

Long-chain polyunsaturated fatty acid nutrition in breastfed and complementary fed South African infants

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“I can do all things through Christ who gives me strength” – Philipians 4:13

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ABSTRACT

Introduction

Adequate intake of essential fatty acids (EFAs) and long-chain polyunsaturated FAs (LCPUFAs) during infancy are important for optimal growth and development. During the first six months of life, the growing infant receives LCPUFAs through breast milk or LCPUFA-supplemented infant formula. Therefore, lactating women should consume adequate amounts of preformed LCPUFAs to ensure adequate transfer of LCPUFAs to the infant through breast milk. However, the introduction of LCPUFA-poor complementary foods at the age of six months may lead to a reduction in blood LCPUFA levels. It is estimated that nearly 22 million infants in low- and middle-income countries are at risk of insufficient intake of LCPUFA. Particularly, LCPUFA intakes from breast milk and complementary foods in South Africa were estimated to be below the recommended intakes. Furthermore, studying different fatty acid (FA) patterns instead of individual FAs considers the different interactions and interrelations that may exist. Thus, in addition to determining the plasma phospholipid FA patterns of infants, the main objective of this study was to assess LCPUFA nutrition of South African infants during breastfeeding and the complementary feeding period.

Methods

In a randomised controlled trial, six-month-old infants from a peri-urban township were randomly selected to receive daily, a small-quantity lipid-based nutrient supplement (SQ-LNS) containing EFAs linoleic acid (LA) and alpha-linolenic acid (ALA) (SQ-LNS); daily SQ-LNS with both EFAs and the LCPUFAs docosahexaenoic acid (DHA) and arachidonic acid (AA) (SQ-LNS-plus), or a control group receiving no supplement. The SQ-LNSs additionally contained micronutrients. Plasma total phospholipid FA composition (% of total FAs) was measured at baseline (n=353) and at 12 months (n=293) (infants with FA data at 6 and 12 months, n=148). Baseline characteristics were assessed to determine associations of total plasma phospholipid FA patterns with feeding practices, growth and psychomotor development. Feeding practices and dietary intakes were assessed using a structured questionnaire and unquantified food frequency questionnaire, respectively. Psychomotor development was assessed using the Kilifi Developmental Inventory (KDI) and anthropometric measurements were measured in all infants.

In a cross-sectional study, red blood cell (RBC) total phospholipids as well as fore-, mid-feed and hind-milk samples of lactating mothers (n=100) of 2-4-month-old infants living in a peri-urban township were assessed. RBC total phospholipid and breast milk FAs were analysed by using quadrupole gas chromatography -electron impact-tandem mass spectrometry (GCMS/MS).

Results

In six-month-old South African infants, infants who received formula milk had higher scores, while breastfed infants had lower scores for the '*high EFAs with low DHA and AA*' and '*high MUFA and nervonic acid*' patterns. Infants who received breast milk, semi-solid foods or cow's milk but not infant formula milk, had higher scores, while formula-fed infants had lower scores for the '*high n-6 LCPUFA*' pattern. Infants who received breast milk and semi-solids had higher scores for the '*trans-FA pattern*'. The '*high MUFA and nervonic acid*' and '*trans-FA*' patterns were positively associated with psychomotor development, while no associations were found with growth.

Results from the intervention study showed the geometric mean (95% CI) plasma total phospholipid DHA and AA contents of 4.1 (4.0-4.3) and 11.5 (11.2-11.8) % respectively. Breastfed infants had significantly higher plasma DHA and AA than their non-breastfed counterparts. Infants receiving the SQ-LNS-plus had significantly higher plasma DHA (4.52 [4.3-4.9]) at 12 months than the infants in the control group (3.8 [3.6-4.0]). The effect size was higher in infants who no longer received breast milk ($\beta = 1.148$ [95% CI= 0.597, 1.699]) than in infants who were still receiving breast milk ($\beta = 0.544$ [95% CI= 0.179, 0.909]). The two SQ-LNSs had no effect on plasma AA.

Breast milk DHA and AA levels of 100 lactating women were (geometric mean [95% CI]) 0.25 (0.24, 3.71) and 0.81 (0.79, 0.83) %, respectively. Breast milk LA and ALA contents were 19.7 (19.1, 20.1) and 0.81 (0.77, 0.88) % respectively. Breast milk ALA and DHA levels were higher in mid-feed milk than in fore-milk while AA levels did not change during a feeding session. Breast milk DHA positively correlated with maternal RBC DHA. The association between breast milk DHA and maternal RBC DHA was stronger in fore-milk and became weaker in mid-feed and hind-milk. Fish consumption was positively associated with higher RBC EPA composition.

Conclusion

The results from this research suggest breast milk is a predominant source of n-6 LCPUFAs at the age of six months, however, South African infants receiving infant formula milk may be at risk of inadequate LCPUFA intake. Furthermore, continued breastfeeding and consumption of LCPUFA-enriched SQ-LNS improved plasma DHA status of infants at 12 months. Infants who no longer receive breast milk, particularly, may benefit most from a SQ-LNS enriched with LCPUFAs. The results from this research further indicate that during breast milk EFA and LCPUFA composition may change within a feed. Also, breast milk DHA was associated with maternal RBC DHA status which confirms that DHA is selectively transferred to the breastfeeding infant within-feed. However, the high content of LA observed in the breast milk of women in this population seemingly reflects a high dietary intake of LA. Thus, it is plausible that lactating women living in a

peri-urban township in South Africa may not be consuming adequate dietary sources for n-3 LCPUFAs.

Keywords: LCPUFA status, breast milk, feeding practices, FA patterns, psychomotor development, lactating women, complementary feeding, small-quantity lipid-based nutrient supplements, breast milk fatty acid concentrations, maternal fatty acid status, fatty acid patterns.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ALA	α - Linoleic acid
BMI	Body mass index
DGLA	Dihomo- γ -linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentanoic acid
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid
FA	Fatty acid
GLA	γ -linolenic acid
Hb	Haemoglobin
HREC	Health Research Ethics Council
HSRC	Human Sciences Research Council
KDI	Kilifi Developmental Inventory
LA	Linoleic acid
LAZ	Length-for-age z-scores
LCPUFA	Long-chain polyunsaturated fatty acids
LNS	Lipid-based nutrient supplement
MUFA	Monounsaturated fatty acids
NWU	North West University
PL	Phospholipid
PUFA	Polyunsaturated fatty acids
RBC	Red blood cell
SPSS	Statistical Package for Social Sciences
TAG	Triglycerides
SQ-LNS	Small-quantity lipid-based nutrient supplement
WAZ	Weight-for-age Z scores
WHO	World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Fatty acids (FAs) are important for the growth and development of infants. FAs provide energy to the human body and have several other structural and physiological functions. FAs have important roles in vision, skin integrity, wound healing, heart health, cognition and immune responses (Chen *et al.*, 2013). The long-chain polyunsaturated fatty acids (LCPUFAs), docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:4n-3) and docosapentanoic acid (DPA, 22:5n-3) are n-3 FAs that are important for growth, development and general health (Mozurkewich *et al.*, 2010). DHA accumulates rapidly in the brain and retina during the early life and therefore has a major role in visual development and cognitive function (Makrides *et al.*, 1995; Birch *et al.*, 2005). With an adequate intake of EPA, infants are assured of improved immunological, inflammatory and metabolic outcomes (Bailey, 2010; Kaur *et al.*, 2011; Patterson *et al.*, 2012). Arachidonic acid (AA, 20:4n-6) is an omega-6 FA that is vital for brain, cognitive and behaviour development (Schuchardt *et al.*, 2010; Hadley *et al.*, 2016). However, while AA also accumulates in the brain, it is also distributed widely throughout other tissues and vital organs in the body (Calder, 2015).

Certain FAs cannot be synthesised by the human body but are obtained directly from the diet because humans lack the *desaturase* enzymes necessary for the synthesis of these FAs *de novo* (Innis, 2003; Innis, 2008). These FAs are linoleic acid (LA; 18:2n-6) and α -linolenic acid (ALA; 18:3n-3) and are known as essential fatty acids (EFAs). Inadequate intakes of LA and ALA result in dermatitis, hair loss, abnormal platelet aggregation and impaired cognitive development (Hansen *et al.*, 1958; Hansen *et al.*, 1963; Kidd, 2007). ALA and LA can be metabolised and converted to the LCPUFAs EPA, DHA, and AA, respectively. However, this process is limited in infants (Innis, 2007; Uauy & Dangour, 2009).

1.1.1 Fatty acid concentrations in breast milk

Breast milk is considered the most suitable form of nourishment for infants during the first six months of life (Sala-Vila *et al.*, 2005; Brenna *et al.*, 2007). Breast milk contains all the vital nutrients, and the lipid fraction is very important in meeting the infant's nutritional needs. Breast milk FAs constitute the biggest fraction of the total energy intake during infancy, providing an average of 44%, energy supply (Grote *et al.*, 2016). LCPUFAs constitute about 2 % of the total fatty FAs in breast milk (Andreas *et al.*, 2015). Thus, breast milk is the sole source of LCPUFAs in exclusively breastfed infants aged zero to six months.

FAs in breast milk are synthesised from the mother's endogenous stores, liver or breast tissue, as well as from her diet. The breast tissue is particularly rich in the lipolytic enzyme lipoprotein lipase. Therefore, during lactation, ingested FAs are absorbed and incorporated into the chylomicrons and then quickly transferred from the plasma into the breast milk (Jensen, 1996). Maternal gestational length, age, stage of lactation, maternal diet and long-term food habits were shown to be associated with the FA composition of breast milk (Larnkjaer *et al.*, 2006; Olafsdottir *et al.*, 2006, Torres *et al.*, 2006; Roy *et al.*, 2012). Hence, the composition of the breastfed infant's lipids in plasma tissues and cell membranes depends on the FA profile of breast milk lipids (Brenna *et al.*, 2007; Antonakou *et al.*, 2013).

Therefore, DHA levels in infant blood were found to be significantly correlated with the DHA levels in both maternal blood and breast milk, which also correlated with each other. In contrast, no correlation was noted for AA (Lauritzen & Carlson, 2011). Furthermore, a decrease in the maternal DHA status over a four-month period was accompanied by a proportional decrease in the infant's status. In addition, a higher product to precursor ratio in infants was noted, which could be a possible indication of the specific transfer of LCPUFA through the breast milk (Otto *et al.*, 2001; Jørgensen *et al.*, 2006).

The fat found in breast milk is variable and has been shown to change during a feed, between fore-milk and hind-milk (Mitoulas *et al.*, 2003; da Cunha *et al.*, 2005). Likewise, the FA composition of breast milk varies during the course of the day, throughout the first year of lactation, as well as from country to country and among regions (Marangoni *et al.*, 2000; Brenna *et al.*, 2007; Grote *et al.*, 2016). It is therefore very important that the different levels of variations are taken into consideration in order to determine the FA composition and, eventually, the FA intake of the breastfed infant (Mitoulas *et al.*, 2003). Although there is currently a lack of knowledge pertaining to infant FA intakes in low- and middle-income countries, researchers may rely on the FA composition of breast milk from well-nourished mothers as a potential guide for dietary recommendations for infants.

1.1.2 Fatty acid nutrition in breastfed infants

During the first six months of life, breast milk is the main source of lipids and FAs for the infant. The World Health Organisation (WHO) recommends that infants should be exclusively breastfed during the first six months of life, with timely introduction of complementary feeding and continued breastfeeding for up to two years and beyond (WHO, 2003). In addition to providing infants with an adequate supply of nutrients, breastfeeding has been associated with reduced risk of infections, obesity, type 2 diabetes and high blood pressure and improved performance in intelligence tests (Koletzko *et al.*, 2005; Agostoni, 2008; Hermoso *et al.*, 2010). During gestation,

the foetus receives LCPUFAs via the placenta from the mother. After birth and during infancy, the infant must also continue to receive an adequate amount of LCPUFAs to ensure optimal visual and cognitive development. Therefore, breast milk is highly recommended as the sole source of LCPUFAs. However, in the event that breastfeeding is not possible, the current recommendation is that infants are provided with infant formulas that have been supplemented with adequate DHA and AA (Koletzko *et al.*, 2008).

1.1.3 Fatty acid nutrition during complementary feeding

After the age of six months, it is important to maintain the balance between breast milk intake and complementary feeding. Even though breast milk provides the infant with much needed LCPUFAs, DHA and AA, the overall volume of breast milk intake decreases in order to allow capacity for nutrient-dense complementary foods (Forsyth *et al.*, 2017). However, inadequate amounts of DHA and AA in the newly introduced complementary foods may result in a reduction of dietary DHA as reflected in blood DHA levels. This reduction may also be due to a simultaneous reduction in breast milk intake in combination with intake of DHA-poor weaning foods (Birch *et al.*, 2005; Rise *et al.*, 2013). Thus, it is important that a continued supply of DHA and AA during the complementary feeding period is maintained and this can be achieved by ensuring that complementary foods contain adequate amounts of FAs. In this regard, new supplements, including micronutrient powders and lipid-based supplements (LNSs) are currently being investigated for their potential to fill the nutrient gaps of infants fed complementary foods, thereby supporting healthy growth and development (Bhutta *et al.*, 2013).

1.2 PROBLEM STATEMENT

The South African Early Childhood Development (SA-ECD) Review (Hall *et al.*, 2016) states that over a fifth (22%) of the children under five years in South Africa are stunted in growth and that stunting is the most prominent form of malnutrition in the country. Research has shown that inadequate EFA intake could be inversely associated with linear growth of children aged between six and 18 months (Adu-Afarwuah *et al.*, 2007; Phuka *et al.*, 2008; Iannotti *et al.*, 2014). In particular, a small quantity lipid nutrient-based supplement (SQ-LNS) has recently been shown to improve linear growth, thereby reducing the incidence of stunting, during a six-month intervention (Matsungu, 2016; Muslihah *et al.*, 2016). It is therefore only recently that strategies of supplementation to combat and prevent malnutrition in other countries have included FAs.

Furthermore, in the past, most data on breast milk FA intakes were restricted to the first week of life (Agostoni *et al.*, 2000; Marangoni *et al.*, 2000). In addition, the changes that occur in breast milk composition during feeds have not been described in South Africa. However, these data may be relevant to assess the relationship between LCPUFA intakes, status and outcomes of early

childhood development, such as psychomotor development. The study of the composition of breast milk, which contains both DHA and AA, has recently become the current bench mark for early nutrition (Forsyth *et al.*, 2017). Therefore, information on the total FA composition of breast milk is important for the evaluation and determination of the physiological FA intake by the infant. Since there is limited knowledge on the lipid composition and amounts of FAs supplied to the infant through lactation in South Africa, there is a need for further studies to determine breast milk LCPUFA composition and LCPUFA intakes from breast milk.

Moreover, the mean red blood cell (RBC) DHA levels decreased by more than 20 % in children fed a diet without fish and rapeseed oil between four and 10 months of age (Libuda *et al.*, 2016). Strategies to optimise the DHA status are therefore needed, particularly during the period of complementary feeding because provision of DHA could potentially support stable DHA blood levels during infancy (Libuda *et al.*, 2016). Despite the very high prevalence of childhood infectious diseases in Southern Africa, the LCPUFA status of infants who are breastfed and those who receive complementary foods remains unknown. There is a scarcity of data on LCPUFA intake particularly in infants who should be assured of an adequate intake of LCPUFA for cognitive development and other positive health outcomes (Colombo *et al.*, 2013). Hence the growing interest in the composition and quality of lipid supply during infancy.

This PhD study will therefore assess the breast milk FA composition of lactating mothers and LCPUFA nutrition of South African infants during breastfeeding and complementary feeding and provide evidence on whether or not there are associations between the EFA status of six-month-old infants and psychomotor development. The results will also show whether a SQ-LNS containing EFAs only (SQ-LNS) and a SQ-LNS containing EFAs and DHA and AA (SQ-LNS-plus) given to infants at the age of six months can improve LCPUFA status by 12 months of age. If proven effective, this approach will be implemented in the developing world, particularly during the complementary feeding period.

1.3 AIMS AND OBJECTIVES

The overall aim of this PhD research was to assess LCPUFA nutrition of South African infants during breastfeeding and complementary feeding and was nested within two different (larger) studies.

Specific objectives

1. To assess plasma FA patterns of six-month-old South African infants and to determine their association with feeding practices, growth and psychomotor development.

2. To investigate the efficacy of the provision of a SQ-LNS containing the EFAs LA and ALA and a SQ-LNS containing EFAs, DHA and AA, from six to 12 months, in improving LCPUFA status of South African infants.
3. To assess the FA composition of breast milk sampled at three time points within-feed, and to determine associations with RBC FA status in lactating South African mothers of 2-4-month-old breastfed infants.

1.4 RESEARCH DESIGN

This PhD project was nested within two larger studies; the first study was a randomised, controlled trial (Tswaka trial) with the aim to investigate the effects of two different novel SQ-LNS on linear growth in infants aged six months to 12 months. Within this larger research study, a cross-sectional analysis of baseline data (six months) was carried out to assess plasma FA patterns [by means of principal component analysis (PCA) and factor analysis] of six-month old infants in relation to their feeding practices, and to determine associations with growth and psychomotor development, to achieve study objective 1. Within this larger study, the PhD project further assessed whether a SQ-LNS containing EFAs, or a SQ-LNS containing EFA, and the LCPUFAs DHA and AA, given to infants aged six months can improve their LCPUFA status by the age of 12 months compared with control infants, to achieve study objective 2. The second larger study was a cross-sectional study, with the objective to assess breast milk LCPUFA composition and the LCPUFA status of lactating women and their infants from the Potchefstroom area in South Africa, to achieve objective 3.

1.5 ETHICAL APPROVAL

The intervention study (Tswaka trial) was approved with ethics numbers EC011-03/2012 and NWU-00011-11-A1 by the Ethical Committee of the Medical Research Council and the North West University (NWU), respectively. The trial is registered as a clinical trial at Clinicaltrials.gov registry (NCT01845610). Ethical approval was also sought from the Health Research Ethics Committee (HREC) of the Faculty of Health Sciences of the NWU for the cross-sectional study and was granted under the ethics number NWU-00016-13-A1. For the cross-sectional study permission was also granted by the Provincial and District Health Departments in the North West Province to recruit mother-infant pairs for this study at local health clinics. Supplementary ethical approval was granted for this PhD project (single study affiliated with larger studies) from HREC of NWU under the ethics number NWU-00333-16-S1.

1.6 RESEARCH TEAM MEMBERS AND THEIR ROLES

Table 1-1 shows the contributions made by each of the research team members

Table 1-1: Research team and contribution to this PhD thesis

Team members	Role
Prof. Marius Smuts	Promoter of the PhD student. Principal Investigator of Tswaka trial. Responsible for design and overall execution of the Tswaka trial. Provided guidance on interpretation of results. Co-author of all manuscripts.
Prof. Jeannine Baumgartner	Co-promoter of the PhD student. Principal Investigator of the cross-sectional study to assess breast milk LCPUFA composition and LCPUFA status of lactating women and their infants from the Potchefstroom area in South Africa. Responsible for design, quality control of laboratory analysis and overall execution of the study. Provided guidance on statistical analysis, interpretation of results and writing of the thesis. Co-author of all manuscripts.
LP Siziba	PhD student. Responsible for the analysis of breast milk LCPUFA composition and data analysis for the cross-sectional study. Assistance with data collection (field work) and data capturing, as well as responsible for statistical analysis of breast milk FA and plasma FA data for the Tswaka randomized controlled trial. Responsible for data analysis, interpretation of results and writing of thesis. First author on all manuscripts.
Dr Linda Malan	Responsible for FA analysis for the cross-sectional study and supervisor of Tsitsi Chimhashu whose Honours project was part of the cross-sectional study investigating red blood cell FA status. Co-author of one manuscript (Chapter 5).
Prof. Mieke Faber	Co-Principal Investigator of Tswaka trial. Questionnaire development, fieldworker training, data coding and analysis for dietary data. Guidance regarding interpretation of results and dietary data on second manuscript. Co-author of two manuscripts (Chapters 3 and 4).
Marinel Rothman	One of the study coordinators of the Tswaka trial. Involved in questionnaire development and training of fieldworkers. Supervised data collection and quality control of dietary data, feeding practices and psycho-motor development. Co-author of one manuscript (Chapters 3 and 4).
Tonderayi Matsungu	One of the study coordinators of the Tswaka trial. Supervised data collection and quality control of anthropometric data. Co-author of two manuscripts (Chapters 3 and 4).
Adriaan Jacobs	Implemented the method for breast milk analysis and performed biochemical analysis of FA analysis in plasma, RBCs and breast milk. Co-author of two manuscripts (Chapter 3 and 5).
Dr Cristian Ricci Biostatistician	Assisted in statistical data analysis and provided guidance on statistical analysis and interpretation. Co-author of one manuscript (Chapter 3).
Tsitsi Chimhashu	Involved in data collection for the cross-sectional study as part of her Honours project which focused on FA status in red blood cells. Co-author of one manuscript (Chapter 5).
Sicelosethu Siro,	Involved in data collection for the cross-sectional study as part of her Honours project. Co-author of one manuscript (Chapter 5).
Dr Jennifer Osei	Involved in data collection for the cross-sectional study as part of her Masters project (focus on iodine concentrations). Co-author of one manuscript (Chapter 5).

The following is a statement from the co-authors confirming their individual role in the research and the three manuscripts.

I declare that as a co-author I have approved the above-mentioned article(s), that my role in the research, as indicated above, is a representation of my actual contribution and that I hereby give consent that the manuscript(s) may be used for Linda Siziba's PhD thesis.



Prof. C. Marius Smuts



Prof. Jeannine Baumgartner



Prof Mieke Faber



Dr. Namukolo Covic



Dr Tonderayi M. Matsungu



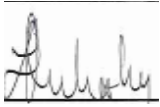
Dr. Marinel Rothman



Dr Cristian Ricci



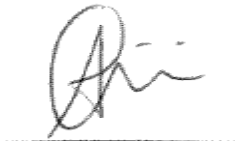
Adriaan Jacobs



Tsitsi Chimhashu



Sicelosethu Siro



Dr Jennifer Osei



Dr Linda Malan

1.7 THESIS OUTLINE

This thesis is presented in article format and is divided into six chapters. All relevant references that were used in chapters one, two, four, five and six are presented according to the requirements specified by the North West University (Potchefstroom Campus). The reference style used in chapter three is in line with the specifications of the journal chosen for publication.

Chapter 1 includes the background and motivation of the PhD research and states the aims and objectives and identified members of the research team and the author's contribution to the research.

Chapter 2 reviews the literature, provides background information for this PhD work and includes information on the importance of LCPUFAs in infancy, as well as LCPUFA nutrition in infants who are breastfed and given complementary foods.

Chapter 3 is the first article titled “**Associations of plasma phospholipid fatty acid patterns with feeding practices, growth and psychomotor development in six-month-old South African infants.**” This manuscript describes the plasma phospholipid FA patterns in six-month-old South African infants and their associations with feeding practices, growth and psychomotor development. The manuscript will be submitted to the Maternal and Child Nutrition Journal. The content and style guidelines are given in Addendum 1.

Chapter 4 is the second article titled: “**Efficacy of novel small-quantity lipid-based nutrient supplements in improving long-chain polyunsaturated fatty acid status of South African infants: A randomized, controlled trial.**” The manuscript describes results on the efficacy of a novel small-quantity lipid-based nutrient supplement in improving LCPUFA status of South African infants by the age of 12 months.

Chapter 5 is the third article titled: “**Breast milk and erythrocyte fatty acid composition of lactating women residing in a peri-urban South African township.**” This manuscript presents new data on the breast milk FA composition in South African women and its associations with maternal FA status; as well as within-feed differences breast milk FA composition.

Chapter 6 summarises the study and provides a brief and general discussion, as well as concluding remarks with reference to the set objectives, limitations of the study and recommendations for future studies.

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CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

Infancy is a critical stage of life for rapid brain growth that requires adequate and proper nutrition. Over the years, researchers have developed a growing appreciation of the value of promoting and supporting breastfeeding in order to improve infant growth and development (Innis, 2014). Any damage that is caused by nutritional deficiencies during early infancy could lead to impaired cognitive development and compromised educational achievement as well as low economic productivity (Kimani-Murage *et al.*, 2011).

Breast milk is universally recognised and recommended as the optimal source of nutrition for infants (Sala-Vila *et al.*, 2005; Brenna *et al.*, 2007). Amongst other nutrients, breast milk comprises fats/lipids which provide energy and importantly, fatty acids (FAs) that are crucial for the development of the central nervous system (Brenna *et al.*, 2007; Koletzko *et al.*, 2008). Of importance are the long chain polyunsaturated fatty acids (LCPUFAs), which cannot be synthesised *de novo* by the infant. These LCPUFAs are especially important for brain development as they have a functional and structural role in infant growth and development. The LCPUFAs DHA and AA are synthesised by elongation and desaturation of the precursor essential fatty acids (EFAs) α -linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6), respectively. However, this process is very limited in infants (Carlson *et al.*, 1986; Innis, 2007). Thus, the supply of LCPUFAs through breastfeeding or enriched formula is vital during neonatal development.

Breastfed infants are ensured of a source of LCPUFAs until they are weaned from breast milk at six months (Birch *et al.*, 2000; Sala-Vila *et al.*, 2005). However, at six months of age, infants are introduced to semi-solid or solid foods and thus are likely to have a reduction in dietary LCPUFAs as reflected in decreased blood levels (Birch *et al.*, 2000). This reduction in the infant's dietary LCPUFA intake may be due to a reduction in consumption of breast milk (Heinig *et al.*, 1993) combined with an increased intake of LCPUFA-poor weaning foods (Jackson & Gibson, 1989; Schwartz *et al.*, 2009).

This chapter elaborates on the importance of LCPUFAs during infancy and reviews the available literature on the FA composition of breast milk and LCPUFA nutrition in infants who receive breast milk, and during the complementary feeding period.

2.2 LIPIDS

Lipids are hydrophobic molecules that are soluble in organic solvents. Lipids are found in mammalian blood, adipose tissue and cell membranes. Lipids have functional and structural roles

in the biological membranes, regulate immune responses and act as an energy reservoir in the human body (Burdge & Calder, 2015). The structure of lipids ranges from simple hydrocarbon chains to more complex molecules, including triacylglycerols (TAGs), phospholipids (PLs) and sterols and their esters. There are different classes of lipids and each class differs according to the following structural components: the number of carbons and double bonds, the branching of the hydrocarbon chain, the position and orientation of double bonds and the addition of polar groups such as choline, inositol and ethanolamine, and glycosylation (Burdge & Calder, 2015).

TAGs and PLs in the diet provide the human body with fatty acids, and usually have saturated fatty acids attached to the carbon atoms. TAGs are made up of three fatty acids linked to one glycerol molecule by an ester bond, while in PLs, the phosphate group is the hydrophilic head attached to the third carbon of the glycerol (C-atom) (Burdge & Calder, 2015). PLs are more likely to bind to the LCPUFAs arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:4n-3), or docosahexaenoic acid (DHA, 22:6n-3); whereas TAGs preferably link to the EFAs linoleic acid (LA; 18:2n-6) or α -linolenic acid (ALA; 18:3n-3). PLs also aid in the easy passage in and out of cells of fat-soluble substances like vitamins and hormones (Whitney & Rolfes, 2018).

2.2.1 Fatty acids

FAs are the smallest functional unit of all lipids. FAs are comprised of a long chain of hydrocarbons with a methyl group at one end and a carboxyl group at the other end. The free methyl group is termed the omega (ω) end while the carboxyl group can form linkages with other molecules such as glycerol.

2.2.1.1 Classification of fatty acids

FAs can be classified according to the length and degree of saturation of the hydrocarbon chain. These characteristics determine how a certain FA will behave in food and during cooking or preparation as well as their contribution to health and disease. The length of the hydrocarbon chain in FAs varies between 2 and 30 carbons (Burdge & Calder, 2015). Different classes of FAs are shown below:

- ❖ Short chain FAs have fewer than 6 carbons
- ❖ Medium-chain FAs have 6 to 12 carbons
- ❖ Long-chain FAs have more than 12 carbons

FAs can also be classified as saturated FAs, monounsaturated (MUFAs) or polyunsaturated (PUFAs), depending on the degree of saturation. On the other hand, the degree of unsaturation is dependent on the number of double bonds. For instance, MUFAs have one and PUFAs have

two or more double bonds (Innis, 2007; Burdge & Calder, 2015). PUFAs can be classified as either n-3 PUFAs with the first double bond at the third carbon relative to the ω -end or n-6 PUFAs with the first unsaturation point at the sixth carbon.

Certain FAs cannot be synthesised *de novo* by humans but are essential for human development and health and thus need to be obtained from the diet. These are known as essential PUFAs and collectively comprise the parent essential FAs (ALA and LA) (Hornstra, 2000). These EFAs ALA and LA, function as precursor FAs for the LCPUFAs DHA and AA, respectively. ALA and LA are converted to their respective LCPUFAs DHA and AA by the *desaturase* and *elongase* enzymes (Figure 2-1). However, this conversion process is very limited in infants (Innis, 2007; Uauy & Dangour, 2009).

In humans, lipids are primarily absorbed in the jejunum. After absorption by the enterocytes, the short- and medium-chain FAs are released directly into the portal bloodstream. The LCPUFAs are re-esterified into TAGs and PLs in the enterocytes, after which they are packaged into lipoproteins, called chylomicrons. These chylomicrons are then secreted into the lymphatic circulation and are channelled into the bloodstream through a thoracic lymphatic duct, thereby bypassing the liver (Burdge & Calder, 2015).

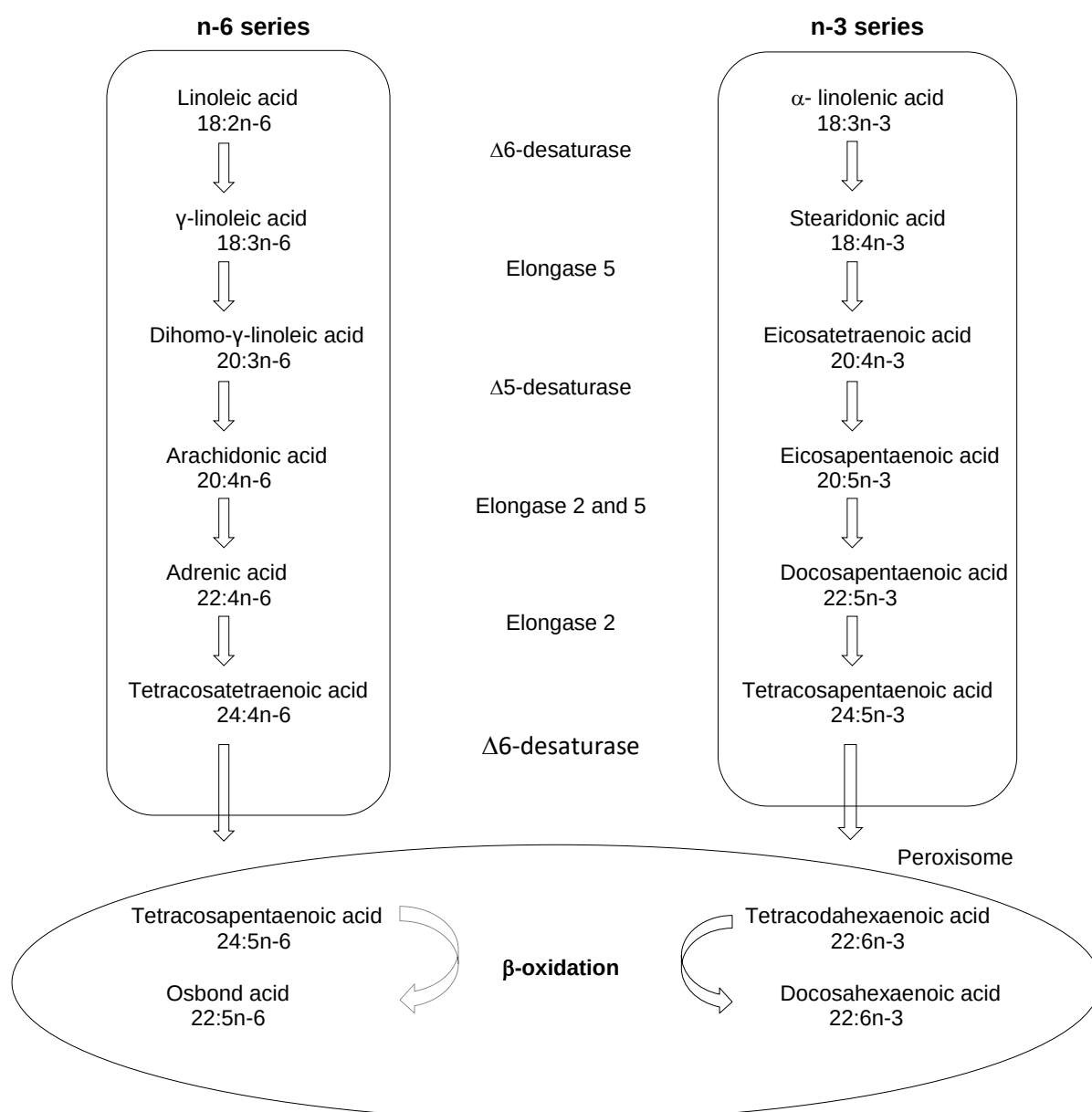


Figure 2-1: Biochemical pathways for the metabolism of n-3 and n-6 fatty acids.

Adapted from: Schmitz and Ecker (2008) and Delplanque *et al.* (2015)

2.3 DIETARY SOURCES OF FATTY ACIDS

Dietary sources of ALA include green leafy vegetables, flax (linseed), walnuts, soy and canola, and the oils produced from these nuts, beans and seeds. LA is predominantly present in plant foods and the richest sources are soybean, corn, sunflower and safflower oils. AA is predominantly found in animal foods and the richest sources are meat, poultry and eggs (Ghosh *et al.*, 2012).

Conjugated linoleic acid (CLA), a mixture of positional and geometrical isomers of linoleic acid (C18:2, *cis*-9, *cis*-12) (Eulitz *et al.*, 1999), is found in ruminant meat and dairy. Oily fish and seafood are the best sources of EPA and DHA. EPA and DHA extracted from algae are also vegetarian sources (Vannice & Rasmussen, 2014). Since most EFAs are plant-derived, the amount of EFAs in breast milk will vary according to the mother's access to plant oils, with a higher intake resulting in higher amounts of EFAs in breast milk. Therefore, a low intake of fat, EFAs and DHA by the mother will be reflected in the breast milk and thus the infant may have inadequate FA intake (Lee *et al.*, 2013; Mulder *et al.*, 2014).

2.3.1 Fatty acid content in relevant foods

It is difficult to quantify FA intake in low income countries because food composition tables generally provide incomplete values for FA content and composition of relevant foods (Michaelsen *et al.*, 2011). The total PUFA, LA and AA contents and ratios in some staple foods are shown in Table 2-1. Rice, wheat and maize are some of the staple foods, but these have low total fat content, traces of ALA and insignificant amounts of LA. Processing and refining these staple foods lowers the fat content even more, thereby reducing the LCPUFA content. Whole-grain cereals do not undergo harsh processing and are therefore considered better sources of EFAs than refined cereals. However, whole-grain cereals are not recommended for infants because they contain higher amounts of phytates and polyphenols (Klish *et al.*, 1998). Phytates and polyphenols inhibit iron absorption. As a result, low iron absorption might contribute to iron deficiency in countries where cereals are a staple food (Brune *et al.*, 1992; Bohn *et al.*, 2008). Despite their low EFA content, cereal grains are largely consumed as one of the important sources of FAs in low-income countries.

