conditions. The WHO recommends cut off values of ± 2 SD which corresponds to the 2.3rd and 97.7th percentile which defines abnormal growth, then the cut off values used by the WHO are the 2nd and the 98th percentile. The WHO percentile cut off values differ from the CDC (Centers for Disease, Control and Prevention) cut off values in that the CDC uses the 5th and the 95th percentile. The percentile cut off values interpretation are as follows: infants and children with a weight for length below the 2nd percentile are classified as low weight for length; children with height for age below the 2nd percentile are classified as children of short stature; and those children with a weight for length over the 98th percentile are classified as children with high weight for length (CDC, 2014c). According with these cut off points, children were on average in the normal range for the measurements of weight and height.

7.1.3 Anthropometric Data From Empirical Study

Table 7.3 - Subject characteristics

Variable	Fe (n=97)			MMN (n=87)			P value
	$\bar{x} \pm SD$	Min.	Max.	$\bar{x} \pm SD$	Min.	Max.	
Age (months)	41.55 \pm 3.16	36.07	47.87	41.40 \pm 3.23	36.03	47.93	0.222
Boys	41.65 \pm 3.13	36.07	47.87	40.99 \pm 3.04	36.03	47.38	
Girls	41.42 \pm 3.23	36.95	47.15	41.92 \pm 3.42	36.36	47.93	
Weight (Kg)	15.49 \pm 2.14	11.5	22.5	15.41 \pm 2.01	12	21.6	0.603
Boys	15.59 \pm 2.02	12.4	21.7	15.71 \pm 1.98	12	21.6	
Girls	15.37 \pm 2.29	11.5	22.5	15.02 \pm 2.02	12.4	19.7	
Height (cm)	97.43 \pm 3.64	90.8	107.5	97.16 \pm 3.94	89	105.2	0.663
Boys	97.9 \pm 3.45	91.3	105.2	97.49 \pm 3.83	91	105.2	
Girls	96.85 \pm 3.81	90.8	107.5	96.74 \pm 4.09	89	104.2	
MUAC (cm)	16.36 \pm 1.31	14	22	16.38 \pm 1.17	14	20	0.474
Boys	16.33 \pm 1.35	14	22	16.47 \pm 1.12	15	20	
Girls	16.41 \pm 1.28	14	20	16.28 \pm 1.24	14	20	

MUAC: middle upper arm circumference.

The MANOVA by gender and group yielded no significant differences between the Fe and the MMN group, for age ($p=0.222$), weight ($p=0.603$), height ($p=0.663$) and MUAC ($p=0.474$) measurements, Wilks' $\lambda= 0.988$, $F = 0.521$, $df = (1,176)$, $p = 0.720$. Weight and height were on average in the normal range: boy 3year old: 14.6 Kg weight, 94.9 cm height; girl 3y: 14.1 Kg weight, 93.9 cm; boy 4y 16.7 Kg weight, 102.9 cm; girl 4 year old 16 Kg, 101 cm height. MUAC under 11.5 cm is defined as acute malnutrition (WHO, 1995). Children were not severely malnourished.

A sub-analysis of weight and height was performed using the following criteria: WHO child growth standards (WHO, 2014), height-for-age, below -2 Z-Score was used to define stunting. The CDC Body Mass Index (BMI) percentiles (a common indicator to assess growth, where a percentile ranking is determined by plotting BMI-for-age) were used to define underweight (less than 5th percentile) (CDC, 2014a). It was found that 2.7% were stunted in the Fe group and 2.3% in the MMN group; and identified underweight children were 5.2% in the Fe group compared to 3.5% in the MMN group. Analyses of these data suggested that children in both groups were no more likely to have stunted growth or be underweight than expected by chance (stunted growth: $X^2 (1, N=183)=0.10$, $p=0.75$; underweight: $X^2 (1, N=183)=0.30$, $p=0.73$).

Percentiles rank the position of a child by indicating what percentage of the reference population a child would equal or exceed. A high percentage would indicate a larger or a taller child, and a low percentage will indicate a smaller or a shorter child. The relevance of the percentiles is because they are common clinical indicators that assess the size and growth patterns of individual children. (CDC, 2014b).

Table 7.4 - Percentiles from pre-school study

Variable	Fe	MMN
	Percentile	Percentile
Weight / Age	50th - 75th	50th - 75th
Height / Age	25th - 50th	25th - 50th
Weight / Height	50th - 75th	50th - 75th

Table 7.5 - Percentage of boys and girls in each group

	Fe (n=97)		MMN (n=87)	
	%	N°	%	N°
Boy	54.6	53	56.3	49
Girl	45.4	44	43.7	38

%, percentage; N°, number

An ANOVA by group showed no significant differences in the percentage of boys and girls between the Fe and the MMN group, $F(1, 182) = 0.052$, $p = 0.820$.

Table 7.6 – Summary of the indices of socio-economic status (SES)

Variable	Fe			MMN			P value
	$\bar{x} \pm SD$	Min	Max	$\bar{x} \pm SD$	Min	Max	
Mother's age (y)	32.45 ± 6.67	18	48	29.36 ± 5.84	20	43	0.001*
Mother's schooling (y)	11.13 ± 3.18	0	16	11.31 ± 2.90	1	16	0.749
Father's schooling (y)	11.42 ± 2.16	1	16	11.87 ± 9.86	0	-	0.749
N° of people living in the house	4.45 ± 2.60	2	19	3.99 ± 1.81	1	11	0.179
N° of rooms in the house	2.16 ± 1.30	1	8	2.08 ± 1.13	1	5	0.431
N° of children (Including child of study)	2.32 ± 1.18	1	6	2.15 ± 1.22	1	6	0.319
Age of the last brother or sister (y)	7.31 ± 5.76	0	23	5.59 ± 5.19	0	20	0.028*

Min, minimum; Max, maximum; n.a, no answer; *significant

Table 7.7 – Details of the indices of SES variables by group

Variable	Fe		MM		P value
	%	N°	%	N°	
Schooling					
Mother's 11 years of schooling	45.4	44	53.5	46	0.749
Father's 11 years of schooling	63.9	62	55.8	48	0.749
Child pre-schooling, attending nursery	63.9	62	67.4	58	0.291
Mother's occupation					
Housewife	58.8	57	59.3	51	0.323
Housewife and working from home	10.3	10	5.8	5	
Housewife and work outside home	10.3	10	5.8	5	
Temporary job	1	1	2.3	2	
Housekeeper	1	1	1.2	1	
Employee in a company	7.2	7	11.6	10	
Student	1	1	1.2	1	
Professional	5.2	5	4.7	4	
Technical professional	1	1	2.3	2	
Self employed	3.1	3	3.5	3	
Labourer	1	1	2.3	2	

Table continued

Variable	Fe		MM		P value
	%	N°	%	N°	
Father's occupation					0.846
Employee in a company	46.4	45	51.2	44	
Student	5.2	5	3.5	3	
Professional	2.1	2	1.2	1	
Technical professional	1	1	2.3	2	
Labourer	2.1	2	7	6	
Self employed	36.1	35	30.2	26	
Military/policeman/soldier/sailor	2.1	2	1.2	1	
Marital status					0.592
Single parent	3.1	3	3.5	3	
Cohabitant	57.7	56	58.1	50	
Married	36.1	35	32.6	28	
Divorced/Separated	3.1	3	5.8	5	
Head of the household					0.236
Father	72.2	70	64	55	
Mother	27.8	27	36	31	
Housing					0.449
Owner	21.6	21	14	12	
Rent	6.2	6	7	6	
Family houseshare	68	66	77.9	67	
Guest	4.1	4	1.2	1	
Predominant material of house floor					0.292
Parquet/wood	3.1	3	3.5	3	
Tiles	16.5	16	22.1	19	
Concrete	78.4	76	74.4	64	
Grit or sand	2.1	2			
Predominant material of house exterior walls					0.869
Brick/concrete	86.6	84	86	74	
Thatch/Clay stone	3.1	3			
Wood	6.2	6	11.6	10	
Predominant material of house ceilings					0.201
Concrete	49.5	48	45.3	39	
Calamine	25.8	25	22.1	19	
Reed	1	1			
Water service					0.926
Installation of water	96.9	94	96.5	83	
Public deposit of water	1	1			
Cistern water	1	1	3.5	3	

Table continued

Variable	Fe		MM		P value
	%	N°	%	N°	
Drainage installation					0.502
Public network inside the house	95.9	93	94.2	81	
Public network outside the house	1	1	1.2	1	
Latrine/silo	2.1	2	3.5	3	
Communal silo	1	1	1.2	1	
Lighting service					0.389
Own installation	93.8	91	95.3	82	
Other public network connection	5.2	5	1.2	1	
Rubbish disposal					0.384
Municipal truck	99	96	100	86	
Other service	1	1			
Own a radio	80.4	78	80.4	78	0.932
Own a TV	100	97	100	86	0.051*
Own a fridge	57.7	56	60.5	52	0.850
Own a phone	88.7	86	89.5	77	0.959
Own a motorcycle/bicycle	25.8	25	25.6	22	0.859
Own a car	9.3	9	10.5	9	0.937

%, percentage; N°, number; *significant

The MANOVA assessing the indices of SES showed that the multivariate effect was not significant by gender, Wilks' $\lambda = .782$, $F(1, 172) = 1.225$, $p = 0.211$, no effect by group, Wilks' $\lambda = .8017$, $F(1, 172) = 1.097$, $p = 0.347$, and significant effect by gender*group interaction, Wilks' $\lambda = .721$, $F(1, 172) = 1.706$, $p = 0.019$. Significant gender and group differences were found in the percentage of head of the household, which were mainly men, and the main source of income of the household (72.2% men v.27.8% women in the Fe group, 64% men v.36% women in the MMN group, $p = 0.036$), and most men were employees of a company (46.2% Fe group v.52.1% in the MMN group), and this shows the patriarchal society which remains in Peru (Gelaye et al., 2010). Significant differences were found in the percentage of schooling years in children (67.4% in the MMN group v.63.9% in the Fe group, $p = 0.007$).

7.2 DIETARY ASSESSMENT

7.2.1 Methods of Dietary assessment

Children's nutrition was determined by the 24-hour recall method, food frequency questionnaire and diet history. The food frequency questionnaire was completed by parents of 182 of the children (Fe group: 43 girls, 53 boys; MMN group: 37 girls, 49 boys). This provided a general overview of the food groups commonly consumed by the children. The diet history interview was conducted by the researcher who interviewed 178-180 of the parents. Completed data for weekday diet history was Fe group: 41 girls, 52 males; MMN group: 37 girls, 48 boys. Completed data for weekend diet history, Fe group: 40 girls, 54 males; MMN group: 36 girls, 48 boys). This provided information about the variation in diet patterns during the week. The 24 hour recall method was used to assess the diet in more detail in a subgroup of 40 children (Fe group: 9 girls, 9 boys; MMN group: 13 girls, 9 boys).

Figure 7.2 - Dietary methods (FFQ, 24 hour recall, Diet history)



24-hour recall analysis

Parents were asked about the foods their children ate the day before at breakfast, mid-morning, at lunch time, mid-afternoon, at dinner time and before going to bed. 24-hour recalls from 40 children were entered into the dietary analysis programme WinDiets version 2008, and from that the nutrient analysis and percentage of energy was determined for macro and micronutrients. Dietary information was collected using probing questions.

Peruvian food was translated and foods that most resembled the Peruvian cuisine were chosen. The food portion sizes handbook was used as reference for estimation of food in grams (FSA, 1988). If no similar food was found then ingredients used in the meal were

searched in the database. The DRVs used were for the UK 2008. The Peruvian DRVs were not immediately available, but were later found to be based on the WHO energy levels (FAO/WHO/UNU, 1985), and the Peruvian food composition tables (INS, 2009) and the energy requirements for the Peruvian population (MS, 2012) were also referred.

Food Frequency Questionnaire (FFQ)

Parents were interviewed about the frequency of foods in a general FFQ of food groups such as biscuits, bread, cereals, fish, meat and meat products, milk, pasta, rice, grains, vegetables, yogurt and miscellaneous food; this was to provide a general frequency of foods. Portion sizes were estimated in this questionnaire. This was followed by a short questionnaire of the foods that were eaten during the last 7 days. Finally a second FFQ by food groups was administered to capture, in detail, more of the dietary patterns. The following scale was used: 1=daily, 2=once per week, 3=twice per week, 4=more than twice per week, 5=rarely/never. FFQ data was transferred to a spreadsheet. When translating foods into Spanish, the most similar foods were selected through an online search of recipes to name the dish accordingly.

Diet History

Diet history information was gathered for one week day and the weekend. Data was then transferred to a spreadsheet. The questions for the diet history interview of weekday food and weekend food were:

1. How many times your child eats during the day?
2. What does your child eat usually during breakfast?
3. What does your child eat during mid-morning?
4. What does your child normally eat at lunch?
5. What does your child normally eat during mid-afternoon?
6. What does your child eat normally at dinner?
7. What does your child normally eat/drink before bed? (optional question).

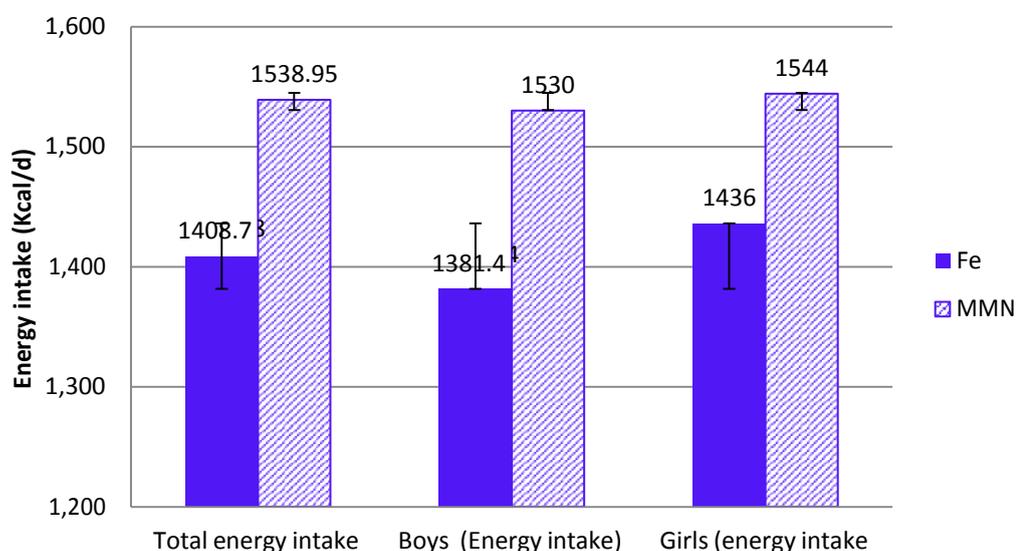
Dietary data information was entered into a database for analysis.

Statistical Analysis

Frequency and percentage of food consumption for the total sample was determined using SPSS software v.20. An independent sample t-test was used to compare groups by energy intake, macronutrient and micronutrient. A two-way Multivariate Analysis of Variance (MANOVA) was used to compare further by gender and group. A two-way Analysis of Variance (ANOVA) was conducted to compare nutrient intake by group and gender.

7.2.2 Results of the 24-hour Recall Diet Analysis

Figure 7.3- Comparison of energy intake by group and gender

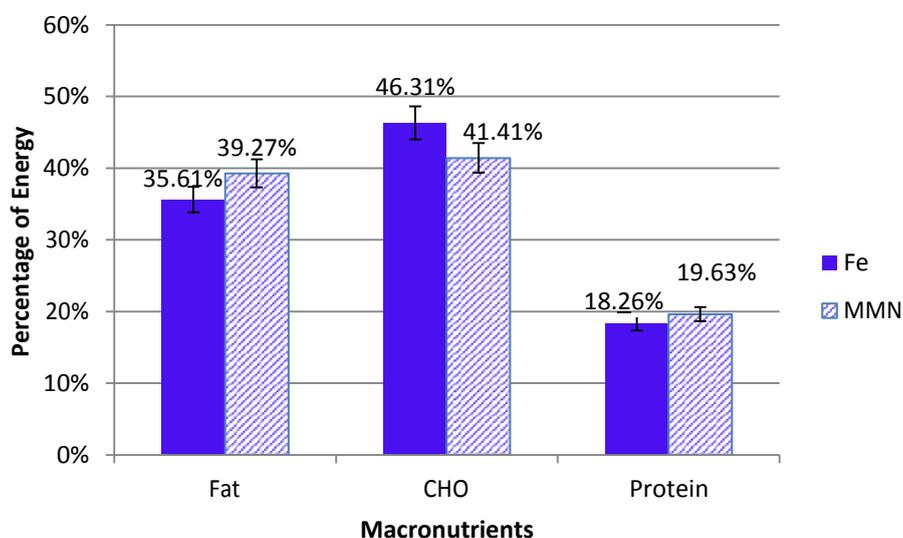


Fe: Iron group; MMN: Multiple micronutrient group

Fe *n*18: boys *n*=9, girls *n*=9; MMN *n*22: boys *n*=9, girls *n*=13

Energy requirements estimated by the 24 hour recall were 1409 Kcal in the Fe group vs 1539 Kcal in the MMN group, $t(38) = 0.783$, $p = 0.438$, which were above the normal levels of 1252 kcal/d (FAO, 2001a) and above the energy requirements for the Peruvian population (MS, 2012), which indicates that the requirements for energy consumption are 1327 Kcal/d in boys and 1252 Kcal/d in girls aged 4 year old. There was also no significant difference by group and gender $F(1,36) = 0.014$, $p = 0.906$.

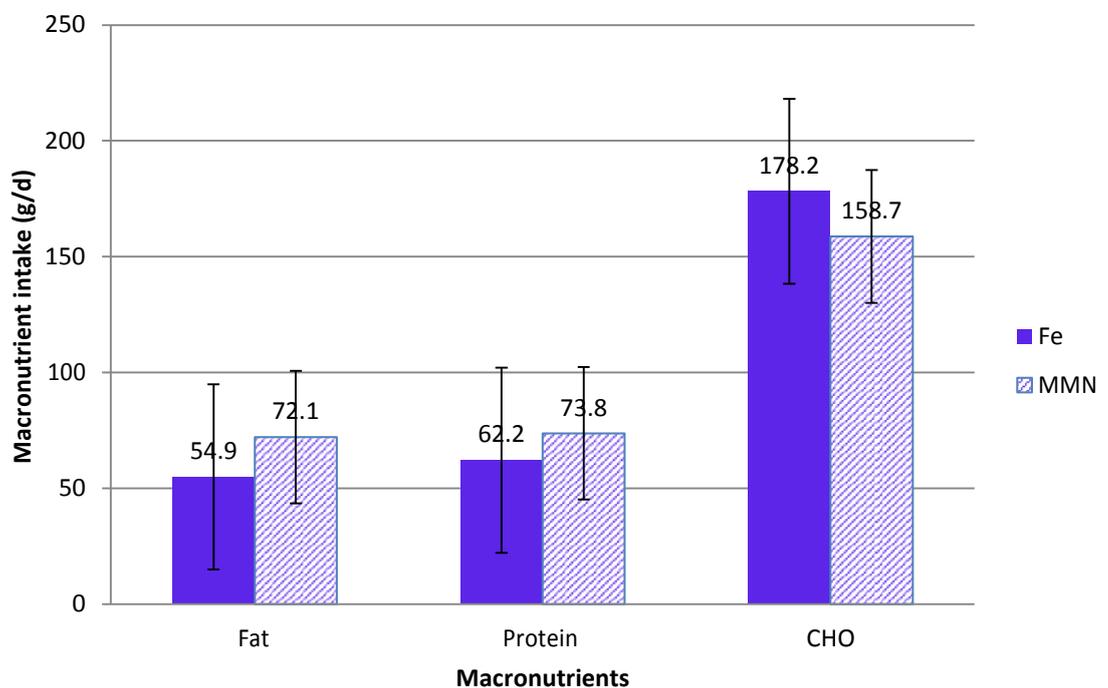
Figure 7.4 - Percentage of energy intake from macronutrients



Fe: Iron group; MMN: Multiple micronutrient group; CHO: carbohydrate

The mean percentage of energy intake from the macronutrients were: 35.61% v 39.27% for fat ($t(38) = .963, p = 0.342$); 46.31% v 41.21% for CHO ($t(38) = 1.302, p = 0.201$), and 18.26% v 19.63% for protein ($t(38) = .624, p = 0.536$), in the iron group v the MMN group. Differences between groups were not significant for these macronutrients. Energy contributions from fat, carbohydrate and protein were within dietary recommendations for the age group.

Figure 7.5 - Macronutrient intake (g/d)



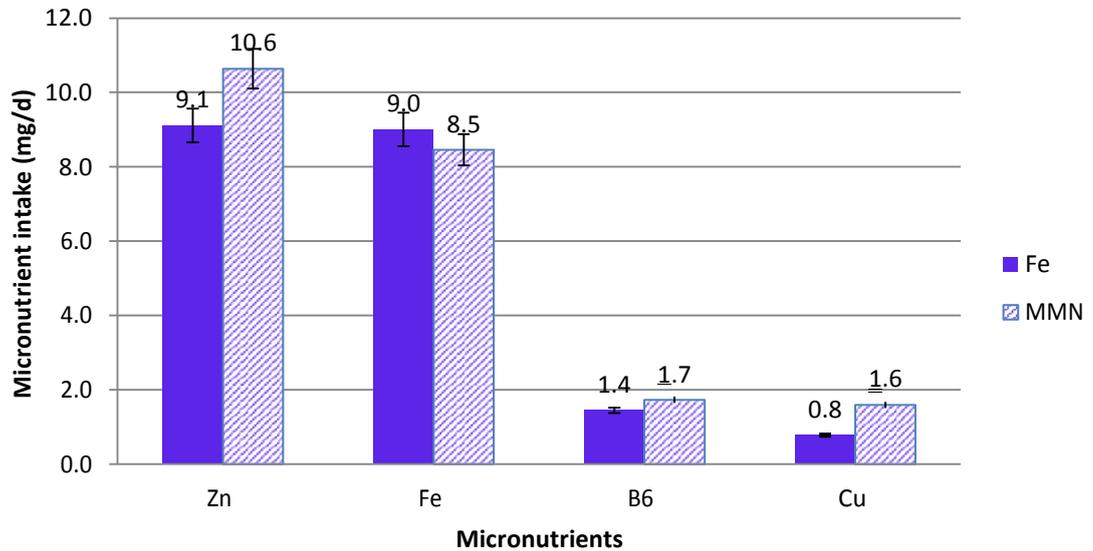
Fe: Iron group; MMN: Multiple micronutrient group; CHO: carbohydrate

This figure shows the estimated amount of macronutrients in grams for both Fe and MMN group which were not significantly different by group, for fat $t(38) = 1.19, p = 0.243$, for protein $t(38) = 1.35, p = 0.184$, for CHO $t(38) = 1.03, p = .31$.

Micronutrient intake/day (mg/d or µg/d)

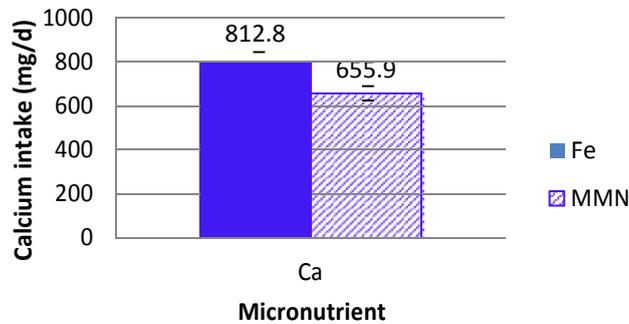
The following figures shows the micronutrient intake per day for main micronutrients involved in cognitive function and those given as a multiple micronutrient supplementation (Zn, Fe, Folic acid, Vitamin C and Vitamin A).