Legumes are also good sources of FAs. Soybean is high in total fat and PUFA, with a higher ratio of LA: ALA of 7:1 in comparison with other legumes (Table 2-1). Consequently, soybean may be considered an important source of EFAs in areas with limited access to animal sources. Red kidney beans and lima beans are also considered good sources of EFAs, particularly ALA. Nonetheless, legumes also contain high amounts of polyphenols (Petry *et al.*, 2010), phytates (Morris & Hill, 1996; Klish *et al.*, 1998) and oligosaccharides (Suarez *et al.*, 1999). As a result, caution must be exercised in including these legumes in the diets of infants and children.

Table 2-1: Fatty acid composition in selected plant foods* (g/100g)

	FATTY ACID			
	Total PUFA	LA	ALA	LA: ALA
Staple food (raw)				
Rice, white flour	0.50	0.46	0.02	23:1
Rice, brown	0.80	0.78	0.03	26:1
Wheat, refined flour	0.50	0.50	0.03	17:1
Wheat, bran	2.30	2.15	0.16	13:1
Maize, whole flour	1.76	1.71	0.05	34:1
Maize, degermed flour	0.63	0.62	0.02	40:1
Sorghum	1.37	1.31	0.07	20:1
Millet	2.13	2.02	0.12	17:1
Teff	1.07	0.94	0.14	7:1
Wild rice (<i>Zizania</i> sp.)	0.68	0.38	0.30	1:1
Legumes (dried, raw)				
Soya bean	12.70	10.99	1.56	7:1
Lima bean	0.80	0.55	0.24	2:1
Haricot bean	1.30	0.50	0.82	0.6:1
Red kidney bean	1.00	0.40	0.63	0.6:1

PUFA- polyunsaturated fatty acids; LA- linoleic acid; ALA, α -linolenic acid

*with data from the Australian NUTTAB published by Food Standard Australia New Zealand (FSANZ 2006) and the USDA National Nutrient Database for Standard Reference, Release 22 (USDA 2009). Adapted from Michaelsen *et al.*, (2011)

In low-income countries where intake of animal foods, including fish, is very low, vegetable oil is also one of the most important sources of FAs (Michaelsen *et al.*, 2011). South Africa is one of the countries in which more than half of the PUFA intake is from vegetable oils (Table 2-2). Palm oil, sunflower oil and sesame oil are some of the regularly used oils, although they are very low in PUFAs and the EFA ALA (Table 2-3). Soybean, canola and rapeseed oils have the highest PUFA content and most balanced LA: ALA ratio. However, rapeseed has been replaced by canola, which was derived from rapeseed using conventional plant breeding techniques to lower the erucic acid content that is detrimental to humans. Nonetheless, they are manufactured and supplied in the highest amounts in many developing countries. Accordingly, they are the most practical food choices in low-income countries (Wolmarans, 2009).

Table 2-2: Sources of PUFA in 13 countries ranked according to GDP (lowest to highest)

Country	Cereals	Starchy roots and pulses	Tree nuts and oil crops	Vegetable oils	Animal source foods*	Fish and seafood
Malawi	57.5	4.7	15.0	18.9	2.8	1.2
Ethiopia	69.9	6.5	6.3	10.1	7.2	0.1
Bangladesh	17.2	2.1	5.0	66.4	3.6	5.8
Burkina Faso	47.3	2.7	20.7	23.9	5.1	0.3
Ghana	25.2	3.9	17.4	41.3	4.1	8.2
India	21.6	3.7	8.3	58.9	6.3	1.3
Vietnam	16.8	0.8	31.8	18.7	26.5	5.4
Bolivia	26.4	1.2	22.0	22.2	27.9	0.3
Indonesia	24.2	0.8	21.5	38.5	8.4	6.7
Guatemala	29.5	1.1	8.5	50.3	10.2	0.4
China	12.2	0.5	9.7	52.7	21.4	3.5
South Africa	28.0	0.3	2.1	57.0	11.7	0.8
Mexico	31.1	0.9	5.8	40.8	20.4	1.1

*Excludes fish and seafood products. PUFA- polyunsaturated fatty acid; GDP- gross domestic product; all values given in percentage of total PUFA; Source: Michaelsen et al. (2011)

Table 2-3: Fatty acid composition of different plant oils (g/100 g)

	Total PUFA	LA	ALA	LA: ALA
Vegetable oil				
Sunflower oil	59.800	39.800	0.200	205:1
Sesame oil	40.000	40.700	0.500	85:1
Palm oil	9.300	9.000	0.200	48:1
Olive oil	10.523	9.762	0.761	13:1
Soybean oil	57.207	50.418	6.789	8:1
Canola oil	28.142	19.005	9.137	1.8:1
Flaxseed oil	67.614	14.246	53.368	1:3.7
Walnuts	48.173	38.093	9.080	1:4.2

Sorted by decreasing LA: ALA ratio; LA- linoleic acid; ALA- α -linolenic acid; PUFA- polyunsaturated fatty acid. With data from the Australian NUTTAB published by Food Standard Australia New Zealand (FSANZ 2006), the USDA National Nutrient Database for Standard Reference, Release 22 (USDA 2009) and the USDA Food composition database. Adapted from Michaelsen *et al.* (2011) and Early Nutrition eAcademy (ENeA, 2016).

While beef, milk, poultry, eggs and seafood are also good sources of n-3 and n-6 FAs, dietary LCPUFA intake from such complementary foods minimal in low-income countries where complementary foods are generally mostly cereal-based. In this regard, South Africa, one of the countries with a higher gross domestic product (GDP), was reported to have an average of dietary

intake of 11.7 % of total PUFA from animal food sources (Michaelsen *et al.*, 2011). Table 2-4 shows the fat content and FA composition of these different animal food sources. In contrast to pork and lamb, beef is a fairly good source of n-3 PUFAs. While poultry and eggs have lower n-6: n-3 PUFA ratios than meat, the amount of PUFAs in animal sources is greatly influenced by their feed (Michaelsen *et al.*, 2011). Fish and seafood are very high in EPA and DHA and are therefore excellent sources of n-3 LCPUFAs. Also, the fatty acid composition of different fish and seafood varies between species (Table 2-5).

Table 2-4: Fatty acid composition of different animal sources of LCPUFAs (g/100 g)

	Total PUFA	LA	ALA	AA	EPA	DHA	n-6 PUFA	n-3 PUFA	n-6: n-3 PUFA
Meat (raw)									
Minced pork (9.4% fat)	1.200	0.980	0.090	0.060	0.000	0.03	1.04	0.12	9:1
Minced beef (10.8% fat)	1.200	0.540	0.130	0.230	0.090	0.02	0.77	0.39	2:1
Minced lamb (6.9% fat)	0.500	0.220	0.110	0.030	0.020	0.01	0.25	0.17	1:1
Beef (grass-fed)	0.064	0.041	0.015	0.006	0.002	0.00			
Pork	0.360	0.300	0.008	0.052	0.000	0.00			
Lamb (trimmed to ¼ fat)	1.700	1.240	0.390	0.070	0.000	0.00			
Liver (veal)	0.818	0.517	0.035	0.266	0.000	0.00			
Poultry (raw)									
Duck, lean and skin	4.50	4.090	0.240	0.103	0.000	□0.01	4.22	0.24	17:1
Quail, flesh and skin	2.60	2.240	0.170	0.100	0.000	0.030	2.34	0.20	12:1
Turkey breast	2.30	2.050	0.160	0.050	0.000	0.010	2.10	0.17	12:1
Chicken breast	1.30	1.130	0.090	0.040	0.000	0.010	1.17	0.11	11:1
Chicken (broilers, fryers)	0.69	0.550	0.020	0.080	0.010	0.030			
Eggs and milk									
Chicken egg, hard boiled	1.000	0.720	0.020	0.190	0.00	0.070	0.91	0.10	9:1
Duck egg, hard boiled	0.900	0.540	0.100	0.310	0.00	□0.010	0.85	0.10	9:1
Milk (full fat)	0.100	0.080	0.030	0.000	0.00	0.000	0.08	0.04	2:1
Cow's milk (3.25% fat)	0.195	0.120	0.075	0.000	0.00	0.000			
Cow's milk (1% milk fat)	0.304	0.300	0.004	0.000	0.00	0.000			
Eggs	1.662	1.555	0.036	0.013	0.00	0.058			

PUFA, polyunsaturated fatty acids; LA, linoleic acid; ALA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Adapted from Michaelsen *et al.* (2011) and ENEA (2016) with data from the Australian NUTTAB published by Food Standard Australia New Zealand (FSANZ 2006) and the USDA National Nutrient Database for Standard Reference, Release 22 (USDA 2009).

Table 2-5: PUFA composition of raw, fresh fish (g/100g fresh fish)

Fish	LA	ALA	AA	EPA	DHA
Cod (Atlantic)	0.005	0.001	0.022	0.064	0.120
Herring (Atlantic)	0.130	0.103	0.06	0.709	0.802
Salmon (Atlantic)	0.172	0.295	0.267	0.321	1.115
Mackerel (Atlantic)	0.219	0.159	0.183	0.898	1.401
Tune (Bluefin)	0.053	0.000	0.043	0.283	0.890
Trout (Mixed species)	0.175	0.155	0.189	0.202	0.528
Cod liver oil	0.935	0.935	0.935	6.898	10.968

LA, linoleic acid; ALA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Source: ENEA (2016) with data from USDA Food Composition Databases

Table 2-6 shows the FA composition of raw and fresh fish as well as different fish species, respectively. Marine species from cold water are generally considered the best sources of n-3 LCPUFA (Monroig *et al.*, 2013; Abedi & Sahari, 2014). Although the freshwater species have comparatively less n-3 LCPUFA, they usually contain higher amounts of ALA. Generally, dietary intakes of LCPUFA vary widely in different populations, depending on the availability of LCPUFA food sources.

Table 2-6: Fatty acid composition of different fish and other animals

	LA	ALA	AA	EPA	DHA	Total n-6 PUFA	Total n-3 PUFA	n-6: n-3 PUFA
Marine fish								
Atlantic herring (<i>Clupea herengus</i>)	0.7	0.3	0.4	7.4	3.9	1.1	11.6	1:10
Cod (<i>Gadus morhua</i>)	1.1	0.2	4.8	14.0	26.3	5.9	40.6	1:6
Thai sardine (<i>Sardinella gibbosa</i>)	1.2	0.5	2.7	6.1	9.7	3.9	16.3	1:4
Cod liver oil	1.8	0.7	1.7	8.9	9.3	3.5	18.9	1:5
Cold-water freshwater fish								
Rainbow trout (<i>Oncorhynchus mykiss</i>)	4.6	5.2	2.2	5	19	6.8	29.2	1:4
Perch (<i>Perca fluviatilis</i>)	1.5	0.5	9.1	8.8	26.5	10.6	35.8	1:3
Roach (<i>Rutilus rutilus</i>)	4.5	2.8	5.5	10.7	14.9	10	28.4	1:3
Atlantic salmon (<i>Salmo salar</i>)	2.7	4.6	4.2	5.1	17.6	6.9	27.3	1:4
Warm-water freshwater fish								
Snakehead (<i>Channa striatus</i>)	8.2	0.5	2.2	0.3	1.5	10.4	2.3	5:1
Common carp (<i>Cyprinus carpio</i>)	7.9	2.9	8.6	8.8	6.5	16.5	18.2	1:1
Nile tilapia (<i>Oreochromis niloticus</i>)	9.0	0.8	1.5	0.8	9	10.5	10.6	1:1
Eel (<i>Monopterus albus</i>)	5.6	0.9	0.7	2.7	0.2	6.3	6.1	1:1
Catfish (<i>Clarias macrocephalus</i>)	6.6	2.7	13.5	3.2	6.7	20.1	12.6	2:1
Climbing perch (<i>Anabas testudineus</i>)	5.4	1.3	12.4	1.1	9.2	17.8	11.6	2:1
Other animals								
Field cricket (<i>Teleogryllus testaceus</i>)	26.6	9.0	0	0	0	26.6	9	3:1
Chinese edible frog (<i>Haplobatrachus rugulosus</i>)	9.2	2.3	10.8	1.7	3.5	20	7.5	3:1
Cambodian spider (<i>Haploelmaalbostriatum</i>)	7.6	3.6	11.2	1.6	1.7	18.8	6.9	3:1

Total n-6 PUFA is the sum of LA and AA and total n-3 PUFA is the sum of ALA, EPA and DHA. PUFA, polyunsaturated fatty acids; LA, linoleic acid; ALA, α -linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. All values are given as percentage of total fatty acids. Source: Michaelsen *et al.* (2011)

2.4 LCPUFA INTAKE DURING INFANCY

As previously mentioned, the conversion of the EFAs, LA and ALA, to AA, EPA and DHA respectively, is very limited in infants (Innis, 2014). Therefore, LCPUFA sources during the first year of life include breast milk and infant or follow-on formula enriched with LCPUFA, as well as complementary foods such as egg, fatty fish, and meat. Highly refined oils from single-cell organisms (certain algal and fungal organisms), eggs or fish as sources of AA and/or DHA, can also be used in infant formulae and as complementary foods. However, this is advisable only if the safety and purity of the specific oil has been authenticated and verified (Koletzko *et al.*, 2008).

2.4.1 Infant feeding recommendations

One of the most important global goals is to achieve optimal nutritional status during infancy and early life (Dykes & Hall-Moran, 2009). Hence programmes to promote breastfeeding and appropriate complementary feeding methods are amongst the most effective interventions to promote healthy growth and development in infants and young children (Jones *et al.*, 2003). Exclusive breastfeeding (EBF) during the first six months of life has been identified as one of the most important preventative interventions for child survival (Sudfeld *et al.*, 2012). The ultimate recommendation is early initiation of breastfeeding, EBF for the first six months of an infant's life, with the timely introduction of complementary food, and continued breastfeeding for up to two years and beyond (WHO & UNICEF, 2003). This is because breast milk has a unique biological influence on the health of the infant as it contains all the necessary nutrients in adequate amounts.

2.4.1.1 Breastfeeding

The World Health Organisation (WHO) describes breastfeeding as the process during which an infant receives breast milk, either directly from the mother's breast or expressed. This definition included instances where the infant receives breast milk exclusively or is being partially breastfed (Newell, 2004). Protection and support of breastfeeding still remains one of the most fundamental activities for infant and child survival worldwide. The *Innocenti Declaration* (WHO & UNICEF, 1990) suggests that women who are already breastfeeding should be protected from any environment or physiological influences that may hinder or discourage them from continuing to breastfeed (Blystad *et al.*, 2010). Over the years, the greatest threat to breastfeeding has been the Human Immunodeficiency virus (HIV) pandemic, which has led to confusion over the appropriate infant feeding guidelines.

2.4.1.1 Importance of breastfeeding

Breastfeeding was shown to have protective effects against child infections (Lamberti *et al.*, 2011; Chu, 2013), including pneumonia (Nkonki *et al.*, 2014), and the development of dental

malocclusion, and to contribute to probable reductions in overweight and diabetes (Kramer & Kakuma, 2002; McCarter-Spaulding, 2004). Previous studies also found associations between breastfeeding and cognitive and neurodevelopment and infant survival (Arifeen *et al.*, 2001; Jain *et al.*, 2002; Rey, 2003; Morley *et al.*, 2004). It is suggested that these benefits and protective effects have a dose-dependent effect, with longer duration of, and more exclusive breastfeeding, being more effective and protective (McCarter-Spaulding, 2004; Allen & Hector, 2005; Victora *et al.*, 2016). A recent meta-analysis (Victora *et al.*, 2016) also reported that breastfeeding improves the survival, health and development of all children. For the lactating mother, breastfeeding was shown to protect against breast cancer, ovarian cancer and type 2 diabetes, and to improve birth spacing. In addition to saving the lives of women, breastfeeding contributes to human capital development. These benefits are found across populations in the high-, middle- and low-income countries. Table 2-7 shows a summary of the other benefits of breastfeeding that have been documented.

Table 2-7: Evidence for health benefits of breastfeeding in infants, children, mothers, in developed countries

Level of evidence ¹	Health outcomes for which breastfeeding is protective		
	Infants and children	Chronic disease in childhood and/or later	Mothers
convincing ²	gastrointestinal illnesses otitis media respiratory tract infections neonatal necrotising enterocolitis		slow maternal recovery from childbirth reduced period of postpartum infertility premenopausal breast cancer Type 2 diabetes
probable ³	asthma and allergy cognitive ability/intelligence some childhood leukaemias urinary tract infection inflammatory bowel disease coeliac disease sudden infant death syndrome	obesity Type 2 diabetes	postmenopausal breast cancer ovarian cancer rheumatoid arthritis
possible ⁴	Type 1 diabetes bacteraemia meningitis dental malocclusion	ischaemic heart disease atherosclerosis risk factors for: • atherosclerosis and heart disease • Type 2 diabetes and metabolic syndrome	maternal depression reduced maternal-infant bonding endometrial cancer osteoporosis and bone fracture no or slow return to pre-pregnancy weight

¹ The classification of evidence of the relationship between breastfeeding and health benefits is based on a comprehensive overview of the evidence base (systematic reviews, meta-analyses, reviews, recent single studies).

² Convincing: evidence of relationship was critically identified in a review and/or shown in meta-analyses to be significant.

³ Probable: most studies have found an association, but confirmation is required in more, or better-designed, studies.

⁴ Possible: too few methodologically-sound studies. Adapted from Allen and Hector (2005).

2.4.2 Breast milk

Breast milk is considered the most suitable form of nourishment for infants during the first six months of life (Sala-Vila *et al.*, 2005; Brenna *et al.*, 2007). Breast milk contains all the necessary nutrients, and the lipid fraction is very important in meeting the infant's nutritional needs. Breast milk FAs constitute the biggest fraction of the total energy intake during infancy, constituting an average of 44% of total energy (Grote *et al.*, 2016).

Breast milk contains several FAs, many of which are present in very low concentrations, with others dominating. LCPUFAs constitute about 2 % of the total fatty FAs in breast milk (Andreas *et al.*, 2015). Approximately 98 % of the lipids in human milk consist of triglycerides, each containing three FAs, of which most are sensitive to maternal nutrition. In mature milk, approximately 85 % of LCPUFAs are in the form of TAGs and 15 % of LCPUFA is in the form of PLs. The short-chain FAs that are found in breast milk are an important source of energy and are

needed for the proper maturation of the gastrointestinal tract of the infant. Breast milk contains predominantly more saturated FAs than MUFAs and PUFAs. Breast milk LA concentrations are higher than ALA, while AA is higher than DHA (Koletzko, 2016). Thus, breast milk is the sole source of LCPUFAs in exclusively breastfed infants aged zero to six months.

From as early as the 90s, observational studies have indicated that children who are breastfed have a greater advantage in cognitive and intellectual development than infant formula-fed children (Forsyth *et al.*, 1998; Hornstra, 2000). Researchers suggest that these beneficial effects involve the role of the LCPUFAs DHA and AA, which are transferred to the infant through breast milk. DHA and AA have both structural and functional roles in the cellular membranes and are very important for physiological and metabolic processes during infancy and beyond (Colombo *et al.*, 2013).

2.4.3 Complementary foods

After a certain age, usually around six months, breast milk as a sole dietary source, no longer meets all the infant's nutritional requirements, hence optimal complementary foods have to be introduced in order to ensure adequate nutrition and growth for the infant (Forsyth *et al.*, 2017a). When infants are introduced to these complementary foods, there is a decrease in the intake of breast milk and infant formula. This may result in a drastic decrease in total dietary intake of LCPUFAs unless the infant is introduced to foods that contain, or are enriched with LCPUFAs (Koletzko *et al.*, 2008). It is advisable to include foods like egg yolk, fish, liver or other LCPUFA-fortified foods (like dairy) during the complementary feeding period (Forsyth *et al.*, 2017b).

While the intake of LCPUFA-enriched food is encouraged during infancy, the LCPUFAs in infant formulas and other dietary products improve the LCPUFA levels in infant plasma and tissue lipids. This biochemical effect can also be seen even months after the period during which these foods would have been consumed (Innis *et al.*, 2001; Innis *et al.*, 2002). Economic limitations, religious beliefs and concerns that infants below the age of one year are unable to digest animal-sourced foods, are some of the factors contributing to limited dietary diversity. For instance, some families still believe that the intake of fish by infants may be associated with allergic reactions (Agostoni *et al.*, 2008a; Perkin *et al.*, 2016). However, infants and young children at a vulnerable age, require energy- and nutrition-dense food so that they are ensured of adequate growth and development, both physically and mentally. It is for this particular reason that the WHO included dietary diversity as a specific recommendation to provide guidance for complementary feeding between the age of 6 and 23 months (WHO, 2003).

Furthermore, the introduction of fish as a complementary food source does not have to be delayed based on concerns regarding allergies. The current expert guidelines state that delaying the

introduction of potentially allergenic foods does not reduce the risk of developing allergy, regardless of atopic heredity. Therefore, since these foods are good sources of LCPUFA, they should not be introduced late in infancy. Besides, evidence suggests that the appropriate and well-timed introduction of fish and other allergenic complementary foods between the ages of 17 and 26 weeks is essential in reducing the risk of allergy (Perkin *et al.*, 2016; Prell & Koletzko, 2016; Muraro *et al.*, 2017).

2.4.4 Feeding practices in South Africa

Inappropriate and inadequate infant feeding practices, like the early introduction of complementary foods, have been identified in various studies conducted in different provinces in South Africa (Kruger & Gericke, 2003; MacIntyre *et al.*, 2005; Faber & Benade, 2007; Ghuman *et al.*, 2009; Ladzani *et al.*, 2011; Goosen *et al.*, 2014; Siziba *et al.*, 2015; Van der Merwe *et al.*, 2015). Indeed, mixed feeding and early introduction of complementary food appear to be the rule rather than the exception in South Africa. Despite a very high (83.0%) breastfeeding initiation rate (Shisana *et al.*, 2013), the percentage of children who are exclusively breastfed decreased with age from 44% of infants aged zero to one month, to 24% of infants aged four to five months, in 2016 (SADHS- National Department of Health, 2017).

Moreover, infants from most low- and middle- income countries are typically introduced to plant-based complementary foods that are insufficient to meet the dietary needs of the growing infants (Forsyth *et al.*, 2016). This may result in a reduction of dietary LCPUFAs as reflected in their blood levels. This reduction may also be due to a simultaneous reduction in breast milk intake (Hoffman *et al.*, 2000; Birch *et al.*, 2005) in combination with intake of LCPUFA-poor weaning foods.

There are limited data on the LCPUFA status of infants in developing countries, particularly South Africa. In their literature review, Huffman *et al.* (2011) found only two studies that have reported data on the FA status of infants and children from developing countries. Despite the inadequate feeding practices in South Africa, the LCPUFA status of breastfed and infants remains unknown. There is also scarcity of data on LCPUFA intakes particularly in infants who should be assured of an adequate intake of LCPUFA for cognitive development and other positive health outcomes. Hence the growing interest in the composition and quality of lipid supply during infancy.

2.5 FATTY ACID REQUIREMENTS AND RECOMMENDATIONS

As previously mentioned, because breast milk is considered an ideal source of nutrients during infancy, it is used as an ideal standard to determine adequate nutrient intakes for infants. In this regard, breast milk LCPUFA concentrations are used to define adequate AA and DHA intakes for

infants. Thus, the adequate intakes of LCPUFA are based on breast milk volume intake, fat content and LCPUFA composition (FAO,2010).

The European Food Safety Authority (EFSA) states that 100mg of DHA/day and 140mg of AA/day can be considered as adequate nutrient intakes of LCPUFA for the majority of infants from birth up until the age of six months (Agostoni *et al.*, 2013). This recommendation is based on evidence showing the importance of LCPUFAs in the growth and development of infants as well as in the compositional requirements of infant and follow-on formula.

Infants require an adequate intake of nutrients, hence the need for Acceptable Macronutrient Distribution Ranges (AMDR). The AMDR give ranges of intakes for specific sources of energy that reduces the risk of chronic diseases whilst providing sufficient intakes of essential nutrients. To achieve a nutritional adequacy of n-3 PUFAs, the FAO/WHO (2008) report suggests an upper-AMRD of 11 %E (percentage contribution of energy) for the total PUFAs in adults and 15 %E for infants aged between six and 12 months of age. The recommendation for infants, in particular, is higher because of a decrease in the bioavailability of Vitamin E. This has been shown to lead to a higher risk of lipid peroxidation, especially when the supply of tocopherol is not increased accordingly. Figure 2-2 shows the daily intake recommendations as stipulated by different international institutions.

Global (FAO, 2010)	• 0 - 6 months : 0.1-0.18%E DHA, 0.2-0.3%E AA • 6 - 24 months: 10-12 mg/kg body weight
Europe (EFSA, 2010)	• 0 - 6 months : 100 mg DHA/day, 140 mg AA/day • 6 - 24 months: 100 mg DHA/day
Early Nutrition Academy (Koletzko et al., 2014)	• 0 - 6 months : 100 mg DHA/day, 140 mg AA/day • 6 - 24 months: 100 mg DHA/day

Figure 2-2: Daily Intake recommendations of LCPUFAs for infants aged 0 to 24 months. Source: ENeA (2016)

2.6 BIOMARKERS OF LCPUFA STATUS

Different blood lipid pools and tissues have been used as biomarkers of intake because the DHA and LA concentrations reflect dietary intake and subsequent metabolism. Over the years,

researchers have used such markers in plasma, red blood cells (RBCs) or adipose tissue when tissue from specific organs of interest is not available (Hodson *et al.*, 2008; Lauritzen & Carlson, 2011). FAs can be assessed as free FAs in constituents of circulating TAGs, serum, RBC membranes, phospholipids or cholesterol esters and/or adipose tissue from different sites in the body. Dietary fat intakes that enter the enterohepatic circulation immediately after a meal, are reflected in some proportions of FAs in the chylomicrons. Therefore, these can be used as short-term markers of fat intake (Arab, 2003). Other plasma and serum measures reflect the dietary intakes of the past few hours (TAGs) or the past few days (cholesterol esters and phospholipid fatty acids). Phospholipid FAs can also reflect weeks of dietary intake.

Adipose tissue is considered the most reliable biomarker of very long-term dietary patterns and metabolic disorders. This is because adipose tissue is not very sensitive to any temporary changes in the diet. The largest fraction of TAGs is found in the adipose tissue and therefore the adipose tissue can be used to determine the TAG status only. However, using adipose tissue as a biomarker is not very practical as there are ethical, financial and practical issues regarding adipose tissue sample collection. In this regard, a whole-blood finger-prick test is considered the most reliable, feasible, cost-effective and practical method for use in investigations that use large sample sizes (Hodson *et al.*, 2008).

Blood consists of plasma, platelets, leucocytes (white blood cells) and RBCs. RBCs comprise the largest fraction of blood and they have a life span of 120 days. RBCs provide a marker that reflects longer-term intake and a more accumulated time period in comparison with serum. RBCs and platelets are accessible through phlebotomy and can also be used as biomarker media for FA analyses (Arab, 2003; Harris & Thomas, 2010). The difference between membrane lipids and storage lipids is that membrane lipids contain a higher proportion of LCPUFAs and rarely reflect any free TAGs.

Plasma can be defined as the liquid component of whole blood. Plasma can reflect FA intake immediately after a meal or a few days after dietary changes. The free FAs found circulating in plasma can be used as a biomarker for the composition of both short- and medium-chain FAs in either the diet or in the adipose tissue. The LCPUFAs are largely incorporated into the necessary constituents of plasma lipoproteins as TAGs, PLs and cholesterol esters (CEs). The TAGs are the most sensitive to any changes in dietary intake of FAs, which may be reflected immediately after a meal. CEs and PLs, on the other hand, are relatively stable and any dietary alterations may be reflected in FA composition within a few days (Arab, 2003; Hodson *et al.*, 2008). Plasma can therefore be used to assess n-3 PUFA status in infants receiving breast milk and complementary foods, especially when PLs are analysed.

2.6.1 Total phospholipid fatty acid composition

PLs have a functional role in the biochemical structure of membranes. PLs are characterised by their physical and biological properties, which improve their specific functions. After ingestion, FAs are incorporated into the PLs *de novo* through the Kennedy pathway (Kennedy & Weiss, 1956), after which the composition of the PLs is changed through a deacylation-reacylation process (Land's cycle) (Lands, 1958). The composition of PLs is directly dependent on the availability of certain FAs, and the PL composition of membranes and tissues also depends on their ability to adapt their structures, depending on the presence of certain FAs (Hodson *et al.*, 2008). Although plasma PL composition can reflect FA composition of the brain tissue (Spector, 2001; Harris *et al.*, 2004), the RBC FA composition is known to be a good indicator of the FA composition of tissues, including the brain (Sanjurjo *et al.*, 1995; Maurage *et al.*, 1998). The RBC membrane PLs can also reflect the levels in the adult heart (Harris *et al.*, 2004).

FAs in blood are exchanged from the lipoprotein coat, RBCs or platelet membrane between PLs. Similarly, FAs that are released into the blood from the adipose tissue can interact with the PLs and are then incorporated into their structures (Hodson *et al.*, 2008). The remodelling time between each FA status biomarker and media differs. While the total platelet PLs may reflect the FA status, the limitation is that there is no identifiable dose response. Plasma PLs and RBC membrane PLs are reliable biomarkers that can be used to determine the FA composition of the PLs of infants (Fekete *et al.*, 2009).

2.6.2 Essential PUFA status and functional status markers

The total amount of various EFAs and LCPUFAs in plasma or RBC PLs is a useful indicator for assessing the essential PUFA status of an individual. However, the plasma content of these essential PUFAs (EFAs and LCPUFAs) does not guarantee that the cells and tissues will use up the FAs properly. Therefore, assessing the functional PUFA status of an individual will require additional status markers. In general, in the event that the essential PUFAs are insufficient and do not meet PUFA requirements, the body will begin to synthesise particular FAs that are hardly present if the EFA and PUFA status are adequate. These particular FAs can therefore be used as status markers for essential PUFAs. For instance, mead acid (20:3n-9) has become the best-known marker of EFA status. If the body has insufficient concentrations of LA and ALA to meet the need for LCPUFA, the synthesis of Mead acid is promoted. Since LCPUFAs hinder the synthesis of Mead acid, the presence of Mead acid reveals a general shortage of all essential PUFAs (Hornstra, 2000).

The essential PUFA status index is the ratio between all essential PUFAs (the sum of all n-3 and n-6 FAs) and non-essential unsaturated FAs (the sum of all n-7 and n-9 FAs). This essential

PUFA status index can be considered as another suitable indicator of the essential PUFA status of an individual. Therefore, a better essential PUFA status is denoted by a higher essential PUFA status index. Furthermore, in the event that there is a functional shortage of DHA, the human body begins to synthesise osbond acid (22:5n-6), which is the LCPUFA of the n-6 series the most equivalent to DHA. However, it is not functionally equivalent to DHA. The ratio between DHA and osbond acid is therefore a reliable indicator of the functional DHA status in stable conditions (Hornstra, 2000).

2.6.3 Fatty acid patterns

To date, researchers have evaluated individual FAs (e.g. AA, EPA, DHA etc.) or groups of FAs (e.g. total n-3 PUFAs), but concentrations of these circulating FAs correlate highly with each other (Imamura *et al.*, 2012). In light of this, individual FAs are expressed as a percentage of total FAs, such that an increase in the proportion of one FA may lead to a decrease in another. This effect may happen in a manner dependent on the FA pool and the specific FA that is regulated (Walker *et al.*, 2015). In addition, some FA are precursors or products that are linked through distinct metabolic pathways. Thus, examining only individual FAs may cause certain interactions or additional effects to be missed (Bigornia *et al.*, 2016).

To overcome such limitations, studying different FA patterns will take into account the different interactions and interrelations. Different pattern analysis techniques in statistics, such as principal component analysis (PCA) and factor analysis can be used for such analysis. PCA uses orthogonal transformation to convert a set of observations of variables that are possibly correlated, such as plasma or RBC FA levels typically are, into a smaller set of variables that are linearly uncorrelated. These transformed and uncorrelated variables are called principal components. These principal components explain the largest possible variation in the original variables and can also, in this case, be called FA patterns (Voortman *et al.*, 2017). PCA generates a scree plot which can be used in conjunction with the Kaiser criterion (Eigenvalues > 1) to determine which components can be retained for analysis. With factor analysis, each factor captures a certain amount of the overall variance in the observed variables, and the factors are then listed in order of how much variation they explain. The relative amount of the variance of the observed variables that a specific factor explains, is expressed as the eigenvalue. The Kaiser criterion states that any factor with an eigenvalue ≥ 1 explains more variance than a single observed variable. In addition, factor loadings obtained from the factor analysis describe how strongly each individual FA contributes to each FA pattern. These factor loadings aid in identifying and defining the distinct FA patterns that will be selected and used for subsequent analysis. Therefore a variable can be considered to be associated with a specific factor (pattern) if the

absolute value of its loading is >0.5 (Macciotta *et al.*, 2015). Distinct FA patterns identified should thus have a clear and technical biological meaning.

In this regard, PCA has been used extensively in nutritional epidemiology to identify innovative FA patterns relevant in to public health (Anderson *et al.*, 2009; Kooijman *et al.*, 2016). Several studies (Warensjö *et al.*, 2006; Anderson *et al.*, 2009; Dahm *et al.*, 2011; Imamura *et al.*, 2012; Bigornia *et al.*, 2016) in the field of public health nutrition have applied this approach to FAs and identified different patterns. Some examples include patterns characterised by high concentrations of n-3 PUFAs or by very long-chain PUFAs. These studies showed associations of different FA patterns with nutrition-related conditions in adults. However, in this PhD study, the factors that were generated presumably represent the quality of the diet of South African infants receiving breast milk and/or complementary foods.

2.7 ROLE OF FATTY ACIDS IN HEALTH

Lipids provide energy and, most importantly, FAs that are necessary for the development of the central nervous system (CNS), but which cannot be synthesised *de novo* by the infant (Brenna *et al.*, 2007). The major functions of FAs are as energy sources and membrane constituents. It is through these principal roles that FAs can influence cellular functions and thus affect physiological responses (Burdge & Calder, 2015). FAs are particularly important for the structure and function of human tissues, immune function and brain and retinal development during gestation and infancy (Koletzko *et al.*, 2011). Figure 2-3 shows a comparative display of how different FAs affect health.