Figure 7.6- Zn, Fe, B6, Cu intake (mg/d)



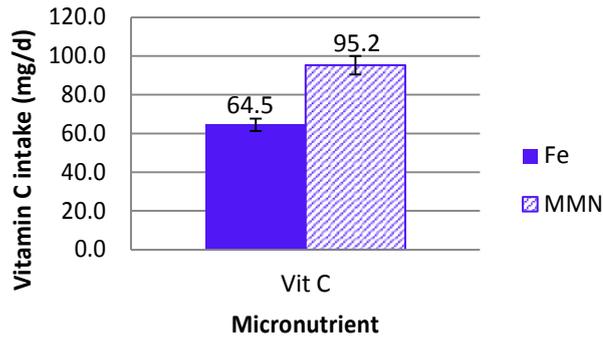
Fe: Iron group; MMN: Multiple micronutrient group, Zn: zinc, Fe: iron, B6: vitamin B6, Cu: copper.

Figure 7.7 - Calcium intake (mg/d)



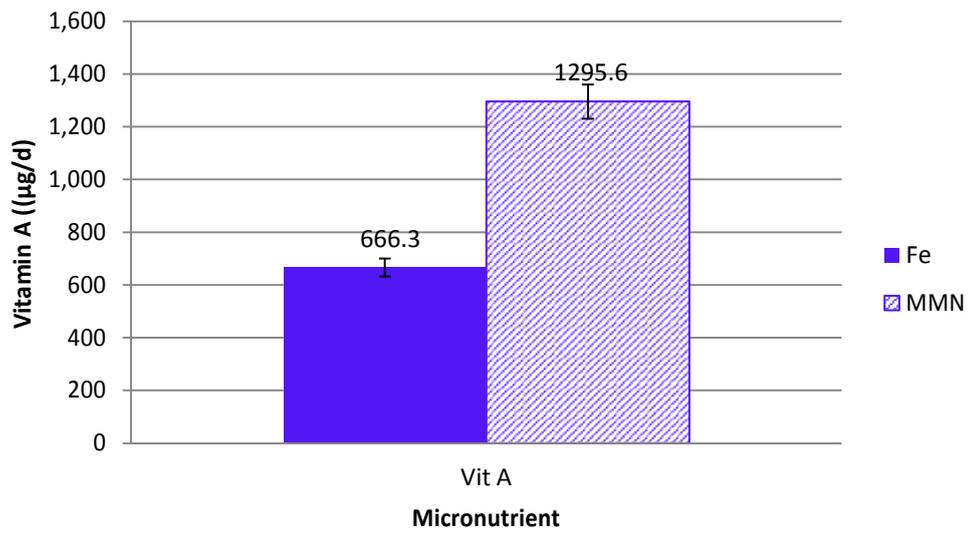
Fe: Iron group; MMN: Multiple micronutrient group, Ca: calcium.

Figure 7.8 - Vitamin C (mg/day)



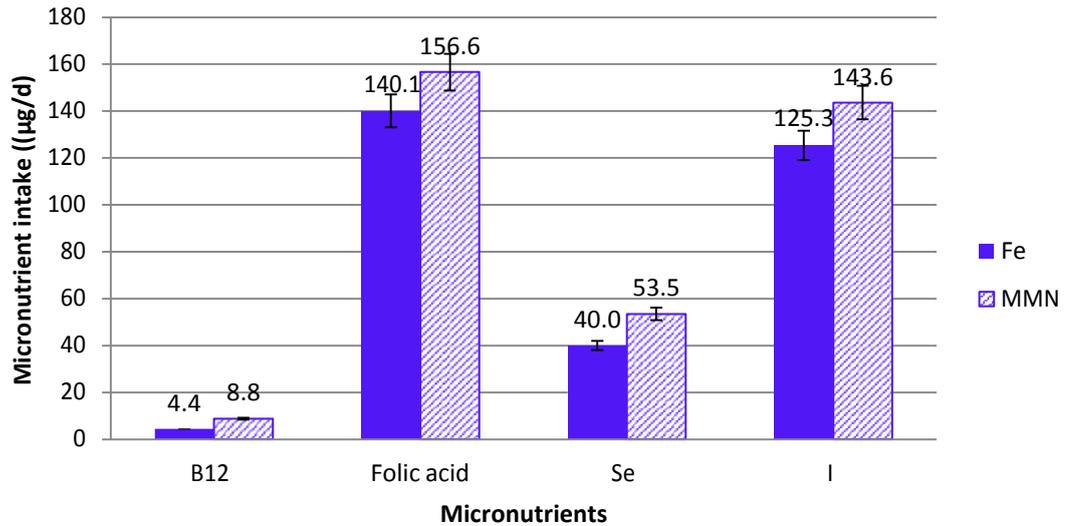
Fe: Iron group; MMN: Multiple micronutrient group, Vit C: Vitamin C.

Figure 7.9- Vitamin A intake ($\mu\text{g}/\text{d}$)



Fe: Iron group; MMN: Multiple micronutrient group, Vit A: Vitamin A.

Figure 7.10- B12, Folic acid, Se, I intake ($\mu\text{g}/\text{d}$)



Fe: Iron group; MMN: Multiple micronutrient group, B12: vitamin B12, folic acid, Se: selenium, I: Iodine.

A two-way MANOVA by gender and group revealed a non-significant multivariate main effect for group, Wilks' $\lambda = .003$, $F(1, 36) = 8.675$, $p = 0.264$; not main effect for gender, Wilks' $\lambda = .202$, $F(1, 36) = .109$, $p = 0.995$; non-significant group-gender interaction, Wilks' $\lambda = .034$, $F(1, 36) = 0.789$, $p = 0.732$. This means that 1 day 24 hour recall revealed no significant differences for micronutrients intake per day.

When comparing energy and nutrients with the Dietary Reference Values, intakes were above recommendations (Table 7.8)

Table 7.8 - Comparison with Reference Nutrient Intake

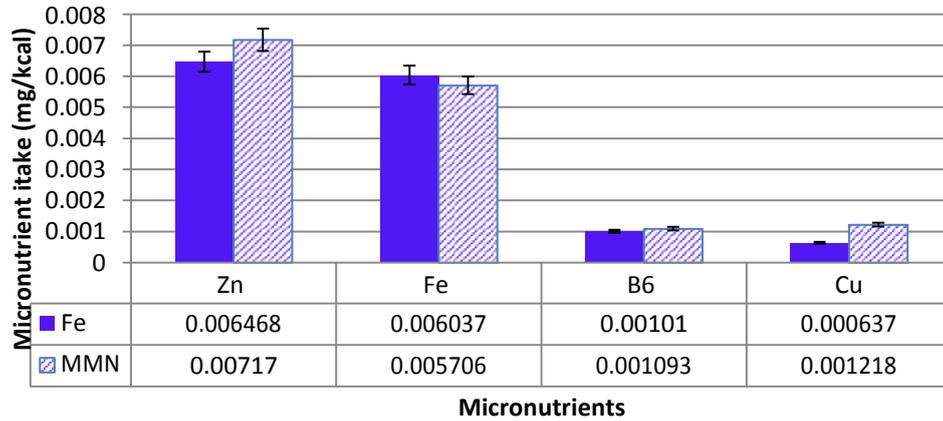
	Mean	Mean	DRVs 4-6y
	Fe	MMN	RNI
Energy(MJ/d)	5.89	6.44	4.9
Fat(Percent of energy)	35.61%	39.27%	less than 35% of food energy
Protein(Percent of energy)	18.26%	19.63%	less than 15% of food energy
CHO (Percent of energy)	46.31%	41.41%	less than 50% of food energy
Zn (mg)	9.11	10.64	6.5
Fe (mg)	9	8.45	6.1
Ca (mg)	812.83	655.86	450
B ₆ (mg)	1.44	1.73	0.9
Vit C (mg)	64.5	95.2	30
B12 (µg)	4.39	8.77	0.8
Folic acid (µg)	140.06	156.64	100
Cu (mg)	0.78	1.59	0.6
Se (µg)	40	53.50	20
I (µg)	125.3	143.64	100
Vit A (µg)	666.3	1295.6	400

Source: Department of Health, L. 1991. Dietary reference values for food energy and nutrients for the United Kingdom. *Committee on Medical Aspects of Food Policy. Report on Health and Social Subjects 41.*

Micronutrient Intake/Energy

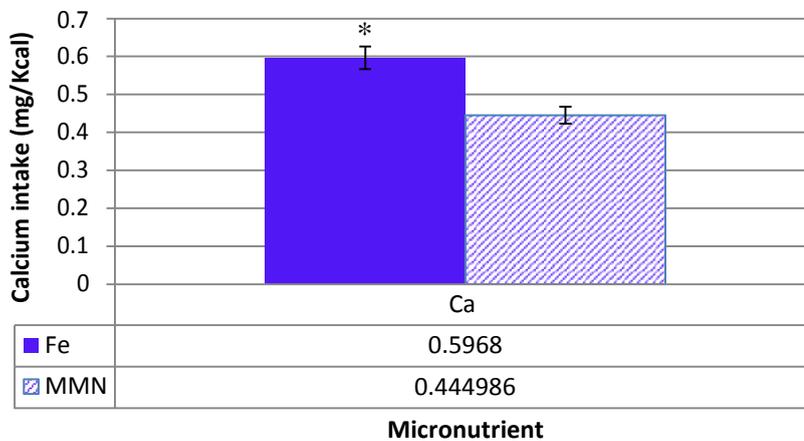
The following figures shows the micronutrient intake over energy for main micronutrients involved in cognition and those given as a supplements (Zn, Fe, Folic acid, Vitamin C and Vitamin A).

Figure 7.11 - Zn, Fe, B6, Cu intake (mg/kcal)



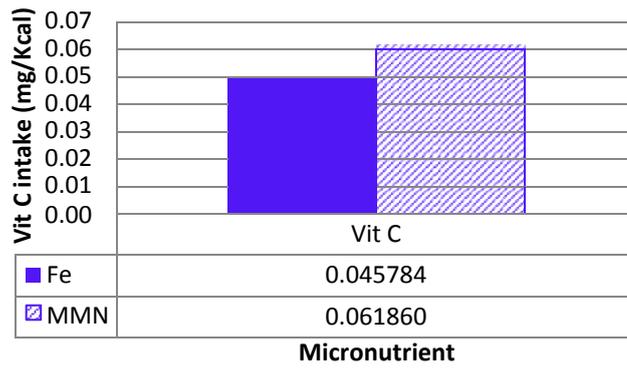
Fe: iron group, MMN: multi-micronutrient group, Zn: zinc, Fe: iron, B6: vitamin B6, Cu: copper.

Figure 7.12 - Calcium (mg/kcal)



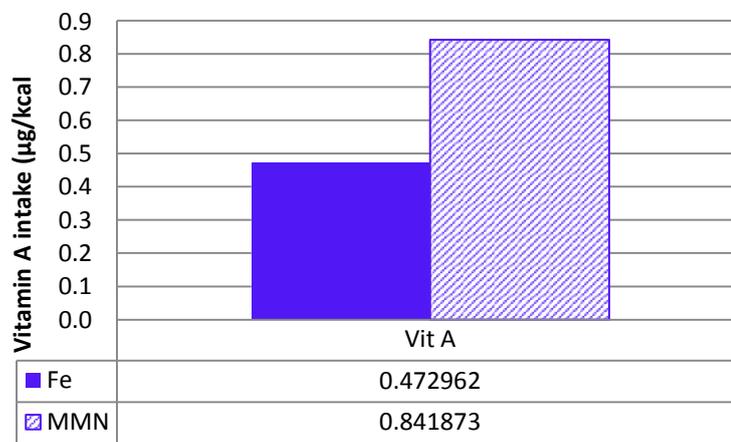
* Significant group differences for Calcium (mg/kcal) ($p=0.027$), Fe: iron group, MMN: multi-micronutrient group, Ca: calcium.

Figure 7.13 - Vitamin C (mg/kcal)



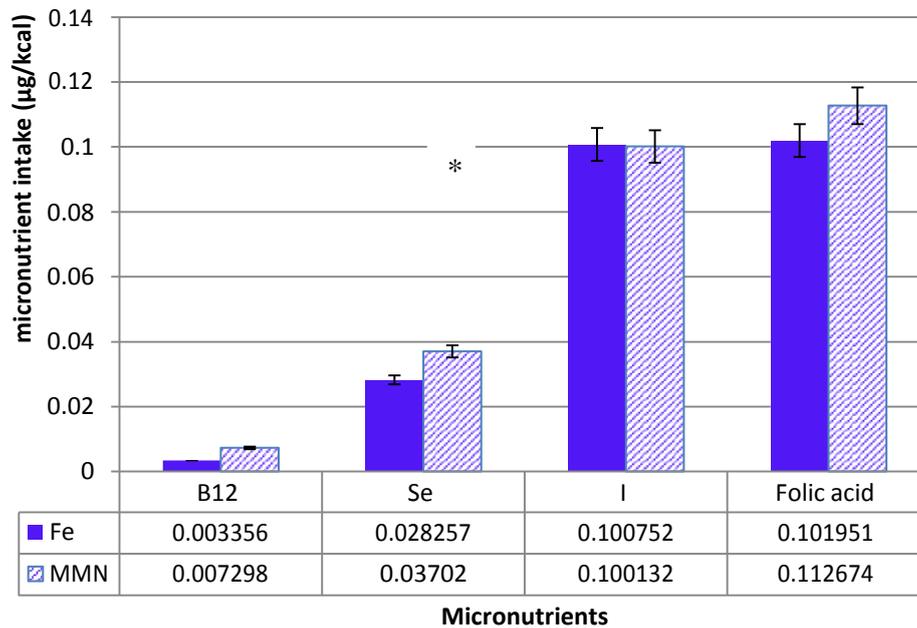
Fe: iron group, MMN: multi-micronutrient group, Vit C: Vitamin C

Figure 7.14 - Vitamin A (µg/kcal)



Fe: iron group, MMN: multi-micronutrient group, Vit A: Vitamin A

Figure 7.15 - B12, Se, I, Folic acid intake ($\mu\text{g}/\text{kcal}$)



* Significant differences by gender and group ($p = 0.030$), Fe: iron group, MMN: multi-micronutrient group, B12: vitamin B12, Se: selenium, I: Iodine, folic acid.

A two-way MANOVA by gender and group revealed a non-significant multivariate main effect for group, Wilks' $\lambda = .654$, $F(1, 36) = 1.427$, $p = 0.222$, except for calcium, Wilks' $\lambda = .654$, $F(1, 36) = 5.315$, $p = 0.027$. Not main effect for gender, Wilks' $\lambda = .738$, $F(1, 36) = .959$, $p = 0.499$, non-significant group-gender interaction, Wilks' $\lambda = .733$, $F(1, 36) = 0.983$, $p = 0.481$, except for selenium, Wilks' $\lambda = .733$, $F(1, 36) = 5.076$, $p = 0.030$. In other words, significant group differences were found for calcium, ($p = 0.027$), significant group-gender interaction for selenium, higher in boys than in girls ($p = 0.030$) and no significant group differences were found for the other micronutrients.

7.2.3 Results of the Food Frequency Questionnaire (FFQ) analyses

The data entries of 182 food frequency questionnaires were completed, giving the following results:

Food frequency questionnaire 1 (FFQ1)

The first food frequency questionnaire and the 7 day questionnaire provided an immediate close-up of the general dietary intake and a synopsis of common foods that children consumed. The frequency of food consumption per group was determined.

Table 7.9 - Frequency of food consumption (FFQ1)

Food products	Frequency of food consumption				
	Daily	Once/wk	Twice/wk	> twice/wk	Rarely
	%	%	%	%	%
Beef	1.1	35.4	30.9	16.6	16.6
Chicken	28.2	5.0	13.8	49.7	3.3
Offal	3.9	33.7	29.8	19.9	12.7
Seafood	1.1	9.9	0.5	0.5	87.9
Milk	95.6		1.1	1.1	2.2
Dairy products	30.4	5.5	11.6	47.5	5.0
Breakfast cereals	35.1	9.5	9.5	36.9	8.9
Legumes	1.1	11.6	44.8	40.9	1.7
Bread	68.1	3.3	4.4	12.6	11.5
Fish	1.1	30	45	21.7	2.2
Rice	94.0		0.5	4.9	0.5
Pasta	14.8	18.7	23.1	41.2	2.2
Peruvian corn	4.5	24.0	23.5	32.4	15.6
Vegetables	55.2	6.1	6.1	29.8	2.8
Fruits	92.8	1.7	1.1	3.3	1.1
Biscuits	13.2	12.6	22	38.5	13.7

The best sources of zinc are seafood, meat, seeds, beans, peas and lentils. Other sources of zinc are milk, dairy foods, bread, and cereal products, such as wheat germ. In our sample, 87.9 % consumed sea food rarely, meat and meat products were consumed regularly, especially poultry, which was more often consumed than red meat. Rice consumption is very common, 94% consumed rice daily, pasta and grains were consumed more than twice per week. There was high consumption of fruits, mainly seasonal fruit.

Food frequency questionnaire 2 (FFQ2)

The second food frequency questionnaire was firstly a short questionnaire of consumption of foods of the past 7 days and this showed that children consumed meat, bread, legumes, and fruits regularly. Secondly, the next questionnaire was more in detail as it provided more information of specific foods by the following food categories: meat and legumes, milk and eggs, cereals derivatives and tubers, vegetables, sugar and fats, fruits and caloric beverages.

Table 7.10 - Frequency of food consumed the past 7 days (FFQ2a)

Food products	Frequency of food consumption					
	Daily	Once/wk	Twice/wk	> twice/wk	Rarely	No reply/unsure
	%	%	%	%	%	%
Beef	1.1	35.8	26.8	12.8	5	18.4
Chicken	19.1	5.1	18	53.9	0.6	3.4
Bread	64	2.8	3.4	14.6	9	6.2
Beans, legumes	1.7	22.5	41	30.9	0.6	3.4
pumpkin	22.1	25	18.6	20.3	4.1	9.9
Eggs	30.3	6.2	13.5	47.8	0.6	1.7
Fruits	49.1	4.8	6.7	29.1	4.2	6.1
Coffee	0.8	6.8	1.5	3	14.4	73.5
Tea	4.5	11.3	10.5	5.3	11.3	57.1

Table 7.11 - Frequency of food consumption (FFQ2b)

Food products	Frequency of food consumption				
	Daily %	Once/wk %	Twice/wk %	> twice/wk %	Rarely %
<i>Meat and legumes</i>					
Beef	0.5	32.4	34.6	17.6	13.7
Chicken	24.0	5	14	53.1	3.9
Seafood		6.9	3.4	2.3	87.4
Fish	1.7	26.3	41.9	26.3	3.9
Pork		16	5.5	13.3	65.2
Sausages		22.5	15.2	20.8	41.6
Offal	3.3	28.3	32.2	21.1	15
Lentils	1.7	38.8	23.6	32	3.9
Lima beans/broad bean	2.3	29.9	21.3	33.9	12.6
<i>Milk and eggs</i>					
Milk	93.3	1.1	0.6	4.4	0.6
Yogurt	32.4	5.5	8.8	49.5	3.8
Cheese	12.6	11.5	14.3	42.9	18.7
Eggs	43.6	1.2	11.0	41.9	
<i>Cereals, derivatives, tubers</i>					
Rice	94.5		0.5	4.9	
Pasta	18.9	22.2	22.8	35	1.1
Bread (white)	65.7	2.8	3.9	9	18.5
Bread (wholemeal)	5.5	5	4.4	6.1	79
Oats	38.9	2.9	16.6	30.9	10.9
Breakfast cereal	18	7.9	14.6	35.4	24.2
Potato	58.5	2.3	6.8	29	3.4
Corn	1.7	16	10.5	15.5	56.4
cooked plantain banana	4.2	6	7.7	11.9	70.2
<i>Vegetables</i>					
Cooked	30.9	3.3	21.5	40.3	3.9
Raw	12.6	7.7	19.2	37.9	22.5
<i>Sugar and fats</i>					
Sugar in beverages	72.3		3.3	19.4	5
Honey	10.6	3.4	7.8	15.1	63.1
Marmalade	3.9	19	19	16.2	41.9
Mayonnaise		20.8	12.8	16.8	48.8
Butter	23.1	18.5	11.6	28.3	18.5
Oil in foods	98.1			1.3	0.6

Meat and legumes,

Meat and meat products consumption was twice per week on average except for pork and sausages. Seafood was rarely consumed whereas pulses were consumed more than twice per week.

Milk and eggs,

93.3% of children consumed milk daily. Yogurt, cheese and eggs were consumed more than twice per week.

Cereals derivatives and tubers,

Rice, potato, bread and oats are consumed on a daily basis by children, while pasta and breakfast cereal are consumed regularly, at least twice per week.

Vegetables,

Cooked vegetables and salads were often consumed more than twice per week and served in stew and soups. Salads were served on the side.

Fruits,

Children consumed a variety of fruits, seasonal and mixed fruit, mainly mandarin, banana, apple, orange, peach, pear. Other fruits were watermelon, papaya, plums, and strawberry. The majority of children consumed a range of fruits and various portions per day, typically 2-3 times per day.

Sugar and fats,

Sugar was added in their beverages very often, honey was rarely consumed, butter spread was added regularly and oil was added to the majority of foods.

Caloric beverages,

Beverages consumed were primarily all types of tea and fruit juice, camomile tea, lemonade, apple juice, orange juice, passion fruit, aniseed tea, herbal tea, fruit juice and hot chocolate.

An additional question was included in the questionnaire about the intake of vitamins; the number of children who consumed vitamins is shown in Table 7.12 .

Table 7.12 - Intake of vitamins

	Fe (n=97)		MMN (n=86)	
	%	N°	%	N°
Intake of vitamins	27.8	27	27.9	24

Fe: Iron group; MMN: Multiple micronutrient group; N°, number; %, percentage

A two-way ANOVA yielded no effect of gender on the additional intake of vitamins, $F(1,178) = 0.608$, $p = 0.437$, no effect of group, $F(1,178) = 0.001$, $p = 0.973$, and no gender-group interaction, $F(1,178) = 0.154$, $p = 0.695$.

Overall, children met dietary recommendations for zinc and majority of nutrients. However, one limitation of the FFQ tend to overestimate energy intake (McPherson et al., 2000).

The FFQ1 and FFQ2a also gathered qualitative information on additional parent's comment of their children's diet with reference to the amount and approximation of food portion size, which supported the local diet pattern.

Some children have soup and main course and others only main course and a cup of milk. If having both meals, generally it was a small portion soup and a medium portion of the main course. Parents were worried that some children refused breakfast, but generally they had milk and mentioned that some children had good appetite, others not. Majority of children consumed plenty of fruits, most drink were herbal tea, fruit juice, occasionally fizzy drink; consumption of quail eggs was popular. In most cases dinner was the same as lunch; occasionally lunch was out of home. Some children rarely had salad. It was the winter season in Peru when data was collected, but most consumed vegetables in soup or stew. Stews and dessert-like rice pudding (mazamorra) were popular. Some children were in private nursery where they received good nutritional food. Some working mothers reported their desire to provide better diet but working shift hours made it difficult for a better care, if late shops were usually closed.

7.2.4 Diet History

Week day diet history

Most children ate a meal and a snack 5 times per day. The table below shows examples of foods given in a week day.

Table 7.13 - Week Day Diet History

Week day dietary pattern	
Breakfast	Oat milk, boiled egg, bottle of milk, hot chocolate, 7 cereals with quinoa, smoothie, bread with cheese or avocado or marmalade or butter, quail eggs, faba beans hot drink, oat porridge with milk, only milk, leftover from previous day, papaya juice, yogurt with cereal.
Mid-morning	Between 2 and 4 fruits: apple, banana, mandarin, passion fruit, boiled eggs, smoothie, bread with butter or marmalade, chicken sandwich, cereal with yogurt, sweet corn with cheese, nursery lunchbox: french fries with egg fried, herbal tea, rice pudding. Other lunchbox: yogurt, cake and mandarin.
Lunch	Beet stew/beans with fish/chicken soup and spaghetti stew/lentils, rice, lettuce salad/ legumes with fried egg, salad/ chicken soup and lentils with salad/pasta with tuna/vegetable soup/
Mid-afternoon	Fruits/bottle of milk and fruit/ cup of milk with bread and butter with marmalade/biscuit and milk/cake and herbal tea/homemade soup/maize porridge or a dessert/ milk with cereal/ small portion of food (same as lunch)/yogurt with cereal.
Dinner	In most cases, dinner was the same as lunch. Cup of milk/fried egg with rice, milk, bread with marmalade/lunch leftover/rice with chicken stew/2 fillet of fried fish with rice/beans with rice/chicken tripe: Chicken stew and herbal tea/lentils with rice and fish/beef stew with rice and potato/chicken soup and pasta/chicken stew with rice/marinade turkey or soup/rice with avocado, vegetable soup with noodles/pasta with tuna/pasta or meat stew.
Eat/drink before going to bed	Cup of milk/ fruit juice/ yogurt

Weekend diet history

The table below shows examples of foods given in a weekend day.

Table 7.14 - Weekend Diet History

Weekend dietary pattern	
Breakfast	Cup of milk/cup of milk and boiled eggs/milk with fried egg/smoothie/milk with cocoa/ leftover e.g bread with pork crackling or hot dog or tuna/ bread with tamales/french fries with egg and oat porridge/yogurt and cake/fruit juice/milk with quinoa/potato with cheese, cereal.
Mid-morning	Fruits/yogurt with cereal/boiled egg/biscuits/rice pudding/scrambled egg with potato/oat porridge.
Lunch	Beans with fried fish/ beef stew and rice/chicken stew and rice/pasta/rice with fried egg/rice with quinoa/rice with stew and broad beans/vegetable soup/soup and main course/ rice with legumes/ rice & offal.
Mid-afternoon	Fruits/biscuits/cup of milk and bread with cheese/small portion from lunch/oat porridge leftover from breakfast/ yogurt/ rice pudding/yogurt with cereal.
Dinner	Same as lunch/lunch leftover/chicken broth/ egg fried rice/ beef stew/chicken puree/milk, rice with chicken/chicken soup and chicken chilli/fried fish with rice/lentils and rice/pasta with chicken/noodle soup/fried chicken with potato and salad.
Eat/drink before going to bed	Bottle/cup of milk/herbal tea/yogurt and cereal/biscuit/beef stew rice/

Diet History qualitative data

From the diet history, most parents reported that children had the same for dinner as they had for lunch, which was a dietary pattern. In some cases, dinner leftovers were given at breakfast also. Children who went to nursery school had a lunch box for the mid-morning.

The following remarks were annotated:

The mother did not cook and ate outside with children. Some mothers gave soup and a main course to their children, but most parents gave only a main course. Some children

slept throughout the afternoon and therefore had nothing until dinner. Some grandparents looked after the child until lunch. Some mothers worked in the evening and left the dinner to be prepared by the husband, so the mother did not know what her child consumed for dinner. Some mothers gave toddler formula “enfagrow” to their children.

7.3 SUMMARY OF ANTHROPOMETRY AND DIETARY ANALYSES

One of the objectives of this study was to evaluate a subsample of 200 children from the cohort study of supplements, with one group supplemented with iron and the other group supplemented with multi-micronutrients including zinc, to measure growth and determine dietary intake patterns and in this manner, to provide evidence of the long-term effect of micronutrient supplementation on growth and also assess nutritional dietary patterns.

Anthropometry

The results from measurement of weight and height revealed that there were no significant differences between the Fe and MMN groups, and that on average the height and weight were in the normal range for the age of the children. Some of the children were below the standard weight and height according to the WHO Child Growth Standards (WHO, 2006a).

The results from the measurement of MUAC showed values in the range 14 to 22, with $\bar{x} \pm SD$ of 16.36 ± 1.31 and 16.38 ± 1.17 for the Fe and MMN groups respectively, revealing that the sample children were considered well nourished (MUAC under 11.5 cm is defined as acute malnutrition (WHO, 1995)), and that there were no significant differences between the groups.

It is recognized that stunting affects 27% of children under 5 year old in low and middle income countries (Thurnham, 2013). In our study the anthropometric data revealed that 2.7% (3 out of 97) were stunted in the Fe group and 2.3% (2 out of 86) in the MMN group; and identified underweight children were 5.2 % (5 out of 97) in the Fe group compared to 3.5% (3 out of 86) in the MMN group. Analyses of these data suggested

that children in both groups were no more likely to have stunted growth or be underweight than we would expect by chance (stunted growth: X^2 (1, N=183)=0.10, $p=0.75$; underweight: X^2 (1, N=183) =0.30, $p=0.73$). Anthropometry data will be discussed further in section 10.2.1.

Dietary Analysis

The results from the 24 hour recall analysis indicated that the children's energy intake were above the reference guidelines for the Peruvian population, and this may indicate a bias toward overestimation of energy intake by the 24 hour recall method (Fisher et al., 2008, Andersen et al., 2004), which may be affected by the parents input of their children's diet, parents may have over reported as they were aware that the diet of their children was assessed. Despite the 24 hour recall method having inherent errors in measuring individual measurement of energy intake, it is still a good tool to assess diet, as it put less burden on the respondents who are able to recall the dietary intake because of the immediacy of the recall period (Coulston and Boushey, 2008, Johnson et al., 1996). In our study, energy and macronutrients were normally distributed and the 24 hour recall showed that most of the energy came from protein and fat which were relatively within the dietary recommendations: Carbohydrate 50%, fat less than 35% and protein 12-15% (FSA, 2006, HN, 2011). 24-hour recalls are commonly used but their limitations for individual assessments due to reduction from daily variation in nutrient intake are well known (Bingham et al., 1997). It is likely that the translation from 24 hour recall to input into the WinDiets database may have added some source of error. Using the WinDiets software had its disadvantages such as the types of comparable food items and the nutritional value of foods in the food composition database which has its limitations namely the natural variability of foods and the coverage of food items (FAO, 2003a). The dietary analyses revealed that overall children met dietary recommendations for zinc and the majority of nutrients above Reference Nutrient Intake (RNI) (Department of Health, 1991) for children aged 4 year old, Zn ((Fe .vs. MMN (9.1 .vs.10.6 mg/d which is above 6.5 mg Zn/d RNI)); Fe ((Fe .vs. MMN (9 .vs.8.5 mg/d which is above 6.1 mg Fe/d RNI)); B6 ((Fe .vs. MMN (1.4 .vs.1.7 mg/d which is above 0.9 mg B₆/d RNI)); Cu ((Fe .vs. MMN (0.8 .vs.1.6 mg/d which is above 0.6 mg Cu/d RNI)); Ca ((Fe .vs. MMN (812.8 .vs.655.9 mg/d which is above 450 mg Ca/d RNI)); Vitamin C ((Fe .vs. MMN (64.5.vs.95.2 mg/d which is

above 30mg VitC/d RNI)); Vitamin A ((Fe .vs. MMN (663.3 .vs. 1295.6 µg/d which is above 400 µg VitA/d RNI)); and showed a good consumption of food sources of zinc from beef, chicken, beans, dairy products and fish, a daily intake of rice, and twice per week of pasta consumption. One limitation of the FFQ is that mothers tend overestimate their children's intake of energy, proteins and lipids (Olinto et al., 1995).

The dietary assessment will be discussed further in section 10.2.2.

CHAPTER 8. –

EMPIRICAL RESEARCH: RESULTS OF THE PSYCHOLOGICAL TESTS

Chapter 8 - EMPIRICAL RESEARCH: RESULTS OF THE PSYCHOLOGICAL TESTS

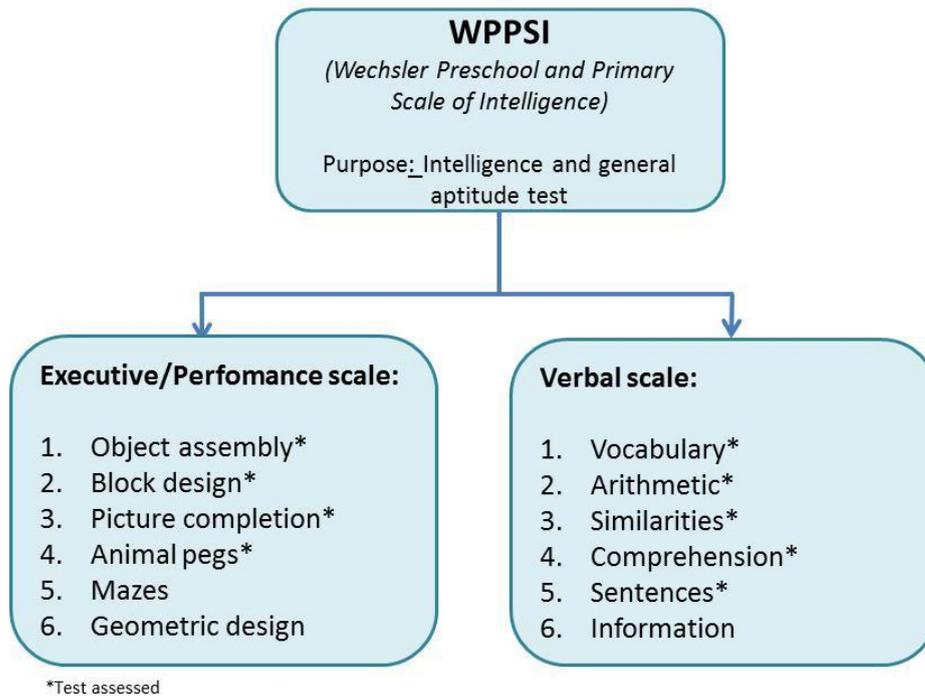
The psychological tests were performed to explore the effect of supplementation on the following aspects of the children's cognitive development: inhibition, working memory, theory of mind development, IQ, and behaviour. This chapter describes the methodology of how the tests were performed and results obtained for the range of psychological tests applied to the sample (WPPSI tests aimed at assessing IQ, simple tests focussing on inhibition, working memory and understanding, and BITSEA tests assessing behaviour). A summary of the results of all psychological tests will be provided at the end of this chapter.

8.1 METHODOLOGY EMPLOYED FOR COGNITIVE TESTS

8.1.1 WPPSI

The Wechsler Preschool and Primary Scale of Intelligence-Revised (WPPSI-R) is a battery of tests applied to 3-7 year old to assess intellectual functioning. The WPPSI-R has two parts, the verbal scale and the executive scale (this is also referred to as the Performance Scale in WPPSI documentation but we will use Executive Scale here). The verbal scale measures language expression, comprehension, listening and the ability to apply these skills to solving problems. The executive scale which assesses nonverbal problem solving, perceptual organisation, speed, and visual-motor proficiency. Each scale has several subtests (Kaufman, 2000) that are illustrated in Figure 8.1.

Figure 8.1 - WPPSI scales



Wechsler Preschool and Primary Scale of Intelligence-Revised (WPPSI-R) subtests were chosen to measure variables of interest such as memory, and attention. Selected subtests were administered for a prorated overall IQ score. 4 subtests in the executive scale were used in the current study: object assembly, block design, picture completion and animal pegs, and 5 subtests in the verbal scale: comprehension, vocabulary, similarities, arithmetic, and sentences. Total executive and verbal scale are shown in Table 8.1. WPPSI Intelligent Quotient (IQ) scores for executive and verbal scale were also determined. In consultation with psychologists from both UCLAN and IIN teams, a table of the WPPSI subtests was created and based on that and, after discussion, the relevant subtests were chosen.

Table 8.1 - WPPSI Executive and verbal assessment

Executive Scale	Assess	Maximum Score
1.Object assembly or Puzzle	Perceptual organization, inductive thinking	32
2.Block design	Ability to abstract conceptualization, analysis and synthesis, spatial visualization	42
3.Mazes	Skill in planning, forecasting, visual motor control, visual motor organization	26
4.Picture completion	Attention to detail, visual perception, ability to distinguish the essential details of the non-essential	28
5.Geometric design	Visuomotor perception, organization and coordination	7 (recognition items) 57 (drawing items) =64
6.Animal pegs	Attention, awareness of purpose, focus, digital and manual dexterity, learning ability	70
Verbal Scale	Assess	
1.Information	Verbal comprehension, range of knowledge, long-term memory involves richness of early environmental	27
2.Vocabulary	Language development, concept formation, learning ability, expressive ability, verbal fluency, word knowledge	47
3.Arithmetic	Knowledge of precalculus, quantitative skills, numerical reasoning ability, attention, concentration	23
4.Similarities	Reasoning by analogy, logical thinking, associative thinking skills	28
5.Comprehension	Social judgment, common sense, practical use of knowledge and judgment in social situations, knowledge of conventional standards of conduct	30
6.Sentences	Short-term memory, mechanical memory, immediate auditory memory, attention, concentration, sequence hearing	37

WPPSI Scoring procedure and Percentile Ranks

The raw score for each WPPSI subtest was converted to a scaled score with a mean of 10 and standard deviation of 3, using the child's chronological age and the respective table from the WPPSI appendix's manual.

In the case of the WPPSI-R IQ values, these are standardised (deviation) IQs with a mean of 100 and standard deviation of 15. To obtain the IQ, the scaled scores on the verbal subtest were then summed and converted to a verbal Intelligent Quotient (IQ), the scaled scores on the performance subtests were summed and converted to a performance IQ, and the scaled scores of all tests were summed and converted to a full IQ. The 3 WPPSI-R IQs are deviation IQs.

The WPPSI tables are the result of standardized data from the WPPSI-R standardization during 1987 to 1989. The sample comprised 1700 American children aged 3 to 7 years old. The percentage of children in the standardization sample in each of the 7 full scale IQ is shown in Table 8.2. It is important to note that the distribution of WPPSI-R IQs is very similar to what is expected in a normal IQ distribution. The classification ratings used to classify the IQ according to the score is shown below.

Table 8.2 - Classification of WPPSI-R Deviation IQ categories and percentage of standardization sample falling in each category

IQ Standard Score Range	Percentile Rank Range	Wechsler Classification	Percentage in standardization sample
130 and above	98 to 99.99	Very superior	2.7
120 to 129	91 to 97	Superior	6.5
110 to 119	75-90	High Average	17.3
90 to 109	25-73	Average	49.4
80 to 89	9 to 23	Low Average	15.7
70 to 79	2 to 8	Borderline	6.4
69 and below	.01 to 2	Intellectual deficient or Extremely Low	2

IQ: Intelligent Quotient. *WPPSI-R use a classification of intellectual deficient

Adapted from Wechsler tests. Percentile ranks and classification ratings (Wechsler, 2014, Aiken, 2004)

WPPSI Materials

The WPPSI materials included the manual, 6 object assembly puzzles (Figure 8.3 - teddy bear, rectangle, car, face, flowers, dog), the block design box (Figure 8.4 - 14 red and white, flat plastic blocks), the animal pegs box with board (Figure 8.4 - 7 blue, 7 white, 6 yellow, and 5 black wooden cylinders), the picture completion book (Figure 8.5), the information, arithmetic (Figure 8.6), vocabulary, similarities book (Figure 8.5) and a stopwatch.

Figure 8.2 - WPPSI materials



Figure 8.3 - WPPSI Object assembly subtest



Figure 8.4 - WPPSI Block design and animal peg subtest



Block design subtest

Animal peg subtest

Figure 8.5 - WPPSI Similarities subtest

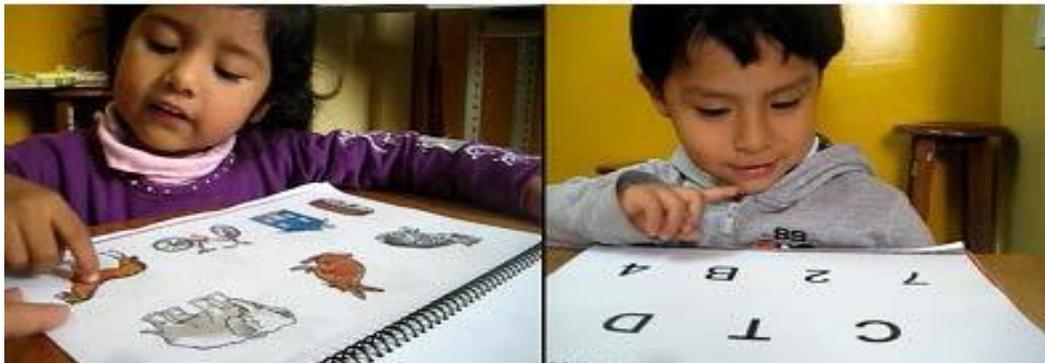


Figure 8.6 - WPPSI Arithmetic subtest



8.1.2 Simple Tests

A protocol was prepared to conduct the tests for inhibitory control (day and night), working memory (9 boxes), and a measure of social cognitive development (theory of mind: false belief task) (See Appendix B33). The set of instructions established in the protocol were applied to every child to maintain uniformity in the test administration.

For the *Day/Night Stroop test* 16 trials were administered in which 8 “day” cards and “8 night” cards were presented according to an established sequence. The Stroop colour test was created by Ridley Stroop in 1935 (Gerstadt et al., 1994). The methodology was similar to the materials used by Gerstadt (1994). 2 set of cards were used, one for the moon and one for the sun (Figure 8.7).

Figure 8.7 - Day and Night cards for the Day/Night Stroop test



The cards were of dimensions 21cm length by 15cm width. The day/night task required inhibitory control of action and learning, children were tested on remembering two rules, which were: to say “day” whenever they saw a black card with the moon and the stars, and to say “night” when a white card with a bright yellow sun was shown. There were 2 training cards and 16 testing cards. The 2 training cards were shown in the order illustrated in Figure 8.8. The testing cards were followed in the order shown in Figure 8.9.

Figure 8.8 - Day/Night training card ordering

2 Training cards	Answer
1. Day	Night
2. Night	Day

Figure 8.9 - Day/Night testing cards ordering

16 Testing cards	Answer
1. Day	Night
2. Night	Day
3. Day	Night
4. Night	Day
5. Night	Day
6. Day	Night
7. Day	Night
8. Day	Night
9. Night	Day
10. Day	Night
11. Night	Day
12. Night	Day
13. Day	Night
14. Night	Day
15. Night	Day
16. Day	Night



If the child had 4 consecutive errors the day/night test was discontinued. A correct answer was given the score of 1, and the incorrect answer, the score of 0. The total score was determined as the number of correct answers over the total of attempts. The mean percentage of correct answers was also determined.

For *the 9 boxes test*,

Materials: The dimensions of the boxes, which were made of cardboard, were 6cm x 6cm, the lid was attached by a string. The lids had different figures drawn on them, in order that the children could differentiate one box from another. Each box had one toy animal inside. Boxes were positioned 6cm apart approximately (illustrated in Figure 8.10). The aim of this task was to remember the box that was opened before and to open a new box to obtain a new animal. The boxes were shuffled behind a card division made of tecnopor material. The child had a maximum of 20 chances to achieve the collection of 9 animals from the 9 boxes. If 5 consecutive errors were made the test was discontinued.

The administration of this test started with the 3 training boxes, in which 3 boxes had each one an animal, children were asked to open a box that has not been opened before. Once one box was opened, the 3 boxes were shuffled behind the card division, then the 3 boxes were presented again.

Figure 8.10 - 3 training boxes and scorecard



This was followed by the 9 boxes test and the same procedure was applied (Figure 8.11). A score of 1 was given if the child opened a box and took the animal from the box (full box), if the child opened a box which had no animal in it (empty box), the score given was zero. This test assessed memory, where children had to open a box that has not been opened previously to find the 9 animals in the less number of attempts. For the 9 boxes test, children were given up to 20 possible attempts.

Figure 8.11 - 9 boxes test and scorecard



9 testing boxes	Score: 1 or 0
1.	
2.	
3.	
4.	
5.	
7.	
8.	
9.	
10.	
11.	
12.	
13.	
14.	
15.	
16.	
17.	
18.	
19	
20	

The Theory Of Mind “false-belief” task

This task evaluated understanding of the change of location (Perner and Lang, 1999). In a standard false belief story, children saw a character (Maria) place a chocolate in one cupboard (green) and then leave to play in the park (see Figure 8.12). Another character (Rosita) then moved the chocolate to another cupboard (blue). When the original character returned (Maria), children answered the experimental questions. “*Now when Maria comes back into the kitchen, where will she look for her chocolate?*”, was the first question; “*Why?*”, was the second question to obtain an explanation of question 1, of why he/she thought that, and to corroborate if the child understood that somebody can have a different mind than him or hers. To generate this conflicting response the child must use theory of mind knowledge to reason that Maria believes that the

chocolate is in the green cupboard because she did not see it moved by Rosita to the other blue cupboard. An aggregate score was created for the theory of mind, performed by summing the score of question 1 and question 2 of the theory of mind to give an aggregate score (range = 0–2).

Figure 8.12 - Theory of mind test



Language and Theory Of Mind are associated (Dunn et al., 1991). Language was important to understand child's answer to the question, in some cases children did not reply and were given a zero score. The procedures described by Perner and Lang (1999) were followed. Theory Of Mind tests assess the child's ability on reasoning and understanding of belief (Bartsch and Wellman, 1989).

8.1.3 BITSEA

Brief Infant-Toddler Social and Emotional Assessment (BITSEA) is a screening test to identify children “at risk” of experiencing social–emotional/behavioural problems for children between 12 and 36 months of age. The BITSEA test (see Figure 8.13) was administered by a psychologist and a trained person in administering the parent form, either in the first or second visit to the clinic. At the end of the test, parent/guardians were given a leaflet of recommendations prepared by the psychologist about aspects of child development, such as stimulating language, correcting behaviour and dealing with tantrums.

Figure 8.13 - BITSEA materials (Manual, BITSEA parent form questionnaire, parent score summary)



The BITSEA questionnaire (See Appendix B34), involves 42 questions on items addressing both social emotional/behavioural problems and social emotional competencies and 2 additional questions for level of worry of behaviour, where parents were asked about their concern on their child’s behaviour and language development. BITSEA standard responses range from 0–2, the response options being: 0 = Not

true/rarely, 1 = Somewhat true/Sometimes and 2 = Very True/Often (Haapsamo, 2009). The two additional questions about behaviour and language concern had response options: 1= I am not worried, 2= I am a little worried, 3= I am worried, and 4= I am very worried. Examples of questions are shown in Table 8.3.

Table 8.3 - Extract from BITSEA questionnaire

Question number	Question	Not true/ rarely	Somewhat true/ Sometimes	Very True/ Often
1	Shows pleasure when he or she succeeds (for example, claps for self)*	0	1	2
4	Is restless and can't sit still §	0	1	2
5	Follow rules*	0	1	2
22	Tries to help when someone is hurt (for example, gives a toy)*	0	1	2
36	Repeats the same action or phrase over and over without enjoyment§	0	1	2
37	Repeats a particular movement over and over (like rocking, spinning)§	0	1	2

*= Question on competence

§= Question on problem behaviour

Additional notes were also made on the parent's notes form.

Scoring BITSEA

Following the BITSEA Examiner's Manual, the parent form was scored with the aid of the parent score summary form (See **Appendix B35**). The Score summary form for the BITSEA form includes the problem domain and total competence scores and a checkbox where the assessor indicates a possible problem, delay, or deficit. If a question was not answered, M was written, meaning missing information. Problem and competence scores were transferred to the appropriate column. Adding the scores in each column the problem total and competence total (raw score) can be determined.