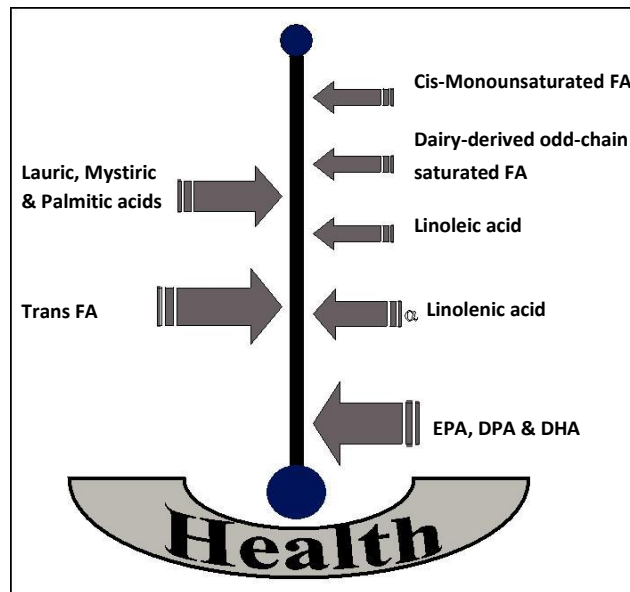


Figure 2-3: Representation of how different fatty acids influence health. The size of the arrow is indicative of a qualitative assessment of the possible effect of the specific fatty acids and the size of the effect it has on health. Source: Calder (2015)

2.7.1 Role of LCPUFA in the development of the CNS

It is widely known that DHA is a nutrient that is critically important for the development of infants. DHA plays an essential role in the structure and function of the retina and the CNS as it accumulates rapidly in these tissues, both *in utero* and during the early stage of infancy (SanGiovanni *et al.*, 2000; Uauy *et al.*, 2003). DHA is further required for the development of the sensory, perceptual, cognitive, and motor neural systems during the “brain growth spurt” (Innis, 2007). The brain is largely dominated by both DHA and AA (Crawford *et al.*, 2003). Substantial amounts of DHA and AA are transferred directly to the foetus via the placenta during pregnancy and through breastfeeding and/or infant formula-feeding after birth (Koletzko *et al.*, 2011). As a result, during the first two years of life, DHA and AA levels rapidly increase to adult levels in the infant’s brain (Sinclair & Crawford, 1972). However, the amount of DHA provided to the infant will vary according to the mother’s dietary intake and the infant formula (Meldrum & Simmer, 2016).

Likewise, AA is also needed for brain growth and is essential for cell division and signalling (Katsuki & Okuda, 1995; Crawford & Broadhurst, 2012). Therefore, the human brain needs both DHA and AA for adequate growth and function. The tissues in the CNS consist of approximately 10 % to 12 % of AA as a percentage of total FAs. Unlike DHA, it seems that the amount of AA

accrued in the CNS tissues is influenced to a larger extent by postnatal age than by dietary supply of AA (Makrides *et al.*, 1994). Gestation and early infancy are therefore the most critical periods during which the rate of brain growth is at its peak and vulnerable to nutritional deficiencies (Martínez & Mougán, 1998; Boucher *et al.*, 2011; Larqué *et al.*, 2013). An inadequate supply of nutrients to the brain during this stage can compromise brain function.

The importance of DHA during the development of the CNS has led to the hypothesis that variations in dietary DHA intake may influence the normal course of brain development. This could, in turn, also affect the functional skills that are controlled and determined by the structure and development of the CNS (Meldrum & Simmer, 2016).

2.7.2 Infant outcomes

Visual development and cognitive development are two major outcomes that are dependent on the LCPUFA status of the infant. Visual development depicts the progression of visual (cerebral) function and retinal (sensory) function. The functional and developmental effects of DHA supplementation can be assessed by making use of developmental scales and other neurophysiologic tests. It is also important to assess the motor development milestones for an accurate evaluation of the infants' growth and development (Colombo, 2001; Cheatham *et al.*, 2006).

2.7.2.1 Visual development

The highest concentrations of DHA and AA are found in the cerebral cortex, particularly in the grey matter within the neural synapse membranes and DHA is also highly concentrated in the retina where it constitutes $\leq 36.4\%$ of total FAs (Ghys *et al.*, 2002; Agostoni, 2008). This is why it is important for vision and learning. High concentrations of DHA are found in the prefrontal cortex because DHA accounts for about 40% of FAs in some areas and is also connected to association and short-term memory (Agostoni, 2008).

Although infants are born with a poorly developed visual system, their vision develops rapidly during the first year of life. Electroretinography is another method of assessing retinal function. It particularly measures the electrical responses of sensory cells in the retina. To assess visual development in infants, studies have evaluated visual acuity, which measures even the smallest detail recognised by the infant's visual system. Visual acuity also measures the clearness of vision. Visual acuity is assessed using different measures of visual development which include visual evoked potential (VEP) or forced choice preferential looking (FPL). VEPs in particular, are electrophysiological responses which are created by the brain in response to certain forms of visual stimulation (SanGiovanni *et al.*, 2000; Uauy *et al.*, 2003). Another measurement that is

used to assess the visual function of depth perception is stereo acuity. This specifically assesses minimum detectable binocular disparity (Eilander *et al.*, 2007) and visual recognition memory (for example, the Fagan Test of Infant Intelligence) (McCann & Ames, 2005).

2.7.2.1.1 LCPUFA status and visual development

An overview of observational studies in infants and their mothers on the associations between LCPUFA status and all infant developmental outcomes is shown in Table 2-8. A total of five observational studies assessing the relationships between the LCPUFA statuses of mother, infant or breast milk LCPUFA levels with visual development, were identified. Three studies (Jørgensen *et al.*, 2001; Krasevec *et al.*, 2002; Jacobson *et al.*, 2008) assessed the association between breast milk phospholipids and visual acuity. Only two studies (Jørgensen *et al.*, 2001; Krasevec *et al.*, 2002) found significant associations between visual acuity of the infant with breast milk DHA at four months and two months, respectively. Jacobson and colleagues (2008) did not find any associations, except between higher cord DHA, as well as the DHA/AA ratio and visual acuity at six months. Two other studies (Innis *et al.*, 2001; Agostoni *et al.*, 2003b) used infant RBC and plasma phospholipids to determine associations with visual development. Both studies found positive correlations between visual acuity and RBC PE DHA at two and 12 months (Innis *et al.*, 2001), as well as between higher plasma AA and visual performance at 12 months (Agostoni *et al.*, 2003b).

Consequently, the relationship between higher cord DHA concentrations with more visual development suggests that there is indeed a need for a significant increase and supply of the specific FAs during the third trimester. These FAs are therefore important for the synaptogenesis in brain and photoreceptor development during the third trimester (Suh *et al.*, 2000; Jacobson *et al.*, 2008). In addition, the researchers found a strong correlation between cord and maternal plasma DHA, which is consistent with the data from five other populations (Otto *et al.*, 1997). This provides strong evidence of the infant's dependence on the mother for an adequate supply of DHA. These data also show the importance of DHA for retinal development and are also consistent with findings from other studies (Gibson *et al.*, 1997; Lauritzen *et al.*, 2004; Jensen *et al.*, 2005) that show that breast milk, during the first days of life, contributes to the infant's development later on in infancy.

Although the correlation between cord and maternal AA was very weak in the study by Jacobson and colleagues (2008), this shows that the infant is less dependent on the mother for the supply of AA. This same pattern has been seen in other populations and Otto *et al.* (1997) ascribe it to larger amounts of AA in most existing maternal diets.

Table 2-8: Observational studies investigating associations between LCPUFA status and infant developmental outcomes

Author year	Country Sample size (n)	LCPUFA assessment	Developmental tests	Findings
Innis <i>et al.</i> (2001)	Canada n = 83	Infant RBC and plasma phospholipids at 2 mo	MDI and PDI (BSID II) at 6 and 12 mo, Speech perception and object search task at 9 mo, FTI at 6 and 9 mo, Visual Acuity at 2, 4, 6 and 12 mo	Significant positive correlations between visual acuity and RBC PE DHA at 2 and 12 mo, with plasma DHA and speech perception at 9 mo. No significant correlations found between LCPUFA status and MDI, PDI, FTI and object search task
Jørgensen <i>et al.</i> (2001)	Denmark n = 39	BM phospholipids	Swept VEPs at 4 mo	Significant associations between visual acuity of the infant and BM DHA
Cheruku <i>et al.</i> (2002)	USA n = 17	Maternal plasma phospholipids at delivery	The sleep recordings using the Motility Monitoring System (MMS)	Significant positive correlations between maternal DHA status and infant sleep states
Krasevec <i>et al.</i> (2002)	Cuba n = 56	BM, plasma and RBC phospholipids at 2 mo	Visual acuity at 2 mo	Positive correlation between BM DHA and visual acuity
Agostoni <i>et al.</i> (2003b)	Italy n = 20	Infant plasma phospholipids at diagnosis of PKU	MDI and PDI (BSID I) at 5 and 12 mo, and VEP at 12 mo	Significantly higher PDI scores at 5 mo associated with higher plasma AA and visual performance at 12 mo in BF infants
Dijck-Brouwer <i>et al.</i> (2005)	Germany n = 317	UA and UV phospholipids at birth	Neonatal neurological examination at day 10-14 after birth	Significant positive correlations between neurological optimality score and AA (UV), EFA (UV) and DHA status (UV and UA)
Colombo <i>et al.</i> (2004)	USA n = 70	Infant and maternal RBC and plasma phospholipids at delivery	Habituation at 4, 6 and 8 mo, Toddler attention at 12 and 18 mo	Infants whose mothers had higher levels of DHA at birth showed significantly accelerated developmental courses in attention across the 1st year
Weiler <i>et al.</i> (2005)	Canada n = 30	Maternal and cord blood phospholipids at birth	lumbar spine 1 – 4, femur and whole-body BMC within 15 d of delivery	Cord RBC LCPUFA was significantly related to infant BMC. Only maternal RBC AA was significantly related to whole-body BMC
Bouwstra <i>et al.</i> (2006)	Germany n = 269	UA and UV phospholipids at delivery	Evaluation of the quality of GMs at 3 mo	Mildly abnormal GMs are associated with a less favourable EFA status in the umbilical artery.
Jacobson <i>et al.</i> (2008)	Canada n = 109	Breast milk, cord and maternal plasma phospholipids	Visual acuity and FTII at 6 mo. Visual acuity, FTII, MDI and PDI (BSID-II) at 11 mo	Significant associations between higher cord DHA and visual acuity and novelty preference at 6 mo, as well as better MDI and PDI scores at 11 mo. No associations between BM DHA and PDI or MDI
Kannass <i>et al.</i> (2009)	USA n = 45	Maternal RBC phospholipids at delivery	Peabody Picture Vocabulary Test–R at 12 mo	Toddlers in both DHA (low and high) groups had similar average look lengths at 12 mo
O'Tierney-Ginn <i>et al.</i> (2017)	USA n = 27	Placental tissue and UV plasma phospholipids	Offspring body composition at day 1 and 6 mo	Early infancy weight gain associated with poor plasma LCPUFA status at birth, and fat deposition in infancy related to changes in placental lipid handling

RBC- Red blood cell; MDI- Mental development index; PDI- Psychomotor development index; BSID II- Bailey's II Scales of Childhood Development; FTII- Fagan Test of Infant Intelligence; PE- Phosphatidylethanolamine; DHA- Docosahexaenoic acid.; AA- Arachidonic acid; LCPUFA- Long chain polyunsaturated fatty acids; BM- Breast milk; UA- Umbilical artery; UV- Umbilical vein; EFA- Essential fatty acids; BMC- Bone Mineral Content; GMs- General Movements

2.7.2.1.2 Maternal LCPUFA supplementation during lactation and effects on visual development of their neonates

Table 2-9 provides an overview of studies which assessed the effect of LCPUFA supplementation during lactation on visual development of the infant. Three studies (Gibson *et al.*, 1997; Lauritzen *et al.*, 2004; Jensen *et al.*, 2005) were identified, that evaluated lactating mothers receiving an LCPUFA supplement or placebo. Duration of supplementation ranged from 12 weeks to four months after delivery. Supplementations used were DHA rich algal oil and fish oil. None of the three studies found any significant differences between groups. However, only one study (Lauritzen *et al.*, 2004) found significant positive correlations between visual acuity and infant LCPUFA status. Supplementing lactating women with DHA was shown to result in an increase in breast milk DHA levels. This is because of the dose-dependent relationship that exists between maternal DHA consumption and DHA levels in breast milk (Gibson *et al.*, 1997; Fidler *et al.*, 2000; Jensen *et al.*, 2005).

In the above-mentioned randomised controlled trials, DHA supplementation during the first four months of lactation increased the DHA content of breast milk as well as the infants' RBC and plasma phospholipid profiles. However, as mentioned earlier, there were no significant effects of DHA supplementation during lactation on any of the indicators of visual development. The associations found suggest that n-3 LCPUFAs, particularly DHA may influence visual maturation. On one hand, it is hypothesised that the duration of supplementation (three to four months) in these studies could have been too short to have a beneficial effect on visual development. Therefore, supplementation could start even before birth, during pregnancy. On the other hand, it could also be that DHA supplementation during lactation does not have a significant effect on visual development of the infant (Eilander *et al.*, 2007).

Table 2-9: The effect of LCPUFA supplementation during lactation on infant developmental outcomes

Author, year Country	Intervention Groups (n)		LCPUFA Level in intervention		LCPUFA assessment	Duration of supplementation	Tests	Findings
¹ Gibson <i>et al.</i> (1997) Australia	A- 0g	n = 12	DHA-rich algal oil with:		Infant RBC and plasma phospholipids at 12 wks	12 weeks	VEP at 12 and 16 weeks, BSID PDI and MDI at 1y	No significant differences between groups for VEP. DHA status associated with MDI only at 1y
	B- 0.2g	n = 10	DHA	43%				
	C- 0.4g	n = 12	n-6 PUFA	1%				
	D- 0.9g	n = 10	SFA	38%				
	E- 1.3g	n = 8	MUFA	18%				
Lauritzen <i>et al.</i> (2004) Denmark	FO	n = 53	FO with:		Infant RBC phospholipids at 4 mo	4 months	Sweep VEP at 2 and 4 mo	No significant differences between groups but VEP positively associated with infant RBC DHA at 4 mo
	OO	n = 44	n-3 LCPUFA	1.5g				
	HF*	n = 49	FO	4.5g				
Jensen <i>et al.</i> (2005) USA			200mg/d AO with:		Infant plasma phospholipids at 4 mo	4 months	Sweep VEPs at 4 and 8 mo, BSID PDI, MDI, GDI, CLAMS and CAT at 12 mo	No significant differences between groups at 12 mo
	AO	n = 83	DHA	41.7%				
			LA	0.8%				
			SFA	44%				
			MUFA	13.6%				
Lauritzen <i>et al.</i> (2005) Denmark	FO	n = 53	4.5g FO with:		Infant RBC phospholipids at 4 mo	4 months	The Infant Planning Test at and MacArthur CDI at 9 mo. Developmental milestones in motor function assessed by interviews at each visit	No association between problem solving and RBC-DHA at 4 mo. Passive vocabulary at 1y significantly lower in FO group. Word comprehension at 1y inversely associated with RBC-DHA at 4 mo
	OO	n = 47	DHA	60%				
	HF*	n = 148	n-3 LCPUFA	1.5g				

¹High DHA-rich algal oil; *High Fish- Used as a reference group; DHA- Docosahexaenoic acid; PUFA- Polyunsaturated fatty acids; SFA- Saturated fatty acids; MUFA- Monounsaturated fatty acids; LA- Linoleic acid; RBC- Red blood cell; VEP- Visual Evoked Potentials; BSID I- Bailey's I Scales of Childhood Development; MDI- Mental development index; PDI- Psychomotor development index; FO- Fish oil. OO- Olive oil; LCPUFA- Long chain polyunsaturated fatty acids; AO- Algal oil; VO- Vegetable oil; LA- Linoleic acid; GDI- Gesell Developmental Inventory; CLAMS- Clinical Linguistic and Auditory Milestone Scale; CAT- Clinical Adaptive Test; CDI- Communicative Development Inventory

2.7.2.1.3 Dietary LCPUFA supplementation during infancy and visual development

Several studies have evaluated the effect of LCPUFA supplemented formula on visual development of the infant (Table 2-10). A total of thirteen studies evaluated visual acuity in addition to other infant developmental outcomes. In these trials, infants were randomised to receive supplements within the first week of birth for different time lengths. Two studies (Birch *et al.*, 1998; Jørgensen *et al.*, 1998) supplemented the infants for four months and two studies (Hoffman *et al.*, 2003; Hoffman *et al.*, 2004) for six months. The duration of supplementation ranged between 30 and 52 weeks in four studies (Makrides *et al.*, 1995a; Birch *et al.*, 2002; Makrides *et al.*, 2002; Birch *et al.*, 2005). Infants were supplemented for 12 months in four studies (Carlson *et al.*, 1996; Auestad *et al.*, 1997; Makrides *et al.*, 2000a; Auestad *et al.*, 2001; Birch *et al.*, 2010). The infant formula that was used in these studies was either enriched with DHA + AA or with DHA alone, compared with a standard or commercial infant formula. Other studies included breastfed infants as a reference group. The LCPUFA supplements were derived from any sources which included fish oil, egg triglycerides or fungal oils.

In four studies (Makrides *et al.*, 1995a; Carlson *et al.*, 1996; Birch *et al.*, 1998; Jørgensen *et al.*, 1998; Makrides *et al.*, 2000a) visual acuity was better in the breastfed reference groups compared with the infant formula-fed infants. In one study (Hoffman *et al.*, 2003), infants who had been weaned from breast milk between four and six months were supplemented with DHA-enriched infant formula. In this study, infants in the supplemented group had better visual acuity at six and 12 months of age. Similarly, another study (Hoffman *et al.*, 2004) that supplemented breastfed infants with DHA-enriched complementary foods showed improved visual evoked potentials at nine and 12 months of age compared with the control group. In addition, five studies (Makrides *et al.*, 1995a; Birch *et al.*, 1998; Birch *et al.*, 2002; Birch *et al.*, 2005; Birch *et al.*, 2010) also found that infants supplemented with DHA-enriched infant formula milk had significantly better visual acuity compared with the control groups (Jasani *et al.*, 2017).

Although better visual acuity was not observed in other studies, it is clearly shown that there is a need for a continued supply of LCPUFA during infancy. Also, the evidence for the beneficial effect of LCPUFA-supplemented infant formula on visual acuity is inconsistent (Koletzko *et al.*, 2008; Delgado-Noguera *et al.*, 2010; Qawasmi *et al.*, 2013). However, the discrepancies in results can be explained by the different methodological limitations, types and/or duration of interventions, dosages and sources of LCPUFA, as well as outcomes measured.

Table 2-10: The effect of LCPUFA supplementation on developmental outcomes in infants

Author, year	Intervention/ groups (n)	LCPUFA level in intervention	LCPUFA assessment	Duration of supplementation	Developmental Tests	Findings
Makrides et al. (1995a)	F1 13 F2 19 HM 23	F1 DHA 0.36 EPA 0.58 GLA 0.27 F2 LA 16.8 ALA 1.6	Infant RBC phospholipids at 6, 16, and 30 wks	30 wks	VEP at 16 and 30 wks	VEP acuities of HM and F1 infants significantly better at 16 and 30 wks. RBC DHA correlated with VEP acuity in all infants at both ages
Agostoni et al. (1995)	F1 27 F2 29 HM 30	F1 DHA 0.3 AA 0.44 F2 LA 11.1 ALA 0.7	RBC and plasma phospholipids at 4 mo	4 mo	Brunet-Lézine development quotient at 4 mo	F1 and HM significantly better develop-mental quotient than F2
Carlson et al. (1996)	F1 19 F2 20 HM 19	F1 DHA 0.1 AA 0.43 F2 LA 21.8 ALA 2.2	Plasma and RBC at 2, 4, 6, 9 and 12 mo	12 mo	Visual acuity at 2, 4, 6, 9, 12 mo	F1 better visual acuity only at 2 mo. HM and F1 had higher grating acuity than F2 infants.
Forsyth et al. (1998)	F1 24 F2 24 HM 27	F1 DHA 0.2 AA 0.35 F2 LA 11.4 ALA 0.7	NA	4 mo	Infant habituation at 4 mo	F1 significantly shorter fixation times. Mean scores on habituation not different
Auestad et al. (1997)	F1 26 F2 28 F3 28 HM 38	F1 DHA 0.12 AA 0.43 F2 DHA 0.23 F3 LA 21.9 ALA 2.2	RBC phospholipids at 2, 4, 6, and 12 mo	12 mo	Sweep VEP and FPL acuity at 2, 4, 6, 9, 12 mo	No significant differences between groups
Willatts et al. (1998)	F1 21 F2 22	F1 DHA 0.2 AA 0.35 F2 LA 11.4 ALA 0.7	NA	4 mo	Problem solving 10 mo	F1 infants had significantly more intentional solutions than F2 infants
Jørgensen et al. (1998)	F1 14 F2 12 F3 11 HM 25	F1 DHA 0.3 GLA 0.5 F2 DHA 0.3 F3 LA 12 ALA 1.2 DHA 0.38 AA 0.4	RBC phospholipids at 1, 2, and 4 mo	4 mo	Sweep VEP 4 mo	HM better than F1. F2 non-significantly better visual acuity than F3. Visual acuity significantly different among all feeding groups. No significant difference among F1, F2 and F3
Scott et al. (1998)	F1 38 F2 33 F3 42 HM 60	F1 DHA 0.12 AA 0.43 F2 DHA 0.23 F3 LA 21.9 ALA 2.2	RBC phospholipids at 2, 4, 6, and 12 mo	12 mo	BSID I PDI and MDI at 12 mo	No significant differences between groups

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Author, year	Intervention/ groups (n)	LCPUFA level in intervention	LCPUFA assessment	Duration of supplementation	Developmental Tests	Findings
Birch et al. (1998)	F1 23 F2 22 F3 23 HM 21	F1 DHA 0.36 AA 0.72 F2 DHA 0.35 F3 LA 14.6 ALA 1.49 DHA 0.29 AA 0.56	Cord blood plasma and RBC phospholipids at 17 and 52 wks	4 mo	FPL and Sweep VEP at 6, 17, 26, 52 wk	F1, F2 and HM significantly better visual acuity than F3 at 6, 17, 26, 52 wk significantly
Lucas et al. (1999)	F1 154 F2 155 HM 138	F1 DHA 0.32 AA 0.3 F2 LA 12.4 ALA 1.1	NA	6 months	Knoblauch, Passamanik, and Sherrards DSI at 9 mo	No significant differences at 9 and 18 mo. But MDI and PDI scores higher in F1 group
Makrides et al. (2000a)	F1 24 F2 23 F3 21	F1 DHA 0.34 AA 0.34 F2 DHA 0.35 F3 DHA 0	Plasma and RBC phospholipids at 16 and 34 wks	12 months	VEP test at 16 and 34 weeks, MDI and PDI at 1y	No significant differences between groups for VEP. HM infants had better VEP acuity at 34 weeks of age. MDI and PDI scores were similar F1, F2 and F3. Plasma AA significantly influenced PDI at 1 year. Plasma and RBC EPA positively associated with PDI
Birch et al. (2000)	F1 19 F2 17 F3 20	F1 DHA 0.36 AA 0.72 F2 DHA 0.35 F3 LA 14.6 ALA 1.49	Plasma and RBC phospholipids at 4 and 12 mo	7 weeks	VEP and FPL at 4 mo and 12 mo	F3 MDI score worse than F1. No significant differences or correlations at 4 and 12 mo
Makrides et al. (2000b)	F1 24 F2 23 F3 21 HM 46	F1 DHA 0.34 AA 0.34 F2 DHA 0.35 F3 LA 16.8 ALA 0.13 DHA 0.2 AA 0.4	Plasma and RBC phospholipids at 6, 16, and 34 wks	34 weeks	Flash VEP at 16 and 34 weeks BSID I PDI and MDI at 12 mo	No significant differences in VEP between groups. MDI and PDI not different or related to plasma DHA
Auestad et al. (2001)	F1 80 F2 82 F3 77 HM 165	F1 DHA 0.14 AA 0.46 Egg F2 DHA 0.14 AA 0.46 F/F F3 LA 20 ALA 2 DHA 0.14 AA 0.46	Plasma and RBC phospholipids at 4 and 12 mo	12 months	Visual acuity at 2, 4, 6, and 12 mo. FTII at 6 and 9 mo. BSID II PDI and MDI at 6 and 12 mo. MacArthur CDI at 9 mo. IBQ at 6 and 12 mo. FPL acuity at 2–12 mo	No differences in developmental tests, visual acuity, and developmental indices

CHAPTER 2: LITERATURE REVIEW

Author, year	Intervention/ groups (n)	LCPUFA level in intervention	LCPUFA assessment	Duration of supplementation	Developmental Tests	Findings
Birch et al. (2002)	F1 30 F2 28	F1 DHA 0.36 AA 0.72 LA 15 ALA 1.5 F2 LA 15 ALA 1.5	Plasma and RBC phospholipids at 17 and 52 wks	46 weeks	VEPs and growth at 6, 17, 26, and 52 wks. Stereo acuity at 17, 26, 39, and 52 wks	F2 had significantly poorer visual acuity at 17, 26, and 52 wks and significantly poorer stereo acuity at 17 wks. Significant correlations between high plasma DHA and better visual acuity and stereo acuity at 17 wks. Significant correlations between high plasma and RBC DHA and better acuity at 52 wks
Bouwstra et al. (2003)	F1 119 F2 131 HM 147	F1 DHA 0.30 AA 0.45	NA	2 months	GMs at 3 mo	Mildly abnormal GMs significantly more prevalent in F2. Normal-optimal GMs more frequent in HM than F1 and F2
Hoffman et al. (2003)	F1 30 F2 31	F1 DHA 0.36 AA 0.72	RBC phospholipids between 4 and 6 mo and at 12 mo	6 months	VEP 4, 6 and 12 mo Stereo acuity at 4, 6, 9 and 12 mo	Significantly better VEP in F1. No significant differences in stereo acuity. Trend of better stereo acuity at 9 and 12 mo. Significant correlations between visual function and RBC DHA at 12 mo
Hoffman et al. (2004)	F1 25 F2 26	F1 130mg DHA/day F2 DHA 0	Plasma and RBC phospholipids at 6 and 12 mo	6 months	VEP and Stereo acuity at 6, 9 and 12 mo	F1 had sig better visual acuity at 9 and 12 mo. VEP at 12 mo correlated with RBC DHA levels. No significant differences in stereo acuity
Ben et al. (2004)	F1 69 F2 52 HM 26	F1 DHA 0.18 AA 0.18 LA 435mg/dm3 ALA 62mg/dm3 F2 LA 435 mg /dm3 ALA 62mg/dm3	NA	6 months	BSID at 3 mo	No significant differences for growth and development between the groups
Ünay et al. (2004)	F1 22 F2 22 HM 23	F1 DHA 0.5	NA	16 weeks	Brainstem auditory evoked potentials (BAEPs) at 1 and 16 wks	Significantly more rapid maturation of auditory brainstem at 16 weeks in F1 than F2
Birch et al. (2005)	F1 42 F2 44	F1 DHA 0.36 AA 0.72 LA 15 ALA 1.5 F2 LA 15 ALA 1.5	RBC phospholipids at 16, 17 and 39 wks	52 weeks	VEP and Stereo acuity at 6, 17, 39 and 52 wks	VEP acuity significantly better in F1 than F2 at 6, 17, 26, and 52 wks. Stereo acuity in F1 significantly better than F2 at 17 wks but not at 39 and 52 wks

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Author, year	Intervention/ groups (n)	LCPUFA level in intervention	LCPUFA assessment	Duration of supplementation	Developmental Tests	Findings
Agostoni et al. (2009)	F1 529 F2 542	F1 400 IU Vitamin D3 20mg DHA F2 400IU Vitamin D3	NA	12 mo	*4 gross motor milestones and **2 early fine motor milestones and ***1 early language milestone	Time to achievement of gross motor milestones did not differ significantly between groups. Time to achievement of fine motor milestones and language was shorter in F1. Significant independent association of the intervention product with a shorter time for sitting without support, fine motor and early language milestones
Birch et al. (2010)	F1 65 F2 59 F3 64 F4 56	F1 DHA 0.96 AA 0.64 F2 DHA 0.64 AA 0.64 F3 DHA 0.32 AA 0.64 F4 DHA 0 AA 0	RBC phospholipids at 4 and 12 mo	12 mo	VEP at 1.5, 4, 9 and 12 mo	F4 significantly poorer visual acuity at 12 mo than F1, F2 and F3. No significant differences in VEP between F1, F2 and F3
Colombo et al. (2011)	F1 20 F2 16 F3 21 F4 15	F1 DHA 0.96 AA 0.64 F2 DHA 0.64 AA 0.64 F3 DHA 0.32 AA 0.64 F4 DHA 0 AA 0	NA	12 mo	Habituation and attention at 4, 6 and 9 mo	F3 and F4 had significantly higher sustained attention. No significant differences between F4 and other groups

*Sitting without support, hands-and-knees crawling, standing alone, and walking alone. **Reaches an object to touch and brings toy to mouth. ***Saying the first comprehensible word composed of 2 syllables (excluding “dada” and “mama”). HM-Human milk group (used as a reference group); LCPUFA- Long-chain polyunsaturated fatty acids; DHA- Docosahexaenoic acid; AA- Arachidonic acid; LA- Linoleic acid; GLA- γ-linolenic acid; ALA- α-linolenic acid; EPA- Eicosapentaenoic acid; RBC- Red blood cell; VEP- Visual Evoked Potentials; MDI- Mental development index; PDI- Psychomotor development index; BSID II- Bailey’s II Scales of Childhood Development; FTII- Fagan Test of Infant Intelligence; CDI- Communicative Development Inventories; BSID I- Bailey’s I Scales of Childhood Development; IBQ- Infant Behaviour Questionnaire; DSI- Developmental Screening Inventory; FPL- Forced-choice preferential looking

2.7.2.2 Neurological and cognitive development

Neurological development and cognitive development are greatly influenced by LCPUFA supply during infancy. Suboptimal LCPUFA accretion in the developing CNS is detrimental to the brain development of the growing infant because it could affect both the infant's emotional and intellectual development (Neuringer *et al.*, 1994). Cognition is a comprehensive word which encapsulates a variety of psychological processes which include memory, learning, reasoning, attention and language. Therefore, cognitive development refers to the changes that occur in different cognitive processes and these changes are monitored over long time periods. Mental or cognitive development of infants is considered multidimensional and nonlinear. This is because it is an outcome or effect of physical growth, neurological maturation, interactions with the environment, and a combination of stimuli (Eilander *et al.*, 2007).

A wide range of tests has been used in different studies to measure different components of brain development. Tests of general movements include the Bayley Scales of Infant Development (BSID I & II) i.e. Psychomotor and Mental Development Indices (PDI & MDI) and Hempel tests. The assessment of the quality of General Movements (GMs) is also a delicate tool for evaluating the effect of nutritional status on neurological development. GMs are characterised by spontaneous movements which involve all parts of the body. Variation, complexity and fluency are features that typically depict normal GMs. However, these features are not present when movements are abnormal. The development of cerebral palsy is envisaged by a clear manifestation of definitely abnormal GMS between the ages of 2 to 4 months (Prechtl *et al.*, 1997; Hadders-Algra & MC Groothuis, 1999; Hadders-Algra *et al.*, 2004). The occurrence of mildly abnormal GMs at that same age, on the other hand, is associated with an increase in the risk of the development of minor neurological dysfunction, attention problems and aggressive behaviour at a later age (Groen *et al.*, 2005).

Psychomotor development is also assessed using the Brunet-Lézine test and more specific assessments include problem-solving and language development tests. As mentioned earlier, these developmental tests assess different components of brain development and, thus, would explain the discrepancies in study results (Carlson & Neuringer, 1999; Uauy *et al.*, 2003; McCann & Ames, 2005). The Gesell Development Inventory (Knobloch *et al.*, 1980) is also used to assess gross motor skills, while language development can also be assessed using the Clinical Linguistic and Auditory Milestone Scale (CLAMS). The MacArthur Communicative Development Inventory is specifically used to assess the development of language and communication skills (Fenson *et al.*, 1994). The visual-motor problem-solving abilities can be assessed by the Clinical Adaptive Test (CAT) (Jensen *et al.*, 2005). The Peabody Picture Vocabulary Test-R (PPVT) is also a

standardised test that measures receptive vocabulary that correlates with IQ (Kannass *et al.*, 2009).

Furthermore, measuring the sleep and wakeful states of infants gives researchers an instrument to assess the functional integrity of the CNS from the time of birth. This is valuable because infant's sleep patterns are an expression of central integrative control (Thoman & Whitney, 1989). There are several processes in the brain which involve both neural and humeral processes in different regions of the brain to generate sleep and wakefulness (Whitney & Thoman, 1993). Sleep-state organisation and CNS function show that changes in sleep architecture are associated with neurological changes during development. Also, unusual sleep patterns are associated with deficits in neurological development (Freudigman & Thoman, 1993; Whitney & Thoman, 1993). In this regard, there is a growing interest in assessing sleep patterns in infants, and sleep recordings can be obtained using the Motility Monitoring System (MMS).

Infant habituation is another simple method that measures memory and cognition in infants. Habituation is described as a decrease in attention to stimulus over several presentations and basically measures looking time. Infant habituation shows the infant's efficiency in processing information, and patterns of habituation are often used to identify differences in attention control. In this regard, characteristics of better attention control include a pattern of a peak fixation (longest fixation) early in the sequence of fixations, followed by a rapid decrease in attention, and the total fixation time being short. Conversely, characteristics of poorer attention control include a pattern of the peak fixation occurring late in the sequence, a slower decrease in attention, and a longer overall fixation time (Forsyth *et al.*, 1998).

2.7.2.2.1 LCPUFA status and neurodevelopment

A total of eight studies (Table 2-8, section 2.5.2.1.1) were identified which assessed the associations between LCPUFA status and cognitive development. Three studies (Innis *et al.*, 2001; Agostoni *et al.*, 2003b; Jacobson *et al.*, 2008), used the BSID I & II to assess neurological function. Significant positive correlations were found between higher plasma AA and higher PDI scores at five months (Agostoni *et al.*, 2003b), as well as between higher cord DHA and better MDI and PDI scores at 11 months (Jacobson *et al.*, 2008). There were no associations found between breast milk DHA and umbilical cord artery/vein phospholipids and PDI or MDI scores.

In addition, one study (Bouwstra *et al.*, 2006) used the Hempel tests to evaluate the quality of GMs. In this study, umbilical cord phospholipids were used as proxy for maternal FA status. At three months of age, infants that were born to mothers with low AA status, had significantly higher levels of abnormal general movement scores. Consistent with these data, Dijck-Brouwer *et al.*

(2005) reported associations between lower neonatal DHA, AA and EFA status with less favourable neurological condition on postnatal days 10-14.

Moreover, infants whose mothers had higher levels of plasma DHA at birth displayed augmented developmental progressions in attention during the first year of life (Colombo *et al.*, 2004). Only one study (Kannass *et al.*, 2009) used the PPVT to measure receptive vocabulary. However, there were no associations found between maternal FA status and attention functioning. Significant positive associations were found between the DHA status of infants and sleep states. These data are consistent with data from another study which suggest that intake of EFAs may regulate sleep (Cheruku *et al.*, 2002).

2.7.2.2.2 Maternal LCPUFA supplementation during lactation and effects on neurodevelopment of their neonates

Four randomised controlled trials (Gibson *et al.*, 1997; Helland *et al.*, 2001; Jensen *et al.*, 2005; Lauritzen *et al.*, 2005) were identified that assessed the effect of supplementing lactating mothers with n-3 LCPUFA, on cognitive development of their infants (Table 2-9, section 2.5.2.1.2). These studies used the BSID I & II, EEG, MacArthur CDI, GDI, CLAMS and CAT neurodevelopmental tests. There were no significant differences between the supplemented groups and control groups except in one study that assessed vocabulary using the McArthur CDI (Lauritzen *et al.*, 2005).