Determination of cut-off points

Because the table of percentiles to compare problem and competence total score were for children below 35 months and in this study children were over 35 months of age. Then the creation of a new cut-off point for children over 35 months of age was required. BITSEA screening cut-points are designed to broadly capture children with potential problems that merit additional follow-up and/or assessment (Briggs-Gowan and Carter, 2007). Standardized values were created from mean scores of problem total from the BITSEA questionnaire. The cut-off points were set at 1 standard deviation, above the mean. Data was transformed and recoded with variable labelled as 1 for children with BITSEA problem and 2 for children without BITSEA problem. The cut off point for problem was set at 1 standard deviation (1), above the mean and the cut off point for good level of competence was set at 1 standard deviation (-1), below the mean. Regarding crosschecked data, 20% of responses (37) of 184 datasets for both the simple and the BITSEA tests were randomly selected and crosschecked by a researcher (EW).

8.1.4 Statistical Methods

All data were collated in excel (version 2010), and transferred to SPSS (version 20) for analysis. Descriptive statistics including means and standard deviations were calculated. In order to analyse the results the following methods were employed, ANOVA, MANOVA and Chi squared.

The interpretation of the effects were examined using p-values and partial eta-squared (η_p^2) results. Partial eta-squared (η_p^2) is a measure of effect size and variance and this was used because it gave an estimate of the degree of association for the sample and usually SPSS software displays the partial Eta squared when looking at the effect size option.

Because Partial ETA squared (η_p^2) is reported in the SPSS output (Levine and Hullett, 2002) then I have converted this to ETA squared (η^2). The distinction between Eta squared (η^2) and partial Eta squared (η_p^2), is explained by Pierce et al. (2004) who defines Eta squared as the proportion of total variation attributable to the factor; partial eta squared is also defined as the proportion of total variation attributable to the factor but it excludes other factors from the total non-error variation. Partial ETA squared (η_p^2) measures the effects of other independent variables and interactions that are partialled out (Richardson, 2011). Partial eta-squared effect size statistics indicate the

proportion of variance of the dependent variable that is explained by the independent variable. In both, ETA squared (η^2) and partial ETA squared (η_p^2), values range from 0 to 1. Cohen developed the following guidelines to interpret ETA results (Pallant, 2010).

Table 8.4 - ETA squared guidelines

Size	Eta squared (% of variance explained)	Cohen's d (standard deviation units)
Small	0.01 or 1%	0.2
Medium	0.06 or 6%	0.5
Large	0.138 or 13.8	0.8

In the current study the conversion of the Partial ETA squared (η_p^2) to ETA squared (η^2) was performed using the following formula:

$\eta^2 = SS_{\text{effect}} / SS_{\text{total}}$ (The ratio of the effect variance (SS_{effect}) to the total variance (SS_{total}) or $\eta^2 = \text{Between-Groups Sum of Squares} / \text{Total Sum of Squares}$ (Becker, 1998) . The result was then compared to Cohen's d benchmark that referred to effect sizes as small ($d = 0.2$), medium ($d = 0.5$), and large ($d = 0.8$) (Lakens, 2013).

For the WPPSI test on intelligence the following methods were employed to analyse the data: Multivariate Analysis of Variance (MANOVA) for verbal and executive subtests; Pearson's correlation to look at the association of additional intake of vitamins, socio-economic status and WPPSI IQ.

For the Day/Night, 9 boxes, and theory of mind tests, a two way Analysis of Variance (ANOVA) was conducted to compare mean scores between the iron and the multi-micronutrient groups. In that analyses age and gender were the independent variables with the dependent variable appropriate for the particular test.

For the BITSEA test, a two way ANOVA, again with age and gender as the independent variables, was performed to compare the problem and competence mean scores. To examine whether the number and percentage of children in each group who presented problem behaviour and good levels of competence was different a Chi squared was used.

A two-way ANOVA was also used also to compare parents 'response regarding their concern about behaviour or language development of their children. Regression analysis was used to test the associations between plasma zinc levels and WPPSI total scale.

8.2 WPPSI TEST RESULTS

Wechsler Preschool and Primary Scale of Intelligence-Revised (WPPSI-R) subtests were chosen to measure variables of interest such as memory and attention. Selected subtests were administered for a prorated overall IQ score, particularly object assembly, block design, picture completion, and animal pegs as well as 5 subtests in the verbal scale: comprehension, vocabulary, similarities, arithmetic, and sentences as described in 8.1.1. The mean scores for each of the 4 subtests from the executive scale are shown in Table 8.5. The mean scores for each subtest of the verbal scale are shown in Table 8.6. The sum of the scores for each scale were used to determined equivalent Executive Intelligent Quotient (IQ) and Verbal IQ result, including gender differences, which are summarised in Table 8.7.

MANOVA analysis was conducted for the executive and verbal scale of the WPPSI test. This was followed by the two-way ANOVA by gender and group, conducted to analyse the intelligent quotient (IQ) scale.

Table 8.5 - WPPSI Executive scale test results

	Fe (n= 93)		MMN(n= 81)		P value	Max. points (N° of questions)
	$\bar{x} \pm SD$		$\bar{x} \pm SD$			
	Boys (n=52)	Girls (n=41)	Boys (n=44)	Girls (n=37)		
Executive subtests						
Object assembly	8.10 ± 3.27	7.78 ± 3.30	8.09 ± 3.24	8.49 ± 2.99	0.470	32 (6)
Picture completion	9.06 ± 2.82	8.90 ± 2.62	9.25 ± 2.54	9.14 ± 2.57	0.960	28 (28)
Block design	10.06 ± 2.49	9.88 ± 2.26	9.98 ± 2.67	10.54 ± 2.82	0.343	42 (14)
Animal peg	9.17 ± 3.20	9.66 ± 3.34	9.41 ± 3.24	9.84 ± 3.43	0.955	70 (28 pegs)

A two-way MANOVA by gender and group revealed a non-significant multivariate main effect for gender, Wilks' $\lambda = 0.988$, $F(4, 167.0) = 0.495$, $p = 0.740$, $\eta_p^2 = 0.012$, for group, Wilks' $\lambda = 0.996$, $F(4, 167.0) = 0.179$, $p = 0.949$, $\eta_p^2 = 0.004$ and non-significant group-gender interaction, Wilks' $\lambda = 0.989$, $F(4, 167.0) = 0.465$, $p = 0.761$, $\eta_p^2 = 0.011$.

Table 8.6 - WPPSI Verbal scale test results

Verbal subtests	Fe(n= 93)		MMN(n= 81)		P value	Max. points (N° of questions)
	Boys (n=52)	Girls (n=41)	Boys (n=44)	Girls (n=37)		
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$		
Comprehension	6.78 ± 1.90	6.80 ± 1.52	6.95 ± 1.68	6.75 ± 1.92	0.687	30 (15)
Vocabulary	5.88 ± 1.86	5.78 ± 1.59**	5.68 ± 1.85	6.83 ± 2.05**	0.028**	47 (25)
Similarities	7.41 ± 2.69	7.72 ± 2.43	7.43 ± 2.50	7.86 ± 2.95	0.887	28 (12)
Arithmetic	8.57 ± 2.27	8.08 ± 2.60	7.75 ± 2.63	7.97 ± 2.70	0.361	23 (23)
Sentences	6.82 ± 2.67	7.95 ± 2.95*	7.20 ± 2.48	7.83 ± 2.87*	0.039*	37 (12)

*Significant differences by gender, \bar{x} : mean, SD: Standard Deviation

**Significant differences by supplemented group and gender

The MANOVA was conducted to assess verbal subtest differences. The multivariate effect was significant by gender, Wilks' $\lambda = .935$, $F(5, 163.0) = 2.271$, $p = 0.050$, $\eta_p^2 = 0.065$, and not significant by gender*group interaction, Wilks' $\lambda = 0.937$, $F(5, 163.0) = 2.206$, $p = 0.056$, $\eta_p^2 = 0.063$. Univariate tests showed that there was significant gender effect differences for the sentences subtest $F(1,167) = 4.323$, $p = 0.039$, $\eta_p^2 = 0.025$, where girls had higher scores than boys. There was not a significant gender difference on the vocabulary subtest $F(1,167) = 3.377$, $p = 0.068$, $\eta_p^2 = 0.020$. Furthermore, univariate tests showed that there was a significant gender*group interaction for the vocabulary subtest $F(1,167) = 4.909$, $p = 0.028$, $\eta_p^2 = 0.029$. Girls in the MMN had higher scores in the vocabulary test than girls in the Fe group. All other verbal subtests scores showed no significant effect $p > 0.05$.

Table 8.7 - WPPSI Intelligent Quotient (IQ) by intervention group, and gender

	Fe (n=93)	MMN (n=81)	Mean difference	P value
	$\bar{x} \pm SD$	$\bar{x} \pm SD$		
Executive IQ	93.95 ± 14.80	94.48 ± 16.75	0.53	0.867
Boys	93.67 ± 14.65	95.48 ± 13.78	1.81	
Girls	94.29 ± 15.16	93.30 ± 19.86	0.99	
Verbal IQ	83.31 ± 9.86	83.38 ± 11.09	0.07	0.924
Boys	83.12 ± 10.30	82.23 ± 10.31	0.89	
Girls	83.56 ± 9.40	84.76 ± 11.94	1.2	
Total IQ score	86.61 ± 12.23	87.90 ± 12.91	1.29	0.479
Boys	86.37 ± 12.02	86.73 ± 12.15	0.36	
Girls	86.93 ± 12.64	89.30 ± 13.79	2.37	

Fe: Iron group; MMN: Multiple micronutrient group; IQ: Intelligent Quotient

Fe: boys n=52, girls n=41; MMN: boys n=44, girls n=37

There was a mean difference of 1.29 between the Fe and the MMN groups for the for the total WPPSI IQ, which was not considered significant ($p=0.479$). Children had a low average total IQ score (80-89), but on the executive scale children had a normal average IQ score (90-99). Three series of two-way ANOVA by group and gender in each one of the WPPSI components (Executive IQ, Verbal IQ and Total IQ score) were performed. A two-way ANOVA for the *Executive IQ* by group and gender yielded no effect by group $F(1,170) = 0.028$, $p= 0.867$, $\eta_p^2 < 0.001$, non-significant effect by gender $F(1,170) = 0.104$, $p= 0.747$, $\eta_p^2 = 0.001$. The group*gender interaction was not significant $F(1,170) = 0.336$, $p= 0.563$, $\eta_p^2 = 0.002$. Similarly for the *Verbal IQ*, there was no effect by group $F(1,170) = 0.09$, $p=0.924$, $\eta_p^2 < 0.001$, non-significant effect by gender $F(1,170) = 0.864$, $p= 0.354$, $\eta_p^2 = 0.005$. The group*gender interaction was not significant $F(1,170) = 0.424$, $p= 0.516$, $\eta_p^2 = 0.002$. Finally for the *total IQ* score, the 2 by 2 ANOVA analysis yielded no effect by group $F(1, 170) = 0.504$, $p= 0.479$, $\eta_p^2 = 0.003$, non-significant effect by gender $F(1,170) = 0.663$, $p= 0.417$, $\eta_p^2 = 0.004$. The group-gender interaction was not significant $F(1, 170) = 0.273$, $p= 0.602$, $\eta_p^2 = 0.002$. These results showed that no significant differences were found for the executive, verbal or total intelligent quotient for the Fe and MMN group.

Positive correlations were however found between the intake of vitamins and WPPSI IQ score in the iron group (Pearson's correlation $r(93) = 0.29$, $p= .005$, $p < 0.01$). The total

WPPSI IQ score was correlated to the child attending nursery school. The correlation was significant at the 0.05 level (2 tailed) in the MMN group, where the WPPSI total IQ score was positively correlated with the mean of the number of children attending nursery school. The Pearson correlation coefficient (.27) indicated a positive correlation between the total intelligence WPPSI score with the children who attended nursery school, $r(86) = .27, p = .013, p < 0.05$. According to Cohen (1998) in Pallant, 2010, the result indicates a small correlation.

When analysing social economic status indicators, the total WPPSI IQ score was also positively correlated with the father's years of education, $r(93) = .32, p = .002, p < 0.01$ and with the mother's years of education, $r(93) = .31, p = .003, p < 0.01$ in the iron group, this implies that children whose parents were more educated, had higher total IQ scores. There was no significant correlation between mother's occupation and the total WPPSI total IQ score for the whole sample, $r(174) = 0.081, p = 0.286$. This association was also weak $R^2 = 0.007$ (0.7%), The mother's occupation explained a non-significant proportion of the variance in WPPSI total IQ score, $R^2 = 0.007, F(1,173) = 1.15, p = 0.286$. Other indicators of family income such as the house's predominant floor material, $r(93) = -0.25, p = 0.017, p < 0.05$ in the iron group, and the possession of devices in the multi-micronutrient group, showed a correlation with total WPPSI IQ score, $r(81) = 0.26, p = 0.020, p < 0.05$.

Whether plasma zinc level related to current WPPSI test performance was examined using regression analysis. Plasma zinc concentration was the predictor variable in the analyses and the WPPSI test score was the dependent variable. Results showed that plasma zinc only accounted for 3.3% of the variation in total WPPSI IQ scores. This means that 96.7% of the variation in total WPPSI IQ scores cannot be explained by plasma zinc alone. There was a correlation between plasma zinc and WPPSI total IQ scores $r(174) = -0.181, p = 0.017$, and the correlation was significant at the 0.05 level (2 tailed). Plasma zinc explained a significant proportion of variance in WPPSI total IQ scores, $R^2 = 0.181, F(1,171) = 5.79, p = 0.017$.

8.3 SIMPLE PSYCHOLOGICAL TEST RESULTS

A series of simple psychological tests (day/night, 9 boxes, theory of mind) were performed to measure how the children used their cognitive skills (as described in section 8.1.2). The results of these tests and statistical analyses are presented in tables 8.8 to 8.16. Two-way ANOVA by gender and group was conducted to compare the control Iron (Fe) group and multi-micronutrient (MMN) intervention group.

8.3.1 Day/Night Test Results

Table 8.8 - Percentage of children who completed the 16 trials task for the day and night test

Group	Percentage (%)	Total Number
Fe (n= 96)	54.6	53
MMN (n= 87)	52.9	46

Fe: Iron group; MMN: Multiple micronutrient group

The percentage of children who completed the 16 trial for the day/night test was 54.6% in the Fe control group vs. the MMN group 52.9%. There was one missing value as 1 child did not complete the Day/Night task.

Table 8.9 - Number of correct answers for those who completed the 16 trial Day/Night test

Completed 16 trial	$\bar{x} \pm SD$	Minimum	Maximum
Fe (n=53)			
Boys (n=29)	11.93 \pm 3.17	4	16
Girls (n=24)	12.58 \pm 3.57	3	16
MMN (n=46)			
Boys (n=27)	12.15 \pm 3.26	3	16
Girls (n=19)	12.47 \pm 3.72	4	16

Fe: Iron group; MMN: Multiple micronutrient group

A two-way ANOVA by group and gender yielded no effect by group $F(1, 95) = 0.006$, $p = 0.938$, $\eta_p^2 < 0.001$, not a significant effect by gender $F(1, 95) = 0.498$, $p = 0.482$,

$\eta_p^2 = 0.005$. There was not a significant group*gender interaction $F(1, 95) = 0.056, p = 0.814, \eta_p^2 = 0.001$. This means that, despite girls having higher scores than boys, for instance in the MMN group the mean number of correct answers for the girls was 12.47 compared to the boys 12.15, the differences for those children who completed the 16 trial day/night task were not significant in the Fe and the MMN group. The converted eta squared for the group was $\eta^2 = 0.000063$ (small effect size by Cohen's d).

Table 8.10 - The percentage of correctness for those who completed the 16 trial day/night test

Completed 16 trial	Percentage (%)	Total Number
Fe (n=53)		
Boys (n=29)	74.57	29
Girls (n=24)	78.65	24
MMN (n=46)		
Boys (n=27)	75.93	27
Girls (n=19)	77.96	19

Fe: Iron group; MMN: Multiple micronutrient group

+The percentage (%) of correctness is defined as the number of correct answers/ the number of attempts (16 trial test).

Similarly, a two-way ANOVA by group and gender on the percentage of correctness yielded no effect by group $F(1, 95) = 0.006, p = 0.938, \eta_p^2 < 0.001$, not a significant effect by gender $F(1, 95) = 0.498, p = 0.482, \eta_p^2 = 0.005$ and the effect by group*gender interaction was not significant $F(1, 95) = 0.056, p = 0.814, \eta_p^2 = 0.001$. In other words, although girls scored higher percentage of correctness than boys in the iron and MMN group, these differences were not significant. The converted eta squared for the group was $\eta^2 = 0.000063$ (small effect size by Cohen's d).

Table 8.11 - Longest correct run day/ night test for the whole sample

Whole sample	$\bar{x} \pm SD$
Fe (n =96)	4.56 ± 5.06
Boys (n=53)	4.20 ± 4.45
Girls (n=43)	5.00 ± 5.74
MMN (n=87)	4.66 ± 5.28
Boys (n=49)	4.37 ± 4.68
Girls (n=38)	5.05 ± 5.99

Fe: Iron group; MMN: Multiple micronutrient group

A two-way ANOVA by group and gender yielded no effect by group $F(1, 179) = 0.019, p = 0.891, \eta_p^2 = 0.001$, not a significant effect by gender $F(1, 179) = 0.916, p = 0.340, \eta_p^2 = 0.005$. There was not a significant group*gender interaction $F(1, 179) = 0.005, p = 0.945, \eta_p^2 = 0.000$. This means that there were no differences for the Fe and MMN group for the children who had the longest correct run for the day/night test. The resulting partial eta squared value is 0.005 which, in Cohen's terms (Pallant, 2010), would be considered a small effect. Cohen classifies 0.01 as a small effect, 0.06 as medium effect and 0.14 as a large effect. The converted eta squared for the group was $\eta^2 = 0.0001$ (small effect size by Cohen's d).

Table 8.12 - Longest correct run for day/ night test for those who completed the 16 trial day/night test

	$\bar{x} \pm SD$
Fe (n=52)	7.75 ± 4.79
Boys (n=29)	6.76 ± 4.35
Girls (n=23)	9 ± 5.12*
MMN (n=46)	7.98 ± 4.96
Boys (n=27)	6.81 ± 4.39
Girls (n=19)	9.63 ± 5.37*

Fe: Iron group; MMN: Multiple micronutrient group; *significant differences by gender

For those children who completed the 16 trial day/night test, there were significant differences by gender, (Fe group, girls ($M=9$, $SD=5.12$), boys ($M=6.76$ $SD=4.35$) v. MMN group, girls ($M=9.63$, $SD=5.37$), boys ($M=6.81$, $SD=4.39$), $P = 0.011$). A two-way ANOVA by gender and group yielded a main effect by gender, $F(1, 94) = 6.749$, $p = 0.011$, $\eta_p^2 = 0.067$, where girls performed the longest correct run for the day/night test than the boys in the day/night test. There was no effect by group $F(1, 94) = 0.125$, $p = 0.725$, $\eta_p^2 = 0.001$, $\eta^2 = 0.0012$ and the group*gender interaction was not significant $F(1, 94) = 0.087$, $p = 0.768$, $\eta_p^2 = 0.001$. This means that significant differences were found between girls in boys in children who completed the 16 trial task, for instance in the MMN group, girls had the longest correct run score 9.63 compared to boys who had a lower score 6.81. The converted eta squared for the differences by gender was $\eta^2 = 0.067$ (medium effect size by Cohen's d).

8.3.2 Nine Boxes Test Results

Table 8.13 - Number of correct answers for the 9 boxes test for the whole sample

	$\bar{x} \pm SD$
Fe (n=97)	8.58 ± 1.12
Boys (n=53)	8.58 ± 1.37
Girls (n=44)	8.57 ± 0.73
MMN (n=87)	8.54 ± 1.21
Boys (n=49)	8.45 ± 1.53
Girls (n=38)	8.66 ± 0.58

Fe: Iron group; MMN: Multiple micronutrient group

A two-way ANOVA by group and gender, yielded no effect by group $F(1, 180) = 0.018$, $p = 0.894$, $\eta_p^2 = 0.000$, non-significant effect by gender $F(1, 180) = 0.308$, $p = 0.580$, $\eta_p^2 = 0.002$. The group-gender interaction was not significant $F(1, 180) = 0.425$, $p = 0.515$, $\eta_p^2 = 0.002$. The converted eta squared for the group was $\eta^2 = 0.000098$ (small effect size by Cohen's d).

Table 8.14 - The percentage of correctness for the 9 boxes test

	Percentage (%)	Total Number
Fe (n=97)	62.05	97
Boys (n=53)	63.92	53
Girls (n=44)	59.79	44
MMN (n=87)	65.39	87
Boys (n=49)	65.70	49
Girls (n=38)	64.98	38

Fe: Iron group; MMN: Multiple micronutrient group

* The percentage (%) of correctness is defined as the number of correct answers/ the number of attempts (20 attempts were permitted in the 9 boxes test).

A two-way ANOVA by group and gender, yielded non effect for the percentage of correctness on the 9 boxes test by group, $F(1,180) = 1.830$, $p = 0.178$, $\eta_p^2 = 0.010$, by gender, $F(1,180) = 0.893$, $p = 0.346$, $\eta_p^2 = 0.005$, by group-gender interaction was also not significant $F(1,180) = 0.437$, $p = 0.509$, $\eta_p^2 = 0.002$. The converted eta squared for the group was $\eta^2 = 0.0099$ (small effect size by Cohen's d).

Table 8.15 - Longest correct run for the 9 boxes test

	$\bar{x} \pm SD$
Fe (n=97)	4.46 \pm 2.05
Boys (n=53)	4.64 \pm 2.11
Girls (n=43)	4.25 \pm 1.89
MMN (n=87)	4.77 \pm 2.20
Boys (n=49)	4.57 \pm 2.38
Girls (n=38)	5.03 \pm 1.95

Fe: Iron group; MMN: Multiple micronutrient group

A two-way ANOVA by group and gender, yielded no effect by group $F(1, 180) = 1.272$, $p = 0.261$, $\eta_p^2 = 0.007$, non-significant effect by gender $F(1, 180) = 0.010$, $p = 0.920$, $\eta_p^2 < 0.001$. The group*gender interaction was not significant $F(1, 180) = 1.826$,

$p= 0.178$, $\eta_p^2 = 0.010$. The converted eta squared for the group was $\eta^2 = 0.0069$ (small effect size by Cohen's d).

8.3.3 Theory Of Mind Test Results (False Belief Task)

Table 8.16 - Aggregate score for the theory of mind test

	$\bar{x} \pm SD$
Fe (n=97)	0.20 \pm 0.49
Boys (n=53)	0.21 \pm 0.49
Girls (n=44)	0.18 \pm 0.50
MMN (n=87)	0.25 \pm 0.55
Boys (n=49)	0.18 \pm 0.49
Girls (n=38)	0.34 \pm 0.63

Fe: Iron group; MMN: Multiple micronutrient group

A two-way ANOVA by group and gender, yielded no effect by group $F(1, 180) = 0.771$, $p= 0.381$, $\eta_p^2 = 0.004$, non-significant effect by gender $F(1, 180) = 0.730$, $p= 0.394$, $\eta_p^2 = 0.004$. The group-gender interaction was not significant $F(1, 180) = 1.406$, $p= 0.237$, $\eta_p^2 = 0.008$. The converted eta squared for the group was $\eta^2 = 0.0042$ (small effect size by Cohen's d).

There was a positive significant correlation between the theory of mind performance and age. The Pearson correlation coefficient (0.255) indicated a positive significant correlation between age (at a cut-off point of 46 months old) and the theory of mind aggregate score in the MMN group, suggesting that children at older age had higher performance on the theory of mind test than children at a younger age. There was also a positive significant correlation at the 0.05 level (2-tailed) between the theory of mind aggregate score and age in the iron group (0.207).

8.4 BITSEA TEST RESULTS

The Brief Infant-Toddler Social and Emotional Assessment (BITSEA) test was used as a screening tool to determine whether a child required a more in-depth assessment of the social-emotional domain. Please refer to section 8.1.3 for full details.

The BITSEA examiner's manual was followed to apply this test. Data were entered into excel files and then imported into SPSS. Range, mean and standard deviation for the problem and competence total score were determined. The Chi square test was used for problem and competence score for boys and girls. A two-way factorial ANOVA was conducted for the language and behaviour concern scale. One way ANOVA was conducted to compare control iron group (Fe) and multi-micronutrients group (MMN) for BITSEA respondents.

8.4.1 BITSEA Respondents

The respondents of the BITSEA questionnaire were diverse, being the mother, father, grandmother, or aunt (See Table 8.17)

Table 8.17 - Identity of BITSEA Respondents

	Respondent of BITSEA forms	Percentage (%)	Total number
Fe (n=97)	Mother	97.9	95
	Father	1	1
	Aunt	1	1
MMN(n=87)	Mother	93.1	81
	Father	4.6	4
	Grandmother	1.1	1
	Mother & Father together	1.1	1

Fe: Iron group; MMN: Multiple micronutrient group

Univariate ANOVA showed that there was no significant differences in the percentage of respondents between the Fe and the MMN groups $F(1, 184)=0.685, p=0.409$.

The table below shows the percentage of children with problem behaviour using the determined cut-off point of 1 standard deviation about the mean

Table 8.18 - Total percentage of children with problem behaviour > 1 Standard Deviation

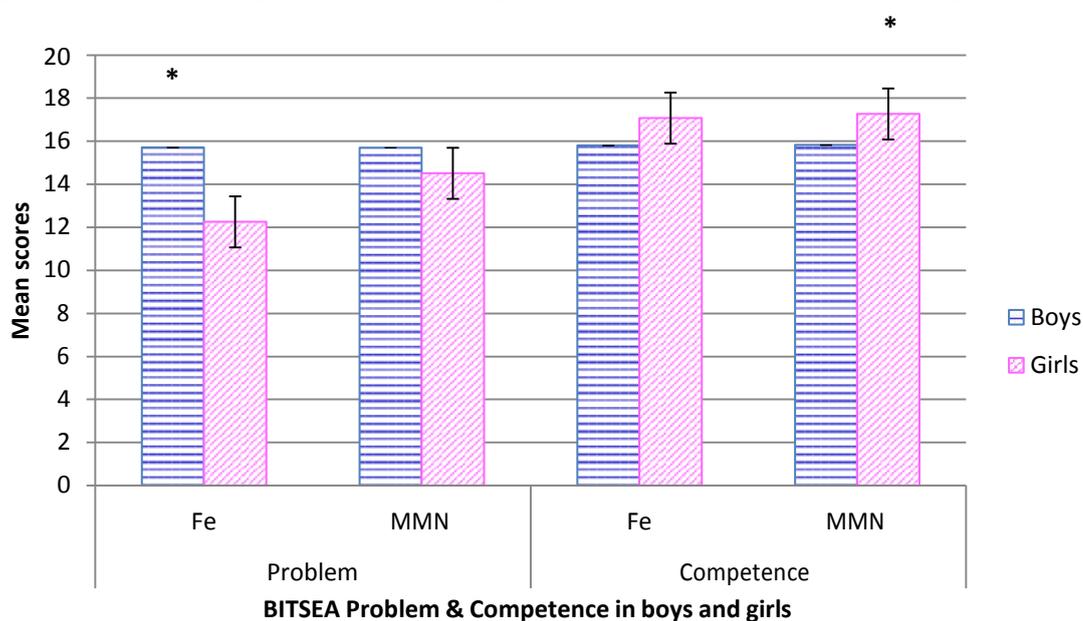
	Total Problem behaviour	Percentage (%)
Fe (n=97)	16	16.5
MMN (n=87)	18	20.7

Fe: Iron group; MMN: Multiple micronutrient group

One way ANOVA showed that total number of children with problem behaviour was not significantly different between the Fe and the MMN group, $F(1, 184) = 0.531, p = 0.467$.

8.4.2 Problem and Competence Scores

Figure 8.14 BITSEA problem and competence mean scores in boys and girls



*significant differences by gender

Table 8.19 - Mean and Standard Deviation for Problem and Competence scores from the 42 item BITSEA questionnaire

	Problem			Competence			P value
	$\bar{x} \pm SD$	Min	Max	$\bar{x} \pm SD$	Min	Max	
Fe (n=97)	14.13 ± 5.99	3	33	16.37 ± 3.21	7	22	0.010 *
Boys (n=53)	15.70 ± 5.85 *	5	33	15.79 ± 2.99	7	21	
Girls (n=44)	12.25 ± 5.65	3	29	17.07 ± 3.37 *	8	22	
MMN (n=87)	15.17 ± 6.31	4	33	16.45 ± 2.54	9	22	0.002 *
Boys (n=49)	15.69 ± 6.78 *	4	33	15.82 ± 2.63	9	22	
Girls (n=38)	14.50 ± 5.67	4	28	17.26 ± 2.19 *	13	22	

Fe: Iron group; MMN: Multiple micronutrient group

*significant differences by gender

A two-way ANOVA on problem scores yielded a non-significant effect by group, $F(1, 180) = 1.570$, $p = 0.212$, $\eta_p^2 = 0.009$; $\eta^2 = 0.0083$. There were significant differences by gender, where boys had higher problem scores than girls, $F(1, 180) = 6.708$, $p = 0.010$, $\eta_p^2 = 0.036$, and there was not a statistically significant group*gender interaction at the $p = 0.210$ level, $F(1, 180) = 1.582$, $\eta_p^2 = 0.009$. The converted eta squared for the differences by gender was $\eta^2 = 0.035$ (small effect size by Cohen's d).

Two by two ANOVA by gender and group on competence scores, yielded a primary effect of gender on competence scores, $F(1, 180) = 10.331$, $p = 0.002$, $\eta_p^2 = 0.054$, such that the average competence in girls was significantly higher than boys. The effect by group was not significant, $F(1, 180) = 0.067$, $p = 0.796$, $\eta_p^2 = 0.001$, $\eta^2 = 0.00035$; the interaction group*gender effect was not significant, $F(1, 180) = 0.041$, $p = 0.840$, $\eta_p^2 < 0.001$. The converted eta squared for the differences by gender was $\eta^2 = 0.054$ (small effect size by Cohen's d).

The partial eta squared was converted to a percentage by multiplying by 100, for instance the partial eta squared (η_p^2), effect size for the differences between the Fe and the MMN group for the competence scores was small, in this case 0.1% explained the variance.

Problem scores between the Fe and the MMN were not significant ($p = 0.212$). However, boys had significantly higher problem scores than girls, ($p = 0.010$). Girls in the MMN group had higher problems scores than girls in the Fe group but differences in scores were not significant ($p = 0.210$).

Competence scores were not significant between the Fe and the MMN group ($p = 0.067$). However, girls had higher competence scores than boys, which was significant ($p = 0.002$). Although, girls in the MMN group had higher competence scores than girls in the Fe group, differences in scores were not significant ($p = 0.840$).

Table 8.20 - Summary of Problem behaviour and competence levels, by gender

	Fe (n=97)				MMN (n=97)			
	Boys (n=53)		Girls (n=44)		Boys (n=49)		Girls (n=38)	
	N°	%	N°	%	N°	%	N°	%
Problem Behaviour BITSEA/P								
Without	41	77.35	40	90.90	38	77.55	31	81.58
With	12	22.64	4	9.09	11	22.44	7	18.42
Competence Levels BITSEA/C								
Good Levels	44	83.02	38	86.36	40	81.63	37	97.37
Poor Levels	9	16.98	6	13.64	9	18.37	1	2.63

Fe: Iron group; MMN: Multiple micronutrient group

For BITSEA/C the number of competent girls in percentage was higher than the number of competent boys, both in the MMN group; girls (97.3%) vs. boys (81.63%) and Fe group; girls (86.36%) vs. boys (83.02%). Comparing BITSEA/P, the number of boys that presented problem behaviour was higher than the number of girls with problem behaviour; Fe group; boys (22.64%) vs. girls (9.09%), and MMN group; boys (22.44%) vs. girls (18.92%).

A total of 4 chi squares (X^2) were conducted to determine the number of boys and girls who presented/ not presented problem behaviour and the number of boys and girls that presented good/poor levels of competence. The chi-square test revealed that there was no significant difference in the number of boys without/with problem behaviour between the Fe and the MMN group. $X^2 (1, N = 184) = 0.001, p = 0.981$. There was no significant difference in the number of girls without/with problem behaviour between the Fe and the MMN group, in other words, the proportion of girls with problem

behaviour in the Fe group vs. the MMN group was not significantly differently, $X^2(1, N = 184) = 1.528, p = 0.216$. There was no significant difference in the number of boys with good/poor levels of competence between the Fe and the MMN group, $X^2(1, N = 184) = 0.034, p = 0.854$. The proportion of girls who had good levels of competence in the Fe group vs. the MMN group was not significantly different, or there was no significant difference in the number of girls with good/poor levels of competence between the Fe and the MMN group, $X^2(1, N = 184) = 3.163, p = 0.075$. Comparing within groups, boys were more likely to have more problem behaviour than girls. Girls were more likely to be more competent than boys.

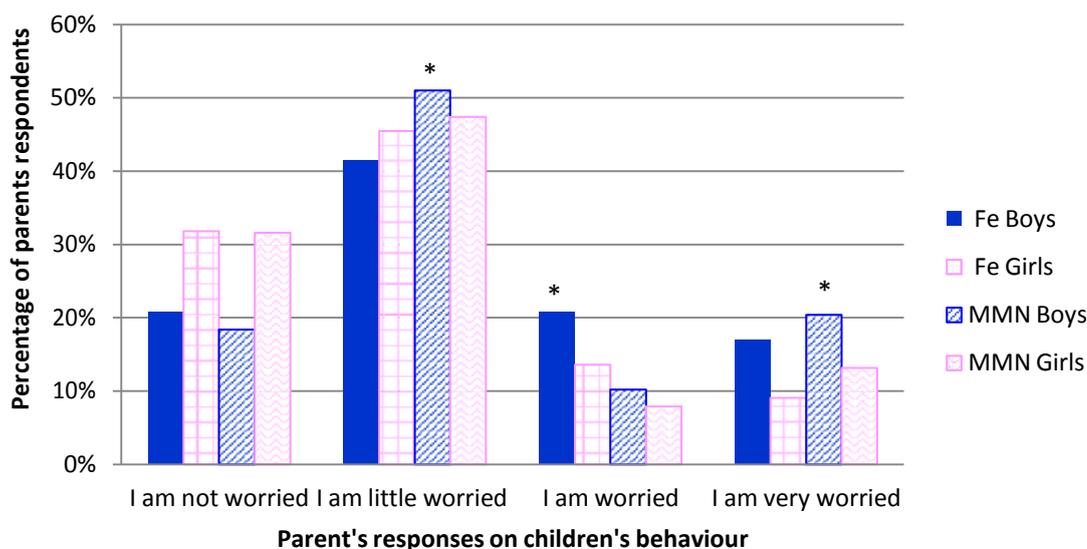
8.4.3 Behaviour Concern

In the question about behaviour concern, where the parents responded about their concern regarding their children's behaviour, the possible options are shown in Table 8.21 and the results in Figure 8.15.

Table 8.21 - Behaviour concern scale

Question	1= Not at all worried	2= A little worried	3= Worried	4= Very worried
How worried are you about your child's behaviour, emotions or relationship?	1	2	3	4

Figure 8.15 - Percentage of respondents who were concerned about behaviour, emotions and child's relationship



Fe: Iron group; MMN: Multiple micronutrient group

*Significant gender differences

Parents in the MMN group were “a little worried” about boys’ behaviour (51%) than girls (47%). In both groups, parents expressed to be “very worried” about their boys’ behaviour than girls, Fe: 17% in boys v. 9% in girls, MMN: 20% in boys v. 13% in girls.

A two-way ANOVA, 2 x 2 by gender and group, yielded no effect for the behaviour concern by group, $F(1,180) = 0.002, p = 0.964, \eta_p^2 = 0.000$; In contrast, the main effect of gender was significant, $F(1,180) = 4.861, p = 0.029, \eta_p^2 = 0.026$, where parents were more concerned on boys’ behaviour than in girls. The interaction of group and gender factors was not significant, $F(1,180) = 0.018, p = 0.892, \eta_p^2 = 0.000$. Thus, parents expressed more concern about their boy’s behaviour.

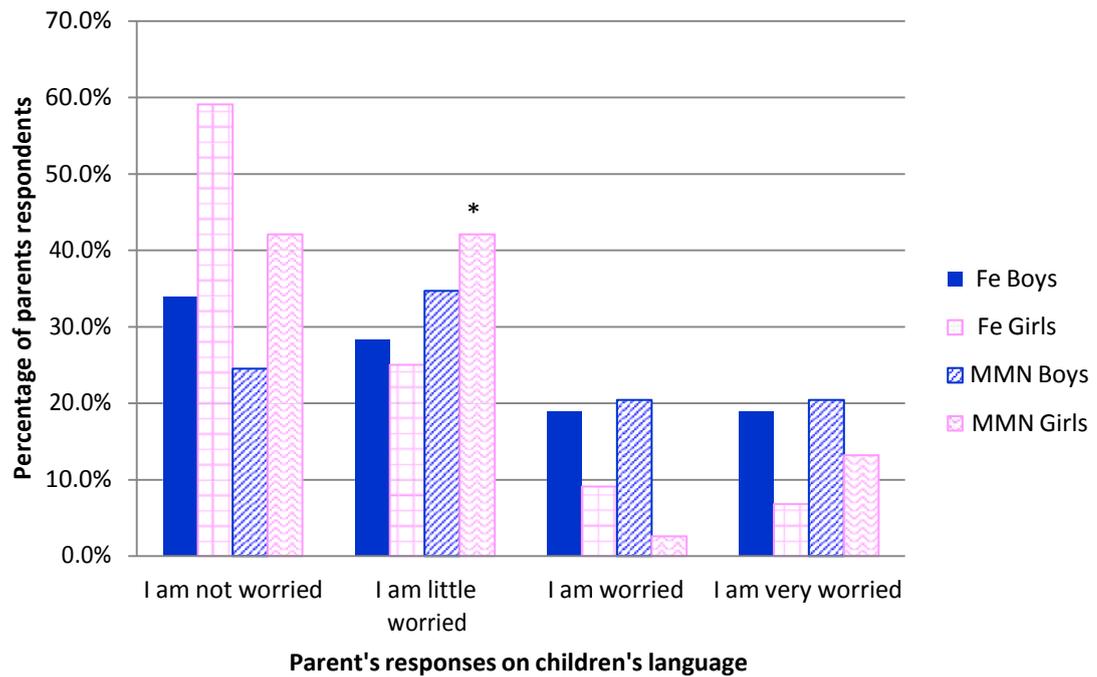
8.4.4 Language Concern

In the question about language concern, where the parents responded about their concern regarding their children’s language, the possible options were in the scale from 1 to 4 and it is shown in the Table 8.22 with the test results shown in Figure 8.16.

Table 8.22 - Language concern scale

Question	1= Not at all worried	2= A little worried	3= Worried	4= Very worried
How worried are you about your child's language development?	1	2	3	4

Figure 8.16 - Percentage of respondents who were concerned about language development of child



Fe: Iron group; MMN: Multiple micronutrient group

*Significant gender differences

Parents in the MMN group expressed to be “a little worried” about their girl’s language development (42.1%) than boys (34.7%). Parents were “worried” about their boy’s language in both groups, Fe: 18.9% in boys v. 9.1% in girls, MMN: 20.4% in boys v. 2.6% in girls, and parents were very concerned about their boy’s language development than girls in both groups, Fe: 18.9% in boys v. 6.8% in girls, MMN: 20.4% in boys v. 13.2% in girls.

A two-way ANOVA yielded no effect for the language concern by group, $F(1,180) = 1.468, p = 0.227, \eta_p^2 = 0.008$; In contrast, the main effect of gender was significant, $F(1,180) = 12.510, p = 0.001, \eta_p^2 = 0.065$. The interaction of these two factors: group and gender was not significant, $F(1,180) = 0.088, p = 0.768, \eta_p^2 = 0.000$. Thus, parents were more concerned about boy’s language development.

Overall the results for the BITSEA test showed that there were significant differences by gender for behaviour and language concern. Parents were more worried about the behaviour and language development of their boys than the girls. There were no significant differences between the iron control group and the MMN group.

8.5 SUMMARY OF PSYCHOLOGICAL TESTS

A range of psychological tests was completed on the study sample in children. A condensed summary table of the main psychological test results is shown below.

Table 8.23 - Total summary of applied psychological tests

Test	Fe		MMN		Compare with score	P value
	Mean	SD	Mean	SD		
WPPSI Total IQ score	86.61	12.23	87.9	12.91	100	0.479
Day/night	7.75	4.79	7.98	4.96	16	0.725
9 boxes	4.51	1.97	4.77	2.2	9	0.331
Theory of mind	0.2	0.49	0.25	0.55	2	0.381
BITSEA Problem	14.13	5.99	15.17	6.31	14	0.212
BITSEA Competence	16.37	3.22	16.45	2.54	16	0.796

No significant score differences were found between the Fe and the MMN group.

For the WPPSI tests no significant differences were found for the overall executive, verbal, or total intelligent quotient between the Fe and MMN group. However, a significant difference was found in one verbal IQ subtest: the vocabulary subtest, between the Fe and MMN group, which may indicate a subtle positive association, and significant gender differences for the verbal IQ subtest: sentences. For the Day-Night test there were no significant differences between the groups, however there were differences between the genders, with girls having higher scores than boys in both groups, for the longest correct run for the Day-Night test on those who completed the 16 test trial. The 9-boxes test and the theory of mind yielded no significant differences. For the BITSEA/P problem scores, differences between Fe and MMN groups were also not significant, though there were significant differences by gender where boys had higher problem scores than girls. Regarding the percentage of children with problem behaviour, this was higher in boys than in girls, the differences were not significant. In the BITSEA/C competence scores there were significant differences by gender with girls having higher competence scores than boys.

The psychological tests will be discussed further in section 10.2.4.

CHAPTER 9. –

EMPIRICAL RESEARCH: RESULTS OF THE CLINICAL ASSESSMENT AND BIOCHEMICAL ANALYSIS

Chapter 9 – EMPIRICAL RESEARCH: RESULTS OF THE CLINICAL AND BIOCHEMICAL ASSESSMENT

In this chapter the details of the methodology used and results of the clinical assessment and biochemical analysis of the children is presented.

9.1 METHODS FOR CLINICAL AND BIOCHEMICAL ASSESSMENT

9.1.1 Clinical Assessment

Clinical assessment was conducted by a paediatrician. This involved the evaluation of the child physical condition and medical history. The following measurements were taken: blood pressure, cardiac frequency, respiratory rate. In the clinical questionnaire parents were asked for the card record of their child. This card had information on vaccinations, weight at birth and for each year. One of the questions in this medical history questionnaire was whether the child had taken supplements at the end of the original study, if so, what the supplement was and for how long this was taken.

The following measurements were undertaken: blood pressure, cardiac frequency and respiratory frequency. The measurement of blood pressure (BP) is generally considered to be an integral part of a clinical examination (Arafat and Mattoo, 1999). It is recommended that children older than 3 year old have their blood pressure measured at least once a year, as part of health screening. The following recommendations were taken into consideration: A correct position of the child while sitting, with legs uncrossed and with cuff at heart level (NGC, 2013). The rest period before the blood pressure measurement, less than 5 minutes, the correct size of the cuff was essential for gaining an accurate recording (RCN, 2011). Systolic (SBP) and diastolic blood pressure (DBP) was measured with a sphygmomanometer; units were expressed in millimetres of mercury (mmHg). Normal blood pressure measurements for children aged 1-5 year old are indicated as 80-110 mmHg for systolic blood pressure and 50-80 mmHg for diastolic blood pressure (UHS, 2014).

With reference to cardiac and respiratory frequency, a stethoscope was used to auscultate heart rate of the child (RCN, 2011) and, respiratory rate. Normal cardiac frequency for a child aged 3-5 year old is 60-140 beats/minute (Fleming et al., 2011).

Normal respiratory rate for a pre-schooler child (3-6 years) is considered to be at a rate of 22–34 breaths per minute or less than 40 breaths per minute in children aged 2-4 year old (Fleming et al., 2011).

9.1.2 Biochemical Assessment

3ml of blood was taken into a vacutainer containing a chelating agent, Ethylenediaminetetraacetic acid (EDTA). Approximate 10 µl blood was taken up into a microcuvette for haemoglobin (Hb) analysis by HemoCue® to measure plasma zinc concentration, of which 1 drop of the sample was used to measure haemoglobin (Hb). The remaining whole blood sample was centrifuged within 2 h of collection at 2500 RPM X 100 for 20-25 minutes to isolate plasma. Using a transfer pipette plasma was transferred from the original tube into the labelled transfer (aliquot) cryovial tube. Plasma samples were labelled with the participant identification code, sample date, and time of centrifuge and stored at –80 °C. at the IIN clinic laboratory. Plasma zinc concentrations were measured using atomic absorption spectrophotometry, the general advantages of this method are the simplicity, sensitivity, precision and cost (Robinson, 1960, Butrimovitz and Purdy, 1977, Halsted et al., 1974). Data was entered into an excel spread sheet and then exported to SPSS for analysis. A total of 180 samples were analysed.

Anaemia was identified using the definition by the World Health Organization, where prevalence of anaemia was defined as the percentage of children with Hb levels below 11 g/dl (WHO, 1996).

Zinc deficiency was defined as zinc concentration <10.7 µmol/L (Gibson, 1990).

The materials used to take blood samples from children are shown in Figure 9.1 and the tourniquet applied to the child. Figure 9.2 shows the calculation of the haemoglobin concentration by the HemoCue method. Figure 9.3 shows the separated plasma which was transferred to a cryovial, labelled and subsequently storage in the fridge at the clinic, see freezer microtube storage box in Figure 9.4.

Figure 9.1 - Laboratory materials and blood specimen collection



Figure 9.2 - HemoCue technique and blood samples centrifugation



Figure 9.3 - Transferring serum/plasma into the cryovial tube.



Figure 9.4 - Plasma sample storage for zinc analysis



9.1.3 Statistical Methods

All data were collated in Excel (version 2010), and transferred to SPSS (version 20) for analysis. Descriptive statistical means and standard deviations were calculated. The interpretation of the effects were examined using p-values. A 2x2 ANOVA was conducted by group and gender for haemoglobin. An independent sample t-test, and ANOVA was used to compare plasma zinc levels across groups.

Hierarchical multiple regression was used to assess the 2 groups Fe and MMN, after controlling for the variables of clinical measurements, weight, height, Socioeconomic status (SES), to predict levels of intelligence (WPPSI total IQ scale).

9.2 RESULTS

9.2.1 Clinical Assessment

The results of the clinical assessment and diagnosis are presented in Table 9.1 and Table 9.2 respectively.

Table 9.1 - Clinical measurements of blood pressure, cardiac frequency and respiratory rate

Variable	Fe (n=96)			MMN (n=84)			P value
	$\bar{x} \pm SD$	Min.	Max.	$\bar{x} \pm SD$	Min.	Max.	
Systolic blood pressure (mmHg)	93.70 ± 4.76	85	110	93.55 ± 5	80	105	0.772
Boys	94.06 ± 5.04	85	110	94.19 ± 5	85	105	
Girls	93.27 ± 4.42	85	105	92.73 ± 4.94	80	105	0.588
Diastolic blood pressure (mmHg)	48.59 ± 3.05	45	60	48.92 ± 2.93	45	60	
Boys *	48.85 ± 3.34	45	60	49.78 ± 2.97	45	60	0.005
Girls	48.30 ± 2.63	45	55	47.84 ± 2.51	45	50	
Cardiac frequency (beats/minute)	73.58 ± 2.56	70	82	73.61 ± 2.46	70	80	0.994
Boys	73.23 ± 2.14	70	78	73.66 ± 2.65	70	80	
Girls	74 ± 2.96	70	82	73.54 ± 2.24	70	78	0.348
Respiratory rate (breaths/minute)	18.22 ± 0.77	17	22	18.10 ± 1.06	14	22	
Boys	18.23 ± 0.85	18	22	18.17 ± 1.17	14	22	
Girls	18.20 ± 0.67	17	20	18 ± 0.91	16	20	

Fe: iron group, boys *n*52, girls *n*44; MMN: multiple micronutrients group, boys *n*47, girls *n*37

* Significant gender differences

For the iron group, the mean systolic blood pressure was 93.70 (SD 4.76) mmHg, and the mean diastolic blood pressure was 48.59 (SD 3.05) mmHg. For the multi-micronutrient group, the mean systolic blood pressure was 93.55 (SD 5.00) mmHg, and the mean diastolic blood pressure was 48.92 (SD 2.93) mm Hg, which were within normal levels for an age of 3-5 years (SBP 80-110, DBP 50-80 mmHg).