In this study (Lauritzen *et al.*, 2005), maternal fish oil supplementation during lactation significantly improved problem solving in girls at nine months, while no such effect was found in boys. Also, fish oil supplementation resulted in a significantly negative effect on vocabulary comprehension at one year in the entire group as well as in boys, and on sentence complexity in boys. This study also showed that passive vocabulary at one year was lower in children whose mothers were supplemented with fish oil compared with those supplemented with olive oil. In girls, infant DHA status at four months positively correlated with problem solving at nine months, while infant DHA status at four months negatively correlated with vocabulary comprehension in boys at 12 months.

Although there were no significant differences between groups, significant positive associations were found between infant DHA status and MDI at one year (Gibson *et al.*, 1997). Also, mature EEG was associated with higher DHA umbilical plasma PLs on the second day of birth although there were no differences observed at three months (Helland *et al.*, 2001). These results show that DHA content in breast milk may contribute to the mental development of the infant. This is consistent with the infant feeding recommendations that support breastfeeding during the first two years of life.

Based on the studies that have been identified, the effects of DHA supplementation during lactation on cognitive development are inconsistent. The study sample used by Gibson *et al.* (1997) was considered too small to detect an effect, while the study by Lauritzen *et al.* (2005) had adequate power but found mixed effects on DHA supplementation during lactation. There were no effects on motor development reported in all four studies. Lauritzen *et al.* (2005) and Jensen *et al.* (2005) reported inconsistent results on the effects of DHA supplementation on language and development. The discrepancy in these results could be due to the different language development tests that were used, as well as the dosage of DHA used in the trials (Eilander *et al.*, 2007).

2.7.2.2.3 Dietary LCPUFA during infancy and neurodevelopment

Thirteen randomised controlled trials were identified that measured neurodevelopmental outcomes, particularly cognitive development (Table 2-10, section 2.5.2.1.3). The dietary interventions used in these studies include egg yolk PLs, LCPUFA derived from fish oil and evening primrose oil, single-cell oils, as well as tuna oil and single-cell oil produced by soil fungus. Five of these studies (Lucas *et al.*, 1999; Makrides *et al.*, 2000a; Makrides *et al.*, 2000b; Auestad *et al.*, 2001; Ben *et al.*, 2004) used the BSID I and II to measure cognitive development at or before one year of age. Results of these studies showed no significant differences between LCPUFA-supplemented and control groups. However, in one study (Lucas *et al.*, 1999), MDI and PDI units were higher in the supplemented group than in the controls. One study (Agostoni *et al.*, 1995) used the Brunet-Léizine developmental quotient to measure cognitive development. In this study, infants who were fed the LCPUFA-supplemented infant formula had significantly better scores in the Brunet- Léizine test compared with those fed a standard infant formula. Significant correlations were found between RBC DHA levels and DQ scores at four months of age.

Another study (Forsyth *et al.*, 1998) assessed cognitive development by measuring infant habituation. In this study, infants in the LCPUFA-supplemented formula group exhibited shorter fixation times; meaning that the infants in the supplemented group had better control of attention. These infants were then followed up in another study (Willatts *et al.*, 1998) to determine whether LCPUFA supplementation also has an effect on problem solving at the age of nine months. In this particular study, infants who received the LCPUFA-supplemented formula, had significantly more problem solutions at nine months, suggesting better information processing and problem solving in infants supplied with adequate amounts of LCPUFA during infancy. Similarly, in another study (Colombo *et al.*, 2011), infants that were fed the highest dose of supplemented infant formula did not differ from any other groups in the time engaged in active stimulus processing. However, in this study LCPUFA supplementation showed positive effects on the infant psychophysiological response and on the quality of attention at four, six and nine months. These data can be explained

by the rapid accretion of LCPUFAs in the cell membranes of the CNS which increases the speed at which infants are able to process information. Infants who exhibit signs of faster processing also tend to have faster reaction times (Forsyth & Willatts, 2002).

The quality of GMS was also used to assess cognitive development at three months of age. While the breastfed infants in this group had normal-optimal GMs, infants who were fed supplemented infant formula showed greater signs of abnormal spontaneous GMs. Similarly, with one study that assessed four gross and two fine motor milestones, the time to achievement of the gross motor milestones did not differ between groups. However, the time to achievement of the fine motor milestones was shorter in the intervention group (Agostoni *et al.*, 2009). Also, Ünay *et al.* (2004) measured the changes in auditory evoked potentials and found that the auditory brainstem response matured more rapidly in infants supplemented with 0.5% DHA during the first 16 weeks of life. These findings show a beneficial effect of LCPUFAs during infancy and that LCPUFA supplementation can be beneficial in the neurodevelopment of infants.

In 2008, Simmer and colleagues, in their Cochrane review, concluded that while LCPUFAs are important during infancy for optimal growth and development, there was very little evidence of the beneficial effects of LCPUFA supplementation on cognitive development. They based their conclusion and findings on a systematic review of the nine randomised controlled trials that have also been identified in this current literature review. Their conclusions were consistent with other systematic reviews (SanGiovanni *et al.*, 2000; Uauy *et al.*, 2003; McCann & Ames, 2005; Eilander *et al.*, 2007; Koletzko *et al.*, 2008) that also evaluated the effect of LCPUFA supplementation on cognitive development in infants and which also concluded that LCPUFAs seem to be of favourable importance in processing information and solving problems during the first year of life (Simmer *et al.*, 2008).

A recent meta-analysis, (Jasani *et al.*, 2017) evaluated randomised controlled trials in which interventions were done between zero and 12 months. It was concluded that these studies do not show any beneficial effects of LCPUFA supplementation on neurodevelopment and cognitive development. Therefore, according to their meta-analysis, supplementing full-term infants with LCPUFA-enriched infant formula cannot be recommended at the present moment. However, significant positive effects of LCPUFA supplementation during early infancy may be seen later on in childhood (Birch *et al.*, 2000; Birch *et al.*, 2007; Drover *et al.*, 2011; Colombo *et al.*, 2013).

2.7.2.3 Growth outcomes

Observational studies found that breast milk DHA was positively associated with growth in preterm infants in Brazil (Tinoco *et al.*, 2009) while no such associations were found in Cuban

(Krasevec *et al.*, 2002) and Burkina Faso (Rocquelin *et al.*, 2003) infants. However, Rocquelin *et al.* (2003) found associations between breast milk FAs and weight gain, in Congo. Weight gain in early infancy was associated with a poor plasma LCPUFA status at birth. Thus, neonatal FA profiles determined from placental tissue and cord blood, could potentially influence the growth trajectory of the infant (O'Tierney-Ginn *et al.*, 2017).

Data on the effect of LCPUFAs on growth and anthropometry are inconclusive. Nonetheless, Gambian infants supplemented with fish oil between three and nine months of age showed an increase in mid-upper-arm circumference and skinfold thickness at 12 months, but not in linear growth (Van der Merwe *et al.*, 2013). There was no effect on weight, length or body mass index observed after supplementing Danish infants from nine to 12 months (Damsgaard *et al.*, 2006). On the other hand, a systematic review indicated that adding both DHA and AA to infant formula milk supports growth; that is similar to infants that are fed unsupplemented infant formula (Makrides *et al.*, 2005), implying no effect on the growth of infants. Similarly, several reviews and meta-analyses (Eilander *et al.*, 2007; Simmer *et al.*, 2008; Delgado-Noguera *et al.*, 2015; Jasani *et al.*, 2017) did not find any significant effects of LCPUFA supplementation on growth of term infants.

2.7.2.4 Other outcomes

2.7.2.4.1 Allergies and immune response

LCPUFAs also have a potential role in the development of the infant's immune system. Infants are born with relatively immature immune systems, and the immune system was shown to mature faster in breastfed infants than in infant formula-fed infants (Field *et al.*, 2001). Over the years, there has been a belief that early introduction of fish to the diet of infants is associated with a potential increase in allergic reactions. Yet, the available evidence suggests that the timely introduction of fish and other allergenic complementary foods, between 17 and 26 weeks, is actually beneficial in reducing the risk of developing allergic reactions (Perkin *et al.*, 2016; Prell & Koletzko, 2016). Likewise, Kull *et al.* (2006) and Alm *et al.* (2009) concur that early introduction of fish in infancy during the complementary feeding period is associated with a reduced incidence of allergic reactions in childhood. However, regardless of the likelihood of LCPUFAs in fish influencing the immune system, other dietary and lifestyle factors are also involved (Kiefte-de Jong *et al.*, 2012)

Based on observational studies, randomised controlled trials and meta analyses, it was concluded that the infant's LCPUFA status seems to have a protective influence on the development of allergies (Koletzko *et al.*, 2014). In their systematic review, Richard *et al.* (2016) found that low DHA concentrations in breast milk are associated with an increased risk of atopic disease in the

infant. Similarly, the ratio of n-6 to n-3 FAs also plays a significant role in this, with high ratios posing negative outcomes and risks. On the other hand, there is still conflicting evidence on the contribution of AA to the development of atopic disease. Richardson and colleagues (2016) also found that supplementation with DHA or a combination of DHA + EPA during lactation improves the markers of allergy and atopic disease in breastfed infants. However, factors like the family history of allergy or atopic disease and the genetic make-up also have to be considered as possible factors which may influence the infant's response to maternal LCPUFA supplementation.

Although a high dose of DHA from nine to 12 months had no effect on the markers of innate immunity or general inflammation in healthy infants, researchers suggest that the immune system matures faster with the supplementation of fish oil, but without apparent reduction in immune activation. Hence, a much higher content of DHA in breast milk was significantly associated with an improved maturation of the infant's immune system (Damsgaard *et al.*, 2007). Also, infants that were fed DHA- and AA-enriched formulas were shown to have immune cell distribution and cytokine profiles (Field *et al.*, 2008) and a higher proportion of antigen-mature lymphocytes similar to those fed breast milk (Foiles *et al.*, 2016). Similarly, two other studies reported that regular consumption of fish during complementary feeding in infants was associated with less allergy, eczema, hay fever and asthma up to toddler ages (Kull *et al.*, 2006; Alm *et al.*, 2009).

2.7.2.4.2 Markers of cardiovascular disease

When compared with infants fed standard infant formula without preformed LC-PUFAs, breastfeeding led to a moderate decrease in heart rate and blood pressure in breastfed infants (ENeA, 2016). The differences in systolic and diastolic blood pressure might be small to negligible, but this reduction may have positive benefits and thus influence the incidence of cardiovascular disease. Although several factors influence the long-term regulation of blood pressure, LCPUFA intake from breast milk is suggested to be one of them (Martin *et al.*, 2005). In this regard, research has shown that markers of cardiovascular diseases persist into adulthood. Infants are vulnerable to several nutritional disturbances, particularly during the period of complementary feeding.

Damsgaard *et al.* (2006), concur that LCPUFAs are associated with cardiovascular markers and, as such, influence the cardiovascular profile of the individual in late childhood and sometimes in adulthood. In their study, fish oil supplementation between nine and 12 months lowered the blood pressure of Danish infants. Enhancement of membrane fluidity and influence of LCPUFA on membrane receptors are some of the mechanisms which were proposed for this. However, more research is needed to determine whether or not contrasting results show that LCPUFAs play such a major role or whether it is just the heterogeneity of methodology.

2.7.3 Lipid based nutrient supplements and effects on infant growth and development

The use of lipid-based nutrient supplements (LNSs), particularly, small quantity lipid-based nutrient supplements (SQ-LNSs), containing both ALA and LA is currently being investigated. Multiple micronutrient-fortified semi-solid pastes that are usually prepared from vegetable oil, ground nut paste, milk, sugar and different micronutrients are also considered as a sub-class of LNSs. LNSs are a cost-effective and affordable product which can be used as a supplement or complementary food with the aim of improving the energy and nutrient content of the infant's diet during weaning (Adu-Afarwuah *et al.*, 2011). Recent research has also shown that inadequate EFA intake is inversely associated with linear growth of children aged between six and 12 months. In particular, SQ-LNSs containing EFAs have recently been shown to improve linear growth thereby reducing the incidence of stunting in Ghana, Haiti and Indonesia in infants aged between six and 12 months (Adu-Afarwuah *et al.*, 2007; Iannotti *et al.*, 2014; Muslihah *et al.*, 2016) respectively. The inclusion of FAs to combat and prevent malnutrition, therefore, is a very recent strategy of supplementation in other countries.

Adu-Afarwuah *et al.* (2007), in their study found that a lower percentage of the non-intervention group of infants could walk independently compared with the intervention groups. All three supplement groups in this study including the infants receiving LNS, showed positive effects on motor milestone development by the age of 12 months compared with the non-intervention group. In addition, another study that was carried out in rural Malawi (Phuka *et al.*, 2008) showed that infants who received LNS daily for 12 months had comparable motor milestone developmental outcomes by the age of 18 months. Also, a greater proportion of the children in the LNS group were able to walk independently by the age of 12 months (Prado *et al.*, 2016b) and 18 months (Prado *et al.*, 2016a). Similarly, LNS interventions showed positive effects on child development, particularly motor and language development at 12, 18 and 24 months in a study by Matias *et al.* (2017). We also previously reported a transient effect of LCPUFA-enriched SQ-LNS on growth and a positive effect on the locomotor outcome in psychomotor development (Smuts *et al.*, 2018).

Although there are studies that have investigated the effects of LCPUFA on motor milestone development and neurological performance in other countries, there are no studies to our knowledge that have tested the hypothesis of any effect of EFAs or LCPUFAs like DHA on motor milestone development in South African infants. This current PhD study did not investigate the effects of the LNS on motor milestone development but included a cross-sectional analysis at baseline to determine the potential associations between FA status, growth and psychomotor development. In addition, it determined whether the provision of a SQ-LNS containing the EFAs ALA and LA, and a SQ-LNS containing EFAs and the LCPUFAs DHA and AA, from six to 12 months can improve LCPUFA status in comparison with control infants.

2.7.4 Effects of diet deficient in LCPUFA in infancy

According to Trumbo *et al.* (2002), a clinical deficiency of ALA and LA manifests itself in the form of neurological abnormalities and poor growth in infants. In addition, an EFA deficiency (EFAD) weakens lipid and energy metabolism, cell membrane structures, lipid signalling pathways and eventually leads to death (Cunnane, 2003). Several FA indices in structural phospholipids have been used as proxies for assessing EFA and LCPUFA status. Historically, Mead acid levels above 0.21% (Siguel *et al.*, 1987), the ratio of Mead to AA > 0.02% (Siguel *et al.*, 1987; Siguel, 1998), the DHA to Osbond ratio (Fokkema *et al.*, 2002; Vlaardingerbroek & Hornstra, 2004), and the essential PUFA status index (Vlaardingerbroek & Hornstra, 2004) have been used to determine essential fatty acid deficiency.

Observational studies in both developed and developing countries suggest that lower levels of DHA in breast milk are associated with higher rates of postpartum depression (Hibbeln, 2002). This can be detrimental to the infant as maternal depression can impact maternal caregiving abilities and thereby affect growth of the infant through inadequate feeding practices. The development of the infant is also compromised as maternal depression can impact interpersonal interactions between mother and infant (Patel *et al.*, 2004; Huffman *et al.*, 2011).

Clinical manifestations in children which may be associated with abnormalities of enzymes involved in phospholipid metabolism include attention-deficit/hyperactivity disorder, dyspraxia, dyslexia and autism. However, these symptoms may be alleviated by provision of dietary LCPUFA supplementation (Young & Conquer, 2005). Neuronal arborisation deficits and synaptic pathological changes, including serotonin and mesocorticolimbic dopamine neurotransmission, and limited regulation of the limbic system by the frontal cortex are also associated with a deficiency of DHA or EPA. This may lead to neurocognitive deficits, anxiety, aggression, and depression later in life (Zimmer *et al.*, 2002; Yao & van Kammen, 2004; Hibbeln *et al.*, 2006).

The accumulation of DHA takes place rapidly in *utero* via the placenta, after delivery through the mother's milk, and later in postnatal life, through the diet. Rats of two or three generations were fed n-3 FA-deficient diets and results showed remarkable decreases in nervous system DHA (Ahmad *et al.*, 2002). A deficiency of DHA in the brain results in a loss of several sensory, behavioural and cognitive functions in both animals and human beings. These functions include remarkable losses in visual sensitivity, visual acuity, visual attention and visual recognition memory, dark-adaptation responses, two-odour discrimination, and spatial navigation memory (Ahmad *et al.*, 2002).

In both humans and animals, the loss of DHA in the CNS is made up for by an increase in adrenic acid (C22:4n-6), osbond acid (C22:5n-6) and sometimes AA. The substitution of DHA by the n-6

FAs may to a certain extent, fulfil the needs of the membrane FAs, but the n-6 FAs and DHA have different functional properties. Thus, the complexities related to this would be consequences of both the loss of DHA and the replacement of DHA by n-6 FAs (Mulder *et al.*, 2014).

LA also plays an important role in the structure of skin ceramides (Feingold & Elias, 2014). Hence, a deficiency of LA will result in a reduction in water barrier and a further reduction in thermal insulation which would then lead to an increase in energy expenditure (Nakamura *et al.*, 2014). This is a precise explanation of how trans-epidermal water loss and complementary heat loss can justify the main symptoms of LA deficiency, which include growth retardation, excessive eating and intense thirst. All the factors could potentially play a role in the inefficient use of dietary calories during EFAD, thereby leading to a reduction in growth efficacy (Smit *et al.*, 2004; Glew & VanderJagt, 2013).

2.8 LCPUFA NUTRITION DURING BREASTFEEDING AND COMPLEMENTARY FEEDING

2.8.1 LCPUFA concentrations in breast milk and LCPUFA status of lactating mothers and their breastfed infants

2.8.1.1 Fatty acids in breast milk

FAs in breast milk are derived from mobilisation of endogenous maternal stores of FAs, synthesis of FAs by the liver or breast tissue, and from the diet (Jensen & Lapillonne, 2009; Lee *et al.*, 2013; Innis, 2014). The breast tissue is particularly rich in the lipolytic enzyme lipoprotein lipase, which catalyses the breakdown of lipids. Thus, during lactation, ingested dietary FAs are absorbed and subsequently incorporated into chylomicrons then transferred quickly from the plasma into the breast milk (Roy *et al.*, 2012; Blackburn, 2014).

In addition, both FAs synthesised in the mammary gland and FAs made available from the maternal plasma are influenced by maternal nutrition (Lee *et al.*, 2013; Innis, 2014). Particularly, the dietary supply of DHA influences the DHA concentrations in breast milk as research has shown that dietary DHA intake is linearly correlated with breast milk DHA (Fidler *et al.*, 2000; Del Prado *et al.*, 2001; Thiombiano-Coulibaly *et al.*, 2003; Peng *et al.*, 2009). However, research from stable isotope studies has shown that 90% of breast milk AA does not come directly from absorbed dietary lipids but from the maternal AA stores in the body.

Short term dietary modifications were also shown to influence the LCPUFA concentrations in breast milk (Sanders & Reddy, 1992; Smit *et al.*, 2000; Brenna *et al.*, 2007). Smaller studies showed that fish-eating populations have higher breast milk DHA concentrations compared with populations that do not consume sea food (Ogunleye *et al.*, 1991; Olafsdottir *et al.*, 2006). Thus

the FA concentrations in breast milk will also vary depending on accessibility to food in a specific region (Yuhas *et al.*, 2006; Brenna *et al.*, 2007). Additionally, food preparation practices also influence maternal LCPUFA status and breast milk levels (Lee *et al.*, 2013). For instance, the breast milk DHA concentrations from two diverse populations in Tanzania were analysed. The breast milk DHA levels were higher in the group of mothers who consumed local fish that was fried in sunflower oil compared with those that ate steamed fish or meat at least once a week (Kuipers *et al.*, 2005).

The amounts of EFAs found in breast milk also vary depending on the mother's access to different vegetable oils. Hence, an increased intake of vegetable oils or plant-derived EFAs would result in higher levels of n-6 and n-3 essential fatty acids (Lee *et al.*, 2013). This was seen in a study that found significant differences in ALA and LA levels in the breast milk of vegans, vegetarians and omnivores (Sanders & Reddy, 1992).

The duration of pregnancy and stage of lactation also play a major role in the fat composition of breast milk (Sala-Vila *et al.*, 2005; Jang *et al.*, 2011). Nearly all LCPUFAs increased significantly ($P < 0.001$) between six weeks and the sixth month of lactation in a study that compared the breast milk FA profiles of lactating mothers (Szabo *et al.*, 2010). Also, maternal LCPUFA status and breast milk levels are not only affected differently by the diet, but are also influenced by smoking, which leads to reduced production of LCPUFAs in the mammary gland (Agostoni *et al.*, 2003a; Agostoni *et al.*, 2008b).

Furthermore, breast milk LCPUFAs are influenced by the variation in the *FADS1* and *FADS2* gene cluster which determines the conversion of LCPUFAs from their parent EFAs (Lattka *et al.*, 2011). Women who are homozygous for the minor allele associated with lower desaturase activity were found to have decreased breast milk DHA. In these women, DHA supplementation or increased fish consumption improved DHA concentrations only in plasma but not in breast milk. These findings imply that increased consumption of fish and the use of supplements may not be beneficial for mothers who are homozygous for the minor allele. The authors speculate that the *FADS* gene cluster is linked to FA transportation in some way. However, the identification of the specific transporter and the genetic regulation needs to be addressed in future studies (Moltó-Puigmartí *et al.*, 2010).

2.8.1.2 LCPUFA concentrations in breast milk

LA concentrations in breast milk have increased three times or more in the USA since the mid-1940s, together with an increased intake of dietary vegetable oil and LA. ALA concentrations have remained constant, resulting in a marked increase in the ratio of LA to ALA (Ailhaud *et al.*, 2006; Delplanque *et al.*, 2015). While the LA concentrations in breast milk increased in Australia from

1981 to 2000, DHA concentrations were shown to have significantly decreased (Gibson *et al.*, 2011). This decrease in DHA may have been due to an increased intake of LA, which then led to a reduction in the conversion of ALA to DHA. This may have also been related to a concomitant decrease in dietary intake of DHA (Stam *et al.*, 2013).

In addition, maternal diet high in carbohydrates and low in fat was shown to result in higher medium-chain triglyceride concentrations in breast milk (Nasser *et al.*, 2010). A high carbohydrate diet also resulted in higher AA, while a high fat diet resulted in higher DHA levels in breast milk (Nyuar *et al.*, 2010). The average DHA concentrations in breast milk vary worldwide, especially in developing countries. In their systematic review, Brenna *et al.* (2007) included 65 studies that reported breast milk DHA and AA concentrations worldwide. They reported mean breast milk AA and DHA concentrations of $0.47 \pm 0.13\%$ and $0.32 \pm 0.22\%$ respectively (% of total FAs). Coastal populations and those that consumed marine food regularly were found to have higher breast milk DHA concentrations.

In comparison with other low-income and middle-income countries, South Africa is among the countries with the lowest DHA and AA breast milk concentrations as shown in Table 2-11. However, the study by Van der Westhuyzen *et al.* (1988) included in the review is an old study and poor technological capability at that time could have led to incorrect estimation of DHA hence the purpose of the current PhD study. Mature breast milk typically contains 34–47% saturated FAs, 31–43% MUFAs, approximately 12%–26% n-6 PUFA and approximately 0.8%–3.6% n-3 PUFA. Palmitic acid (C16:0) is one of the saturated FAs and contributes approximately 17%–25% of all the breast milk FAs (Delplanque *et al.*, 2015). Breast milk levels of AA are fairly stable while breast milk levels of DHA are variable and largely determined by maternal blood levels, as well as influenced by the diet (Gibson *et al.*, 1997; Jensen *et al.*, 2000).

Table 2-11: Breast milk DHA and AA concentrations found in studies from low-income and middle-income countries ranked according to DHA level

Country	Infant age (mo)	Sample size	DHA	AA	Reference
Pakistan	12	8	0.06	0.24	Smit <i>et al.</i> (2000)
Rural South Africa	6.5	18	0.10	1.00	Van der Westhuyzen <i>et al.</i> (1988)
Enshi, China	2-18	9	0.15	0.35	Dodge <i>et al.</i> (1999)
China	3	23	0.18	0.51	Xiang <i>et al.</i> (2005)
Nigeria, Niger	0.3-6	34	0.20	0.51	VanderJagt <i>et al.</i> (2000)
Nigeria, Niger	0.3-16	89	0.20	0.52	Knox <i>et al.</i> (2000)
Urban South Africa	6.8	12	0.20	0.60	Van der Westhuyzen <i>et al.</i> (1988)
Belize	>0.3	6	0.21	0.44	Van Beusekom <i>et al.</i> (1990)
Xichang, China	2-18	10	0.22	0.52	Dodge <i>et al.</i> (1999)
Mexico	1-12	46	0.26	0.42	Yuhas <i>et al.</i> (2006)
Tanzania	>0.3	11	0.27	0.60	Muskiet <i>et al.</i> (1987)
Beijing, China	2-18	10	0.28	0.63	Dodge <i>et al.</i> (1999)
Nigeria	0.1-0.5	28	0.32	0.58	Okolo <i>et al.</i> (2000)
Panama	0.5-1	8	0.32	0.52	Rueda <i>et al.</i> (1998)
Nigeria	6-7	15	0.33	0.44	Okolo <i>et al.</i> (2000)
Nigeria	2-3	20	0.34	0.56	Ogunleye <i>et al.</i> (1991)
China	1-12	50	0.35	0.49	Yuhas <i>et al.</i> (2006)
Dominican Republic	0.75	7	0.40	0.50	Van Beusekom <i>et al.</i> (1993)
Suriname	>0.3	20	0.41	0.58	Muskiet <i>et al.</i> (1987)
Cuba	2	52	0.43	0.67	Krasevec <i>et al.</i> (2002)
Curao	>0.3	47	0.43	0.71	Muskiet <i>et al.</i> (1987)
Saint Lucia	1	12	0.53	0.58	Boersma <i>et al.</i> (1991)
Congo	5	102	0.55	0.44	Rocquelin <i>et al.</i> (1998)
Philippines	1-12	54	0.74	0.39	Yuhas <i>et al.</i> (2006)
Dominican Republic	>0.3	6	0.91	0.33	Van Beusekom <i>et al.</i> (1990)

All values given as percentage of total fatty acids

Adapted from Brenna *et al.* (2007) and Michaelsen *et al.* (2011)

2.8.1.3 LCPUFA status of lactating mothers and their breastfed infants

Crawford *et al.* (1976) coined the term “biomagnification,” and this is described as infants exhibiting a higher plasma lipid and RBC LCPUFA profile in comparison with their mothers. If biomagnification occurs through the placenta, preformed AA and DHA are preferentially obtained from the mother and transferred to the foetus for nourishment (Crawford *et al.*, 2003).

Although the AA status of the infant can represent more than twice the amount of AA of the mother, these levels are not dependent on the availability of its precursor LA in the mother. Thus, if the LA content in breast milk is stable, this implies that the conversion of AA is not meeting the foetal demands for AA (Crawford *et al.*, 2003).

According to Farquharson *et al.* (1993), there is a rapid decline in infant adipose tissue DHA concentrations noted after the birth. This led to the hypothesis that DHA accumulated in adipose tissue actually functions as a DHA reservoir to supply the infant quantitatively with much larger amounts after birth than during pregnancy. Breast milk supplies the infant with the much-needed LCPUFAs for development. However, the relative contribution of breast milk towards the LCPUFA status of the infant will depend particularly on the LCPUFA content of milk (Innis, 2000; Marangoni *et al.*, 2000; Montgomery *et al.*, 2003). In addition, it has been shown that maternal plasma FA concentrations are reflected in breast milk (Sprecher *et al.*, 1995; Marangoni *et al.*, 2000; Jensen & Lapillonne, 2009). These observations could be suggestive of the fact that *n*-3 FAs are to a certain degree, transferred to breast milk directly.

In addition, significant correlations were found for LA, ALA and DHA between the plasma and the breast milk of Italian women (Marangoni *et al.*, 2000). However, a study that was done in Indian women found no such correlations and only weak correlations between the overall content of *n*-6 and *n*-3 PUFA in milk and plasma (Kilari *et al.*, 2009). Lauritzen *et al.* (2001), concurred that the DHA levels in infant blood significantly correlate with the DHA levels in both maternal blood and breast milk, which also correlate with each other. In contrast, no correlation has been noted for AA.

In 2006 (Jørgensen *et al.*), in an observational study carried out in 17 mothers and their term infants, a decrease in the maternal DHA status was found over a four-month period and was accompanied by a proportional decrease in the infants' status. Also, a higher LCPUFA to precursor ratio in infants was noted, which could be a possible indication of the specific transfer of LCPUFA through the breast milk. These variations in the composition of PUFA in breast milk may be a reflection of the mother's metabolic adaptation to ensure an adequate supply of LCPUFA to the foetus and the breastfeeding infant (Otto *et al.*, 2001; Jørgensen *et al.*, 2006).

Table 2-12 shows the average breast milk concentrations of DHA and AA during the first year of lactation. Breast milk DHA concentrations can be increased by supplementing lactating mothers with fish oil (Harris *et al.*, 1984), DHA-rich oil (Jensen *et al.*, 2000; Hawkes *et al.*, 2002) or *n*-3 LCPUFA rich eggs (Jensen *et al.*, 2000), or by increasing maternal consumption of fatty fish (Urwin *et al.*, 2012). In these studies, maternal plasma and breast milk DHA concentrations increased subsequently in response to DHA intake. Also, maternal supplementation during lactation resulted in a dose-dependent increase in infant plasma and RBC DHA (Gibson *et al.*,

1997; Jensen *et al.*, 2000; Brenna & Lapillonne, 2009). Therefore, an increase of DHA consumption during lactation will also result in an increase in breast milk DHA, which is consequently reflected in the infant DHA status.

Table 2-12: Average DHA and AA concentrations in breast milk over the first year of lactation

LCPUFAs	1 month	2 months	3 months	6 months	9 months	12 months
DHA	0.25±0.11	0.24±0.11	0.26±0.09	0.30±0.15	0.25±0.11	0.34±0.18
AA	0.51±0.16	0.52±0.13	0.52±0.10	0.52±0.15	0.51±0.10	0.50±0.10

Values given as percentage of total fatty acids; DHA- Docosahexaenoic acid; AA- Arachidonic acid; Adapted from: ENeA (2016)

It has also been shown across different populations that at delivery and at the age of three months, biomagnification is not dependent on the maternal AA status. This suggests that biomagnification is a biological process and a uniform AA status is achieved in infants only at the expense of their lactating mothers (Otto *et al.*, 2001; Kuipers *et al.*, 2007). Infants with low birth weight were shown to have the lowest levels of AA, while pre-term infants were shown to have lowest DHA levels. Therefore, it is plausible that, for optimal infant growth and development of the child, a certain level of infant prenatal AA status should be maintained (Crawford *et al.*, 2003).

In contrast to biomagnification, when maternal DHA status is very high, the infant's RBC-DHA status is usually lower. This process is known as "bioattenuation". At low maternal DHA status there seems to be a strongly controlled interaction between DHA and AA but the complete opposite at high DHA maternal status (Kuipers *et al.*, 2011; Luxwolda *et al.*, 2014). At higher maternal DHA status, bioattenuation could potentially inhibit an abundant transfer of DHA across the placenta, which could possibly lead to competition with AA.

Furthermore, the maternal RBC-AA status was found to be consistently higher at three months after delivery (Luxwolda *et al.*, 2012) than at birth. However, the RBC AA infant status tends to be within the same range as or higher than the mother's RBC AA status at delivery. However, from birth up until the age of three months, the RBC AA status of the mother increases while there is a decrease noted in the infant's RBC AA status. This decrease could be as a result of the reduced rate at which LA is converted to AA in the infant. This is because after delivery, the infant's capacity to synthesise LCPUFA decreases radically and has been shown to decrease even further with gestational age at birth (Carnielli *et al.*, 2007).

Pakistani infants were reported to have lower RBC DHA levels in relation to breast milk concentrations (Smit *et al.*, 2000). On the other hand, at six months of age, Cambodian infants had lower baseline blood levels of LA, comparable ALA levels and higher levels of AA + EPA +

DHA compared with Italian infants (Agostoni *et al.*, 2007). There are limited data on the LCPUFA status of lactating mothers and their infants in developing countries, particularly South Africa.

2.8.2 LCPUFA intakes from breast milk during lactation

According to Hartmann *et al.* (2000) and Mitoulas *et al.* (2003), the fat concentration in breast milk is influenced by the stage of lactation and also varies and changes during the course of each feed between fore-milk and hind-milk. As a result, the FA composition of breast milk changes and varies during the course of the day, throughout the first year of lactation, as well as from country to country. Therefore, in order to estimate the FA intake of breastfed infants, it is important to take into account the different levels of variations. The LCPUFA composition of breast milk and LCPUFA intakes of breastfed infants are also important because this will provide direction in defining the adequate nutrient intake of infants (Grote *et al.*, 2016).

Hind-milk total lipids were found to increase by up to three times during the first three months of lactation. In this regard, breast milk DHA was found to be higher in hind-milk than in fore-milk (Marangoni *et al.*, 2000; da Cunha *et al.*, 2005). Regarding the influence of lactation on the FA variations, a marked decrease in the breast milk FA concentrations of lactating women was observed between the first and second month, with an increase starting only at nine months (Mitoulas *et al.*, 2002). Similarly, Agostoni *et al.* (2003a) found a decrease in the DHA concentrations during the first months of life. In contrast to this, Michaelsen *et al.* (1994) and Mandel *et al.* (2005) observed a similar increase only after the ninth month of lactation, while Szabo *et al.* (2010) reported an increase in the breast milk lipid profiles of lactating women between week six and the sixth month of lactation.

During the period between birth and the age of six months, the average intake of lipids in fully breastfed infants amounts to 21.42 g/day. This essentially results in a remarkable intake of 3.9 kg of lipids supplied to the breastfed infant during the first six months of life (Grote *et al.*, 2016; Koletzko, 2016). In relation to this, lactating women need to attain a daily intake of at least 200 mg of DHA to supply the fully breastfed infant with at least 0.3% of DHA through breast milk. In this way, the infant is ensured of a daily supply of 100 mg of DHA/day which is considered favourable for meeting the infant's metabolic needs (Koletzko *et al.*, 2014)

The EFA supply in exclusively breastfed infants depends on both breast milk composition and intake. However, breast milk intakes have been shown to be lower in developing countries than in developed countries (Forsyth *et al.*, 2016). As a result, this has led to insufficient intake of EFAs in infants. In developing countries, infants that are partially breastfed are often given high-carbohydrate, low-fat complementary foods before they reach the age of six months. This has

been associated with decreased breast milk intakes in infants, which further aggravates EFAD (Forsyth *et al.*, 2017b).