Respiratory rates were also within normal levels for a child aged 3-6 year old (22-34 breaths/min). Similarly cardiac frequency (beats/minute) levels were in the normal range of 60-140 beats/minute for children aged 3-5 year old.

The multivariate effect was significant by gender, Wilks' $\lambda = .946$, $F(1, 175) = 2.432$, $p = 0.049$, for the diastolic blood pressure ($p=0.005$) where measurements were higher in boys than in girls. There was no effect by group, Wilks' $\lambda = .992$, $F(1, 175) = 0.338$, $p = 0.852$, and not significant effect by gender*group interaction for blood pressure, cardiac frequency and respiratory rate, Wilks' $\lambda = .979$, $F(1, 175) = .930$, $p=0.448$.

Further, results from the hierarchical multiple regression was used to assess the 2 groups, after controlling for the variables of clinical measurements, weight, height, Socioeconomic status (SES), to predict levels of intelligence WPPSI total IQ scale. In the iron group: R^2 change was 52.8% which means that 52.8% of the variance is explained by the variables on clinical measurements blood pressure, respiratory, cardiac frequency and haemoglobin ($F(34, 54) = 1.77, p = 0.03$). 41.4% of the variance is explained by the variables of SES, ($F(26, 62) = 1.69, p = 0.05$). The variance that was explained by SES was statistically significant in the iron group, with a significant contribution made by the material of the house floor ($beta = -0.25, p = 0.05$), age of the child ($beta = 2.35, p = 0.0$), and MUAC measurement ($beta = 0.521, p = 0.03$). In the MMN group, clinical measurements blood pressure, respiratory, cardiac frequency and haemoglobin explained 34.7% of the variance ($F(34, 39) = 0.61, p = 0.93$). 32.2% of the variance was explained by the variables of SES which did not make a significant contribution, ($F(26, 47) = 0.86, p = 0.66$).

Table 9.2 - Healthy children, anaemic children and other diagnoses

	Fe (n=97)						MMN (n=86)					
	Boys (n=53)		Girls (n=44)		Total		Boys (n=49)		Girls (n=37)		Total	
	%	N°	%	N°	%	N°	%	N°	%	N°	%	N°
Healthy	73.6	39	81.8	36	77.7	75	63.3	31	78.4	29	69.8	60
Anaemic	13.2	7	9.1	4	11.3	11	16.3	8	10.8	4	14	12
Bronchitis	-	-	2.3	1	1	1	8.2	4	10.8	4	9.3	8
Rhinitis	13.2	7	6.8	3	10.3	10	12.2	6	-	-	7	6

Fe: iron group; MMN: multiple micronutrients group

The percentage of children who were healthy, anaemic or had bronchitis or rhinitis did not differ significantly by group, $X^2(3, N=183) = 7.52, p = .057$, or by gender $X^2(3, N=183) = 7.52, p = .096$.

9.2.2 Biochemical Assessment

Anaemic children were identified using the reference values in Table 9.3 . The results of the haemoglobin and plasma zinc concentration analyses are shown in Table 9.4 to Table 9.7.

Table 9.3 - Anaemia reference values

Population	Anaemia			
	Non Anaemia*	Mild	Moderate	Severe
Children 6-59 m	110 or higher	100-109	70-99	Lower than 70

* Haemoglobin in grams per litre (WHO, 2011).

Table 9.4 - Haemoglobin (Hb) measurements

Variable	Fe (n=96)			MMN (n=85)			P value
	$\bar{x} \pm SD$	Min.	Max.	$\bar{x} \pm SD$	Min.	Max.	
Hb(g/dl)	11.83 ± 0.83	10	13.8	11.96 ± 1.0	8.8	14.3	0.334
Boys	11.77 ± 0.80	10	13.8	11.83 ± 1.08	8.8	14.3	
Girls	11.89 ± 0.87	10.1	13.8	12.13 ± 0.90	10.6	13.7	

Fe: iron group, boys *n*53, girls *n*43; MMN: multiple micronutrients group, boys *n*48, girls *n*37.

No significant differences were found for haemoglobin measurements between the Fe group (M= 11.83 g/dl, SD =0.83) v. the MMN (M=11.96 g/dl, SD = 1) by independent sample *t*-test, *t* (179) = 0.97, *p* = 0.334. Further analysis using 2x2 ANOVA by group and gender, revealed no main effect by group, *F* (1,177) =1.12, *p*= .29, by gender *F* (1,177) =2.38, *p*= .13, and by group*gender interaction *F* (1,177) = .43, *p*= .52.

Table 9.5 - Percentage and number of anaemic children

Haemoglobin (g/dl)	Fe (n=96)				MMN (n=85)				P value
	Boys (n=53)		Girls (n=43)		Boys (n=48)		Girls (n=37)		
	%	N°	%	N°	%	N°	%	N°	
< 11 g/dl Anaemic	11.3	6	11.6	5	16.7	8	10.8	4	0.302
≥ 11 g/dl Non anaemic	88.7	47	88.4	38	83.3	40	89.2	33	0.915

Fe: iron group; MMN: multiple micronutrients group

The percentage of anaemic children was higher in the MMN group (14.1%) than the Fe group (11.5%), but differences were not significant by group : $X^2 (1, N=181)=0.29$, $p=0.59$).

Table 9.6 - Plasma [Zn] converted to $\mu\text{mol/L}$

Variable	Fe (n=96)			MMN (n=84)			P value
	$\bar{x} \pm \text{SD}$	Min	Max	$\bar{x} \pm \text{SD}$	Min	Max	
Plasma zinc ($\mu\text{mol/L}$)	18.99 \pm 4.26	10.71	29.07	18.83 \pm 4.05	10.71	33.66	0.792
Boys	19.18 \pm 4.38	11.48	29.07	19.11 \pm 4.65	11.48	33.66	
Girls	18.76 \pm 4.16	10.71	26.78	18.45 \pm 3.12	10.71	23.72	

Fe: iron group, boys $n=52$, girls $n=44$; MMN: multiple micronutrients group, boys $n=48$, girls $n=36$.

An independent-sample t -test indicated that plasma zinc was not significantly different for the iron group ($M=124.12\mu\text{g/dl}$, $SD=27.86$) than the multiple micronutrient group ($M=123.04\mu\text{g/dl}$, $SD=26.50$), or in $\mu\text{mol/L}$ for the iron group ($M=18.99 \mu\text{mol/L}$, $SD=4.26$) *v.* the multiple micronutrient group ($M=18.83\mu\text{mol/L}$, $SD=4.05$), $t (178) = 0.265$, $p = 0.792$. Further analysis by the two way ANOVA by group and gender yielded no main effect by group, $F (1,176) = .10$, $p= .76$, by gender $F (1,176) = .75$, $p= .39$, and by group*gender interaction, $F (1,176) = .04$, $p= .85$.

As zinc deficiency was defined as zinc concentration levels below $10.7 \mu\text{mol/L} \sim 69.93 \mu\text{g/dl}$ (Gibson, 1990) or less than $68 \mu\text{g/dl}$ (López de Romaña et al., 2005). No zinc deficiency on average in the current study in preschool children, except that more girls had plasma zinc concentrations in the lower range, particularly those who were only supplemented with iron, but differences between groups were not significant ($p=0.558$). Girls *vs.* boys (23% *vs.* 21% in the iron group, 14% *vs.* 17% in the multiple micronutrient group).

Table 9.7 - Lower vs higher plasma [Zn] concentrations per group

	Fe (n=95)						MMN (n=84)						P value
	Boys		Girls		Total		Boys		Girls		Total		
	%	N°	%	N°	%	N°	%	N°	%	N°	%	N°	
Plasma zinc ($\mu\text{g/dl}$)													
$\geq 70 \leq 95$	21.2	11	23.3	10	22.1	21	16.7	8	13.9	5	15.5	13	0.558
$> 95 \leq 220$	78.8	41	76.7	33	77.9	74	83.3	40	83.8	31	84.5	71	0.641

Fe: iron group, boys $n=52$, girls $n=43$; MMN: multiple micronutrients group, boys $n=48$, girls $n=36$

The average percentage of children in the lower range of plasma zinc concentrations was larger in the Fe group (22.1%) than in the MMN group (15.5%). Plasma zinc sub-analysis using the cut-off point of plasma zinc concentration below 70µg/dl ~ 10.7µmol/L revealed that zinc deficiency was 1% (1 out of 95) in the Fe treated group and 1.2 % (1 out of 84) in the MMN treated group, with no significant difference between groups: $X^2(1, N=179)=0.08, p=0.93$).

When regression analysis was conducted to evaluate the plasma zinc levels and intelligence score (WPPSI total IQ) for the whole sample, this resulted in the model fitting of $R^2=0.033$ (3.3%). R square was not close to 1 which means the relationship was not very strong. The regression equation for total IQ was:

$$\text{WPPSI total IQ} = 0.083 * \text{Plasma zinc} - 97.4$$

Equations can be used to predict any value of Y for any value of X. When regression analysis was conducted per each group, the R squared was also not close to 1. Model fitting for the Fe group was: $R^2=0.050$ (5%), and for the MMN group was: $R^2 = 0.018$ (1.8%). Regression equations were:

$$\text{Fe: WPPSI total IQ} = -0.098 * \text{plasma zinc} + 98.4$$

$$\text{MMN: WPPSI total IQ} = -0.065 * \text{plasma zinc} - 96.1$$

9.3 SUMMARY OF CLINICAL AND BIOCHEMICAL ASSESSMENT

This study found cases of anaemia in both Fe (11.5%), and MMN group (14.1%) and more girls had plasma zinc concentrations in the lower range particularly those who were only supplemented with iron. Identified Zn deficiency was under 1.2%, with no significant difference between groups. Clinical assessment measurements were within the normal range. The clinical and biochemical assessment will be discussed further in 10.2.3

CHAPTER 10. –

GENERAL DISCUSSION

Chapter 10 - GENERAL DISCUSSION

The first two aims of this thesis were to perform a comprehensive review of literature on zinc intake, status and health outcomes, and to perform meta-analyses on this data so as to identify gaps in knowledge for a range of age groups. This was described in chapters 3, 4 and 5. While some studies report a positive effect of zinc intake/status on cognitive function, others report mixed results, and the evidence regarding the effect of zinc intake or status on cognitive function is lacking and inconclusive. Although the meta-analysis of studies conducted in children showed no effect of zinc supplementation on any of the identified cognitive domains, there were some small indicators of improvements in cognition following zinc supplementation when all studies across all population groups are considered as a whole. However there was a paucity of well-designed long term trials investigating the relationship between zinc intake/status and cognitive function in humans. These findings will be discussed in section 10.1, how well the aims were achieved, together with the implications.

The third aim of this thesis was to investigate long term impact of zinc supplementation during infancy on cognition, one of the health outcomes from the first part considered to be of particular importance and with only a limited number of studies. This was described in chapters 6, 7, 8 and 9, considered two groups, one supplemented with iron alone, and the other with multiple micronutrient supplementation. We observed no significant difference between the two groups in terms of anthropometry, weight, height and stunting. Similarly both groups met dietary recommendations for zinc and the majority of nutrients. In terms of the cognitive tests we observed little difference between the 2 groups, with the exception of some specific subtests. We found some cases of anaemia in both groups, with low levels of zinc deficiency, and clinical measurements in the normal range. These findings will be discussed in section 10.2, how well the aim was achieved, together with its implications.

The overall thesis discussion is summarised in section 10.3, and the limitations of the work carried out in this thesis is assessed in section 10.4. Finally we give recommendations and further directions to extend this work in section 10.5.

10.1 SYSTEMATIC REVIEW AND META-ANALYSES

This study was part of a series of systematic reviews undertaken by the EURRECA network of excellence, which was established to review and align the methodologies for the derivation of micronutrient dietary reference values (DRVs), in Europe (van't Veer et al., 2013). Zinc was selected as a priority micronutrient because of the significant heterogeneity in zinc DRVs across Europe and of the inadequacy of zinc intake especially in the most vulnerable groups (Lowe et al., 2013).

To fully understand the role of the different systematic reviews, first the I-S association was assessed in all life stage groups, then the I-S-H association was assessed for various health outcomes for each life stage group. In order to examine these associations, both RCTs and observational (cohort and cross-sectional) studies were utilized. RCTs were included because they are considered to be evidence of the highest grade in the hierarchy of study designs, as they intend to avoid bias by using random sample assignment (Pogue and Yusuf, 1998), although observational studies tend to overestimate treatment effects (Concato et al., 2000), these were included to provide a robust evidence of the I-S-H interrelationships.

The search protocol used status biomarkers and intake methods that were identified as being “best practice” (van't Veer et al., 2013, EURRECA, 2013). Health outcomes were selected using an eminence approach (Dhonukshe-Rutten et al., 2010, Fairweather-Tait, 2011, Serra-Majem et al., 2009, Roman-Vinas et al., 2009). Regarding biomarkers of status, although plasma and serum, urine and hair zinc concentrations were included as markers of zinc status, only plasma and serum zinc concentration were reported frequently and data was sufficient to be used in the meta-analysis. There is a paucity of adequate zinc biomarkers, but plasma zinc is currently the most widely used and accepted biomarker of zinc status, despite poor sensitivity and imperfect specificity (Hambidge, 2003). Plasma zinc is an insensitive indicator of zinc status in children. For example children with low plasma zinc may benefit from zinc supplementation and some children with normal plasma zinc status and poor growth may also have growth-limiting zinc deficiency and exhibit a growth response to zinc supplementation (Fung et al., 2002). Serum zinc levels also tend to diminish with age and differences in serum zinc levels between males and females persist even in old age (Rea, 1989). Interpretation of the changes in plasma zinc concentration as an index of zinc absorption

has also definite limitations (Solomons and Jacob, 1981). Serum or plasma zinc concentration is considered the best available biomarker for populations at risk of zinc deficiency (de Benoist et al., 2007). The studies that were included in the zinc systematic review reported plasma zinc as a measurement of zinc status.

The dose response approach for the zinc intake and zinc status is a novel approach; main findings from the zinc systematic review (I-S relationship) were that doubling the zinc intake, increased serum/plasma zinc status by 9% in children, by 3% in pregnant women, by 1% in lactating women and by 6% in adults. Zinc dietary recommendations have relied mainly on the factorial approach, which mostly depends on physiological data, where this data is limited in populations such as infants. This method combines zinc requirement to replace obligatory losses with additional needs of zinc such as foetal growth during pregnancy and growth and development from childhood to adulthood. In this method there is a need to consider zinc bioavailability as a modifiable factor by the amount of phytate content in foods. The zinc intake-status-health dose response method is a complementary approach that consists of examining zinc intake levels that will result in optimal health and disease prevention, such as the measurement of zinc biomarker in response to zinc dietary intake or the assessment of clinical disease health outcome/endpoint in relation to zinc intake or status. However the difficulty of this approach is the lack of a sensitive zinc biomarker (Lowe et al., 2013). In order to derive dietary reference values the most robust data need to be integrated using a systematic approach that take into consideration the micronutrient, the population and the health outcome (Dhonushe-Rutten et al., 2013, Matthys et al., 2011). The new generated zinc systematic review in intake-status association provided useful information for dietary zinc recommendations which, together with the factorial approach, could be used as qualitative or quantitative evidence when setting future zinc recommendations.

The systematic review of the relationships between dietary zinc intake and/or status with health outcomes yielded a large number of studies for each identified health outcome, however there was great heterogeneity in the way the health outcomes were reported between different studies. This meant that for many of the health outcomes, meta-analysis of the relationship with zinc intake or status was not possible, except for two notable exceptions which were growth and cognitive function. Findings from the cognitive function review in children and adults evaluating the relationship between zinc intake/status and indices of cognitive function through a meta-analyses showed no

significant Intake-Status/Health association on indices of intelligence, executive function and motor outcome. However, if considering all studies that were included in the review but not in the meta-analysis, namely 6 studies in children (Gibson et al., 1989, Cavan et al., 1993, Tamura et al., 2003, Caulfield et al., 2010, Christian et al., 2010, Pongcharoen et al., 2012, Gao et al., 2008, Lam et al., 2008, Murray-Kolb et al., 2012), then there is some indication of a positive association for I/S-H relationship. The zinc systematic review in cognitive function showed that some studies report a positive effect of zinc intake/status on cognition (Penland et al., 1997, Sandstead et al., 1998, Gewa et al., 2009, Tupe and Chiplonkar, 2009, Ortega et al., 1997, Stoecker et al., 2009, Hubbs-Tait et al., 2007, Umamaheswari et al., 2011, Maylor et al., 2006), and others reported mixed results, namely that a positive effect is reported on one subtest and negative in the others (Gibson et al., 1989, Cavan et al., 1993, Tamura et al., 2003, Caulfield et al., 2010, Christian et al., 2010, Pongcharoen et al., 2012, Gao et al., 2008, Lam et al., 2008, Murray-Kolb et al., 2012).

Data extracted from studies undertaken in the adult and elderly and in the pregnant and lactating women were too limited to convincingly assert overall conclusions with reference to cognition however data was more readily available from studies in children. When data from the included studies was synthesised on the effect on zinc supplementation on cognition the results were equivocal, where some reported a positive association and others not. The association between zinc intake/status and cognitive outcomes for the different population groups are outlined below.

10.1.1 Children

The review in studies in children identified the effect of zinc supplementation on cognitive outcome and the psychological tests used in each study. For instance, the study by Penland et al. (1997) found a significant improvement in performance ($p < 0.05$) subtests from the Cognition Psychomotor Assessment revised (CPAS-R) in measurements of visual perception (design matching), visual memory (delayed design matching), concept formation (oddity) and gross motor skills (tapping and tracking). Sandstead et al. (1998) also found that zinc with micronutrients improved more performance, motor skills (circular tracking test) and concept formation and abstract reasoning (oddity shapes) ($p < 0.01$) than with micronutrients or zinc alone. A significant association between zinc intake and higher gains in digit span (DS) total test scores was reported by Gewa et al. (2009), where a child with a daily high intake of

zinc, gained 0.73 more points in the DS total test. Zinc supplementation and zinc rich foods significantly increased the scores for memory and the Raven progressive matrices ($p<0.05$) in adolescent girls (Tupe and Chiplonkar, 2009). Murray-Kolb et al. (2012), found a significant overall effect of zinc supplementation on intelligence and executive function test in children aged 7-9 year old. Hubbs-Tait et al. (2007) reported that zinc explained significant variance ($p=0.01$) in McCarthy scales on children's abilities verbal score and Umamaheswari et al. (2011) reported a significant improvement in verbal ($p=0.05$), non-verbal memory ($p<0.01$) and intelligent quotient ($p=0.05$) score after zinc supplementation of 5mg/day in the form of syrup in children aged 9-11 year old and only verbal memory ($p<0.01$) was improved in children aged 6-8 year old.

10.1.2 Pregnant Women

For pregnant women, only one study was selected where Stoecker et al. (2009) found a positive correlation between plasma zinc status and the Raven coloured progressive matrices test, which is a non-verbal validated measure of cognitive function (Raven, 2000) ($r=0.27$, $p<0.008$).

10.1.3 Adults and Elderly

For adults, significant positive associations were found for spatial working memory ($p=0.030$) (Maylor et al., 2006) with intakes of 15 and 30 mg/d of zinc. Ortega et al. (1997) found a positive correlation between intakes of zinc from the diet and the Mini Mental State ($r=0.1349$, $p<0.05$) and Lam et al. (2008) reported lower plasma zinc status were related to poorer performance on test of concentration in women aged 60-94 year old ($p=0.008$). Taking all of the above studies, there is some small evidence on the positive association of zinc and cognitive function.

With all studies taken together (adults and children) there is some evidence of a positive association of zinc and cognitive function, but further high quality randomized controlled trials are needed to examine this association.

In summary, zinc systematic reviews and meta-analyses, provided estimates of the dose-response relationships using data on zinc intake and biomarkers of zinc status from intervention and observational studies. In terms of the purpose of this systemic review, the first aim of this thesis was achieved. The systematic reviews also highlighted the

need for further research in vulnerable populations and met the second aim of this thesis.

10.2 EMPIRICAL RESEARCH

The third aim of this thesis was to evaluate the effect of multiple micronutrients including zinc on cognitive, social-emotional, growth, and anaemia in pre-school children. As shown in the systematic review and meta-analysis, there were only limited studies available, particularly in health outcomes, hence the need for further studies to corroborate the findings from the systematic review. There are several aspects of the empirical study that merit discussion.

The study design allowed us to approach this study in 2 stages, firstly the clinical, biochemical and anthropometric assessment, and secondly the nutritional assessment and psychological tests. This study design was built from an initial intervention study in infants, carried out by UNICEF and IIN in 2010, details of which are shown in Section 6.2. There is evidence that Peruvian children from poor background in the periurban city of Lima may be zinc deficient as their diet consumptions have inadequate zinc content (Duggan et al., 2003). In the current study, children were randomly assigned to either iron (Fe) or multi-micronutrient (MMN) supplements at infancy. Of these children, 200 were randomly selected to be tested at 4 year old from the Fe and the MMN groups.

The purpose of the study was to investigate whether micronutrient supplementation including zinc had a long term impact on cognitive and socio-emotional development in pre-school Peruvian children, in comparison to children who received only iron supplements. Research on the beneficial effects of micronutrient nutrition and supplementation for brain function and behaviour is important because micronutrient deficiencies continue to be a modern health concern in both developing and developed countries. The evidence for the effect of multiple micronutrients on cognition is limited (Christian and Tielsch, 2012). For instance, the review by Allen et al. (2009) demonstrated a significant positive effect of multiple micronutrient supplementation on motor outcome but not on mental outcome in children. This was shown in 4 studies (Faber et al., 2005, Black et al., 2004, McGrath et al., 2006, Adu-Afarwuah et al., 2007)

that were not part of the meta-analysis. The systematic review in RCTs by Eilander et al. (2010) showed that the pooled effect from 17 studies that assessed the effect of multiple micronutrient on cognitive performance in children aged 5 to 16 year old, was not significant neither for the fluid intelligence (ability to reason) nor the crystallized intelligence (knowledge built over time). However there were 4 studies (Manger et al., 2008, Schoenthaler et al., 1991, Vazir et al., 2006, Wang et al., 2003) that did show significant overall effect for academic performance ($p=0.044$). Similarly the review by Best et al. (2011) revealed that some studies did report a positive effect of multi-micronutrient fortification on cognitive outcomes of memory (Osendarp et al., 2007, Van Stuijvenberg et al., 1999), fluid intelligence and other cognitive outcomes (Solon et al., 2003).

This research attempted to understand more fully the effect of micronutrient supplementation on functions attributed to the central executive which are involved in a child's development. In this study evaluation of the executive functions in Peruvian children was conducted through a series of psychological tests. Nutritional assessment was also conducted through anthropometry, biochemical zinc analysis, clinical assessment, and dietary assessment. I have confidence in the data from the empirical study as a whole, as it was collected by trained psychologist, paediatrician, nurse and health worker.