The average breast milk intake at the age of six months has been measured to be 854g per day in exclusively breastfed infants (Butte *et al.*, 2002; WHO, 2003). It is estimated that FAs comprise 4.2 % of breast milk, and based on these data, exclusively breastfed infants consume an average of 169 mg per day of AA and 115 mg per day of DHA at the age of six months. Moreover, breastfeeding is recommended for up to two years and beyond. Therefore, it is estimated that at the age of 12 months, breast milk intake ranges between 600 and 900 g per day. This will provide infants with an intake of AA ranging between 118 and 178 mg per day. The average estimated intake of AA is approximately 12-18 mg/kg/day when adjusted for body weight using weight-for-age percentiles (Hadley *et al.*, 2016). However, breast milk provides an amount of 95.6 mg/day AA at 1 month, 109.6 mg/day at 2 months, with a progressive and sharp decrease at 3 months (101.1 mg/day) and 6 months (58.7 mg/day), respectively (Grote *et al.*, 2016).

Furthermore, Mitoulas *et al.* (2003) measured the FA composition of breast milk to determine the average daily FA intake of infants in Western Australia. The results of this study showed significant differences in the proportions of FAs between mothers and during the first year of lactation. In contrast to this, the amount of FA intake of the infants over the 24-hour period did not differ during the first year of lactation. It was only the intakes of ALA and DHA in infants that differed significantly from the mothers' intakes

Previous studies have documented the importance of FAs in early infancy. Older studies also concluded that researchers could rely on the FA composition of well-nourished mothers as a guide to make dietary recommendations for infants. The few relevant studies that were done included populations from other countries; however, in South Africa there is limited information about the LCPUFA intakes of infants as well as the LCPUFA variations in breast milk.

2.8.2.1 Recommended LCPUFA intakes for lactating women

During breastfeeding, an adequate intake of LCPUFA is required for lactating women to ensure adequate LCPUFA intake through breast milk for the growing infant. Even though infants are able to convert the EFAs LA and ALA into their respective LCPUFAs AA and DHA, this conversion process is very limited and the amount of LCPUFAs is inadequate to meet the infant's requirements (Koletzko *et al.*, 2001). Based on the application of stable isotope techniques in infants, researchers estimate that approximately only 6% of the plasma AA is replenished daily through endogenous synthesis (Demmelmair *et al.*, 1995). Therefore, for infants to be ensured of an adequate LCPUFA supply through breast milk, breastfeeding women are advised to consume preformed DHA and AA during lactation (Carnielli *et al.*, 2007).

In this regard, the FAO (2010) recommends DHA intakes of between 190-210 mg/d to ensure adequate supply to the infant while also maintaining maternal DHA status. Considering the clear linear relationship between dietary DHA and breast milk DHA, researchers concluded that a regular and daily intake of 200 mg DHA leads to the required breast milk DHA content of 0.3%, which will provide a daily supply of 100 mg DHA/day to infants (Fidler *et al.*, 2000). Figure 2-4 shows similar recommendations by different governing bodies. These recommendations are similar to those for pregnant women. Hence the recommendation to consume a minimum of two portions of fish per week still remains. However, the use of fish oil supplementation is encouraged only in women who do not consume fish.

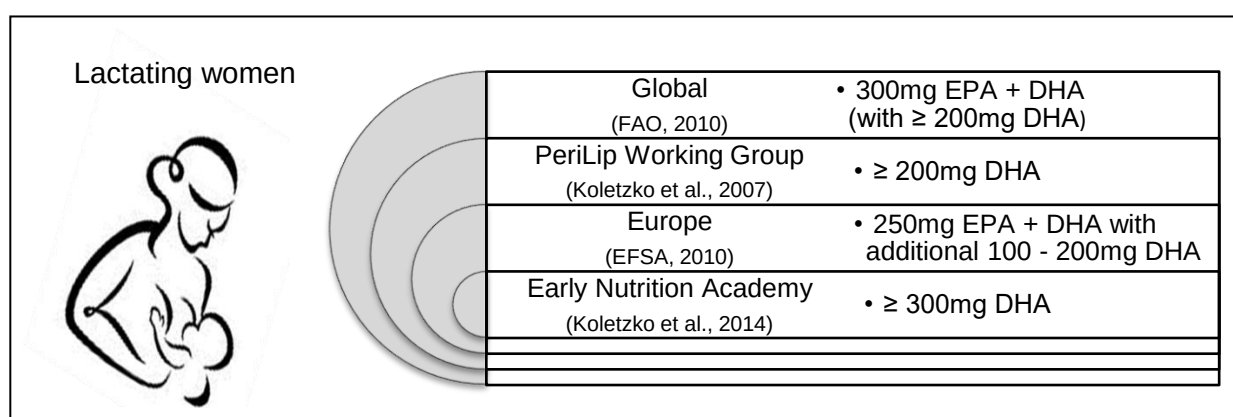


Figure 2-4: Recommended LCPUFA intakes for lactating women. Source: ENeA (2016)

2.8.3 LCPUFA and infant formula milk

In as much as breast milk is the set gold standard for nutrient intake during infancy, many attempts have been made to mimic its composition in order to get an “ideal” breast milk substitute (infant formula) for infants. It has been decades since DHA and AA have been added to different infant formulas with the sole purpose of duplicating the nutritional supply and functional effects achieved through breastfeeding (Koletzko *et al.*, 1989; Makrides *et al.*, 1995b). The stipulation of the current compositional requirements for infant formula according to the expert groups are that the addition of DHA to infant formula is optional provided that the content of AA is equal to, or higher than the DHA content (Brenna *et al.*, 2007; Koletzko *et al.*, 2011). This in essence, “mimics” the composition of human breast milk.

2.8.3.1 Addition of LCPUFAs to infant formulas

The addition of LCPUFA in infant formula emerged in the early 80s and was based on the important role of DHA in early brain development (Brenna, 2016). Older studies (Makrides *et al.*,

1994; Farquharson *et al.*, 1995) found higher levels of LCPUFA in the cerebral cortex of breastfed infants compared with infants who received standard infant formula. Based on the known importance of DHA and AA during infancy, today LCPUFA-enriched infant formulas are common world-wide (Hadley *et al.*, 2016)

Based on the world-wide averages and recommendations of DHA and AA content in breast milk, most infant formulas contain 0.2-0.4 % of total FAs as DHA and between 0.35-0.7 % of total FAs as AA (Brenna *et al.*, 2007). However, these recommendations are still under discussion. Current suggestions are that all commercial infant formulas should contain equal or higher levels of preformed AA than DHA content in order to ensure an adequate status of both DHA and AA in non-breastfed infants (Hadley *et al.*, 2016). According to Brenna *et al.* (2007), infant formula typically provides 140 mg/day and 100 mg/day of AA and DHA respectively.

2.8.3.2 Legislation of infant formula composition

There are different binding regulations regarding the composition of infant formulas, and these apply within the jurisdiction of national governments through the ministry of health, or the Department of Health in South Africa. For instance, the Food and Drug Administration develops the food regulations in the USA, while in Europe, the EFSA advises the European Commission, which gives directives and regulations. The WHO and FAO established an inter-governmental agency known as the Codex Alimentarius Commission. The Codex Alimentarius Commission is responsible for regulations relating to food at a global level and their recommendations are based on the latest science in all relative areas (Kent, 2014).

2.8.3.2.1 European law

As previously mentioned in section 2.5, based on the international expert groups (Koletzko *et al.*, 2014), the EFSA stipulates adequate intakes of 100 mg/d of DHA and 140 mg/d of AA for infants aged zero to six months and 100 mg/d DHA for infants aged 6 to 24 months (Agostoni *et al.*, 2013). Regarding infant formula, the EFSA stipulates that all infant and follow-up formulas must contain 20 to 50 mg DHA/100 kcal, while the addition of AA was not finalised. Therefore, assuming that infant formula contains an average fat content of 5.2 g per 100 Kcal, this recommendation would add up to a total of 0.38-0.96% DHA content of FAs. This is actually higher than the 0.2-0.4% total DHA that is found in most of the infant formulas that are enriched with DHA. Regardless of this, this particular infant formula enriched with DHA is marketed for term infants in Europe and also contains equal or even higher levels of preformed DHA compared to breast milk DHA content (Koletzko *et al.*, 2015).

However, it has been hypothesised that the approach of FA intake through infant formula could result in more noticeable differences in the infant's DHA status than when DHA is incorporated directly through maternal supplementation during pregnancy. Therefore, this could result in more characteristic effects on the development of the infant (Schwartz *et al.*, 2010). This hypothesis raises a great deal of concern regarding the second six months of life because some complementary foods usually provide inadequate amounts of LCPUFAs during the period of rapid neuronal tissue growth. Hence the increased risk of suboptimal DHA supply during the critical period of introduction of complementary foods (Libuda *et al.*, 2016).

2.8.3.2.2 Addition of both DHA and AA to infant formula

The addition of DHA in infant formula has been accepted by many experts, paediatricians and governing bodies; however, the addition of AA to infant formulas still meets with significant disapproval (Koletzko, 2016). Koletzko *et al.* (2015) suggest that the addition of only preformed DHA, and not AA to infant formulas, is a novel approach that has not been tested and clinically evaluated. However, LCPUFA (DHA + AA)-supplemented infant formula had significant positive effects on the quality of attention in infants between four and nine months of age (Colombo *et al.*, 2011), while LCPUFA supplementation in infant formula milk did not influence performance on the standardised tests of language and performance at 18 months (Colombo *et al.*, 2013). In this regard, there is evidence of the safety and suitability of infant formula containing both DHA and AA as it also aids in normalising the LCPUFA status of the infant. Therefore, preclinical and clinical evaluation of the effects, suitability and safety of infant formula without added AA would be required (Koletzko *et al.*, 2015; Brenna, 2016; Hadley *et al.*, 2016).

2.8.3.2.3 LCPUFA composition of standard infant formula in South Africa

Many studies have reported that both term and preterm infants who are fed infant formulas that contain ALA and LA, but are not supplemented with LCPUFA, tend to have depleted LCPUFA concentrations in both plasma and RBCs (Forsyth & Willatts, 2002). Owens *et al.* (2013), proposed that all infant formulas in South Africa must meet the global standards recommended by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), the international expert group that was commissioned by the Codex Alimentarius Commission in November 2004. Therefore, it is mandatory for all infant formulas to contain sufficient amounts of nutrients and there is generally no brand that can be considered as better than another.

The ESPGHAN committee (Koletzko *et al.*, 2005) stipulates that all formulas should contain LA and ALA. A recent Cochrane review (Jasani *et al.*, 2017) reported that giving full-term infants with LCPUFA-supplemented infant formula showed no beneficial or harmful effects on infant

developmental outcomes. Therefore, there is a need for further research before routine supplementation is recommended.

The Codex Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants (Codex Stan 72-1981; Amendment: 1983, 1985, 1987, 2011, 2015 and 2016. Revision: 2007) supports the addition of DHA but as an optional ingredient as long as it is provided with AA and with a guidance upper level (GUL) of DHA of 0.5% of FAs. The Codex also states that *“if DHA is added to infant formula, AA contents should reach at least the same concentration as DHA. The content of EPA, which can occur in sources of LC-PUFA, should not exceed the content of DHA. National authorities may deviate from the above conditions, as appropriate for the nutritional needs.”* Table 2-13 shows the amounts of LA and ALA required in infant formulas according to the Codex Standard for Infant Formula and Formulas. Also, the Codex Stan 72-1981 recommends minimum and maximum values of 5:1 and 15:1, respectively (Codex Alimentarius Commission, 2007).

Table 2-13: Essential fatty acid quantities in infant formulas

Linoleic acid		Minimum	Maximum	GUL
	mg/100 kcal	300	-	1 400
	mg/100 kJ	70	-	330
α -Linolenic acid				
	mg/100 kcal	50	N.S.*	-
	mg/100 kJ	12	N.S.	-

*Not specified

2.8.4 LCPUFA intakes during complementary feeding

After a certain age, usually around six months, breast milk as a sole dietary source no longer meets all the infant's nutritional requirements, hence optimal complementary foods have to be introduced in order to ensure adequate nutrition and growth for the infant (WHO & UNICEF, 2003). Complementary foods are defined as every solid or liquid other than breast milk, infant formula or follow-on formula. In this regard, complementary feeding is associated with major changes in both macronutrient and micronutrient intake. A common assumption is that the primary factor for dictating complementary feeding, is an increase in need for energy and protein, but some micronutrients are most likely to become limited earlier than macronutrients (Hemsworth *et al.*, 2016).

As mentioned previously in section 2.5.3, there is a dramatic decrease of n-3 FA intake during the complementary feeding period (Michaelsen *et al.*, 2009). It is important that infants are

ensured of an adequate supply of FAs, particularly DHA, during this period of rapid growth. Several studies have shown that children with an inadequate intake of dietary DHA, performed badly in developmental tasks, particularly those that measured brain development (Kuratko *et al.*, 2013). Therefore, the newly introduced foods during the complementary feeding period should make up for the decreased supply of DHA and AA from breast milk (Grote *et al.*, 2016).

Forsyth *et al.* (2016) recently showed estimates of DHA and AA intakes of children aged between six and 36 months in developing countries. The median AA and DHA intakes from breast milk and food were 64.0 mg/d and 48.9 mg/d respectively while South Africa's median intakes were 74.0 mg/d and 38.3 mg/d, respectively. The median AA and DHA intakes from complementary foods only were 17.9 mg/d and 14.6 mg/d respectively while South Africa's median intakes were 34.4 mg/d and 14.6 mg/d. The countries with the lowest intake of DHA and AA from complementary foods also had the lowest gross national income and highest birth rates. In this regard, plant-based complementary foods are consumed in most low- and middle-income countries and are insufficient to meet the dietary needs of the growing infants. Therefore, meat, poultry, fish and eggs need to be included in the diet of infants after the age of six months.

There are several potential PUFA sources that can be provided to infants. For instance, breastfed infants who received DHA-enriched egg yolks four times per week from six to 12 months, had higher RBC DHA levels at 12 months than did those fed standard egg yolks or no egg yolks at all (Makrides *et al.*, 2002). Also, at one year of age, RBC DHA was found to be 24 % higher in infants receiving formula supplemented with DHA and AA compared with infants receiving commercial infant formula milk (Hoffman *et al.*, 2003). Consistent with these results, Hoffman *et al.* (2004) randomised breastfed infants to receive either one jar per day of weaning foods containing DHA-enriched egg yolk, or control baby food, between six and 12 months. By 12 months, those receiving the enriched food showed an increase in RBC DHA.

Furthermore, a daily dose of one teaspoon of fish oil remarkably increased the n-3 PUFA level in RBC membranes over a period of three months (Damsgaard *et al.*, 2007). Also, regular consumption of fish during complementary feeding improved the EPA and DHA status of infants by the age of 10 months. Therefore, it was concluded that the provision of oily fish with complementary food can prevent reductions in the infant's DHA status (Libuda *et al.*, 2016). In this regard, it is recommended that children eat at least two portions of fatty fish a week in order to enhance their intake of EPA and DHA; and that it is advisable to introduce fish early in their diet particularly during the complementary feeding period to ensure regular consumption of fish throughout life (Smuts & Wolmarans, 2013).

Regardless of a diverse complementary food diet that is higher in meat, eggs and fish that contribute substantially to the diet, the unfavourable effect of introducing complementary foods

low in AA is also apparent. For instance, the average intake of AA drastically decreased from 72 mg/day at six months to 24 mg/day at nine months. In addition, predominantly breastfed infants had higher AA intakes at three months (103 mg/day) compared with 9-month-old (24 mg/day) infants whose diet comprised of 20% breast milk (Schwartz *et al.*, 2009; Schwartz *et al.*, 2010).

Moreover, a new strategy and promising approach is the use of the LNS which is also enhanced with multiple micronutrients and extra energy. These LNSs can be mixed with complementary food at the time of consumption (Muslihah *et al.*, 2016). In this regard, the improvements that were seen in linear growth (explained in section 2.7.2.3) in the study population from Ghana (Adu-Afarwuah *et al.*, 2007), could be related to the EFA content in the LNS. Although these researchers did not evaluate the change in FA status, it is suggested that providing a daily dose of LNS as a complementary food to infants aged between six and 12 months, can promote linear growth and possibly yield other health benefits in populations where complementary feeding is inadequate. Therefore, it is plausible that the provision of LNS containing EFAs in infants that are fed complementary foods could potentially improve the LCPUFA status of those infants of low socio-economic backgrounds.

2.9 THE SITUATION OF FATTY ACID NUTRITION IN SOUTH AFRICA

Breastfed infants are ensured of an adequate supply of LCPUFAs through breast milk provided that lactating women consume an adequate amount of dietary LCPUFAs (Koletzko *et al.*, 2001). It is therefore important to ensure that lactating women have an adequate LCPUFA status and consume an adequate dietary supply of LCPUFAs to cater for their needs as well as those of the infants. As previously mentioned the addition of DHA to infant formulas in South Africa is optional, while the addition of AA to infant formulas has not yet been regulated. Despite the inadequate feeding practices in South Africa, the LCPUFA status of breastfed infants and those given complementary foods, remains unknown; yet infants should be assured of an adequate intake of LCPUFAs for optimal neurodevelopment, growth and other positive health outcomes. Hence the relevance of this PhD study.

2.10 CONCLUSION

In conclusion, LCPUFAs have been shown to have very important protective, structural and functional roles. There is strong evidence that LCPUFAs are very important for human development *in utero* and in infancy, as well as throughout the life cycle. Breastfed infants are ensured of a source of LCPUFAs from breast milk until they are weaned at six months. However, the need for a continued supply of preformed LCPUFAs beyond weaning from exclusive

breastfeeding, is undetermined and may even be of more importance to infants exposed to earlier introduction of LCPUFA-poor complementary foods or less breast milk up to the age of 12 months.

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CHAPTER 3

Associations of plasma total phospholipid fatty acid patterns with feeding practices, growth and psychomotor development in six-month-old South African infants

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CMS, JB, MF and NC conceptualised and designed the study; CMS was the principal investigator of the Tswaka study and had overall responsibility for data collection.

MF was the co-principal investigator of the Tswaka study, contributed to the study design and provided guidance on analysis of feeding practices.

MR and TMM were the study co-ordinators of the Tswaka study and helped to execute the study.

JB, MR, TMM and LPS executed the study and collected data.

AJ developed the method for breast milk analysis and performed biochemical analysis; CMS provided guidance on the development of the method and biochemical analysis and LPS quantified FAs.

LPS, CR and JB performed statistical analyses; CMS, JB and CR provided guidance on statistical analysis and interpretation.

LPS wrote the first draft of the manuscript and all authors read, revised and edited the manuscript.

This manuscript is formatted according to the guidelines of Maternal & Child Nutrition

ABSTRACT

The objective of this study was to assess plasma fatty acid (FA) patterns of six-month-old South African infants and to determine their association with feeding practices, growth and psychomotor development.

Plasma total phospholipid FA composition (% of total FAs) of six-month-old infants (n=353) from a peri-urban township was analysed, and principal component and factor analysis were performed to identify plasma FA patterns. Feeding practices, anthropometric measurements and psychomotor development scores were determined.

Four major plasma phospholipid FA patterns were identified: A '*high EFAs with low DHA and AA*', a '*high n-6 LCPUFA*', a '*high monounsaturated FA (MUFA) and nervonic acid*', and a '*trans-FA*' pattern. Formula feeding was associated with higher, while breastfeeding was associated with lower scores for the '*high EFAs with low DHA and AA*' and '*high MUFA and nervonic acid*' patterns. On the other hand, breastfeeding, the consumption of cow's milk and the consumption of semi-solid foods were associated with higher scores, while formula feeding was associated with lower scores for the '*high n-6 LCPUFA*' pattern. Breastfeeding and the consumption of semi-solids were also associated with higher '*trans-FA*' pattern scores. The '*high MUFA and nervonic acid*' and '*trans-FA*' patterns were positively associated with psychomotor development scores.

In six-month-old South African infants, we identified distinct plasma FA patterns that presumably represent the FA quality of their diet, and that are associated with psychomotor development. Our results suggest that breast milk is an important source of n-6 LCPUFAs and formula-fed infants may be at risk of inadequate LCPUFA intake.

Keywords: LCPUFA status, breast milk, feeding practices, FA patterns, psychomotor development

INTRODUCTION

Infancy is a critical period of rapid neurodevelopment that requires adequate and proper nutrition. Thus, poor nutrition during the first 1000 days of a child's life may lead to cognitive and behavioural deficits later in life (Prado & Dewey, 2014). Long-chain polyunsaturated fatty acids (LCPUFAs) are important structural components of membrane phospholipids in the central nervous system (CNS), the retina and other tissues (Crawford & Broadhurst, 2012). The LCPUFAs arachidonic acid (AA; C20:4n-6) and docosahexaenoic acid (DHA; C22:6n - 3), are particularly needed for the development and functioning of the CNS.

During the third trimester of pregnancy, DHA and AA are transferred to the foetus via the placenta. Their rapid accretion in the CNS continues throughout the early stages of infancy (Carver, Benford, Han, & Cantor, 2001). Although DHA and AA can be synthesised *de novo* from their parent essential fatty acids (EFAs), alpha linolenic (ALA; C18:3n-3) and linoleic acid (LA; C18:2n-6) respectively, this conversion process is very limited in infants (Uauy & Dangour, 2009). The European Food Safety Authority (EFSA) considers 100 mg of DHA/day and 140 mg of ARA/day as adequate intakes of LCPUFA for most infants from birth up until the age of 6 months (Agostoni et al., 2013). The World Health Organization (WHO & UNICEF, 2003) recommends exclusive breastfeeding (EBF) for the first six months of life and timely introduction of nutritionally adequate and safe complementary foods thereafter, with continued breastfeeding for up to two years and beyond. Breast milk is the main source of LCPUFAs in breastfed infants, and the relative fatty acid (FA) composition of breast milk is associated with maternal plasma FA status influenced by maternal diet (Innis, 2014).

In South Africa, inappropriate feeding practices, such as mixed feeding and early introduction of complementary foods were found to be the rule rather than the exception (Faber, Laubscher, & Berti, 2016). With a very high prevalence of HIV in South Africa, inappropriate infant feeding practices in the HIV-positive population are often driven by a desire to protect the infant from HIV. However, there is a higher risk of HIV transmission associated with these feeding practices (Zulliger, Abrams, & Myer, 2013).

Despite a high (83%) breastfeeding initiation rate (Shisana et al., 2013), the percentage of South African children reported to be exclusively breastfed in 2016, decreased with age from 44% in infants aged zero to one month, to 24% in infants aged four to five months (SADHS-National Department of Health, 2017). Moreover, infants from most low- and middle- income countries are typically introduced to plant-based complementary foods that are insufficient to meet the dietary needs of growing infants (Forsyth, Gautier, & Salem, 2016). As a result, infants are introduced to complementary foods that may result in a reduction of dietary LCPUFAs as reflected in their blood

FA profiles. This reduction may also be due to a simultaneous reduction in breast milk intake in combination with intake of LCPUFA-poor weaning foods (Rise et al., 2013).

There are limited data on the LCPUFA profiles of infants in developing countries. Despite inappropriate feeding practices in South Africa, the LCPUFA status of infants who are breastfed and those who receive complementary foods remains unknown; yet infants should be assured of an adequate intake of LCPUFAs for optimal neurodevelopment, growth and other positive health outcomes (Colombo et al., 2013). Previously, FA have been reported and expressed individually as a relative measure of the percentage of total FAs. However, as these FAs are mutually dependent, a change in the proportion of one FA may influence the proportions of many other FAs (Imamura et al., 2012; Voortman et al., 2017). To overcome this limitation in the current study, principal component analysis (PCA) and factor analysis were used to define specific plasma phospholipid FA patterns of six-month-old peri-urban South African infants. Therefore, the aim of the present study was to determine the associations of plasma phospholipid FA patterns with feeding practices, growth and psychomotor development in six-month-old South African infants.

Key messages

Six-month old breastfed South African infants living in a peri-urban setting have adequate EFA status.

Plasma total phospholipid FA patterns presumably represent the FA quality of an infant's diet, and are associated with psychomotor development

South African infants fed infant formula are at risk of inadequate DHA and AA intake, while breastfed infants are ensured of n-6 LCPUFA intake.

Lactating mothers need to consume adequate dietary n-3 LCPUFAs to ensure that breastfed infants are guaranteed of an adequate supply of n-3 LCPUFAs through breast milk.

METHODS

Participants and study site

This cross-sectional study was performed using baseline data of the Tswaka trial, which was a randomised, controlled trial designed to investigate the effects of two small-quantity lipid-based nutrient supplements (SQ-LNS) on linear growth in infants aged six to 12 months. The Tswaka trial was conducted during the period of September 2013 and January 2015 in the Jouberton area

of the greater Matlosana Municipality in the North West Province, South Africa. The trial was carried out on six-month-old infants and their mothers, who were recruited from five different clinics in the study area.

Infants were enrolled in the study at the age of six months. Infants were excluded if they had never previously been breastfed; had experienced severe known congenital abnormalities; were severely anaemic (haemoglobin < 7 g/dL) or severely wasted (weight-for-length Z-score < -3 SD); had other diseases that were referred for hospitalisation by the clinic staff; had known allergies and/or intolerances to peanuts and/or soy, cow's milk protein, and/or fish; were receiving special nutritional supplements as part of feeding programmes, or were not born as singletons.

Data collection and measurements

At enrolment, the infants' Road to Health booklets were used to confirm their age. A 4 mL venous blood sample was drawn into EDTA-coated vacutainers from the antecubital arm area or dorsal area of the hand. Venous blood samples were centrifuged for 10 minutes at 2000 g and 100 µl plasma aliquoted and stored at 4°C on site. These samples were transported daily from the study site to the main laboratory at the Potchefstroom campus of the North West University for storage at - 80 °C until analysis.

If a blood sample could not be obtained from the antecubital arm area or dorsal area of the hand, a blood sample was taken by means of a finger prick only for the haemoglobin (Hb) measurement. The Hb concentrations were determined using the HemoCue system (HemoCue 201+; HemoCue® AB). Anaemia was defined as Hb <11 g/dL. Weight and recumbent length were measured according to WHO (1995) standardised techniques. Infants were weighed by a research nurse, without clothing to the nearest 0.01 kg using a digital baby scale (Seca model 354, GmbH & Co. KG., Hamburg, Germany, maximum 20 kg). Recumbent length was measured to the nearest 0.1 cm (Seca 416 infantometer; Seca GmbH & Co. KG., Hamburg, Germany). The WHO growth standards (2006) were used to calculate age and gender-specific Z-scores for weight and length.

Breast milk (fore-milk) samples (5 mL) were collected at baseline between 8:00 a.m. and 12:00 noon from mothers who were still breastfeeding at the time of sample collection and who were willing to provide a sample. Samples were collected in sterile polypropylene tubes by manual expression of the breast that was not used at the last feed. Breast milk samples were aliquoted and stored at -80°C until analysis.

Feeding practices

Information on their breastfeeding and complementary feeding practices was collected from participants. A structured questionnaire was developed, based on the WHO (2010) guidelines for assessing infant and young child feeding practices, and was used to collect this information. Some of the indicators included for this study, were breastfeeding practices at the time of the study, exclusive breastfeeding up to the age of six months and whether the infant had been introduced to infant feeding formula. Descriptive qualitative information on the usual consumption of foods by the infants over the past seven days was collected using a set of unquantified food frequency questions as explained in detail by Rothman et al. (2018). These questions have also been used in other similar studies (Faber et al., 2016; Smuts et al., 2005).

Psychomotor development

The Kilifi Developmental Inventory (KDI) (Abubakar, Holding, Van Baar, Newton, & van de Vijver, 2008), which was developed and evaluated in Africa using materials and activities that both parents and infants can relate to, and a parent rating scale developed for South Africa, were used to assess psychomotor development, as previously described (Osei et al., 2017). While the tools were developed by a qualified psychologist, for the purpose of this study, we included only those activities applicable to six-month-old infants. Prior to the commencement of the trial, the KDI was translated into the local language by a team member who was proficient in speaking and writing the language. The KDI was specifically used to assess locomotor development and fine motor skills by means of a parental report and direct observation by fieldworkers.

In this study, the KDI was used to determine different developmental scores for locomotor skills and eye-hand coordination. Locomotor skills were assessed using different activities, which included movement in space, static and dynamic balance, as well as motor coordination. While the infant's ability to manipulate objects and engage in activities requiring fine motor coordination were the basis of eye-hand coordination assessments. All activities were scored separately and ranked according to the infant's ability to perform the specific task, as explained in detail by Rothman et al. (2018) and Osei et al. (2017). In addition, a questionnaire on parent rating of motor development was used for parental assessments. This questionnaire allowed each caregiver to rate the infants' gross motor developmental milestones.

Fatty acid analysis

Fatty acid extraction from plasma samples

Lipids were extracted from plasma samples with chloroform: methanol (2:1 vol: vol) by using a modification of the method of (Folch, Lees, & Sloane-Stanley, 1957). Lipid extracts were concentrated using nitrogen gas, and thin-layer chromatography (silica gel 60 plates without

fluorescent indicator, 10x20 cm; Merck) was used to separate neutral lipids from the phospholipids. Phospholipids were eluted using petroleum ether: diethyl ether: acetic acid (90:30:1, vol: vol: vol) and a pinch of 2,5-Bis(5-tert-butyl-benzoxazol-2-yl)thiophene. The lipid fraction containing the phospholipids was removed from the thin-layer chromatography plate and trans-methylated with methanol: sulphuric acid (95:5, vol: vol) at 70°C for 2 hours. This led to the formation of fatty acid methyl esters (FAMES). The FAMES were extracted using hexane and water. The organic layer was aspirated, evaporated, redissolved in hexane, and analysed by gas chromatography–electron ionisation mass spectrometry. All solvents used during the extraction procedure contained 0.01% butylated hydroxytoluene.

Fatty acid extraction from breast milk samples

Fatty acids from breast milk samples were derivatised and extracted using a modification of the methods described by Liu, Mühlhäusler, and Gibson (2014) and Folch et al. (1957). Aliquots of breast milk samples were subjected to direct trans-methylation by incubation with methanol: sulphuric acid (95:5; vol: vol) at 70°C for 3 hours. Samples were cooled to room temperature, after which the derivatised lipids were extracted with methanol, hexane and water, followed by centrifugation at 1200 × g for 5 minutes. The top phase, containing lipid and fatty acid methyl esters (FAMES), was aspirated and evaporated to dryness using nitrogen gas. Samples were redissolved in chloroform: methanol: saline (86:14:1, vol: vol: vol) and subjected to thin-layer chromatography (silica gel 60 plates without fluorescent indicator, 10x20cm; Merck) using petroleum ether: diethyl ether: acetic acid (90:30:1, vol: vol: vol) and a pinch of 2,5-Bis(5-tert-butyl-benzoxazol-2-yl)thiophene as the mobile phase. The separated FAMES fraction was visualised under UV light and removed from the thin-layer chromatography plate. An extraction with methanol: sulphuric acid (95:5, vol: vol), hexane and water followed, after which the top hexane phase containing the FAMES was aspirated and evaporated to dryness under nitrogen gas. The FAMES were redissolved in a small volume of hexane and analysed by gas chromatography-electron ionisation mass spectrometry. All solvents used during the extraction procedure contained 0.01% butylated hydroxytoluene.

Analysis by gas chromatography mass spectrometry

Samples were analysed on an Agilent Technologies 7000 GC/MS Triple Quad system comprising an Agilent 7890A gas chromatograph equipped with an Agilent G7001B triple quad mass spectrometer (Agilent Technologies). The gas chromatography separation of FAMES was carried out on an HP-88 capillary column (100 m x 0.25 mm x 0.20 µm; Agilent Technologies) by using helium as the carrier gas at a flow rate of 2.2 mL/min. Initial inlet temperature was held at 70 °C for 0.02 minutes, after which it was ramped to 270 °C at 500 °C/min. The mass spectrometer

source was maintained at a temperature of 230 °C. A sample volume of 1 µL was injected, and split ratios of 80:1 for plasma samples and 10:1 for breast milk samples were used. The oven temperature was maintained at 50 °C for 1 minute, then ramped to 170 °C at 30 °C/min, then from 170 °C to 215 °C at 2 °C/min, after which it was ramped to 230 °C at 4 °C/min. The temperature was then held isothermally at 230 °C for 7 minutes. The total analysis time was 38.25 minutes. Mass spectrometry with 70 eV electron-ionisation was carried out in multiple-reaction monitoring mode, with at least two transitions per compound. The FAMES were quantified using MassHunter Quantitative Analysis software (Version B.05.02, Agilent Technologies). FAME peaks were identified and calibrated against a standard reference mixture of 33 FAMES (Nu-Check-Prep) and two single FAME standards (Larodan Fine Chemicals AB). Relative percentages of FAs were calculated by expressing the concentration of a given FAME as a percentage of the total concentration of all FAMES identified in the sample (Baumgartner et al., 2012).

Statistical methods

Data were checked for normal distribution using the Kolmogorov–Smirnov test and visual inspection of histogram plots. Categorical variables were reported as frequencies and percentages. Skewed variables (individual FAs) were log transformed. Data were described using arithmetic means and standard deviation if normally distributed; the geometric means were used elsewhere. A PCA and factor analysis of the correlation matrix of Blom's normal ranks was used to determine the patterns of association between plasma FA (Blom, 1958). The Kaiser criterion (Eigenvalues > 1) and the scree plot visual inspection were used to define the factors to be retained. To name factors, variable loadings with an absolute value higher than 0.5 were considered. Finally, associations of plasma phospholipid FA pattern scores with feeding practices, growth and psychomotor development were evaluated using a generalised linear model adjusted for sex, age and Hb. The models determining the associations with psychomotor development were adjusted for Hb and LAZ, as Hb concentrations and LAZ were found to be significant predictors of psychomotor development (Rothman et al., 2018). All statistical tests were two-tailed, and type-I error rate was set to 5%. Statistical analysis was performed using SAS® (version 9.4; SAS Institute, Inc, Cary, NC).

Ethical considerations

The Tswaka trial was registered at <http://clinicaltrials.gov> as NCT01845610. The study was approved by the ethics committees of North West University (NWU) (NWU-00001-11-A1) and the South African Medical Research Council (SAMRC) (EC-01-03/2012), adhered to the principles of the Declaration of Helsinki and followed good clinical practice (GCP) guidelines. The North West Provincial Department of Health and Social Development also reviewed the trial protocol and the

trial was further registered with the Directorate for Policy, Planning and Research. Informed consent was obtained from the parents/legal guardians of the infants before any study-specific procedures were performed.

RESULTS

Baseline characteristics and fatty acid status of infants aged 6 months, and breast milk FA composition

Of the 750 infants enrolled in the Tswaka trial, plasma samples for total phospholipid FA analysis were available from 353 infants, who thus comprised the study sample. Table 3-1 shows the characteristics of these infants. The study sample comprised 53% (n = 187) male and 47% (n = 166) female infants, with a mean age of 6.2 ± 0.3 months. A third (30%, n = 104) of the infants in the study were stunted.

Table 2 shows the plasma total phospholipid FA composition of all infants, and separately in non-breastfed and breastfed infants at the time of the survey, as well as the FA composition in available breast milk samples. The geometric mean (95% CI) plasma ALA and LA contents in all infants were 0.12 (0.11, 0.13) and 21.4 (21.1, 21.7) % respectively. The plasma DHA and AA contents were 4.1 (4.0, 4.3) and 11.5 (11.2 - 11.8) % respectively. The plasma total n-6 PUFA content was six times higher than plasma total n-3 PUFA. The ratio of DHA to osbond was 5.7 (5.5, 6.0) %. The infants in this study had an essential PUFA status index and a plasma omega-3 index of 3.8 (3.7, 3.8) and 4.4 (4.3, 4.6) % respectively.