10.2.1 Anthropometry

The growth of a child is an indicator of health (WHO, 1995, Gelandar, 2006) and there are factors that affect growth including family history, gender, nutrition, sleep and health status (USDA, 2013). Children recruited onto the current study were from a poor background from the outskirts of Lima and growth was measured during infancy and at the pre-school age. It is important to monitor child growth and prevent growth failure especially during the critical period of the first 2 years of life. Usually children who had growth failure, tend to have poorer cognitive and schooling outcomes later in life and their future potential to be productive members of society is also reduced (Martorell and Nguyen, 2010).

The anthropometric data in the current study revealed that the two groups were comparable at the beginning of the initial (2010) study, having a height-for-age that did not differ significantly between the Fe and the MMN groups. Subsequently, the follow up study at 3-4 years failed to reveal any significant improvement in growth between the two supplementation groups. However, many of the children were below the standard weight and height according to the WHO Child Growth Standards (WHO, 2006a). The normal weight and height for a child aged between 3 to 4 years are 14.6-16.7 kg and 94.9-102.9 cm in boys and 14.1-16 kg and 93.9-101.6 cm for girls (WHC, 2013). However measurements in Peruvian children differ, for instance Checkley et al. (2003) reported that Peruvian children at 24 months of age, were 2.5 cm shorter than the US National Center for Health Statistics/World Health Organization growth reference. This concurs with the current study where the children fell between the 25th-50th percentile for the height for age percentile (de Onis et al., 2007).

MUAC has been used in developing countries as an inexpensive and useful tool for detecting child malnutrition (Roy, 2000). The advantage of MUAC is the portability of the tape and that a single cut-off value (12.5 or 13 cm) can be used for children under 5 years of age (WHO, 1995). In our study data MUAC did not differ significantly between the Fe and MMN groups. MUAC measurements for the Fe and the MMN group were over 16 cm. The World Health Organization (WHO) defines severe acute malnutrition as a mid-upper arm circumference (MUAC) < 11.5 cm, or a weight-for-height z-score (WHZ) below -3 (Mogeni et al., 2011). Comparing our MUAC measurements in pre-schoolers it can be deduced that overall children were not malnourished.

10.2.2 Quality of the Children's Diet

In the current study, diet was assessed using the 24 hour recall (n=40 analysed by WinDiets), food frequency questionnaire and diet history. This was a descriptive qualitative analysis to determine dietary patterns of Peruvian young children. It was noted from the general dietary data that children's diets were comprised of cereals in the morning, cooked soup or rice based dish with animal based protein source (most commonly chicken) for lunch, and for dinner the majority had lunch leftovers. Children had a high consumption of fruits (especially seasonal fruits), up to 7 portions per day. Fruits and vegetables are important sources of various nutrients including vitamin A and C, and fibre (BCC, 2013).

The 24-hour recall showed that intake of zinc and iron did not differ between the 2 groups ($p = 0.264$), and Peruvian nutritional guidelines. Energy requirements were above normal levels and energy contributions from macronutrients were within dietary recommendations. This was an unexpected finding because micronutrient deficiency and anaemia are still common in Peru (López de Romaña et al., 2005, Penny et al., 2004), particularly amongst low income families. The sample population did come from the outskirts of Lima, where there is a history of nutritional deficiencies. However, the study sample appeared to have an adequate diet. This may be a beneficial consequence of the improvement of the Peruvian economy and strong economic performance in recent years, with an average Gross domestic product (GDP) growth rate of 6.4% between 2002 and 2012, during this decade, Peru's income per capita has increased by more than 50% (Bank, 2013).

There were no significant differences for other micronutrients that are associated with cognition such as iodine, folate, selenium. A recent systematic review by (Bougma et al., 2013) have shown that iodine deficiency had a substantial impact on mental development but more well-designed RCTs that includes the role of iodised salt, are necessary to quantify iodine's contribution to delayed children's mental development. In Peru, iodization of salt is mandatory at 30-40 ppm. (Internut, 2013). As one group received iron supplementation, limited evidence exists on children's mental performance and psychomotor development that suggests that iron supplementation in infants may positively influence children's psychomotor development, but it does not appear to change their mental development or behaviour (Szajewska et al., 2010). Current evidence indicates that the effect of MMN supplementation on children's intellectual development seems beneficial but a thorough evaluation of this evidence is still lacking (Eilander et al., 2010).

Pre-school children have high nutrient requirements relative to their size as they are growing and developing rapidly, and are usually very physically active (BCC, 2013). From 4-6 years, requirements for energy, protein, all the vitamins and minerals increase except for vitamins C, D and iron (HN, 2011). Good regular dietary habits are the best way to ensure optimal mental and behavioural performance in children (Bellisle, 2004). From the FFQ, Peruvian children consumed abundant cereals and grains, similar to the findings by Bermudez and Tucker (2003) in dietary patterns in Latin American

populations that indicated that main food contributors of energy were cereals and root and tubers. Rice was consumed very often and it is known that insufficient concentrations of zinc and iron are found on this staple (Johnson et al., 2011, Mayer et al., 2008). In addition, cereals have a high phytate content that can inhibit zinc absorptions (Krebs, 2000, Lonnerdal, 2000). From the diet history undertaken, a dietary pattern was that dinner was the same as lunch, dinner is usually a filling meal in the Peruvian tradition, consisting of different types of dishes made with potatoes, corn, meat, or seafood (country, 2013, IMB, 2010). Zinc and iron food sources came from chicken (49.7%) on average consumed more than twice per week, beef (30.9%) twice per week, offal (29.8%) twice per week, and legumes (40.9%) more than twice per week.

Regarding diet and supplementation, a good diet in addition to micronutrient supplementation will add to the intake of zinc/iron and influence the response of plasma status.

10.2.3 Clinical and Biochemical Assessment

Blood pressure, cardiac frequency and respiratory rate are key vital signs that are used to assess the physiological status of children in clinical settings (Fleming et al., 2011). In the current study, blood pressure, respiratory rate, cardiac frequency and heart rate were measured. The results for the children were all in the normal range. For the respiratory rate, (<40/min) according to the WHO reference, and from 60-140 beats/min, and on the 1st centile based on published centile charts (Fleming et al., 2011). Measurement of SBP is preferred to DBP because of its greater accuracy and consistency (De Swiet et al., 1989).

To define anaemia, the WHO cut off points were used, where children aged 6 months to 5 years are considered to be anaemic if their haemoglobin levels are below 11 g/dl (WHO, 2008), and low zinc was defined as a serum concentration below 10.7 µmol/L or less than 70 µg/dL (Smith et al., 1979) . Haemoglobin levels were used to estimate the incidence of anaemia in children of 3-4 year old in the population of Villa El Salvador, Peru; anaemia was defined by blood haemoglobin level, as either present (<11.0 g/dL) or absent (>11.0 g/dL). Results from the current study revealed that 10% of the children in the iron supplementation group and 14% of the children in the MMN group were anaemic based on haemoglobin concentrations.

The biochemical analysis of plasma zinc indicated that the children in both groups had normal levels of plasma status, and no zinc deficiency was noted. Iron and zinc interact and affect both iron and zinc status (Wasantwisut et al., 2006) so perhaps the comparable levels of zinc observed in both groups is showing this in our study also. However, on average, girls have lower plasma zinc concentrations than boys, but differences by gender were also not significant ($p=0.39$), (mean and SD) in the MMN group, girls (18.45 ± 3.12), boys (18.83 ± 4.05).

In the original supplementation study in infants in 2010, iron and multiple micronutrient supplementation had a positive effect in reducing anaemia in both groups and lead to an improvement of biochemical indicator of micronutrient status, plasma zinc in the MMN group, after 6 months of supplementation (Zavaleta personal communication). In the current study, no significant differences in either haemoglobin ($p = 0.334$) or plasma zinc concentrations ($p = 0.792$) were found in either Fe or MMN groups. Our findings are similar to the study by Penny et al. (2004) that reported no significant differences in plasma zinc status ($p=0.97$) nor haemoglobin levels ($p=0.18$) when comparing children who were given zinc alone (10 mg Zn/d) or zinc plus vitamins and minerals against placebo, however, they did observe significant changes in plasma and haemoglobin concentrations from baseline to 6 months ($p<0.0001$). Significant differences in change of zinc status and haemoglobin concentrations were also reported by Smuts et al. (2005), that assessed 1134 infants from 4 countries (Indonesia, Peru, South Africa and Vietnam) who were randomized in 4 groups to receive either weekly multiple micronutrient ($n 283$), daily multiple micronutrient ($n280$), daily iron ($n288$) and placebo ($n283$). In this study a difference in plasma changes were observed between placebo .vs. daily multiple micronutrient, $p<0.05$, and differences in changes in haemoglobin concentrations was noted between placebo .vs. daily iron, daily multiple micronutrient and weekly multiple micronutrient. Another study by Rosado et al. (1997), which was a double-blind randomized community trial, reported that zinc levels were increased significantly in 219 Mexican pre-schoolers when given 20 mg Zn/d alone in the form of zinc methionine ($16.8 \mu\text{mol/L}$), and when given combined 20 mg Zn/d plus 20 mg Fe/d as ferrous sulphate ($18.3 \mu\text{mol/L}$) for 12 months. This resembles our study which reported average similar plasma zinc concentrations of $18.9 \mu\text{mol/L}$ when children were given iron alone and $18.8\mu\text{mol/L}$ when given multiple micronutrients. The efficacy of daily multiple micronutrients in improving plasma zinc status ($14.7 \mu\text{mol/L } p<0.05$) has also been shown by Untoro et al. (2005) in infants aged

6-12 months old that were given placebo, daily iron alone, daily/weekly multiple micronutrient supplementation and significant changes in plasma zinc status from baseline for all four groups were also reported.

10.2.4 Psychological Tests

A wide range of psychological tests were used to evaluate cognitive function in pre-school children, especially intelligence, executive function and social-emotional behaviour. These tests were WPPSI, simple tests (day/night, 9 boxes and theory of mind), and BITSEA, where each test has its own applicability therefore a variety of tests were used. The application of a battery of cognitive tests to young Peruvian children is, to the knowledge of the author, the first time this has been employed, with the idea being that overlap between tests provides potential corroboration of results. Additionally the theory of mind, 9 boxes and BITSEA tests had never been applied to such a sample before, and the protocols for application were developed in collaboration with IIN. To understand how these functions may influence cognitive development, each of the tests will be considered in turn.

WPPSI

The concept of intelligence has always been difficult to define and the concept remains elusive and debatable. All cultures acknowledge individual differences in the degree to which people exhibit intelligent behaviour, which means the way people approach and solve problems, one definition being “fundamental faculty” which includes, judgment, practical sense, initiative, and adaption to circumstances (Weinberg, 1989). Measuring cognitive performance involves cognitive factors such as cognitive efficiency, stores of acquired knowledge and thinking abilities, whereas non-cognitive factors are facilitator-inhibitor, which are internal and external (Flanagan et al., 2000). IQ scores are prominent indicators of a child's level of cognitive functioning. Early learning (learning that occurs outside the home and before school entry) or attendance at pre-school has been associated with positive benefits in children (Sylva and Wiltshire, 1993). In our study we also found a correlation between children attending nursery and a higher IQ ($r=.27, p=0.013$).

WPPSI evaluates intelligence, according to Colom et al. (2010), the areas of the brain considered most important for human intelligence are the discrete regions of the

dorsolateral prefrontal cortex and the parietal cortex. In other words the frontal and parietal lobes likely comprise the main processing areas for intelligence. The frontoparietal network may also be relevant for both intelligence and working memory (Gray and Thompson, 2004).

For the WPPSI tests, the main results were that a significant difference between the iron and the multi-micronutrient group was found in only one verbal IQ subtest: the *vocabulary* subtest ($p=0.028$), where girls in the multi-micronutrient group had higher vocabulary scores than girls in iron group, which may indicate a subtle positive association. In the study by Quereshi, mean verbal IQs for boys were significantly ($p<.05$) higher than those for girls (Quereshi and Seitz, 1994), in contrast, in the current study, gender differences were found for the mean verbal IQ subtest named *sentences*, where girls had higher scores than boys ($p=0.039$).

Regarding the WPPSI subtests, as each scale (Verbal/Performance) comprises 5 subtests and 2 optional subtests, in our study not all 12 subtests were applied, children were tested on 4 subtests from the verbal and 5 subtests from the executive/performance scale and a prorated overall IQ score was obtained. One of the subtests was object completion / puzzles, which are tasks that provide immediate feedback about mastery (meeting some goal or performance standard), or no mastery, and in this manner, providing information relevant to intellectual competence (Stipek and Iver, 1989).

In the current study the impact of socioeconomic status (SES) on the indicators of cognitive functioning was assessed and revealed that WPPSI IQ score was positively correlated with parent's education ($p<0.01$) and family income which is a traditional indicator of SES, was also correlated with the WPPSI test children's performance ($p<0.05$). Family income and parental occupation has been shown to be highly correlated with academic achievement (McLoyd, 1998). Children are dependent on others, and therefore they depend on their family's economic circumstances. Children experiencing poverty during their preschool and early school years are more likely to have low performance rates and experience learning disabilities and developmental delays (Brooks-Gunn and Duncan, 1997). Poor and low-SES children had been shown to have lower academic performance than non-poor and middle class children (McLoyd, 1998).

There were some overlaps for measurement of attention and memory however the range of cognitive tests performed gave us corroboration of the findings from the WPSSI tests by using the other tests too, namely the day/night, 9 boxes, and theory of mind tests (discussed below). This leads to further confidence in the results obtained.

Simple Tests

For the Day-Night Stroop test children needed to hold two rules in mind: they were instructed to say “day” when shown a black card with a picture of a white moon and stars, and to say “night” when shown a white card with a picture of a yellow sun (Gerstadt et al., 1994). This test measures inhibition, namely the ability to suppress a dominant automatic response, but inhibition also involves interference control, directed forgetting, emotional control, and motor control (Nigg, 2000). However a lack of inhibitory control and susceptibility to interference are limiting factors in pre-school children’s performance (Anderson, 2010). Also a child’s ability to inhibit a dominant response, as measured by the Day-Night Task, improves with age (Smith et al., 2013). The Day-Night test has previously been used in other studies for children aged 3.5 to 7 years (Diamond et al., 1997, Gerstadt et al., 1994), and this is a reason why this test was considered in this study. In our study, differences between the Fe and MMN groups were not significant ($P = 0.768$). However, for those who completed the 16 trial test of the Day-Night Stroop test, there were significant gender differences, where girls had higher scores than boys in both groups ($P = 0.011$).

The 9-boxes test exercised the working memory and attention of the child and activated part of the memory system involving use of short-term memory, in the prefrontal cortex (Kane and Engle, 2002). Short-term memory is defined as a type of memory used to retain information for a short time that includes the working memory component, which is a sort of mental workspace or sketchpad in the mind that is used to manipulate information in consciousness. Working memory is a more complex construct than short-term memory (Engle et al., 1999) and its measurements is important as it plays a causal role in children’s developing skills and knowledge, mainly in the area of literacy (Gathercole and Alloway, 2004). The measure of working memory at school entry (4-5 y in the UK) has been found to predict success in national assessments of scholastic abilities up to 3 years later (Gathercole et al., 2003). Because, working memory is a system that underlies human thought processes and maintains and stores information in

the short term (Baddeley, 2003), it is likely that some tested children did not store information about which box was opened previously, children often wanted to find the animals but forgot the question task. For this test, no significant differences were found between the Fe and MMN groups ($P = 0.894$). When the longest correct run of the test was assessed this also yielded no significant differences ($P = 0.138$).

The day/night and 9 boxes tests evaluate inhibitory control and working memory respectively. Inhibitory control and working memory act as basic executive functions which make possible the development of more complex executive functions like problem-solving (Carter et al., 2003b). Executive function is an umbrella term for a set of mental inter-related processes that link past with present experiences, and is used to perform a series of activities which are responsible for a defined goal such as directed behaviour, including planning, initiation of activity, organized search, strategizing, self-regulation, mental flexibility, attention, remembering details, use of feedback, managing time and space, and impulse control (Welsh et al., 1991, Anderson, 2002). It seems to be regulated by the prefrontal regions of the frontal lobes, but mixed evidence that does not support a one-to-one relationship between executive functions and frontal lobe activity (Alvarez and Emory, 2006). Working memory (WM) and inhibition are practically inseparable constructs that comprise the core of executive function (Best et al., 2009). Children between the ages of 3 to 5 year old frequently display a spurt in performance on tasks of inhibition and WM.

The theory of mind is the ability to attribute mental states to oneself and others (Wimmer and Perner, 1983). The question of why children generally fail false belief tests up to the age of four still remains. The development of children's theory of mind may be possibly explained by the result of an increasing working memory capacity but this is not yet explored (Davis and Pratt, 1995). The theory of mind task is complex because it requires the integration of two representations; of the situation; the reality and the false belief (Halford, 1993). Thus, conflicting ideas need to be understood concurrently (Flavell et al., 1990). The theory of mind is the ability to explain and predict other people's behaviour by attributing to them independent mental states such as beliefs and desires (Gallagher and Frith, 2003), it is likely that this task proved challenging to young Peruvian children as the majority failed the task, and this may be due to the intricacy of the task and understanding of the question.

There is a strong relationship between the theory of mind and language development (Wilde, 2005, Astington and Jenkins, 1999), some children did not know colours (e.g. the 2 cupboards, one was green, other was blue), which made the task difficult, others were shy to speak, and others pointed their answer. The understanding of belief and, relatedly, understanding of mind, exhibit genuine conceptual change in the preschool years, and it is widely accepted that by 4 years of age most normally developing children have acquired an understanding of mind (Wellman et al., 2001), this means that a child is able to understand a person's actions in terms of that person's desires, thoughts, emotions and beliefs, which could be either true or false beliefs (Astington, 1993, Mitchell, 1996, Cutting and Dunn, 1999). However, in the current study, most children (aged 36-48 months old) did not understand the theory of mind question, though, the older children performed better in the task. Most children thought the question was "*where is the chocolate located?*" the question was: "*Where do you think Maria will look for her chocolate?*" Most of the answers were "*the chocolate is in the blue cupboard*", so maybe children thought "*where is the chocolate?*" and so the answer will be in the blue cupboard. If asking the question in another way: "*where did Maria leave her chocolate?*" then the child answered correctly, it was in the green cupboard (the child said green). It may be speculated that the failure on the theory of mind/working memory/inhibitory task was likely due to the maturity of the brain regions or a prefrontal deficit.

An initial trial of the theory of mind maybe suggested using firstly some deceptive items, such as the example of the Crayola crayon box that contained sticks instead of crayons, and the child asked "What is in the crayon box?"(Frye et al., 1995). This is a suggested pre-test which could have been used and this could have been followed by the theory of mind test that was administered in this study, involving the chocolate item. One observation at the clinic was that conditions of peacefulness during the test were not always achieved, especially with so many children at the clinic, the doors were opened or test interrupted on occasion, which may have affected child concentration and the test delivery. According to Evans (2006), the physical environment can have an influence on child development, for instance Wachs (1978) reported that male children aged 12-14 mo living in noisier homes had deficits in intellectual functioning, whereas older male children aged 15-23 mo seemed unaffected by noise levels in the home.

Piaget is known for his observations that children think differently about things at different ages (Grossman, 2012). Our study was conducted in pre-school children, Piaget's interpretation was that preschool children are still fundamentally intuitive, or "preoperational" (Houde, 2000), hence at the preoperational stage. Developing a complete understanding of the effect of zinc will likely involve understanding its effect in these different cognitive development stages (sensorimotor, pre, concrete and formal operational), rather than simply categorising them as children.

BITSEA

The Brief Infant-Toddler Social Emotional Assessment (BITSEA) assessed the child's behaviour. Briggs-Gowan et al. (2013) suggests that the BITSEA test may be a valuable tool to aid screening, identification, and assessment of early social-emotional/behavioural problems. One component of BITSEA is the social-emotional competence, and assessment of competence is critical to identify children whose developmental expectations are not meeting the social emotional domain (Briggs-Gowan and Carter, 2006). Findings in the current study showed significant differences in the BITSEA competence (BITSEA/C) scores, for example that girls had higher competence scores than boys ($p = 0.002$). This is in line with the gender differences indicated by Briggs-Gowan et al. (2004), that states that (BITSEA/C) differed with gender, with lower competence scores in boys than girls in each age group and with higher BITSEA problems (BITSEA/P) scores in boys than girls. From the present study, no differences were found between the Fe and MMN group for the BITSEA problem (BITSEA/P) scores ($p = 0.212$). The BITSEA test is usually applied to children aged 12-35 mo, and in Turkey the BITSEA test was applied to children from 12-42 mo (Karabekiroglu et al., 2009). In the present study, BITSEA analysis was based on developed cut off points for children aged 36-48 mo.

One component of BITSEA assessed Problem Behaviour and the social-emotional aspect, (BITSEA/P). There is evidence that nutritional deficits can have an impact on individual behavioural development via psychosocial pathways (Wachs, 2000), for instance, one pathway is the role of nutrition in parent-child interaction such that undernourished parents will have less energy to provide adequate stimulation to their children (Grantham McGregor et al., 1984). Zinc nutritional deficit is believed to affect

children's behaviour; however, the evidence is inconclusive (Grantham-McGregor and Ani, 1999).

As a general comment, the application of the cognitive tests would not be described as problematic for such a young sample, though, as described above, there were inconveniences in the application process. However since we were applying the test with a trained psychologist, it was possible to overcome these difficulties, and consequently have confidence in the data obtained.

To the best of our knowledge the 9 boxes, theory of mind, day/night tests and BITSEA tests had not been often applied to young Peruvian children (no publishable studies), whereas WPPSI has been used before as a measurement of intelligence (Caulfield et al., 2010, Salinas-Pielago et al., 1998). The present research findings were in line with the results by Taneja et al. (2005) that zinc supplementation in young children aged 12-18 months did not affect the mental or psychomotor development index scores.

The current study also measured risk factors for cognitive function and social-emotional development, these were: maternal depression, malnutrition, micronutrient deficiency. Risk factors that affect about 25% of young children in developing countries are: malnutrition, inadequate stimulation or learning activities and micronutrient deficiency (iron, iodine), other risk factors are maternal depression, violence exposure (Walker et al., 2007). IQ scores have also been associated with the social environmental risk factors, hence more attention should be placed to the complex of environmental contexts of the child (Sameroff et al., 1987). Furthermore, brain development is affected by a less stimulating, less emotional and less supportive environment or deprivation of positive experiences, which will have an impact on cognitive, social and behavioural delays. These children will have difficulty in dealing with complex situations in adulthood (WHO, 2009) and these factors are especially applicable in developing countries (Walker et al., 2007).

Impact of Family structure, family background

Socio-economic status, data on SES indicators (education, occupation) was also assessed. Family background has a significant impact on the development of theory of mind (Cutting and Dunn, 1999). In Peru and in many less-developed countries, Patriarchal patterns still remain (Berkman et al., 2002). Regarding the mother's

occupation, there is little evidence that maternal employment during a child's infancy may harm children's development (Cooksey et al., 2009). Han et al. (2001) indicated a negative effect of early maternal employment on children's cognitive and behavioural outcomes over time, mainly at 1st-year. An adverse effect of mother's employment on children's intellectual ability of 4 year old children was also shown by (Desai et al., 1989) but only in boys from higher income families. In our study only 8.2 % of mothers were working part-time and the relationship between mother's occupation and cognitive function intelligence outcome WPPSI total IQ score was not significant ($p= 0.286$), where 0.7%, of the variance in WPPSI total IQ score is explained by the mother's occupation, thus in our study we can conclude that the mothers occupation did not have an effect on cognition.