Two thirds (66%, n = 236) of infants included in this study were still breastfeeding at the time of the study. However, of only 164 breastfed infants (47%) with FA data available, mothers provided a breast milk sample. There were significant differences between the plasma FA composition of infants that were still breastfeeding and those that were no longer receiving breast milk ($p < 0.05$) (Table 3-2). Breastfed infants had significantly higher plasma DHA (4.73 [4.57, 4.90]) and AA (13.08 [12.83, 13.34]), but lower plasma LA (20.4 [20.1, 20.8]) and ALA (0.09 [0.08, 0.09]) compared with non-breastfed infants.

The breast milk samples (n = 380) collected from the mothers whose infants were participating in the Tswaka study contained $47.4 \pm 5.3\%$ saturated FAs, $27.5 \pm 3.6\%$ monounsaturated FAs (MUFAs) and $24.1 \pm 4.8\%$ PUFAs. The ALA and LA contents were 0.7 (0.6, 0.7) and 20.6 (20.1, 21.0) % respectively. The DHA and AA contents were 0.2 (0.2, 0.3) % and 0.8 (0.7, 0.8) % respectively

Table 3-1: Characteristics of six-month-old infants with plasma total phospholipid fatty acid data available

Characteristic		Plasma FAs available (n = 353)			<i>P</i> ¹ value
		<i>n</i>	%	Mean ± SD	
Males		187	53.0		0.478
Females		166	47.0		
LAZ				-1.4±1.1	0.944
Stunting	Stunted (LAZ < - 2 SD)	104	29.5		0.595
	Not Stunted (LAZ ≥ - 2 SD)	249	70.5		
WLZ				0.5±1.1	0.376
Wasting	Wasted (WLZ < - 2 SD)	1	0.3		0.934
	Not wasted	352	99.7		
Overweight	Overweight (> +2 WLZ)	13	3.7		0.228
	Not Overweight	340	96.3		
WAZ				-0.6±1.2	0.512
Underweight	Underweight (WAZ < - 2SD)	36	10.2		0.475
	Not Underweight	317	89.8		
Hb (g/dL)				11.3±1.1	0.414
Hb Status	Anaemic (Hb < 11 g/dL)	126	35.7		0.324
	Not anaemic	227	64.3		

FA - Fatty acids; LAZ - length-for-age Z-score; WLZ - weight-for-length Z-score;

BAZ- BMI-for-age z-scores; WAZ- weight-for-age z-score; Hb - haemoglobin

¹Chi-square (categorical variables) and independent t-test (categorical and continuous variables) procedures used to determine differences between groups (plasma FAs available and not available). Level of significance $p < 0.05$

Table 3-2: Breast milk and infant plasma total phospholipid fatty acid composition of six-month-old South African infants (% of total fatty acids)

FATTY ACID		Infant plasma (n = 353)	Plasma non-BF infants (n = 117)	Plasma BF Infants (n = 236)	Breast milk (n = 380)
SFA		44.5 (44.4, 44.7) ¹	44.6 (44.3, 44.8)	47.5 (44.4, 44.7)	47.4 ± 5.26 ²
MUFA		11.5 (11.3, 11.7)	13.2 (12.9, 13.6) *	10.7 (10.5, 10.9)	27.5 ± 3.8
PUFA		42.9 (42.7, 43.1)	41.2 (40.9, 43.9) *	43.7 (43.5, 43.9)	24.1 ± 4.8
n-9 fatty acids					
C20:3n-9	Mead acid	0.00 (0.00, 0.00)	0.01 (0.01, 0.01) *	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
n-3 fatty acids					
C18:3n-3	ALA	0.12 (0.11, 0.13)	0.24 (0.22, 0.26) *	0.09 (0.08, 0.09)	0.65 (0.63, 0.68)
C20:5n-3	EPA	0.27 (0.25, 0.28)	0.33 (0.31, 0.36) *	0.24 (0.22, 0.25)	0.06 (0.06, 0.68)
C22:5n-3	DPA	0.86 (0.84, 0.88)	0.81 (0.78, 0.85) *	0.88 (0.85, 0.91)	0.14 (0.14, 0.15)
C22:6n-3	DHA	4.11 (3.98, 4.26)	3.12 (2.97, 3.27) *	4.73 (4.57, 4.90)	0.24 (0.23, 0.25)
n-6 fatty acids					
C18:2n-6	LA	21.4 (21.1, 21.7)	23.4 (22.9, 23.9) *	20.4 (20.1, 20.8)	20.6 (20.1, 21.0)
C20:4n-6	AA	11.5 (11.2, 11.8)	8.85 (8.61, 9.10) *	13.1 (12.8, 13.3)	0.76 (0.74, 0.77)
C20:3n-6	DGLA	2.31 (2.26, 2.37)	2.30 (2.21, 2.40)	2.31 (2.25, 2.38)	0.55 (0.54, 0.57)
C22:4n-6	Adrenic acid	0.66 (0.65, 0.68)	0.57 (0.55, 0.60) *	0.71 (0.69, 0.73)	0.19 (0.19, 0.19)
C22:5n-6	Osbond acid	0.72 (0.70, 0.74)	0.66 (0.63, 0.70) *	0.75 (0.72, 0.78)	0.08 (0.08, 0.08)
Total fatty acids					
Σn-3 ³	n3 PUFA	5.56 (5.43, 5.70)	4.70 (4.53, 4.88) *	6.04 (5.88, 6.20)	1.18 (1.15, 1.22)
ΣLC n-3 ⁴	n3 LCPUFA	5.39 (5.25, 5.54)	4.43 (4.26, 4.61) *	5.94 (5.78, 6.11)	0.50 (0.48, 0.51)
Σn-6 ⁵	n6 PUFA	37.7 (37.5, 37.9)	33.8 (36.5, 37.2) *	38.1 (37.9, 38.4)	22.8 (22.3, 23.3)
ΣLC n-6 ⁶	n6 LCPUFA	15.8 (15.5, 16.2)	12.9 (12.8, 13.3) *	17.5 (17.2, 17.8)	2.00 (1.97, 2.04)
Fatty acid ratios					
Total (n6/n3)	n6: n3 PUFA	6.78 (6.61, 6.96)	7.83 (7.51, 8.16) *	6.31 (6.13, 6.50)	19.3 (18.6, 19.9)
Total LC (n6/n3)	n6: n3 LCPUFA	2.93 (2.87, 3.00)	2.93 (2.81, 3.04)	2.94 (2.86, 3.02)	4.03 (3.90, 4.16)
Mead/AA	Mead/AA	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
LA/ALA	LA: ALA	176.5 (166.6, 186.9)	97.4 (91.3, 103.8) *	237.3 (226.8, 248.3)	31.4 (30.1, 32.8)
DHA/Osbond	DHA: Osbond	5.73 (5.47, 6.02)	4.70 (4.34, 5.09) *	6.33 (5.99, 6.70)	2.97 (2.84, 3.09)
	EPA: DHA	0.06 (0.06, 0.07)	0.24 (0.22, 0.26) *	0.05 (0.05, 0.05)	0.25 (0.24, 0.26)
Essential PUFA status index ⁷		3.75 (3.67, 3.84)	3.11 (3.02, 3.22) *	4.11 (4.02, 4.20)	0.88 (0.86, 0.91)
Omega 3 index ⁸		4.43 (4.29, 4.57)	3.49 (3.33, 3.65) *	4.99 (4.83, 5.15)	0.31 (0.30, 0.32)

FA – Fatty acid; BF – Breastfeeding; LA - Linoleic Acid; ALA- α- Linoleic Acid; AA- Arachidonic acid; DGLA- Dihomo-γ-linolenic acid; EPA- Eicosapentaenoic Acid; DPA- Docosapentanoic acid; DHA- Docosahexaenoic acid; PUFA- Polyunsaturated Fatty Acids; LCPUFA- Long-chain polyunsaturated fatty acids

* Significant differences between BF and non-BF infants' plasma total phospholipid FA composition (p < 0.05), assessed using general linear models adjusted for age and sex.

¹ FAs were log transformed, values presented as Geometric Mean (95% CL), all such values

² Mean±SD all such values

³ Sum of all n-3 FAs

⁴ Sum of all n-3 long-chain fatty acids (20 - 22 carbons)

⁵ Sum of all n-6 fatty acids

⁶ Sum of all n-6 long-chain fatty acids (20 - 22 carbons)

⁷ Ratio between all essential PUFAs (the sum of all n-3 and n-6 FAs) and all non-essential unsaturated fatty acids (the sum of all n-7 and n-9 FAs)

⁸ Sum of EPA and DHA

Plasma total phospholipid fatty acid patterns

Four latent factors were retained according to the Kaiser criterion (50% cumulative variance of all 29 FAs) (Table 3-3). Factor loadings > 0.5 and < -0.5 were used to define each FA pattern. Factor 1 correlated positively with essential PUFAs and negatively with the n-3 and n-6 LCPUFAs AA and DHA. For this reason, the first factor was named the '*high EFAs with low DHA and AA*' pattern. Factor 2 was named the '*high n-6 LCPUFA*' pattern as it had positive loadings with the major n-6 LCPUFAs. Factor 3 was characterised by '*high MUFA and nervonic acid*' while factor 4 was characterised by '*trans-FAs*'.

Table 3-3: Factor loadings of individual plasma total phospholipid fatty acid patterns

FATTY ACID		FACTOR 1	FACTOR 2	FACTOR 3	FACTOR 4
		<i>High EFAs with low DHA and AA</i>	<i>High n-6 LCPUFA</i>	<i>High MUFA and nervonic acid</i>	<i>Trans-FA</i>
C14:0	Myristic acid	-0.08512	0.31146	-0.07953	0.38901
C16:0	Palmitic acid	0.54233	0.12284	0.05214	0.07235
C16:1	Palmitoleic acid	0.02023	0.65337	0.50995	0.16399
C18:0	Stearic acid	-0.41741	0.18208	-0.60562	-0.05663
C18:1n-7c	Vaccenic	-0.25638	0.24497	0.40845	0.0189
C18:1n-9T	<i>Trans</i> -Vaccenate	-0.29611	0.38145	0.0973	0.66839
C18:1n-9	Elaidate	-0.20819	0.4299	0.08623	0.69548
C18:1n-9c	Oleic acid	0.70669	0.19602	0.44031	0.10626
C18:2n-6	LA	0.68539	-0.36829	-0.39032	-0.12903
C18:3n-3	ALA	0.84873	-0.12337	0.18656	-0.00985
C18:3n-6	GLA	0.64956	0.40874	0.16755	-0.21754
C18:4n-3	Stearidonic acid	0.31164	-0.02208	-0.18744	0.29924
C20:0	Arachidic acid	-0.28515	-0.64462	0.29343	-0.03793
C20:1n-9	Eicosenoic acid	0.67964	-0.1959	0.08684	-0.10552
C20:2n-6	Docosadienoic acid	-0.26369	0.20097	-0.38591	-0.20464
C20:3n-9	Mead acid	0.52956	0.56111	0.29702	-0.09632
C20:3n-3	Eicosatrienoic acid	0.61321	-0.08161	-0.05916	0.23473
C20:3n-6	DGLA acid	-0.01261	0.54763	0.23014	-0.36373
C20:4n-6	AA	-0.83172	0.21304	-0.05638	-0.0299
C20:5n-3	EPA	0.40659	-0.01193	0.28877	0.17838
C22:0	Behenic	-0.28517	-0.71415	0.48086	-0.01943
C22:1n-9	Erucic acid	-0.01195	-0.19268	0.25176	-0.00831
C22:3n-3	Docosatrienoic acid	0.21827	-0.00046	-0.04247	0.33888
C22:4n-6	Adrenic acid	-0.48409	0.56814	0.20476	-0.3436
C22:5n-3	DPA	-0.25676	0.28583	0.28335	-0.15873
C22:5n-6	Osbond acid	-0.27148	0.60172	0.24431	-0.33654
C22:6n-3	DHA	-0.68521	-0.11894	-0.05851	0.24021
C24:0	Lignoceric acid	-0.34656	-0.62771	0.50642	0.02366
C24:1n-9	Nervonic acid	-0.39641	-0.50198	0.59426	0.02275
Eigen value		6.26	4.45	2.83	1.99
Total variance (%)		22	15	10	7

LA - Linoleic Acid; ALA- α - Linoleic Acid; GLA – γ -linolenic acid; DGLA - Dihomo- γ -linolenic acid; AA - Arachidonic acid; EPA - Eicosapentaenoic acid; DPA - Docosapentaenoic acid; DHA - Docosahexaenoic acid; LCPUFA – Long-chain polyunsaturated fatty acids; FA – Fatty acid; MUFA – Monounsaturated fatty acids

Factor loadings >0.5 contribute to defining each factor and are highlighted in grey

Associations between baseline characteristics and plasma total phospholipid fatty acid patterns

Associations of baseline characteristics with the FA patterns are presented in (Table 3-4). Boys had significantly higher scores for the '*high n-6 LCPUFA*' pattern than girls ($p = 0.034$). None of the patterns were associated with growth measurements.

More than a third of the infants (34.6%, $n = 122$) were anaemic. Hb concentrations correlated positively with the '*high EFAs with low DHA and AA*' pattern ($p < 0.001$) and negatively with the '*high n-6 LCPUFA*' ($p < 0.001$) pattern scores. Thus, anaemic infants had higher factor scores for the '*high n-6 LCPUFA*' [0.20 (0.02, 0.37)] pattern compared with the non-anaemic infants [-0.10 (-0.24, 0.02)].

Table 3-4: Associations between infant characteristics at six months and plasma total phospholipid fatty acid patterns

CHARACTERISTICS		FACTOR 1			FACTOR 2		FACTOR 3		FACTOR 4	
		N	High EFAs with low DHA and AA ¹ LS (95% CL)	p value ²	High n-6 LCPUFA ¹ LS (95% CL)	p value ²	High MUFA and nervonic acid ¹ LS (95% CL)	p value ²	Trans FA ¹ LS (95% CL)	p value ²
Gender	Female	187	-0.03 (-0.18 , 0.12)	0.604	-0.12 (-0.27 , 0.03)	0.034	0.08 (-0.07 , 0.24)	0.154	0.04 (-0.11 , 0.19)	0.486
	Male	166	-0.03 (-0.12 , 0.17)		0.10 (-0.04 , 0.25)		-0.07 (-0.21 , 0.07)		-0.03 (-0.18 , 0.11)	
LAZ			0.07	0.204	-0.004	0.938	-0.045	0.437	0.031	0.596
Stunting	Stunted (LAZ < -2SD)	104	-0.12 (-0.32 , 0.07)	0.127	0.09 (-0.11 , 0.28)	0.266	0.15 (-0.05 , 0.34)	0.082	-0.09 (-0.28 , 0.10)	0.268
	Not stunted	249	0.05 (-0.07 , 0.17)		-0.04 (-0.17 , 0.08)		-0.06 (-0.18 , 0.07)		0.04 (-0.08 , 0.17)	
WLZ			-0.036	0.199	-0.037	0.145	-0.042	0.338	0.006	0.540
Overweight	Overweight (>+2 WLZ)	13	-0.06 (-0.61, 0.48)	0.817	-0.33 (-0.88, 0.21)	0.220	0.31 (-0.23, 0.86)	0.251	-0.43 (-0.98, 0.11)	0.114
	Not overweight	340	-0.0 (-0.10 , 0.11)		0.01 (-0.09, 0.12)		-0.01 (-0.23, 0.86)		0.12 (-0.09, 0.12)	
WAZ			0.011	0.864	-0.037	0.560	-0.072	0.257	0.024	0.699
Underweight	Underweight (WAZ < -2SD)	36	0.09 (-0.24 , 0.42)	0.551	-0.07 (-0.41 , 0.26)	0.671	0.14 (-0.19 , 0.47)	0.399	-0.13 (-0.46, 0.20)	0.404
	Not Underweight	317	-0.01 (-0.12 , 0.10)		0.00 (-0.11 , 0.11)		-0.01 (-0.12 , 0.10)		0.02 (-0.09 , 0.13)	
Hb (g/dL)			0.221	<0.001	-0.209	<0.001	0.09	0.120	0.003	0.959
Hb status	Anaemic (Hb < 11g/dL)	126	-0.29 (-0.47, -0.12)	<0.001	0.20 (0.02 , 0.37)	0.007	-0.09 (-0.27, 0.09)	0.220	0.01 (-0.16, 0.19)	0.844
	Not anaemic	227	0.16 (0.04, 0.29)		-0.10 (-0.24, 0.02)		0.05 (-0.08, 0.18)		-0.01 (-0.14, 0.12)	

FA - Fatty acids; LA - Linoleic acid; ALA- α - Linoleic acid; AA- Arachidonic acid; EPA- Eicosapentaenoic acid; DPA- Docosapentanoic acid; DHA- Docosahexaenoic acid; LCPUFA- Long-chain polyunsaturated fatty acids. LAZ - length-for-age Z-score; WLZ - weight-for-length Z-score; BAZ- BMI-for-age z-scores; WAZ- weight for age z-score; Hb - haemoglobin.

¹Least squares (LS) means (95% Confidence Limits) of factor scores all such values

²P value is the differences between the factor scores in each category, determined by general linear model with factor scores as outcome variables and characteristics as fixed factor, adjusted for sex, age and Hb (growth parameters).

Associations between infant feeding practices and total phospholipid fatty acid patterns

Associations of feeding practices with FA patterns are summarised in Table 3-5. More than two thirds (67%, n=236) of the infants were still breastfeeding at the time of the study (six months). Almost half (47%, n=167) of the infants had been introduced to formula milk and most (93%, n = 329) of the infants had already been introduced to semi-solids at the time of the study. Only 8% (n=27) of the infants were still exclusively breastfed at six months.

Infants who were receiving infant formula milk had significantly higher factor scores for the '*high EFAs with low DHA and AA*' FA pattern (0.62 [0.50, 0.74]) than infants who were not receiving infant formula milk (-0.60 [-0.71, -0.48]). In contrast, infants who were still breastfed (exclusively and non-exclusively) had lower factor scores than infants who were no longer breastfed ($p<0.001$).

Infants who were still breastfed, consumed cow's milk or semi-solid foods had significantly higher scores for the '*high n-6 LCPUFA*' FA pattern than their counterparts, while infants who received formula milk had significantly lower factor scores (-0.25 [-0.40, -0.10]) than infants who did not receive formula (0.22 [0.08, 0.37]).

Infants who received infant formula milk had significantly higher, while infants who were still breastfed had significantly lower scores for the '*high MUFA and nervonic acid*' FA pattern than their counterparts.

Infants who were still breastfed or received semi-solid foods had significantly higher factor scores for the '*trans-FA*' pattern than their counterparts.

Associations between phospholipid fatty acid patterns and infant psychomotor scores at six months

The results of a general linear model to examine the relationship between plasma phospholipid FA patterns and psychomotor scores are shown in Table 3-6. The mean (95% CI) scores for the different psychomotor developmental categories were as follows: eye-hand coordination sub-scale: 20.4 (20.1, 20.7), locomotor skills sub-scale: 16.4 (16.1, 16.6), and parent rating: 20.1 (19.8, 20.4). The maximum possible scores for the combined psychomotor score and eye-hand coordination sub-scale are 53 and 27, respectively. Factor scores for the '*high MUFA and nervonic acid*' pattern were positively associated with locomotor scores ($p=0.035$). Factor scores for the '*trans-FA*' pattern were positively associated with both eye-hand coordination ($p=0.009$) and locomotor scores ($p=0.015$).

Table 3-5: Associations between feeding practices and plasma total phospholipid fatty acid patterns

FEEDING PRACTICE		N	FACTOR 1		FACTOR 2		FACTOR 3		FACTOR 4	
			<i>High EFAs with low DHA and AA</i>	<i>p value</i> ²	<i>High n-6 LCPUFA</i>	<i>p value</i> ²	<i>High MUFA and nervonic acid</i>	<i>p value</i> ²	<i>Trans-FA</i>	<i>p value</i> ²
BF	Yes	236	-0.54 (-0.63, -0.47) ¹	< 0.001	0.11 (-0.02, 0.24)	0.003	-0.18 (-0.30, -0.05)	< 0.001	0.10 (-0.03, 0.23)	0.009
	No	117	1.11 (0.98, 1.21)		-0.22 (-0.40, -0.04)		0.36 (0.18, 0.54)		-0.20 (-0.38, -0.02)	
EBF	EBF up to 6 mo	27	-0.68 (-1.05, -0.31)	< 0.001	-0.22 (-0.59, 0.16)	0.254	0.06 (-0.32, 0.44)	0.770	-0.24 (-0.62, 0.14)	0.187
	Stopped EBF	326	0.05 (-0.05, 0.16)		0.01 (-0.10, 0.12)		-0.00 (-0.11, 0.11)		0.02 (-0.09, 0.13)	
FM	Yes	167	0.62 (0.50, 0.74)	< 0.001	-0.25 (-0.40, -0.10)	< 0.001	0.13 (-0.02, 0.29)	0.014	-0.02 (-0.17, 0.13)	0.556
	No	182	-0.60 (-0.71, -0.48)		0.22 (0.08, 0.37)		-0.13 (-0.27, 0.02)		0.03 (-0.12, 0.17)	
Cow's milk	≥ 4 days/week	23	-0.01 (-0.12, 0.10)	0.995	0.56 (0.15, 0.96)	0.006	0.24 (-0.13, 0.08)	0.215	0.03 (-0.38, 0.43)	0.919
	seldom/never	326	-0.01 (-0.42, 0.40)		-0.04 (-0.15, 0.07)		-0.03 (-0.17, 0.66)		0.00 (-0.10, 0.11)	
Semi-solid foods	Yes	329	-0.00 (-0.11, 0.11)	0.847	0.02 (-0.08, 0.13)	0.017	0.02 (-0.09, 0.13)	0.376	0.03 (-0.07, 0.14)	0.033
	No	21	-0.05 (-0.48, 0.39)		-0.52 (-0.96, -0.09)		-0.19 (-0.63, 0.25)		-0.46 (-0.89, -0.02)	
Fats	Yes	194	-0.02 (-0.16, 0.12)	0.888	0.06 (-0.09, 0.20)	0.224	0.01 (-0.13, 0.16)	0.734	-0.05 (-0.20, 0.08)	0.186
	No	155	-0.01 (-0.17, 0.16)		-0.08 (-0.24, 0.09)		-0.02 (-0.18, 0.14)		0.09 (-0.07, 0.24)	

FA - Fatty acid; LCPUFA- Long chain polyunsaturated fatty acids; MUFA - Monounsaturated fatty acids; BF - Breastfeeding; EBF- Exclusive breastfeeding; BM – Breast milk; FM - Formula milk.

¹Least squares (LS) means (95% Confidence Limits) all such values.

²P value is for the difference between the means of the factor scores in the different categories, determined using general linear model with factor score as outcome variable and feeding practice as fixed factor, adjusted for sex and age.

Table 3-6: Associations between Hb, LAZ and plasma phospholipid fatty acid patterns and psychomotor development at 6 months

Model	Psychomotor Development					
	Eye-hand co-ordination		Locomotor skills		Parent rating	
	β estimates	p value	β estimates	p value	β estimates	p value
Hb	-0.354	0.046	-0.071	0.626	-0.112	0.448
LAZ	0.567	0.002	0.412	0.005	0.585	<0.001
<i>High EFAs with low DHA and AA</i>	-0.159	0.412	-0.108	0.502	0.215	0.186
<i>High n-6 LCPUFA</i>	-0.004	0.982	0.003	0.986	-0.091	0.574
<i>High MUFA and nervonic acid</i>	0.097	0.612	0.334	0.035	0.229	0.153
<i>Trans-FA</i>	0.450	0.009	0.384	0.015	0.207	0.194

FA - Fatty acids; LA - Linoleic Acid; ALA- α - Linoleic Acid; AA- Arachidonic acid; EPA- Eicosapentaenoic Acid; DHA- Docosahexaenoic acid;

PUFA- Polyunsaturated Fatty Acids; LCPUFA- Long-chain polyunsaturated fatty acids; LAZ - length-for-age Z-score; Hb - haemoglobin

General linear model adjusted for Hb and LAZ.

Hb concentration was a significant predictor, $p < 0.05$ (Rothman et al., 2018)

LAZ is a significant covariate, $p < 0.05$ (Rothman et al., 2018)

DISCUSSION

In this study, we identified four major plasma total phospholipid FA patterns that were associated with feeding practices and psychomotor development in six-month-old South African infants from a peri-urban township: A '*high EFAs with low DHA and AA*' pattern, a '*high n-6 LCPUFA*' pattern, a '*high MUFA and nervonic acid*' pattern, and a '*trans-FA*' pattern. Formula feeding was associated with higher, while breastfeeding was associated with lower scores for the '*high EFAs with low DHA and AA*' and '*high MUFA and nervonic acid*' patterns. On the other hand, breastfeeding, the consumption of cow's milk and the consumption of semi-solid foods were associated with higher scores, while formula feeding was associated with lower scores for the '*high n-6 LCPUFA*' pattern. Breastfeeding and the consumption of semi-solids were also associated with higher '*trans-FA*' pattern scores. The '*high MUFA and nervonic acid*' and '*trans-FA*' patterns were positively associated with psychomotor development scores of infants. No associations were found with growth.

The infants in this present study had an adequate EFA and LCPUFA status overall, based on several FA indices in structural phospholipids that have been used as proxies for assessing EFA and LCPUFA status, such as the essential PUFA status index, the DHA to osbond acid (22:5n-6) and the omega 3 index (Hornstra, 2000; Vlaardingerbroek & Hornstra, 2004). However, infants who were no longer breastfed at the time of the study had a significantly lower LCPUFA status.

Infants who were still receiving breast milk, consumed semi-solid foods or cow's milk, but not infant formula milk at the time of the survey had higher factor scores for the '*high n-6 LCPUFA*' pattern. This FA pattern was characterised by high plasma contents of the LA metabolite dihomo- γ -linolenic acid (DGLA; C20: 3n-6), the very long-chain n-6 PUFAs adrenic (C22:4n-6) and osbond acid (C22:5n-6). Similar to AA, adrenic acid accumulates rapidly after birth during the period of the brain growth spurt in infants (Hadley, Ryan, Forsyth, Gautier, & Salem, 2016). The observation that this continued breastfeeding-associated FA pattern was characterised by n-6 LCPUFAs but not by n-3 PUFAs suggests that maternal intake of n-3 LCPUFAs, i.e. from fatty fish, is very low in this population. Thus, as a balanced supply of the LCPUFAs AA and DHA is important for adequate accumulation of AA and DHA in the growing brain during infancy (Novak, Dyer, & Innis, 2008), breastfeeding women are advised to consume at least 200mg of dietary DHA to provide breast milk with a DHA content of at least 0.3%. This will ensure that a breastfed infant is guaranteed to receive a daily supply of 100 mg DHA/day which is enough to meet their metabolic needs (Koletzko, 2016).

In addition, continued breastfeeding and the consumption of semi-solid foods were both associated with higher scores for the '*trans-FA*' pattern. This FA pattern comprised only trans-FAs with factor loadings above 0.5. However, other studies have used lower factor loading cut-offs to define patterns. Thus, if a lower cut-off had been used to define each pattern, then the n-3 LCPUFAs would have been included in this pattern. Furthermore, this was the only pattern with positive loadings for DHA and other n-3 LCPUFAs. Also, it is plausible that some of the infants who were breastfed or who had already started eating semi-solids were receiving a considerable amount of n-3 LCPUFAs through breast milk or semi-solid foods, respectively. Therefore, we speculate that, since DHA is a major LCPUFA associated with growth and development in infancy, this could have contributed to the positive association observed between the '*trans-FA*' pattern and psychomotor development. Nonetheless, only 2% of our study population had started eating fish. The introduction of complementary foods results in a decrease in breast milk and formula intake which in turn results in a drastic decrease in total dietary intake of LCPUFAs (Rise et al., 2013). Thus, it is advisable that good food sources of LCPUFAs such as egg yolk, fish, liver or other LCPUFA fortified foods (like dairy) should be included in the complementary feeding diet (Muraro et al., 2017; Perkin et al., 2016; Prell & Koletzko, 2016).

In South Africa, all infant feeding formulas contain the EFAs LA and ALA, but the addition of DHA as an ingredient in infant formula is optional (Codex Alimentarius Commission, 2007). Despite the known importance and need for AA during infancy, the addition of AA to infant formulas has not yet been regulated. Thus, similarly with other studies (Forsyth & Willatts, 2002; Makrides, Neumann, Byard, Simmer, & Gibson, 1994), the factor scores for the '*high EFAs with low DHA and AA*' pattern were higher in infants who were receiving formula milk, while being lower in

infants who were still exclusively or non-exclusively breastfed. These results suggest that formula-fed infants may not synthesise enough LCPUFAs to allow adequate accretion in the brain at a rate that is comparable with and corresponds to breastfed infants who receive LCPUFAs through breast milk. Therefore, because preformed LCPUFAs are necessary and because of the acknowledged importance of the LCPUFAs DHA and AA during infancy, LCPUFA-enriched formulas are now common worldwide (Hadley et al., 2016).

On the other hand, the formula feeding-associated pattern of *'high EFAs with low DHA and AA'* was positively associated with Hb concentrations. A possible explanation for this could be that the infants in this study were receiving mostly iron-fortified infant formula milk. In contrast, factor scores for the continued breastfeeding-associated pattern of *'high n-6 LCPUFA'* were higher in anaemic than non-anaemic infants. This is in agreement with data from Europe (Eussen, Alles, Uijterschout, Brus, & Van Der Horst-graat, 2015), China (Luo et al., 2014) and South Africa (Faber, 2007), showing that the prevalence of anaemia is lower in infants who receive infant formula. Breast milk contains highly bioavailable iron but in low concentrations. Therefore, the infants' iron status during the first six months is dependent on the infants' iron stores at birth, which in turn are dependent on maternal iron status during pregnancy. Therefore, the WHO (2009) recommends that infants with healthy birth weight must be supplied with iron at the age of 6 months by the introduction of iron-rich complementary foods. Therefore, it is plausible that while fortified infant formula milk may not provide the much needed LCPUFAs, it may be a good source of iron in these children.

Moreover, formula feeding was positively, while continued breastfeeding was negatively associated with the *'high MUFA and nervonic acid'* FA pattern. This pattern was characterised by high loadings of palmitoleic acid (C16:1n-7), which is one of the two predominant MUFAs found in infant formula milk. However, the potential purpose and impact of MUFA supply to the immune system and other functional outcomes have not yet been explored in infants and their nutritional relevance remains unknown (Delplanque, Gibson, Koletzko, Lapillonne, & Strandvik, 2015). Although found in very low levels in formula milk, nervonic acid is a major very long-chain FA important for myelination, brain growth and development (Innis, Sprecher, Hachey, Edmond, & Anderson, 1999). It may also be synthesised endogenously in new-borns and could therefore have contributed to the positive association observed between the *'high MUFA and nervonic acid'* pattern and psychomotor development. However, its relevance in dietary supply still remains speculative (Delplanque et al., 2015).

In the present study, there were no associations with growth, although research suggests that EFAs are important for optimal linear growth (Adu-afarwuah et al., 2007) and in older children (Jumbe et al., 2016). It is unclear why associations between growth and the plasma FA patterns

were not observed in this study, but the data from human studies attempting to make a connection between FA intake and linear growth is mixed (Jumbe et al., 2016).

A limitation of this study is the low success rate of 64% (480/750) in drawing blood at baseline. Furthermore, of the successfully drawn blood samples, adequate amounts for FA analysis were only available from 353 infants (47%), since iron status analyses had the highest priority. This led to a reduced sample size being used for the analyses done in this study. Data used in this current study were collected in one area of the North West province in South Africa. Thus, our results cannot be generalised to infants in all areas of South Africa. Also, the cross-sectional and observational nature of this study restricts the possibility of drawing conclusions on causation, particularly between FA status and psychomotor development. However, one of the strengths of this study is the use of the PCA and factor analysis to determine FA patterns. The latent factors retained were characterised by high factor loadings of different FAs, hence the strict criterion that was used to define the patterns. The application of PCA is an innovative approach that can be used in future studies to determine distinct patterns considering the complexity of individual FA data (Imamura et al., 2012). Therefore, in light of the present study, a combination of FAs may potentially be important in determining the quality of the diet of infants in future studies.

In conclusion, this study identified four distinct plasma FA patterns in six-month-old South African infants. Our findings suggest that formula-fed infants are at risk of inadequate DHA and AA intake, while breastfed infants are likely ensured an adequate supply of n-6 LCPUFAs. However, it is plausible that lactating women do not consume adequate dietary n-3 LCPUFAs, which might lead to an insufficient supply of n-3 LCPUFAs through breast milk. The number of infants reported to have been introduced to fish, was very small (2%). Thus, mothers and caregivers should be advised to give their infants at least two portions of fatty fish a week to enhance their intake of EPA and DHA (Smuts & Wolmarans, 2013) during the complementary feeding period. These findings reinforce the importance of the WHO recommendation for EBF up to the age of six months, timely introduction of LCPUFA-rich complementary foods and continued breastfeeding for up to two years and beyond.

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CHAPTER 4

Efficacy of novel small-quantity lipid-based nutrient supplements in improving long-chain polyunsaturated fatty acid status of South African infants: A randomised, controlled trial

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ABSTRACT

Introduction: Long-chain polyunsaturated fatty acid (LCPUFA) intakes from breast milk and complementary foods in six- to 36-months-old South African infants were recently estimated to be below recommendations. Thus, fortifying complementary foods with essential fatty acids (EFAs) and LCPUFAs may be necessary to ensure adequate supply for optimal growth and development.

Objectives: To investigate the efficacy of small-quantity lipid-based nutrient supplements (SQ-LNS) containing EFAs with or without LCPUFAs in improving LCPUFA status in South African infants fed complementary food.

Methods: In a randomised controlled trial, six-month-old infants (n=750) were randomised to receive: 1) daily SQ-LNS containing the EFAs linoleic acid (LA) and alpha-linolenic acid (ALA) (SQ-LNS), 2) daily SQ-LNS with EFAs and the LCPUFAs arachidonic acid (AA) and docosahexaenoic acid (DHA) (SQ-LNS-plus), or 3) no supplement (control group). Both SQ-LNSs contained micronutrients. Plasma total phospholipid FA composition (% of total FAs) was measured at baseline (n=353) and at 12 months (n=293) (infants with FA data at 6 and 12 months, n=148).

Results: At baseline, geometric mean (95% CI) plasma DHA and AA were 4.1 (4.0-4.3) and 11.5 (11.2-11.8) % respectively, with significantly higher plasma DHA and AA in breastfed than non-breastfed infants. Infants receiving the SQ-LNS with EFAs and LCPUFAs had significantly higher

plasma DHA (4.52 [4.3-4.9]) at 12 months than the controls (3.8 [3.6-4.0]), with a higher effect size in infants who no longer received breast milk ($\beta = 1.148$ [95% CI= 0.597, 1.699]) than in infants who were still breastfeeding ($\beta = 0.544$ [95% CI= 0.179, 0.909]). There was no effect of either of the two SQ-LNSs on plasma AA. Consequently, infants receiving the SQ-LNS with EFAs and LCPUFAs had a significantly lower plasma n-6 to n-3 PUFA ratio at 12 months than control infants did.

Conclusion: Our study suggests that the provision of SQ-LNS containing EFAs and LCPUFAs is efficacious in improving plasma DHA status. Those infants, particularly, who are no longer breastfed may benefit most from LCPUFA-enriched SQ-LNS.

INTRODUCTION

The long-chain polyunsaturated fatty acids (LCPUFAs) docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (AA; 20:4n-6) are critically important for infant growth and development (Carlson & Colombo, 2016). During gestation, DHA is preferentially transferred to the foetus across the placenta. After birth, endogenous synthesis of both DHA and AA can also occur by conversion of the essential fatty acids (EFAs) α -linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6), respectively. This conversion, however, is limited during infancy and sufficient amounts cannot be converted to meet the requirements of the growing infant (Innis, 2007). Therefore, breast milk and LCPUFA-enriched infant formula are the sole source of LCPUFAs during the first six months of life (Koletzko *et al.*, 2008).

After six months of age, breast milk alone no longer meets all the nutritional requirements of the infant. It is for this reason that the World Health Organisation (WHO & UNICEF, 2003) recommends timely introduction of complementary foods at the age of six months with continued breastfeeding for up to two years and beyond. A decrease in intake of breast milk or infant formula, together with the introduction of LCPUFA-poor complementary foods, results in a reduction of dietary DHA and AA supply, which is reflected in blood levels (Libuda *et al.*, 2016). This can be corrected by the introduction of foods that contain or are enriched with LCPUFAs (Michaelsen *et al.*, 2009). Thus, the European Food Safety Authority (EFSA) recommends an intake of 100 mg of DHA/day for the majority of infants from six to 24 months of age (Agostoni *et al.*, 2013).

Forsyth *et al.* (2016) recently showed that the median estimates of AA and DHA intakes from both breast milk and complementary foods in South African infants, are lower than the recommended intakes. In this regard, plant-based complementary foods, consumed in most low- and middle-income countries, are insufficient to meet the dietary needs of growing infants. Therefore, meat, poultry, fish and eggs need to be included in the diet of infants after the age of six months (Michaelsen *et al.*, 2011).

Moreover, there are new supplements, including micronutrient powders and lipid-based supplements (LNSs), that are currently being investigated for their potential to fill the nutrient gaps in infants fed complementary foods, thereby supporting healthy growth and development (Bhutta *et al.*, 2013; Muslihah *et al.*, 2016). These LNS interventions have shown positive effects on linear growth (Adu-Afarwuah *et al.*, 2007) and child development, particularly motor (Phuka *et al.*, 2008; Prado *et al.*, 2016a; Prado *et al.*, 2016b) and language development (Matias *et al.*, 2017) at 12, 18 and 24 months, in some, but not all studies. However, these studies did not determine the efficacy of LNS in improving LCPUFA status.

In South Africa, there are limited data on the LCPUFA status of infants and no trials have addressed whether complementary foods high in LCPUFA can improve infant LCPUFA status during late infancy. This study therefore investigated the efficacy of the provision of a SQ-LNS containing the EFAs LA and ALA and a SQ-LNS containing EFAs, DHA and AA from six to 12 months in improving LCPUFA status of South African infants.

METHODS

Participants and study site

This study was nested within the randomised, controlled Tswaka trial which investigated the effects of two different small-quantity lipid-based nutrient supplements (SQ-LNSs) on linear growth and psychomotor development in infants aged six months (Smuts *et al.*, 2018). The study was done in the Jouberton area of the greater Matlosana Municipality in the North West Province, South Africa, from September 2013 to January 2015. The trial was conducted in six-month-old infants who were recruited from the study area by fieldworkers speaking the local language, who explained the expectations of the study to the mothers, after which willing mothers signed informed consent forms. Infants were excluded from the trial if they had (i) not been previously breastfed; experienced (ii) severe obvious congenital abnormalities; (iii) severe anaemia (haemoglobin < 7 g/dL) or (iv) severe malnutrition (weight-for-length Z-score < -3.00 SD); (v) other diseases that were referred for hospitalisation by the clinic staff; (vi) known allergies and/or intolerances to peanuts and/or soy, milk, and/or lactose and/or fish; (vii) received or were receiving special nutritional supplements as part of feeding programmes; (viii) not been born as singletons; (xi) been known to be HIV-positive; (x) or were subject to any plans to move out of the study area within the following seven months into the trial.

Sample size

A total of 750 infants was enrolled into the Tswaka trial (250 infants per group), to allow for an expected dropout rate of 25%. The calculations for the Tswaka sample size were based on the expected variance in growth as the primary outcome of the six-month intervention. All infants included in the Tswaka trial, therefore, had an equal chance of being included in the fatty acid analyses. However, only infants who had plasma FAs available at six and/or 12 months were included in this study.

Intervention

Infants were randomly allocated to two treatment groups and a control group at the age of six months, using block randomisation. Infants in the treatment groups received either (1) a fortified fat-based paste with EFAs (SQ-LNS) or (2) a fortified fat-based paste with EFAs, DHA and AA, as well as phytase and various other nutrients (SQ-LNS-plus). The control group did not receive any SQ-LNS for the duration of the trial (age 6-12 months) but was given a six months' supply of SQ-LNS after completing the trial. The nutritional profiles of the SQ-LNS and SQ-LNS-plus are shown in Table 4-1. Randomisation lists were prepared by the trial statistician using NQuery version 7. Colour-coding was used for identification of a particular group for implementing and monitoring purposes.

The SQ-LNS products were produced and manufactured internationally by DSM and UNILEVER. Prior to the Tswaka trial, the acceptability of the SQ-LNS products was tested based on the mother's own acceptance and her perception of the infant's acceptance (Rothman *et al.*, 2015). The caregivers were advised to mix the supplement with the infants' usual first complementary feed of the day. The infants were given one sachet (20g) of the SQ-LNS to eat daily, for six months. The caregivers were also advised to use the product exclusively for the research participant. Apart from not receiving supplements, the control group and the two treatment groups were treated the same way.

Data collection and measurements

The infants' details were recorded on a recruitment form and the health card was used to verify the infants' age at enrolment. Information on the socio-economic status at baseline, bi-monthly infant feeding practices and infant morbidity during the previous seven days, was collected using a detailed questionnaire. Assessment of acceptability assessment was determined by use of a questionnaire during the exit interview.

A 4 mL venous blood sample was drawn into EDTA-coated vacutainers from the antecubital arm area or dorsal area of the hand at the age of six months. To determine haemoglobin concentrations using the Hemocue system (Hemocue 201+; HemoCue® AB), an aliquot from whole-blood samples obtained was used. If blood drawing was not successful, a capillary blood sample was taken by means of a finger prick. Anaemia was defined as Hb < 11 g/dL. Venous blood samples were centrifuged for 10 minutes at 2000g and 100ul plasma aliquoted into 500ul microfuge tubes and stored at 4 °C. These samples were transported daily from the study site to the main laboratory at the Potchefstroom campus of the North West University for storage at -80 °C until analysis.

Weight and recumbent length were assessed according to the WHO (1995). Infants were weighed by a research nurse, without clothing to the nearest 0.01 kg, using a calibrated digital baby scale (Seca model 354, GmbH & Co. KG., Hamburg, Germany, maximum 20 kg). Recumbent length was measured to the nearest 0.1 cm (Seca 416 infantometer; Seca GmbH & Co. KG., Hamburg, Germany). Anthropometry data were entered using EpiData 3.1. The SAS macros for the WHO Child growth standards (2006) were used to calculate age and gender-specific Z-scores for weight and length.

Table 4-1: The energy and nutrient content of the two SQ-LNS products used in the study¹

	SQ-LNS	SQ-LNS-plus
Amount, g (1 portion)	20	20
Energy, kcal	114	113
Energy density, kcal/g	5.7	5.7
Protein, g	3.0	3.7
% calories from protein	10%	13%
Fat, g	8.0	8.8
% calories from fat	63%	70%
Essential fatty acids		
Linoleic acid (LA), g	1.5	1.8
α -Linolenic acid (ALA), mg	265	348
LA/ALA ratio	5.7	5.0
Long-chain polyunsaturated fatty acids		
Docosahexaenoic acid, mg ²	-	75
Arachidonic acid, mg	-	75
Micronutrients:³		
Vitamin A, μ g	200	200
Vitamin D, μ g	2.5	2.5
Vitamin E, mg	2.5	3.8
Vitamin K, μ g	7.5	7.5
Thiamine, mg	0.25	0.25
Riboflavin, mg	0.25	0.25
Niacin, mg	3	3
Pantothenate, mg	1.0	1.0
Vitamin B6, mg	0.25	0.25
Biotin, μ g	4.0	4.0
Folate (B9), μ g	80	80
Vitamin B12, μ g	0.45	0.45
Vitamin C, mg	23.3	103
Calcium, mg	250	396
Iodine, μ g	45	45
Iron, mg	5.8	5.8
Zinc, mg	6.2	6.2
Copper, mg	0.28	0.28
Selenium, μ g	8.5	8.5
Magnesium, mg	-	30
Manganese, mg	-	0.6
Phosphorus, mg	-	230
Potassium, mg	-	257
Choline (from lecithin), mg	2.0	7.8
L-Lysine, mg	-	160
Phytase, FTU	-	200

¹ SQ-LNS and SQ-LNS-plus are small-quantity lipid-based nutrient supplements; The SQ-LNS and SQ-LNS-plus products were provided by Unilever R&D (Vlaardingen, Netherlands) and DSM Nutritional Products Ltd (Kaiseraugst, Switzerland), respectively. SQ-LNS was manufactured by Unilever R&D Vlaardingen B.V. and packed by Budelpack B.V.; SQ-LNS-plus was manufactured by GC Rieber Compact India Pvt. Limited. 20 g LNS products were packed in sachets containing a daily ration and did not require any preparation prior to consumption. SQ-LNS-fortified fat-based paste with EFAs and contained 1.6 g of skimmed milk powder; SQ-LNS-plus-fortified fat-based paste with EFAs, DHA and AA and 4.6 g of skimmed milk powder, as well as phytase and various other nutrients

² Fish oil also contained 17 mg eicosapentaenoic acid (EPA)

³ From added micronutrient mix, excluding micronutrients from other raw material sources
FTU, phytase activity units

Fatty acid analysis

Plasma fatty acid analysis

Lipids were extracted from plasma samples with chloroform: methanol (2:1 vol: vol) by using a modification of the method of Folch et al. (1957). Lipid extracts were concentrated using nitrogen gas, and thin-layer chromatography (silica gel 60 plates without fluorescent indicator, 10x20 cm; Merck) was used to separate neutral lipids from the phospholipids. Phospholipids were eluted using petroleum ether: diethyl ether: acetic acid (90:30:1, vol: vol: vol) and a pinch of 2,5-Bis(5-tert-butyl-benzoxazol-2-yl)thiophene. The lipid fraction containing the phospholipids was removed from the thin-layer chromatography plate and trans-methylated with methanol: sulphuric acid (95:5, vol: vol) at 70°C for 2 hours. This led to the formation of fatty acid methyl esters (FAMES). The FAMES were extracted using hexane and water. The organic layer was aspirated, evaporated, redissolved in hexane, and analysed by gas chromatography–electron ionisation mass spectrometry. All solvents used during the extraction procedure contained 0.01% butylated hydroxytoluene. Samples were analysed on an Agilent Technologies 7000 GC/MS Triple Quad system comprising an Agilent 7890A gas chromatograph equipped with an Agilent G7001B triple quad mass spectrometer (Agilent Technologies). The gas chromatography separation of FAMES was carried out on an HP-88 capillary column (100 m x 0.25 mm x 0.20 µm; Agilent Technologies) by using helium as the carrier gas at a flow rate of 2.2 mL/min. Initial inlet temperature was held at 70 °C for 0.02 minutes, after which it was ramped to 270 °C at 500 °C/min. The mass spectrometer source was maintained at a temperature of 230 °C. A sample volume of 1 µL was injected, and split ratios of 80:1 for plasma samples were used. The oven temperature was maintained at 50 °C for 1 minute, then ramped to 170 °C at 30 °C/min, then from 170 °C to 215 °C at 2 °C/min, after which it was ramped to 230 °C at 4 °C/min. The temperature was then held isothermally at 230 °C for 7 minutes. The total analysis time was 38.25 minutes. Mass spectrometry with 70 eV electron-ionisation was carried out in multiple reaction monitoring mode, with at least two transitions per compound. The FAMES were quantified using MassHunter Quantitative Analysis software (Version B.05.02, Agilent Technologies). FAME peaks were identified and calibrated against a standard reference mixture of 33 FAMES (Nu-Check-Prep) and two single FAME standards (Larodan Fine Chemicals AB). Relative percentages of FAs were calculated by expressing the concentration of a given FAME as a percentage of the total concentration of all FAMES identified in the sample (Baumgartner *et al.*, 2012).

Data management and statistical methods

All data were checked for normal distribution and the presence of outliers using the Shapiro-Wilk test, Q-Q plots and visual inspection of histogram plots. Skewed variables were log transformed

before data analysis and are presented as geometric mean with 95% confidence intervals (95% CI). Normally distributed data were expressed as mean $\pm$ SD. One-way ANOVA and Pearson Chi-square tests were used to compare baseline characteristics between randomisation groups, of infants that had plasma FAs available at six and 12 months, as well as of those that had FAs available at 12 months only. One-way ANOVA and independent t-tests were used to determine differences in FA composition at six and 12 months for infants who were breastfed and those who were no longer breastfed. Differences in FA compositions at 12 months by intervention group were assessed using general linear models. In the first model, differences were adjusted for age and sex. Continued breastfeeding at 12 months was a significant predictor of FA status and was therefore added as a covariate in the second model. The third model was adjusted for baseline FA status, age, sex and continued breastfeeding at 12 months. The dependent t-test was used to determine differences between breastfed and non-breastfed infants within randomisation groups. P values < 0.05 were considered significant. Statistical analysis was performed using IBM SPSS Statistics (version 25; IBM Co).

Ethical considerations

The Tswaka trial was registered at <http://clinicaltrials.gov> as NCT01845610. The study was approved by the ethics committees of North West University (NWU) (NWU-00001-11-A1) and the South African Medical Research Council (SAMRC) (EC-01-03/2012), adhered to the principles of the Declaration of Helsinki and followed good clinical practice (GCP) guidelines. The North West Provincial Department of Health and Social Development also reviewed the trial protocol and the trial was further registered with the Directorate for Policy, Planning and Research. Informed consent was obtained from the parents/legal guardians of the infants before any study-specific procedures were performed

RESULTS

Of the 750 infants enrolled in the Tswaka trial, plasma samples for FA analysis were available from 353 and 293 infants at baseline (six months) and end-point (12 months), respectively (Figure 2-1). However, only 148 infants had plasma total phospholipid FA data available at both time points (six and 12 months). Baseline characteristics and feeding practices of infants with plasma total phospholipid FA data available are shown in Table 2. A third (30%, n = 87) of these infants were stunted. Overall, 71% (n = 209) were breastfed at baseline (six months) and more than half (56%, n = 163) were still breastfeeding at 12 months. The prevalence of anaemia at baseline was 36% (n = 103). There were no significant differences between the randomisation groups (Table 4-2) in any of the characteristics except in sex distribution and prevalence of overweight, amongst

the infants that had plasma total phospholipid FA data available at both time points and at 12 months only.

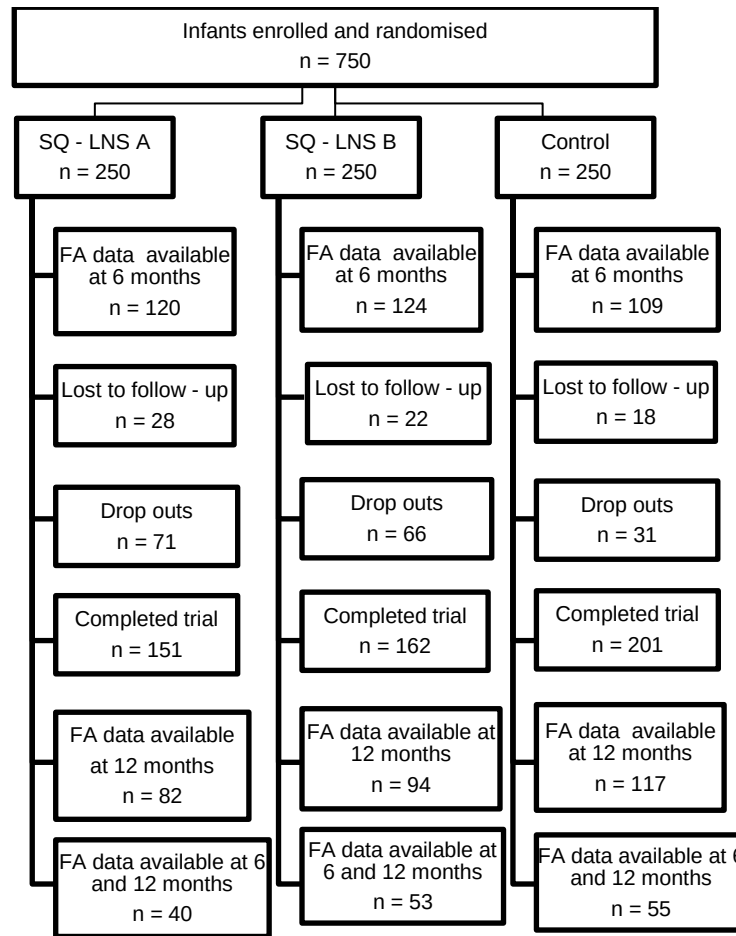


Figure 4-1: Flow diagram of participant randomisation, lost to follow-up and FA data availability throughout the study. SQ-LNS, small-quantity lipid-based nutrient supplement.

Table 4-2: Baseline characteristics of infants with plasma FAs available at baseline and at endpoint

Baseline Characteristics	Infants with FA available at both 6 and 12 months				Infants with FA available at 12 months			
	SQ-LNS (n = 40)	SQ-LNS-plus (n = 53)	Control (n = 55)	p ²	SQ-LNS (n = 82)	SQ-LNS-plus (n = 94)	Control (n = 117)	p ²
Age (m)	6.2±0.3 ¹	6.2±0.2	6.2±0.3	0.332	6.2±0.3	6.2±0.2	6.2±0.2	0.990
Males [n%]	13 (32.5)	31 (58.5)	26 (47.3)	0.046	28 (34.1)	54 (57.4)	58 (49.6)	0.008
Females [n%]	27 (67.5)	22 (41.5)	29 (52.7)		54 (65.9)	40 (42.6)	59 (50.4)	
LAZ	-1.58±1.21	-1.62±1.03	-1.31±1.10	0.285	-1.47±1.19	-1.56 ±0.98	-1.31±1.11	0.232
Anthropometric indices [n (%)]								
Stunting LAZ <-2 SD	18 (45.0)	17 (32.1)	12 (21.8)	0.057	29 (35.4)	28 (29.8)	30 (25.6)	0.335
WLZ	0.50±0.90	0.70±1.21	0.59±1.03	0.659	0.57±0.90	0.74±1.20	0.57±1.12	0.478
Wasting WLZ <-2 SD	1 (2.5)	0 (0)	0 (0)	0.261	1 (1.2)	0 (0)	0(0)	0.275
Overweight >+2 WLZ	0 (0)	2 (3.8)	1 (1.8)	0.437	1 (1.2)	5 (5.3)	5 (4.3)	0.336
WAZ	-0.72±0.98	-0.56±1.29	-0.45±1.22	0.545	-0.58±0.98	-0.48±1.16	-0.45±1.29	0.732
Underweight (WAZ< - 2 SD)	2 (5.0)	9 (17.0)	5 (9.1)	0.160	4 (4.9)	11 (11.7)		
Hb (g/dL)	11.27±1.28	11.28±1.17	11.40±1.03	0.810	11.50 ±1.47	11.36 ±1.28	11.50 ±1.37	0.726
Anaemic (Hb < 11 g/dL)	8 (20)	12 (22.6)	14 (25.5)	0.953	32 (39.0)	33 (35.1)	41 (35.0)	0.819
BF at baseline [n%]	27 (67.5)	38 (71.7)	40 (72.7)	0.610	60 (73.2)	67(71.3)	82(70.1)	0.894
BF at endpoint [n%]	20 (52.6)	31 (58.5)	31 (56.4)	0.390	48 (58.5)	52 (55.3)	63 (53.8)	0.679

FA - Fatty acids; LAZ - length-for-age Z-score; WLZ - weight-for-length Z-score; BAZ- BMI-for-age z-scores; WAZ- weight-for-age z-score; Hb –haemoglobin; BF - Breastfeeding.

SQ-LNS and SQ-LNS-plus are small-quantity lipid-based nutrient supplements; The SQ-LNS and SQ-LNS-plus products were provided by Unilever R&D (Vlaardingen, Netherlands) and DSM Nutritional Products Ltd (Kaiseraugst, Switzerland), respectively. SQ-LNS was manufactured by Unilever R&D Vlaardingen B.V. and packed by Budelpack B.V.; SQ-LNS-plus was manufactured by GC Rieber Compact India Pvt. Limited. 20 g LNS products were packed in sachets containing a daily ration and did not require any preparation prior to consumption.

SQ-LNS-fortified fat-based paste with EFAs; SQ-LNS-plus-fortified fat-based paste with EFAs, DHA and AA, as well as phytase and various other nutrients

¹ Mean±SD (all such values)

² p value for differences between categories by randomisation groups determined using one-way ANOVA or Pearson Chi-square test

Plasma total phospholipid DHA and AA (% of total FAs) for all infants who had plasma FA data available at baseline (six months) were 4.1 (4.0-4.3) and 11.5 (11.2-11.8) %, respectively. Plasma DHA and AA in infants who were still breastfeeding and those who no longer received breast milk at six months according to their respective randomisation groups, are shown in Figure 4-2. Plasma DHA and AA at baseline were significantly lower in non-breastfed than breastfed infants in all groups ($p < 0.001$).

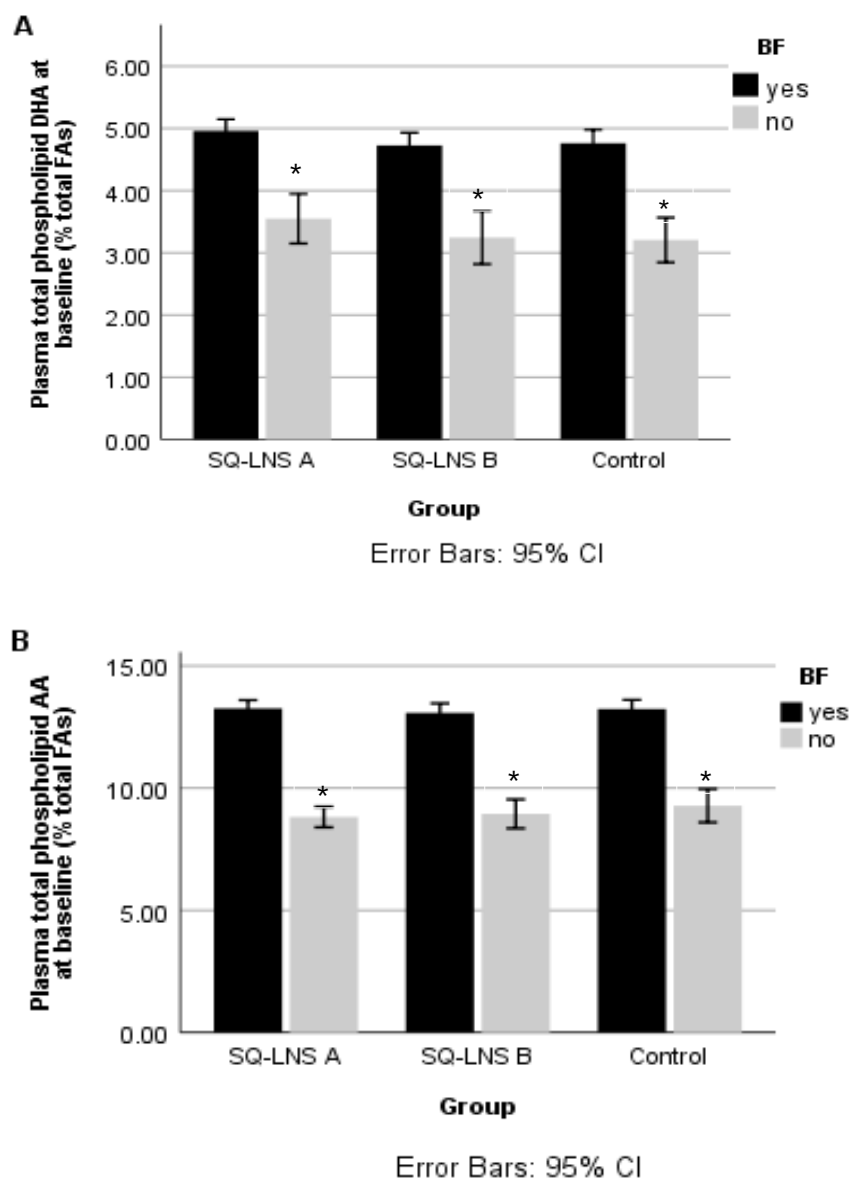


Figure 4-2: Geometric means (95% CI) plasma total phospholipid DHA and AA (% total FAs) in infants who were still breastfeeding and those who were no longer receiving breast milk at baseline (six months). *Indicates significant differences between breastfed and non-breastfed infants within groups ($p < 0.001$). General linear model and dependent t-test used to determine differences between breastfed and non-breastfed infants. DHA - Docosahexaenoic acid; AA - Arachidonic acid; BF- Breastfed

Table 4-3 shows the geometric mean (95% CI) of the selected total phospholipid FAs for all the infants with plasma FA data available at baseline (6 months, n = 353) and all infants with plasma FA data available at endpoint (12 months, n = 293). There were no significant differences between groups at baseline, except in plasma DHA: osbond and n6: n3 LCPUFA ratios ($p < 0.05$).

Infants receiving the SQ-LNS with EFAs and LCPUFAs (SQ-LNS-plus), had significantly higher plasma DHA (4.5 [4.3-4.9]) at 12 months than the control infants (3.8 [3.6-4.0]), with an effect size [β (95% CI)] of 0.819 (0.480, 1.158) (adjusted for age and sex). The effect of higher plasma DHA remained significant even after additional adjustment for continued breastfeeding at 12 months ($\beta = 0.823$; 95% CI= 0.507, 1.139), and for baseline DHA ($\beta = 0.714$, 95% CI= 0.305, 1.123). In addition, the infants receiving the SQ-LNS with EFAs and LCPUFAs had lower plasma LA (21.8 [21.1 - 22.4]) compared with their control counterparts (22.6 [22.0-23.3]) ($\beta = -0.812$; 95% CI= -1.592, -0.033). This effect remained significant when additionally adjusting for continued breastfeeding at 12 months ($\beta = -0.808$, 95% CI= -1.595, -0.021), but was no longer significant when further adjusting for baseline plasma LA ($p = 0.161$).

There was no effect of either of the two SQ-LNS on plasma AA. Consequently, infants receiving the SQ-LNS with EFAs and LCPUFAs had a significantly lower plasma n-6 to n-3 PUFA ratio at 12 months of age ($\beta = -1.039$; 95% CI= -1.565, -0.513). The effect of the SQ-LNS on the other FA profiles was consistent even when the models were additionally adjusted for continued breastfeeding at 12 months and for respective baseline FA status.

In all models, breastfeeding at 12 months was a significant predictor of plasma FA status at 12 months ($p < 0.05$). The overall estimated mean adherence to the intervention (proportion of days when the supplements were consumed) was 94.1% and 94.4% in the SQ-LNS and SQ-LNS-plus respectively. Based on the reported average weekly consumption, most of the infants in the SQ-LNS (78.8%) and SQ-LNS-plus (78.2%) groups consumed all of the weekly supply of the supplements in ($p > 0.05$).

Table 4-3: Fatty acid composition at baseline (six months) and endpoint (12 months) by randomisation

FAs AVAILABLE	SQ-LNS n	SQ-LNS-plus n	Control n	p^1	p^2	p^3
At baseline (BL)	120	124	109			
At endpoint (EP)	82	94	117			
At both BL and EP	40	53	55			
LA						
Baseline	21.32 (20.08 - 21.32) ^a	21.32 (20.08 - 21.32)	21.32 (20.49 - 21.32)	0.028	0.035	0.161
Endpoint	22.87 (22.19 - 23.57)	21.75 (21.11 - 22.42) **	22.64 (21.97 - 23.33)			
ALA						
Baseline	0.12 (0.09 - 0.12)	0.11 (0.09 - 0.11)	0.12 (0.09 - 0.12)	0.528	0.225	0.925
Endpoint	0.12 (0.11 - 0.14)	0.12 (0.10 - 0.13)	0.12 (0.11 - 0.13)			
AA						
Baseline	11.47 (10.59 - 11.47)	11.47 (10.59 - 11.47)	11.35 (10.27 - 11.35)	0.822	0.673	0.611
Endpoint	11.13 (10.69 - 11.58)	11.35 (11.02 - 11.70)	11.24 (10.91 - 11.58)			
EPA						
Baseline	0.27 (0.26 - 0.27)	0.26 (0.22 - 0.26)	0.25 (0.21 - 0.25)	0.087	0.109	0.698
Endpoint	0.28 (0.26 - 0.30)	0.31 (0.28 - 0.34)	0.28 (0.26 - 0.30)			
DHA						
Baseline	4.30 (3.89 - 4.30)	4.05 (3.59 - 4.05)	3.89 (3.45 - 3.89)	0.000	0.000	0.000
Endpoint	3.74 (3.49 - 4.01)	4.52 (4.30 - 4.85) **	3.78 (3.59 - 3.97)			
n6: n3 PUFA						
Baseline	6.48 (5.98 - 6.48)	6.82 (6.29 - 6.82)	6.95 (6.29 - 6.95)	0.000	0.000	0.000
Endpoint	7.46 (7.02 - 7.92)	6.23 (5.87 - 6.61) **	7.24 (6.95 - 7.53)			
n6: n3 LCPUFA						
Baseline*	2.80 (2.58 - 2.80)	2.97 (2.74 - 2.97)	3.00 (2.77 - 3.00)	0.000	0.000	0.001
Endpoint	3.06 (2.88 - 3.25)	2.61 (2.50 - 2.71) **	3.03 (2.91 - 3.15)			
LA: ALA						
Baseline	174.16 (142.59 - 174.16)	181.27 (148.41 - 181.27)	170.71 (137.00 - 170.71)	0.770	0.449	0.810
Endpoint	177.68 (160.77 - 196.36)	183.09 (170.71 - 204.38)	186.76 (170.71 - 204.38)			
DHA: Osbond						
Baseline*	6.23 (5.31 - 6.23)	5.58 (4.66 - 5.58)	5.25 (4.57 - 5.25)	0.000	0.000	0.000
Endpoint	5.31 (4.75 - 5.92)	7.53 (6.82 - 8.33) **	5.20 (4.75 - 5.69)			
EPA: DHA						
Baseline	0.06 (0.05 - 0.06)	0.06 (0.05 - 0.06)	0.06 (0.05 - 0.06)	0.546	0.451	0.313
Endpoint	0.07 (0.06 - 0.08)	0.06 (0.06 - 0.08)	0.07 (0.06 - 0.08)			
Essential PUFA index ⁴						
Baseline	3.81 (3.52 - 3.81)	3.74 (3.45 - 3.74)	3.63 (3.42 - 3.63)	0.769	0.840	0.936
Endpoint	3.93 (3.78 - 4.09)	3.85 (3.66 - 4.05)	3.89 (3.74 - 4.05)			
Omega 3 index ⁵						
Baseline	4.61 (4.17 - 4.61)	4.34 (3.93 - 4.34)	4.22 (3.74 - 4.22)	0.000	0.000	0.001
Endpoint	4.05 (3.78 - 4.34)	4.95 (4.66 - 5.25) **	4.13 (3.93 - 4.34)			

FAs – Fatty acids SQ-LNS–Small-quantity lipid-based nutrient supplement; FA- Fatty acid; BL- Baseline; EP- Endpoint LA - Linoleic Acid; ALA- α- Linoleic Acid; AA- Arachidonic acid; EPA- Eicosapentaenoic Acid; DHA- Docosahexaenoic acid; PUFA- Polyunsaturated fatty acids; LCPUFA- Long-chain polyunsaturated fatty acids

^aFAs were log transformed before all analysis and values are presented as geometric means and 95% CI (all such values). The values provided at BL and EP are representing all children with FA data available at six months and all children with FA data available at 12 months

*Significant differences between three groups at BL

**Significant differences between SQ-LNS-plus and control group at EP

¹Differences in endpoint FA status between the groups determined by using a general linear model adjusting for age and sex.

²Differences in endpoint FA status between the groups determined by using a general linear model adjusting for age, sex, and continued breastfeeding at 12 months
Continued breastfeeding until 12 months was a significant predictor of fatty acid status at endpoint

³Differences in endpoint FA status between the groups determined by using a general linear model adjusting for age, sex and BL FA status

⁴Ratio between all essential PUFAs (the sum of all n-3 and n-6 FAs) and all non-essential unsaturated FAs (the sum of all n-7 and n-9 FAs)

⁵Sum of EPA and DHA

A significant interaction was observed between the SQ-LNS-plus and breastfeeding at 12 months for plasma DHA ($p = 0.042$), but not AA ($p = 0.151$). Thus, we determined separately the effects of SQ-LNS on plasma DHA in infants who continued to be breastfed at 12 months and those who no longer received breast milk (

Figure 4-3). Adjusting for sex and age, we found a significant effect of SQ-LNS-plus for higher plasma DHA at 12 months compared with the control group in both, the infants who were still breastfeeding ($p = 0.006$) and those who no longer received breast milk ($p < 0.001$). The effect size was $\beta = 0.544$ (95% CI= 0.179, 0.909) in infants who were still breastfeeding and $\beta = 1.148$ (95% CI= 0.597, 1.699) in infants who no longer received breast milk. Infants who continued to breastfeed at 12 months, had significantly higher plasma DHA than those who were no longer receiving breast milk within their respective groups ($p < 0.001$).