10.2.5 Hypothesis of Empirical Study

The hypothesis for the study was that children given multiple micronutrient supplements during infancy will have higher cognitive and behaviour function scores than those given iron alone, and that differences between groups for cognitive tests will be significant. As discussed in 10.2.4, analysis of the cognitive test data revealed significant group differences ($p=0.028$) only for the cognitive WPPSI subtest vocabulary, but not significant for the remaining battery of tests. This concurs with the study by Chellappa and Karunanidhi (2012) in adolescents (17-19yrs) where daily supplementation with iron (60mg/d), zinc (30mg/d), and iron plus zinc (60 mg Fe/d, 30mg Zn/d) for 4 months, benefitted only certain cognitive tests (mental speed, when given Fe or Zn alone or both FeZn; visual memory, when given both FeZn supplementation). In their study adolescents who received iron or iron plus zinc scored higher than when receiving zinc alone, following a 4 month supplementation period. Contrast this with the current study where infants were supplemented for 6 months from the age of 6-17 months. It may be important to consider the timing and length of supplementation, since brain growth during infancy and early childhood may have a greater potential for impact through diet than brain growth during foetal life (Gale et al., 2004), and zinc is critically important for cognitive development during early childhood (Coulson and Vitetta, 2009).

In summary, the empirical study has been performed successfully on a sample of 200 children in Peru, providing very useful information in terms of growth and cognitive development following supplementation, fulfilling the third aim of the thesis.

10.3 OVERALL SUMMARY

From the series of zinc systematic literature reviews, databases were built to provide a resource for deriving DRVs. The reviews, collation and ultimately the analyses of data was a valuable strength in this first phase of this research. Intake-status was found to be a strong relationship and the majority of studies included in this category were Randomised controlled trials (RCTs), which are considered the most rigorous way of determining whether a cause-effect relationship exists between treatment and outcome (Sibbald and Roland, 1998). The broad systematic review on intake and status (I-S) provided an estimate of the dose-response relationship between zinc intake and plasma/zinc status. Changes in plasma zinc concentration were assessed as a function of the amount of additional zinc provided. In children doubling of zinc intake increased plasma/serum level by 9% (Moran et al., 2012a). During pregnancy and lactation doubling the zinc intake increased zinc concentration in serum or plasma by 3% and by 1% respectively (Moran et al., 2012a) and in adults and elderly doubling of zinc intake increased plasma/serum zinc by 6% (Lowe et al., 2012). Analysis of the intake-status-health (I-S-H) outcome for zinc highlighted the paucity of data on the relationship between zinc and cognitive function in human, which was a very important identified gap in knowledge and there was a need to investigate cognitive function health outcome in children and adult population. The systematic review results on zinc and cognitive function revealed that there was no significant overall effect of zinc supplementation on any of the cognitive outcomes assessed in children (intelligence, executive function and motor skills). The series of zinc systematic review and meta-analyses was the starting point to explore the I/S-H association in a field setting, then the empirical study was undertaken to evaluate this health outcome of interest.

Results of the empirical study in Peru showed that, with the exception of vocabulary subtest verbal IQ WPPSI scale in girls, there were no significant differences in the cognitive test scores between children that received MMN and those that received Fe alone during infancy. For the vocabulary subtest verbal IQ WPPSI scale, higher scores

were seen in girls from the MMN group when compared with the girls in the Fe group. In contrast, there were some significant differences by gender for the following psychological tests: sentences subtest verbal IQ WPPSI scale, Day/night test, BITSEA Competence (BITSEA/C) scores, where girls had higher scores than boys and BITSEA Problem (BITSEA/P) scores, where boys had higher problem scores than girls.

Thus, findings from the psychological tests from this study indicate that Multi-micronutrient supplements including zinc (MMN) had no long term additional effects on cognitive and social-emotional development in Peruvian preschool children function at preschool age, compared to Fe alone, except for one subtest from the WPPSI verbal scale, which was the vocabulary subtest. Other co-variables should be taken into consideration such as the critical period of micronutrient deficiency, the timing and length of micronutrient supplementation and the response to psychological and socio-environmental factors.

MMN has been shown to be effective in preventing anaemia, iron, and zinc deficiencies. In our field study MMN including zinc was used to evaluate cognition, and as zinc status levels were in the normal range, then our finding support the efficacy of MMN supplementation to improve zinc status (López de Romaña et al., 2005). From the initial study, children in the MMN group have improved plasma zinc status. Regarding the dietary pattern of Peruvian pre-school children, a distinct pattern was that children usually had for dinner the same food from lunch or with a slightly variation. Left overs were quite common, a large variety of fruit consumption intake, all sorts of herbal tea and in some cases children had soup and a main course for lunch. The empirical study highlighted the low performance of pre-schoolers, which may shed new light on the need to stimulate cognitive processes in young children.

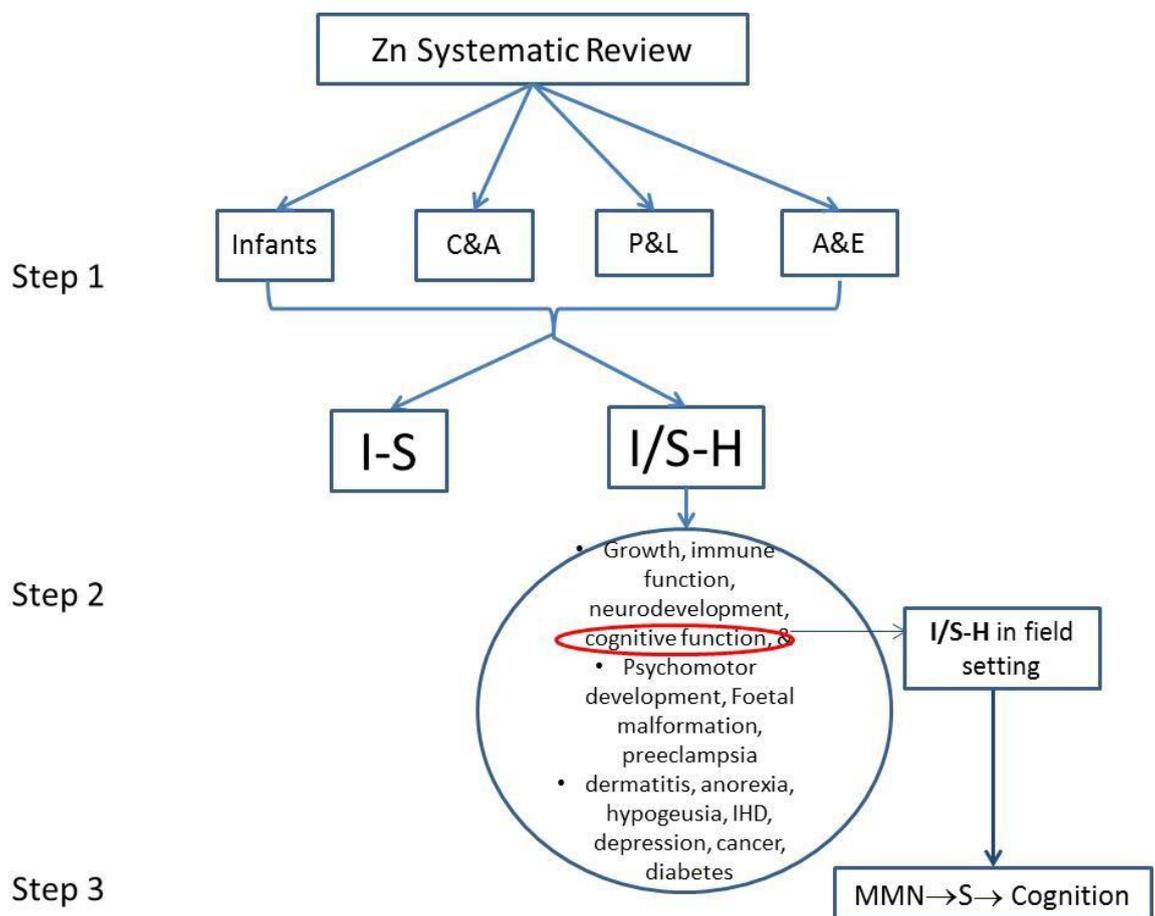
Linking the systematic review with the empirical study, the empirical study corroborated the findings from the systematic review with reference to growth. The zinc systematic review that looked at the effect of zinc supplementation in children's growth aged 1-8 year old, found that there was no effect of zinc supplementation on growth. The empirical study also found that multiple micronutrient supplementation had no additional long term effect on growth when compared with iron supplementation alone. Regarding the cognitive health outcome, the zinc systematic review on indices of cognitive function found no significant effect of zinc supplementation on intelligence,

executive function and motor outcome of the 6 studies in children included in the meta-analysis. However when considering all 18 studies from all populations, 9 studies reported a positive effect of zinc supplementation and cognitive function. The empirical study was set to explore further the effect on cognition. The findings on the empirical study also showed no significant effect at long term of given multiple micronutrient supplementation during infancy on cognitive function in pre-school children.

10.3.1 PhD Synthesis

Figure 10.1 shows the summary of this thesis in 3 steps: a series of systematic reviews was achieved in various populations, findings from these reviews, showed a very clear I-S association, mainly from the randomised controlled trials in all clusters, the opposite occurred for the I/S-H association (Step 1).

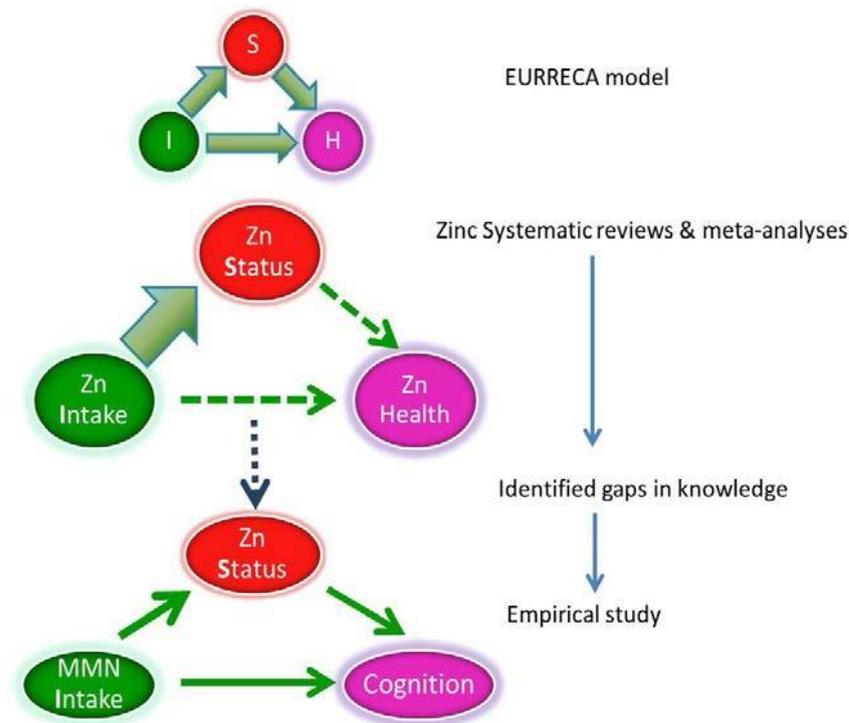
Figure 10.1 - PhD Synthesis



C&A: Children and adolescents; P&L: Pregnant and lactating women; A&E: Adult and Elderly; I-S: Intake-Status relationship; I/S-H: Intake/Status-Health; MMN: Multi-micronutrients; S: Status.

From the pool of health outcomes, the zinc systematic review showed limited studies in a priority health outcome which was cognitive function (Step 2). A new study was designed to test the existing model from step 1 and to evaluate one health outcome where gaps in knowledge were identified, in a population group. Then an international collaboration was built to test the I-S-H model in a field setting (Step 3).

Figure 10.2 - Application of the Intake-Status-Health model



The figure above shows the developed EURRECA model of the association approach for all micronutrients of the inter-relationships between intake-status-health outcomes relationships, using “best practice” guidelines that recommend measuring food intake using a validated 24 hour recall preferably on at least 2 non-consecutive days, FFQ and dietary records for a minimum of 3 days, using plasma zinc concentration as the most common reported biomarker of zinc status and a set of health indicators associated with zinc deficiency. The undertaken zinc systematic review revealed that there was robust evidence for defining the intake-status relationship, where plasma zinc concentration responded consistently to zinc supplementation both in children and adults, than the relationship between status-health and intake-health. However, limited numbers of studies were found for intake/status-health associations for which the data could be compared due to the diversity of the methodological approaches and outcome measures

reported. This provides a rich area for future research and exploration of micronutrients with regard to health outcomes in vulnerable populations, using more standardised methodology.

This study is the first of its kind exploring indices of cognitive function and diet in Peruvian children. The battery of cognitive tests applied in Peruvian children could be applied to other children from deprived background to measure different cognitive outcomes, diet and behaviour.

This research has contributed to the field interest regarding nutrition, micronutrients and cognitive function, with particular reference to zinc. Undertaking further research to examine in more detail the relationship between micronutrient intake, status and cognitive function in combination with other external factors including parental education, provision of environmental stimuli to their children, will be most beneficial to vulnerable groups of society, especially those of lower socio-economic status and may influence policy makers.

10.4 LIMITATIONS

There are many methodological issues inherent to the empirical research, which is a great challenge (Sandstead and Lofgren, 2000, Penland, 2000), which are individual differences, gender and age that are extremely important concerns when designing a research and analysing results to assess the relationships between nutrient intake and behaviour and cognitive function.

Since child growth references from WHO were used, it is recognized the methodological limitations on this reference data, such as different anthropometric measure and different cut-off points. WHO or NCHS/WHO growth references were developed by the United States National Center for Health Statistics (NCHS), and were based on national survey data collected in the 1960s and 1970s. These references are past four decades, and data collected was only in one country, the United States. Thus there are conceptual, and practical problems in using WHO growth references (Wang et al., 2006), such as using patterns of child growth of a single country as a standard for all

other countries, meaning comparing growth in USA children with growth in Peruvian children.

In the empirical study a true control group was not present as both groups were treated, one group received iron and the other group received multi-micronutrients including zinc. Consequently the difference between the 2 groups was zinc+folicacid+vitaminC+vitaminA. In the original study design by Zavaleta et al., it was considered inappropriate to give no supplements to children in a population where there is high prevalence of iron deficiency anaemia (Creed-Kanashiro et al., 2000). Hence, one group was given iron supplements and the other group was given multi-micronutrients including zinc. Outside of the study children were not routinely given iron.

With reference to the sample of children, this may not be representative of the population as a whole, particularly of the most impoverished areas of the district of VES or nutritionally deficient children, with respect to social economic status and parental education.

With respect to dietary methods, one limitation was that the food frequency questionnaire did not collect information on portion sizes. The use of the FFQ was one general model but other types of questionnaire could have been developed specific to the population and of easy use for parents' response. There could have been errors in data collection as these are inherent of using these diet methods. Another limitation was the use of the WinDiets software where not all international foods are listed, so meals were compared with those that were more similar or by adding individual ingredients, also not all food portion sizes information was provided in the software package, hence the food portion size handbook was referred to (Crawley, 1988). It is evident that the compositional value of food composition databases differ on quality and it is difficult to make international comparisons because of variations in both nomenclature and the composition of foods; these are recognized source of errors in the food composition databases. The known limitations of the food composition databases are as follows: the variation in the composition of foods, the partial or limited coverage of either food items or nutrients, an inappropriate database (the UK and the US food composition tables are probably the most common databases used worldwide because of their availability in computerized form and the ample coverage of foods), errors in using the database, the differences in software and ultimately the limitations involved in

measuring food intake (FAO, 2003b). With reference to the 24 hour recall, this requires an experienced trainer in administering the interview, and in this case the author conducted the interview and was not an expert. Also it should be noted that in our study, the 24 hour recall was collected for 1 day and according to EURRECA “best practice” guidelines, a minimum of 3 days are recommended for dietary assessment.

Regarding biochemical analysis, haemoglobin is important as low values indicate a deficiency of iron, but this biomarker is neither specific nor very sensitive for the detection of iron deficiency (Hambidge, 2003). Some limitations and pitfalls in our study are recognized, such as using the plasma/serum zinc as a biomarker and haemoglobin as a marker of iron deficiency anaemia, instead of using the ferritin marker (μL). Due to cost limitations, only plasma/zinc was analysed in the sampled children, and not a ferritin analysis.

The lack of observable benefit of zinc on cognitive scores may have several explanations. There may be no effect of micronutrient including zinc on cognition; 6 months of micronutrient supplementation during infancy may be insufficient time for treatment effects to unfold; or micronutrient deficiency may affect specific behaviours or processes that are not detected with the series of psychological tests performed (WPPSI, day/night, 9 boxes, theory of mind, BITSEA). It is also possible that the effect of zinc on cognitive function may have been limited by its interaction with other trace minerals and vitamins, such as iron, folic acid, vitamin A, vitamin C.

As for the specific cognitive tests, the WPPSI-R scale is a psychometric test that has been shown to be a good reliable tool to evaluate intelligence and cognitive development on preschool children (Meio et al., 2001, Lichtenberger, 2005, Kaufman, 1990). However this test is not free of source of errors, for example Whitten et al. (1994) stated that incorrect IQs assigned by examiners resulted in one potential diagnostic error. 3 sources of errors identified were: examiner carelessness, inadequate training, and complex scoring procedures. In the current study, the examiner was a qualified experienced psychologist. Regarding the interpretation of the WPPSI test, the analysis by Gyurke et al. (1990) supports the interpretation of separate Verbal and Executive/Performance Scales and of a Full Scale Score. Bishop and Butterworth (1979), observed that children who refused to co-operate with one or more of the

WPPSI subtests were more likely than not to develop normally. In the current study, not all children were cooperative but most completed the administered WPPSI subtests and some children had lower scores for psychological tests, a low score in preschool children may also be due to tiredness and daytime sleepiness, e.g. sleep problems, fatigue and reduced alertness which has been shown to be caused by sleep disruption (Simola et al., 2010, Sadeh, 2007).

BITSEA is usually applied to children aged from 1-3 years of age. Children in our study were on average over 3 years of age. An alternative test could be applied in a future study, such as the Child Behaviour Checklist for 1.5 to 5 years of age (Achenbach and Edelbrock, 1983a).

The subsample (20% of the original sample, Fe n=97, MMN n=87) was representative of the original sample of 902 children from the children population living in Villa El Salvador. There was a good level of compliance as parents brought their children to the second session of the tests or those who failed to attempt were visited at their homes for a reminder. I believe it could be possible for the data to be generalized to the children living in Villa El Salvador and to children of Lima living on the coast.

10.5 RECOMMENDATIONS, AND FUTURE DIRECTIONS

The first 3 years of life are crucial in child development, as the child's brain is most sensitive to external factors such as the environment; consequently an adequate stimulation and nutrition are necessary at this stage. A balanced healthy diet and following practical nutritional guidelines in food preparation for 3-4 year old are advisable Crawley (2006). Efforts should be made to reduce malnutrition; adequate attention should be paid to nutrition in health and development policies (Pelletier et al., 1995).

With reference to children's cognitive performance, children would benefit from increased cognitive scores if they attended a nursery school, or receive suitable additional stimulation at home. The promotion of early psychosocial stimulation and adequate nutritional status could increase children's cognitive development (Santos et al., 2008). Intervention programs aiming to improve maternal knowledge, reduce

maternal stress, and promote maternal supportiveness of the infant during mother–child interaction may improve child cognitive development (Wacharasin et al., 2003). Because stunting has been associated with adverse cognitive development in childhood (e.g. children who were stunted at infancy and at childhood had lower scores tests than non-stunted children), then policy makers should focus on children’s catch-up growth leading to improvement of a child’s physical and intellectual development (Crookston et al., 2010).

The empirical study, as a follow-up to the original intervention study which involved supplementation of the sample population, revealed relatively low levels of stunting, anaemia, and zinc deficiency. This is a potentially important finding for future policy decisions in Peru, particularly regarding whether to provide supplementation, and moreover for what period of time. Infants in this study were supplemented for 6 months, so there could be a potential to advocate a small further increase in the length of supplementation together with providing nutrition education to parents, regarding rich sources of zinc and iron.

For children of the age range considered in this study, the chosen series of tests are recommended as employable by suitably trained personnel, and were not problematic. Should the sample age range rise much beyond that considered in our study, the choice of test should be revised to consider other tests (see Limitations above).

Implication of our findings for further research

Our study offers a contribution to the state of the art of our understanding of the nutrition of Peruvian children from the periphery of Lima and the influence of mother’s emotions and well-being on child’s cognitive development. Practical implications would be to improve the child’s environment and stimulate learning, provide nutritional education on sources of zinc and iron from the diet and, if considered, referral to a psychologist or to a language/behaviour development specialist.

Findings from the cognitive socio-emotional development, through the BITSEA test, assessment, implies that assessment of cognitive behaviour is fundamental to education (Geake and Cooper, 2003). Implications for education could be drawn from the psychological and nutritional assessment. Exploration of other areas of cognitive

behaviour should be considered. Identifying any possible deficit or to examine items associated with autism spectrum disorders for a pattern of problem behaviour.

Practical implications of this study will be to endeavour to provide education around the sources of zinc in the diet, nutrition at pre-school age, psychological advice to stimulate learning, and a happy environment for the child. As has been shown that the disadvantages of early childhood malnutrition last at least till adolescence, children present poor IQ levels, cognitive function and school achievement and higher behavioural problems (Grantham-McGregor, 1995).

Furthermore, it is important to focus on early development because evidence exists that disadvantaged children in developing countries who do not reach their developmental potential are less likely to be productive adults (Grantham-McGregor et al., 2007), and this has economic implications for the country. If science and politics listen to each other as in the case of the school breakfast in the Peruvian Andes by (Jacoby et al., 1998), then first improving the child's nutritional status, will also enhance cognition and improve their future education prospects.

Novel contributions

Novel contributions are the database created for the zinc systematic review and the findings of the Intake-Status/Health association through the dose response approach which may be a useful tool to deriving future dietary recommendations for zinc.

- The identified cognitive health outcome and the limitation of studies in zinc health outcomes that led to design and carry out the empirical study evaluating the long term impact of multiple micronutrients in cognitive function in pre-school children.
- The battery of cognitive tests applied in Peruvian children to obtain measurements of various cognitive domains.

Future Research

Further work on micronutrient research is needed, firstly to examine the effect of zinc micronutrient supplementation in combination with other micronutrients, and secondly to examine how zinc deficient populations respond to zinc supplementation/fortification (Black, 1998).

It would be interesting to see if there is an increase of intelligence over a period of time of the sample pre-school children tested and at later point in life, similar to the study by (Liu et al., 2012). A forthcoming study could assess the supplementation for a longer period to see any possible full benefit of zinc.

Future research is needed on all aspects of cognitive function, e.g. cognitive decline in elderly and cognitive development in children with inclusion of social-emotional outcomes and to consider external factors to improve the intellectual functioning especially of culturally deprived children. The Child Behaviour Checklist which is applicable for children aged 1.5 - 5 years of age (Achenbach and Edelbrock, 1983b) could be used in future study and the day/night test could be conducted on a computer.

More well-controlled trials studies would be needed to evaluate the critical period for zinc during early foetal neurodevelopment (Bhatnagar and Natchu, 2004) and during the stages of cognitive development. The evidence for improved neuropsychological performance among zinc-supplemented children is increasing (Black, 2003b), but further work is necessary to duplicate current studies and elucidate the effect on academic performance. Further research is required on the long term effect of zinc on cognitive development and psychomotor outcome, behaviour and malnutrition and to consider the effects of poverty on children's cognitive development, which occur early (Brooks-Gunn and Duncan, 1997). Consequently future work should look at poverty and the child's environment and how these influence their cognitive development and further research would be necessary to explore the I/S-H association in cognition in children.

Finally, critical issues to be considered for future research would include: single v. multiple micronutrient, the period of supplementation and the duration of supplementation, together with environmental indicators and psychological and social

factors (Wasantwisut, 1997). The period of supplementation of children remain a window of opportunity for more research in brain development and long-term studies could provide a valuable tool in public health policy.

CHAPTER 11. –

REFERENCES

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