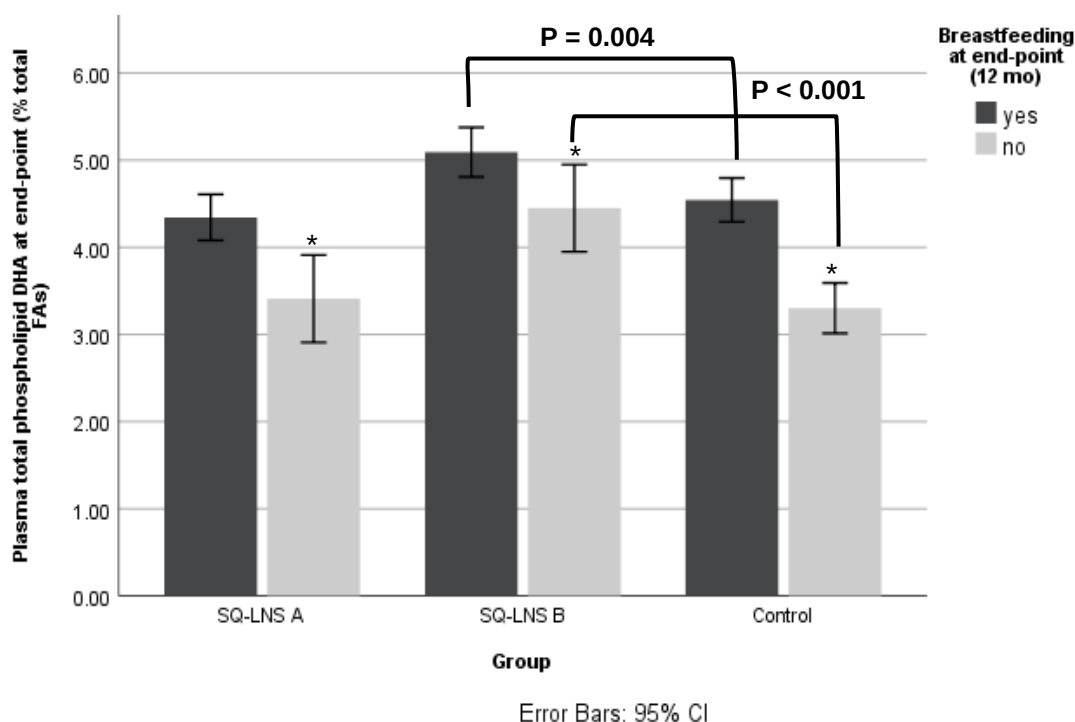


Figure 4-3: Geometric means (95% CI) plasma total phospholipid DHA at end-point (12 months) by randomisation groups for all infants with FA data available who no longer received breast milk and who continued being breastfed at 12 months. Number (n) of infants breastfed at 12 months: SQ-LNS = 48 SQ-LNS-plus = 52 Control = 63. Number of infants (n) no longer receiving breast milk: SQ-LNS = 31 SQ-LNS-plus = 42 Control = 52. *Indicates significant differences between breastfed and non-breastfed infants within groups ($p = 0.000$). General linear model and dependent t-test used to determine differences. DHA - Docosahexaenoic acid

DISCUSSION

In this randomised controlled trial in six-month-old South African infants, the provision of a SQ-LNS containing EFAs and the LCPUFAs DHA and AA resulted in higher plasma total phospholipid DHA at 12 months compared with control infants, with no effect on plasma total phospholipid AA. Thus, the SQ-LNS containing EFAs and LCPUFAs led to a significantly lower plasma n-6 to n-3 PUFA ratio at 12 months. Those infants, particularly, who were no longer breastfed during the complementary feeding period benefited from the LCPUFA-enriched SQ-LNS.

Our study suggests that the provision of an SQ-LNS containing EFAs and LCPUFAs is efficacious in improving plasma DHA status, without changing plasma AA. This beneficial effect remained significant even after adjusting for continued breastfeeding at 12 months and baseline DHA. This is in agreement with studies that were done in high-income settings, showing that other LCPUFA-enriched formulas or dietary products can effectively improve the LCPUFA levels in infant plasma and tissue lipids during the complementary feeding period (Makrides *et al.*, 2002; Hoffman *et al.*, 2003; Hoffman *et al.*, 2004). However, while the LCPUFA-enriched SQ-LNS resulted in higher plasma DHA status in infants who were still breastfeeding at 12 months, the SQ-LNS was of particular benefit to those infants who were no longer receiving breast milk, with a stronger effect size. In non-breastfed infants the LCPUFA-enriched SQ-LNS resulted in these children having a DHA status similar to that of breastfed infants in the control group. Thus, it is important to take into consideration that optimal complementary foods introduced during this period should make up for the decreased supply of LCPUFAs from breast milk (Grote *et al.*, 2016).

On the other hand, plasma total phospholipid AA was not affected by the LCPUFA-enriched SQ-LNS even though it contained EFAs and both DHA and AA. At six months of age, breastfeeding was associated with higher plasma total phospholipid DHA and AA, but only 66% of infants were still breastfed at this time point. We previously assessed the breast milk FA composition in this study population (Siziba *et al.*, chapter 3) and found a median [interquartile range] breast milk DHA content of 0.23 [0.19-0.30] %, which is considerably lower than the worldwide mean content of $0.32 \pm 0.22\%$ reported in a meta-analysis by Brenna *et al.* (2007). On the other hand, we also found a breast milk AA content of 0.76 [0.66-0.87], which is higher than the reported $0.47 \pm 0.13\%$ in the same meta-analysis. Therefore, we speculate that it is possible that the higher AA status in the infants at baseline was caused by the higher AA content found in breast milk. Thus, there was likely no change in AA after consumption of the SQ-LNS-plus as plasma AA levels were already high.

However, even though the SQ-LNS did not affect plasma AA, Brenna (2016) proposes that there is insufficient evidence for excluding AA from the diets of infants who are consuming DHA. Also,

several trials have shown the importance of adding AA to the diets of infants even for DHA-dominated outcomes (Brenna, 2016). Moreover, there have been no trials that have evaluated the effects of AA in the absence of DHA. Thus, the potential health benefits of supplementation with both DHA and AA cannot be attributed to DHA alone. It is logical, therefore, to ascribe these benefits to the variables that are used in most of these studies, particularly the combination of both DHA and AA (Hadley *et al.*, 2016). This is because the combination of DHA and AA has shown clear benefits for cognitive development, visual function, as well as blood pressure, even beyond the period of supplementation into early childhood (Forsyth *et al.*, 2003; Colombo *et al.*, 2011). Consequently, not only should complementary foods be introduced at the age of six months, but a continued supply of DHA and AA should be maintained during this period of critical growth and development (Forsyth *et al.*, 2017). Considering the low continued breastfeeding rate and the relatively low levels of breast milk DHA, there is a potential need to introduce LCPUFA-rich complementary food or LCPUFA-enriched supplements, i.e. in the form of a SQ-LNS, in this setting.

The SQ-LNS which contained only the EFAs LA and ALA did not affect plasma phospholipid DHA and AA in infants in this study. We attribute this to the limited conversion processes of DHA and AA from their precursor EFAs ALA and LA respectively by a series of desaturation and elongation processes (Innis, 2014). Also, the n-6 FA LA competes with ALA, an n-3 FA, for the same elongation and desaturation pathway. Therefore, the conversion of EPA to DHA is decreased by high dietary intake of LA (Gibson *et al.*, 2013). High plasma total phospholipid LA is a characteristic of the infants included in this study (Siziba *et al.*, chapter 3). In addition, of the many studies to date, none has found an equivalent DHA and AA status in growing infants fed the 18-carbon EFAs compared with infants fed LCPUFAs, and this is particularly true for DHA (Carlson & Colombo, 2016). Also, the LNSs that have been used in the past were designed to provide in addition to other micronutrients, energy, protein, macro-minerals and EFAs but not LCPUFAs (Hess *et al.*, 2015). Thus, to our knowledge, this is the first study of its kind to have the LCPUFAs DHA and AA as additional ingredients in the SQ-LNS. We also previously reported a transient effect of SQ-LNS-plus on growth and a positive effect on the locomotor outcome in psychomotor development (Smuts *et al.*, 2018). Hence, the enrichment of LNS with LCPUFAs may improve DHA status, as well as yield other health benefits as previously shown in similar settings. Nonetheless, further research is needed to evaluate different formulations of SQ-LNS, possibly with higher amounts of ALA and reduced ratios of LA:ALA, which could be beneficial in further examining the potential of the SQ-LNS to improve both EFA and DHA status.

A strength of this study is the analysis of plasma FA as a marker of LCPUFA intake and status as opposed to erythrocytes and other lipid fractions. The analysis of plasma phospholipids has greater within-individual variability (Harris & Thomas, 2010). Also, plasma FAs are a more

sensitive marker of recent dietary intake in comparison with the erythrocytes (Harris *et al.*, 2013); the plasma phospholipid fraction is therefore preferred if rapid changes are of interest (Cao *et al.*, 2006; Harris *et al.*, 2007). On the other hand, a limitation of this study is that blood was successfully drawn from only 68.1% (350/514) of the infants at 12 months who completed the study, and only 57% (293/514) had an extra plasma aliquot available for plasma FA analysis. This led to a reduced sample size for the analyses done in this study.

In conclusion, our study suggests that continued breastfeeding and consumption of LCPUFA-enriched SQ-LNS from six to 12 months of age can result in higher plasma DHA status of infants at 12 months of age; suggesting that infants who are no longer breastfeeding can benefit most from LCPUFA-enriched SQ-LNS. Therefore, the provision of SQ-LNS containing LCPUFAs during the complementary feeding period is a potential strategy to ensure adequate intakes of LCPUFAs, particularly of DHA.

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CHAPTER 5

Breast milk and erythrocyte fatty acid composition of lactating women residing in a peri-urban South African township

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ABSTRACT

Introduction: Adequate intake of essential fatty acids (EFAs) and long-chain polyunsaturated fatty acids (LCPUFAs) in lactating women is important to ensure adequate transfer of EFAs and LCPUFAs to breast milk for their developing infants. Data on breast milk fatty acid (FA) composition in South African lactating women in relation to their FA status, as well as on potential compositional changes within-feed, are limited.

Objective: The aim of this study was to assess the FA composition of breast milk sampled at three time points within-feed, and to determine associations with red blood cell (RBC) FA status in lactating South African mothers of 2-4-month-old breastfed infants.

Method: FA composition (% total FAs) was measure using gas chromatography-mass spectrometry in RBC total phospholipids, as well as fore-, mid-feed and hind-milk samples of lactating mothers (n=100) of 2-4-month-old infants living in a peri-urban township

Results: The geometric mean (95% CI) breast milk DHA and AA were 0.25 (0.24, 3.71) and 0.81 (0.79, 0.83) % overall, respectively. The saturated FA and MUFA contents were 37.7 (37.3, 38.1) and 28.5 (27.9, 28.8) % overall, respectively. The maternal RBC DHA and AA contents were 3.8 (3.6, 4.0) and 15.4 (14.8, 16.1) % respectively. Breast milk ALA levels were higher in mid-feed ($p = 0.017$) and hind-milk ($p = 0.003$) than in fore-milk. In addition, DHA was higher in mid-feed ($p = 0.050$) and hind-milk ($p = 0.003$) than in fore-milk. AA levels remained constant within-feed. Breast milk DHA was positively correlated with maternal RBC DHA while AA was not. Fish consumption was positively associated with higher RBC EPA composition.

Conclusion: Our results in South African lactating mothers indicate that breast milk EFA and LCPUFA composition may change within-feed, with an increase in ALA and DHA within a breastfeeding session, while LA and AA remained unchanged. Furthermore, breast milk DHA was associated with maternal RBC status, while breast milk AA did not. Thus, our results suggest that DHA is preferentially transferred to breast milk within-feed through biomagnification.

INTRODUCTION

Breast milk is an ideal source of nutrients for the new born infant. Its unique nutritional composition and non-nutritive bioactive components have been associated with favourable developmental outcomes and survival in infants fed breast milk (Ofstedal, 2012). Breast milk comprises fats/lipids (45%-55%) which provide energy amongst other nutrients. Furthermore, breast milk contains a wide variety of lipid components, particularly fatty acids (FAs), many of which are present in very low concentrations while others are dominating (Delplanque *et al.*, 2015). For instance, breast milk contains predominantly more saturated FAs than monounsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs). Additionally, with regards to the essential FAs (EFAs), breast milk linoleic acid (LA; 18:2n-6) concentrations are higher than α -linolenic acid (ALA; 18:3n-3). On the other hand, arachidonic acid (AA; 20:4n-6) is higher than docosahexaenoic acid (DHA; 22:6n-3) (Koletzko, 2016).

The lipid content in breast milk is more variable within-feed than other micronutrients. Notably, hind-milk may contain two or three times the concentrations of fat found in fore-milk (Saarela *et al.*, 2005). Previously, total lipids were shown to be higher in hind-milk than in fore-milk during the first three months (Marangoni *et al.*, 2000). Only one study (da Cunha *et al.*, 2005) reported higher levels of ALA and DHA in hind-milk than in fore-milk, which was in contrast to what has been reported by other older studies (Daly *et al.*, 1993; Gibson & Kneebone, 1980; Hall, 1979). However, the observed higher fat content in hind-milk than fore-milk could be of biological benefit to the infant. During a feed, infants may initially get breast milk that is higher in essential water-soluble micronutrients, while those infants who are hungrier and drink more would obtain an increasingly higher fat content to satisfy their caloric needs (Keating *et al.*, 2013).

FAs in breast milk are derived from mobilisation of endogenous maternal FA stores, synthesis of FAs by the liver or breast tissue, and from the diet (Demmelmair & Koletzko, 2017). Notably, the amounts of EFAs found in breast milk were shown to vary depending on the mother's access to different vegetable oils. Consequently, an increased intake of vegetable oils or plant-derived EFAs may result in higher levels of n-6 and n-3 EFAs (Lee *et al.*, 2013). Furthermore, DHA supplementation in lactating women has been shown to influence DHA concentrations in breast milk (Kim *et al.*, 2017). In contrast, the slow turn-over maternal body pools of AA have been shown to be the major source of AA in breast milk (Szabo *et al.*, 2010). Furthermore, Lauritzen and Carlson (2011), concurred that the DHA levels in both maternal blood and breast milk correlate with each other, while no correlation was noted for AA.

In their meta-analysis, Brenna *et al.*, (2007) reported mean breast milk AA and DHA concentrations of $0.47 \pm 0.13\%$ and $0.32 \pm 0.22\%$ respectively (% of total FAs) respectively. Compared with other low-income and middle-income countries, South Africa was among the countries with the lowest reported DHA and AA breast milk concentrations (Van der Westhuyzen *et al.*, 1988). However, the available data on breast milk FA composition in South African women is old and no recent data are available, hence the purpose of the current study.

Moreover, researchers suggest that the beneficial effects of breastfeeding in infants involve the role of the LCPUFAs DHA and AA, particularly for cognitive and intellectual development during infancy and beyond (Colombo *et al.*, 2013). Thus, breast milk supplies the infant with the much-needed LCPUFAs for growth and development. To our knowledge, there are no recent data available on the FA composition of breast milk in South African lactating women. As breast milk composition is dynamic and not only varies between mothers and populations but also within-feed (Ballard & Morrow, 2013), we assessed the FA composition of breast milk sampled at three time points within-feed and determined associations with red blood cell (RBC) FA status in lactating mothers of 2-4-month-old breastfed infants living in a township in the North West province of South Africa.

METHODS

Participants

This cross-sectional study included a convenient sample of 100 lactating women (age ≥ 18 years old) and their infants (aged 2-4 months) residing in two peri-urban settlements (Ikageng and Promosa) on the fringes of Potchefstroom in the North West province of South Africa. However, for the purpose of this study, we included only information regarding the lactating women. The majority of residents in these two townships are of Black African descent with high rates of unemployment and low socio-economic status. Recruitment of subjects was done by trained fieldworkers at local health clinics in Ikageng and Promosa. Lactating women were included in the study if they were: 1) generally healthy with no known history of thyroid disease (relevant to other outcome measures); 2) currently breastfeeding; and 3) not using any iodine-, iron- or n-3 fatty acid-containing supplements.

Data and biological sample collection

Lactating mothers (and their infants) were invited to the metabolic clinic at the Centre of Excellence for Nutrition at the North West University, South Africa, where the study procedures were conducted between 08:00 a.m. and 12:00 noon. Upon arrival at the study site, they were provided with detailed information about the study in their home language (Afrikaans, English or

Setswana) by a trained fieldworker; and they signed informed consent in the presence of a fieldworker. A standard breakfast was also served to the lactating mothers at arrival and before collection of all biological samples and other information. A detailed questionnaire was used to collect information on socio-economic characteristics, consumption of LCPUFA containing foods, use of supplements (currently and during pregnancy), breastfeeding practices and other information described previously (Osei *et al.*, 2016). Breastfeeding practices were divided into three categories; 1) Exclusive breastfeeding (EBF); 2) Predominant breastfeeding; 3) Partial breastfeeding (WHO, 2010).

Height and weight of the women were measured using standard anthropometric techniques (Faber & Benade, 2007). Mothers were asked to remove their shoes, empty their pockets and wear minimal clothing when all measurements were being taken. Height was measured using a rigid stadiometer and recorded to the nearest 0.5 cm. Weight was measured on a high capacity electronic flat scale (Seca 813; Germany) and recorded to the nearest 0.1 kg. Height and weight were used to calculate BMI, and categories were determined according to the WHO BMI classification standards.

Breast milk samples (ca. 5 ml of fore, mid-feed and hind-milk), were obtained by manual expression following a sample collection process adapted from Neville *et al.* (1984) as described previously by Dold *et al.* (2016). Prior to obtaining a fore-milk sample, each mother was asked to clean her breast with a wet cloth; after which they expressed a breast milk sample before breastfeeding. The infant was then put to the breast to suckle for 2-2.5 minutes after let-down, judged as the time when the baby began to swallow actively. Another 5 ml sample was then collected to obtain the mid-feed sample, after-which the infant was let to suckle on the breast until satisfied. The hind-milk sample was collected after the feed. For the interpretation of results, these three categories were used during analysis (fore-, mid-feed and hind-milk). To control for the natural changes that occur in breast milk, the mothers who were included in the study had similar habitual diets as they were residing in the same area. Breast milk samples were aliquoted and stored at -80 °C until analysis.

A 5 ml sample of venous blood was drawn from the mothers into EDTA-coated evacuated tubes (Becton Dickinson). Separation of red blood cells and plasma was done by centrifuging the blood immediately (500 x g for 15 min at room temperature), after which the plasma was aliquoted. For FA analysis, red blood cells were washed twice with 0.15 molNaCl/L and centrifuged at 500 x g for 10 min to remove the buffy coat.

Fatty acid analysis

FA analysis in red blood cells was carried out at the Centre of Excellence for Nutrition, at the North West University (NWU) using Gas Chromatography–Mass Spectrometry (GC-MS) and the methodology was adapted from the method described by (Baumgartner *et al.*, 2012).

Fatty acid extraction from red blood cells

Lipids were extracted from RBC samples with chloroform: methanol (2:1 vol: vol) by using a modification of the method of Folch *et al.* (1957). Lipid extracts were concentrated using nitrogen gas, and thin-layer chromatography (silica gel 60 plates without fluorescent indicator, 10x20 cm; Merck) was used to separate neutral lipids from the phospholipids. Phospholipids were eluted using petroleum ether: diethyl ether: acetic acid (90:30:1, vol: vol: vol) and a pinch of 2,5-Bis(5-tert-butyl-benzoxazol-2-yl)thiophene. The lipid fraction containing the phospholipids was removed from the thin-layer chromatography plate and trans-methylated with methanol: sulphuric acid (95:5, vol: vol) at 70°C for 2 hours. This led to the formation of fatty acid methyl esters (FAMES). The FAMES were extracted using hexane and water. The organic layer was aspirated, evaporated, redissolved in hexane, and analysed by gas chromatography–electron ionisation mass spectrometry. All solvents used during the extraction procedure contained 0.01% butylated hydroxytoluene.

Fatty acid extraction from breast milk samples

FAs from breast milk samples were derivatised and extracted using a modification of the methods described by Liu *et al.* (2014) and Folch *et al.* (1957). Aliquots of breast milk samples were subjected to direct trans-methylation by incubation with methanol: sulphuric acid (95:5; vol:vol) at 70°C for 3 hours. Samples were cooled to room temperature, after which the derivatised lipids were extracted with methanol, hexane and water, followed by centrifugation at 1200 × g for 5 minutes. The top phase containing lipid and fatty acid methyl esters (FAMES), was aspirated and evaporated to dryness using nitrogen gas. Samples were redissolved in chloroform: methanol: saline (86:14:1, vol: vol: vol) and subjected to thin-layer chromatography (silica gel 60 plates without fluorescent indicator, 10x20cm; Merck) using petroleum ether: diethyl ether: acetic acid (90:30:1, vol: vol: vol) and a pinch of 2,5-Bis(5-tert-butyl-benzoxazol-2-yl)thiophene as the mobile phase. The separated FAMES fraction was visualised under UV light and removed from the thin-layer chromatography plate. An extraction with methanol: sulphuric acid (95:5, vol: vol), hexane and water followed, after which the top hexane phase containing the FAMES was aspirated and evaporated to dryness under nitrogen gas. The FAMES were redissolved in a small volume of hexane and analysed by gas chromatography-electron ionisation mass spectrometry. All solvents used during the extraction procedure contained 0.01% butylated hydroxytoluene.

Analysis by gas chromatography mass spectrometry

Samples were analysed on an Agilent Technologies 7000 GC/MS Triple Quad system comprising an Agilent 7890A gas chromatograph equipped with an Agilent G7001B triple quad mass spectrometer (Agilent Technologies). The gas chromatography separation of FAMES was carried out on an HP-88 capillary column (100 m x 0.25 mm x 0.20 μ m; Agilent Technologies) by using helium as the carrier gas at a flow rate of 2.2 mL/min. Initial inlet temperature was held at 70 °C for 0.02 minutes, after which it was ramped to 270 °C at 500 °C/min. The mass spectrometer source was maintained at a temperature of 230 °C. A sample volume of 1 μ L was injected, and split ratios of 80:1 for RBC samples and 10:1 for breast milk samples were used. The oven temperature was maintained at 50 °C for 1 minute, then ramped to 170 °C at 30 °C/min, then from 170 °C to 215 °C at 2 °C/min, after which it was ramped to 230 °C at 4 °C/min. The temperature was then held isothermally at 230 °C for 7 minutes. The total analysis time was 38.25 minutes. Mass spectrometry with 70 eV electron-ionisation was carried out in multiple-reaction monitoring mode, with at least two transitions per compound. The FAMES were quantified using MassHunter Quantitative Analysis software (Version B.05.02, Agilent Technologies). FAME peaks were identified and calibrated against a standard reference mixture of 33 FAMES (Nu-Check-Prep) and two single FAME standards (Larodan Fine Chemicals AB). Relative percentages of FAs were calculated by expressing the concentration of a given FAME as a percentage of the total concentration of all FAMES identified in the sample (Baumgartner *et al.*, 2012).

Statistical methods

Visual inspection of histogram plots and the Kolmogorov–Smirnov test were used to check data for normal distribution. Normally distributed variables were described using the arithmetic mean and standard deviation, non-normally distributed variables were log transformed and described by the geometric mean and geometric 95% confidence interval (CI). Categorical variables were reported as frequencies and percentages. A generalised linear model based repeated measures analysis was used to evaluate differences in individual FAs between fore-, mid-feed and hind-milk. Furthermore, a generalised linear model was used to evaluate the relation between maternal RBCs (independent variables) and overall breast milk FAs by tertiles (dependent variables). The analysis of the residuals was used to check for linear model assumptions. Additionally, a non-linearity test was performed using a Wald test applied to the coefficient of the quadratic component. In case of non-linearity, log transformation was used, and residual analysis was repeated. Results from the linear model analyses were presented as slope estimates and 95% CI. Exponential values were calculated and presented for log transformed variables. The p-value for trend was calculated interpolating the tertiles. The relation between maternal RBCs and breast milk FAs was then analysed separately with each breast milk fraction. Partial correlations between

selected individual maternal RBC FAs and FAs in each breast milk fraction, were reported. All models were adjusted for maternal age, BMI, Hb status, HIV status and number of children. All statistical tests were two-tailed, and type-I error rate was set to 5%. Statistical analyses were performed using SAS® (version 9.4; SAS Institute, Inc, Cary, NC) and IBM SPSS Statistics (version 25; IBM Co).

Ethical considerations

The study was carried out in agreement with the guidelines stipulated by the Declaration of Helsinki involving human subjects. The study was approved by the Health Research Ethics Committee of the NWU (NWU-00016-13-A1). The North West provincial and district health departments also reviewed the study protocol and granted permission to recruit mother (-infant) pairs for the study at the local health clinics. Written informed consent was obtained from all participating women before any study-specific procedures were done.

RESULTS

A total of 100 lactating women were included in the study whose characteristics are shown in Table 5-1. The women were aged 27.8 ± 6.8 years. More than a third (31%) of the women were overweight and 27% were obese. A total of 67 women (67%) practised exclusive breastfeeding at the time of the study.

Table 5-1: Characteristics of lactating women:

CHARACTERISTICS		%	Mean $\pm$ SD
Lactating women (n=100)			
Age (years)			27.8 $\pm$ 6.8
Number of children			2.2 $\pm$ 1.2
HIV-positive		22	
Smoking or snuff	At the time of study	10	
	Before pregnancy	8	
	Primary	5	
Education	Secondary	90	
	Tertiary	4	
	Other (University, Diploma etc)	1	
Employed	Yes	11	
	No	89	
Mode of delivery	Vaginal	79	
	Caesarean	21	
Height (m)			1.6 $\pm$ 0.1
Weight (kg)			66.5 $\pm$ 14.7
BMI	Underweight (BMI < 18.5)	3	
	Normal weight (18.5 $\leq$ BMI $\leq$ 24.9)	39	
	Overweight (25.0 $\geq$ BMI $\leq$ 29.9)	31	
	Obese (BMI $\geq$ 30)	27	
Breastfeeding practice	Exclusive	67	
	Predominant	9	
	Partial	24	

BAZ: body mass index-for-age z-scores, HIV: human immunodeficiency virus, BMI: body mass index.

Predominant breastfeeding: the infant's predominant source of nourishment has been breast milk (including milk expressed or from a wet nurse as the predominant source of nourishment). However, the infant may also have received liquids (water and water-based drinks, fruit juice) ritual fluids and ORS, drops or syrups (vitamins, minerals and medicines).

Partial or mixed feeding: the infant is given some breast feeds and some artificial feeds, either milk or cereal, or other food or water

Of the 100 women enrolled in the study, RBC, fore-, mid-feed and hind-milk samples were available for FA analysis from 98, 80, 85 and 74 women, respectively (Table 5-2). The geometric mean (95%CI) RBC ALA and LA contents (% total FAs) in all women were 0.1 (0.1, 0.1) and 9.9 (9.7, 10.3) % respectively. The maternal RBC DHA and AA contents were 3.8 (3.6, 4.0) and 15.4 (14.8, 16.1) %, respectively. The ratio of DHA to osbond acid was 3.5 (3.3, 3.8) %. The women in this study had an essential PUFA status index and a plasma omega-3 index of 2.3 (2.3, 2.4) and 4.2 (4.0, 4.4) % respectively. None of the women's characteristics and eating habits were associated with individual FAs in breast milk and RBCs (data not shown) with the exception of EPA. Women who reported to consumed fish often (n = 3) had a significantly higher EPA composition (0.69 [1.8, 0.9]) compared to those who reported to never (n = 14; 0.36 [0.3, 0.4]; p = 0.023), rarely (n =19; 0.33 [0.29, 0.37]; p = 0.005) or sometimes (n = 56; 0.37 [0.34, 0.41]; p = 0.017) eat fish.

Palmitic acid (C16:0) was the predominant saturated FA in breast milk and RBCs. Analysis of within-feed differences indicated that saturated FAs were higher in mid-feed ($p = 0.003$) and hind-milk ($p = 0.002$) than in fore-milk (Figure 5-1A).

Oleic acid (C18:1n-9) was the predominant MUFA in breast milk, while palmitoleate (C16:1n-7) was the predominant MUFA in RBCs. Total MUFAs were significantly higher in fore-milk than in mid-feed ($p < 0.001$) and hind-milk ($p = 0.001$, Figure 5-1B).

Of the n-3 PUFAs, ALA levels were higher in mid-feed ($p = 0.017$) and hind-milk ($p = 0.003$) than in fore-milk (Figure 5-1C). In addition, DHA was higher in mid-feed ($p = 0.50$) and hind-milk ($p = 0.003$) than in fore-milk (Figure 5-1D).

Adrenic acid was higher in fore-milk than in the mid-feed ($p = 0.00$), and the only n-6 FA for which there was a significant difference within-feed (Figure 5-1G).

Table 5-2: Red blood cell total phospholipids and fore-, mid-feed and hind-milk fatty acid compositions in South African lactating women

FATTY ACID		MATERNAL RBC (n=98)	FORE-MILK (n=80)	MID-FEED (n=85)	HIND-MILK (n=74)
C14:0	Myristate	0.23 (0.20, 0.26) ¹	10.13 (9.50, 10.79)	10.49 (9.85, 11.17)	10.70 (10.04, 11.42)
C16:0	Palmitate	20.6 (20.03, 21.23)	20.38 (19.88, 20.90)	20.07 (19.59, 20.56)	19.95 (19.44, 20.48)
C18:0	Stearate	15.9 (15.6, 16.3)	6.51 (6.21, 6.82)	6.24 (5.99, 6.50)	6.07 (5.81, 6.35)
C20:0	Eicosanoic	0.36 (0.35, 0.37)	0.15 (0.14, 0.15)	0.13 (0.13, 0.14)	0.14 (0.13, 0.15)
C22:0	Behenic	1.50 (1.45, 1.70)	0.14 (0.13, 0.15)	0.14 (0.13, 0.15)	0.14 (0.13, 0.16)
C24:0	Lignoceric	4.64 (4.50, 4.78)	0.12 (0.11, 0.13)	0.12 (0.11, 0.13)	0.13 (0.11, 0.14)
ΣSFA		43.4 (42.7, 44.2)	46.24 (45.24, 47.26)	46.81 (45.72, 47.92)	46.99 (45.89, 48.11)
C16:1n-7	Palmitoleate	20.6 (20.03, 21.23)	2.88 (2.68, 3.08)	2.92 (2.73, 3.12)	2.95 (2.76, 3.15)
C18:1n-7	Vaccenate	0.96 (0.93, 0.99)	1.57 (1.50, 1.65)	1.56 (1.49, 1.63)	1.56 (1.49, 1.64)
C18:1n-9	Oleate	10.05 (9.82, 10.29)	24.10 (23.43, 24.79)	23.21 (22.55, 23.88)	23.02 (22.34, 23.72)
C20:1n-9	Eicosenoic	0.21 (0.20, 0.21)	0.30 (0.28, 0.31)	0.28 (0.27, 0.29)	0.28 (0.27, 0.30)
C22:1n-9	Erucic	0.10 (0.10, 0.11)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
C24:1n-9	Nervonic	3.57 (3.49, 3.66)	0.07 (0.06, 0.08)	0.07 (0.07, 0.08)	0.08 (0.07, 0.08)
ΣMUFA		15.2 (14.9, 15.5)	29.04 (28.18, 29.92)	28.15 (27.31, 29.01)	28.00 (27.15, 28.88)
C18:2n-6	Linoleate	9.97 (9.66, 10.29)	19.46 (18.62, 20.35)	19.74 (18.94, 20.57)	19.75 (18.93, 20.61)
C18:3n-6	GLA	0.09 (0.09, 0.10)	0.17 (0.15, 0.19)	0.18 (0.16, 0.19)	0.18 (0.16, 0.19)
C20:2n-6	Docosadienoic	0.38 (0.36, 0.39)	0.43 (0.40, 0.46)	0.42 (0.40, 0.45)	0.43 (0.40, 0.46)
C20:3n-6	DGLA	1.63 (1.57, 1.70)	0.62 (0.59, 0.66)	0.62 (0.59, 0.65)	0.63 (0.59, 0.67)
C20:4n-6	AA	15.44 (14.82, 16.07)	0.81 (0.77, 0.85)	0.81 (0.77, 0.85)	0.82 (0.78, 0.85)
C22:4n-6	Adrenic	4.04 (3.91, 4.17)	0.19 (0.07, 0.08)	0.18 (0.017, 0.19)	0.19 (0.18, 0.20)
C22:5n-6	Osbond	1.08 (1.03, 1.13)	0.08 (0.07, 0.08)	0.08 (0.07, 0.08)	0.08 (0.07, 0.08)
Σn-6 PUFA		28.92 (28.24, 29.61)	21.85 (20.96, 22.77)	22.10 (21.26, 22.97)	22.15 (21.29, 23.04)
Σn-6 LCPUFA		22.75 (22.10, 23.42)	2.15 (2.06, 2.25)	2.13 (2.05, 2.22)	2.16 (2.07, 2.26)
C18:3n-3	ALA	0.11 (0.10, 0.11)	0.79 (0.71, 0.89)	0.84 (0.16, 0.19)	0.84 (0.75, 0.94)
C18:4n-3	Stearidonic	0.01 (0.01, 0.01)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
C20:3n-3	Eicosatrienoic	0.6 (0.05, 0.06)	0.04 (0.04, 0.05)	0.04 (0.04, 0.04)	0.04 (0.04, 0.05)
C20:5n-3	EPA	0.37 (0.34, 0.40)	0.06 (0.06, 0.07)	0.07 (0.06, 0.08)	0.07 (0.06, 0.08)
C22:5n-3	DPA	1.87 (1.79, 1.94)	0.14 (0.13, 0.15)	0.15 (0.13, 0.16)	0.15 (0.13, 0.16)
C22:6n-3	DHA	3.81 (3.63, 4.01)	0.24 (0.22, 0.25)	0.26 (0.24, 0.29)	0.27 (0.25, 0.30)
Σn-3 PUFA		6.50 (6.24, 6.76)	1.34 (1.24, 1.46)	1.42 (1.31, 1.54)	1.44 (1.32, 1.57)
Σn-3 LCPUFA		6.35 (6.10, 6.60)	0.50 (0.47, 0.54)	0.54 (0.49, 0.58)	0.55 (0.50, 0.60)
ΣPUFA		39.6 (38.7, 40.5)	23.25 (22.30, 24.25)	23.61 (22.71, 24.53)	23.67 (22.75, 24.63)
n6: n3 PUFA		4.45 (4.28, 4.63)	16.27 (15.17, 17.45)	15.54 (14.42, 16.74)	15.38 (14.21, 16.64)
n6: n3 LCPUFA		3.60 (3.45, 3.77)	4.28 (4.02, 4.56)	3.98 (3.66, 4.31)	3.93 (3.58, 4.31)
MEAD: AA		0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
LA: ALA		10.11 (9.79, 10.43)	24.53 (22.18, 27.13)	23.60 (21.48, 25.93)	23.42 (21.23, 25.84)
DHA: Osbond		3.53 (3.32, 3.76)	3.06 (2.82, 3.32)	3.44 (3.14, 3.77)	3.57 (3.20, 3.99)
EPA: DHA		0.10 (0.09, 0.10)	0.26 (0.24, 0.29)	0.25 (0.23, 0.28)	0.24 (0.22, 0.27)
EPUFA status ²		2.34 (2.28, 2.39)	0.80 (0.75, 0.85)	0.84 (0.80, 0.88)	0.85 (0.80, 0.90)
Omega 3 index ³		4.21 (4.01, 4.41)	0.30 (0.28, 0.33)	0.33 (0.30, 0.37)	0.35 (0.31, 0.38)

FA – Fatty acid; LA - Linoleic Acid; ALA- α- Linoleic Acid; AA- Arachidonic acid; DGLA- Dihomo-γ-linolenic acid; EPA- Eicosapentaenoic Acid; DPA- Docosapentanoic acid; DHA- Docosahexaenoic acid; PUFA- Polyunsaturated Fatty Acids; LCPUFA- Long-chain polyunsaturated fatty acids

¹FAs were log transformed, values presented as Geometric Mean (95% CL), all such values

² Ratio between all essential PUFAs (the sum of all n-3 and n-6 FAs) and all non-essential unsaturated fatty acids (the sum of all n-7 and n-9 FAs)

³ Sum of EPA and DHA

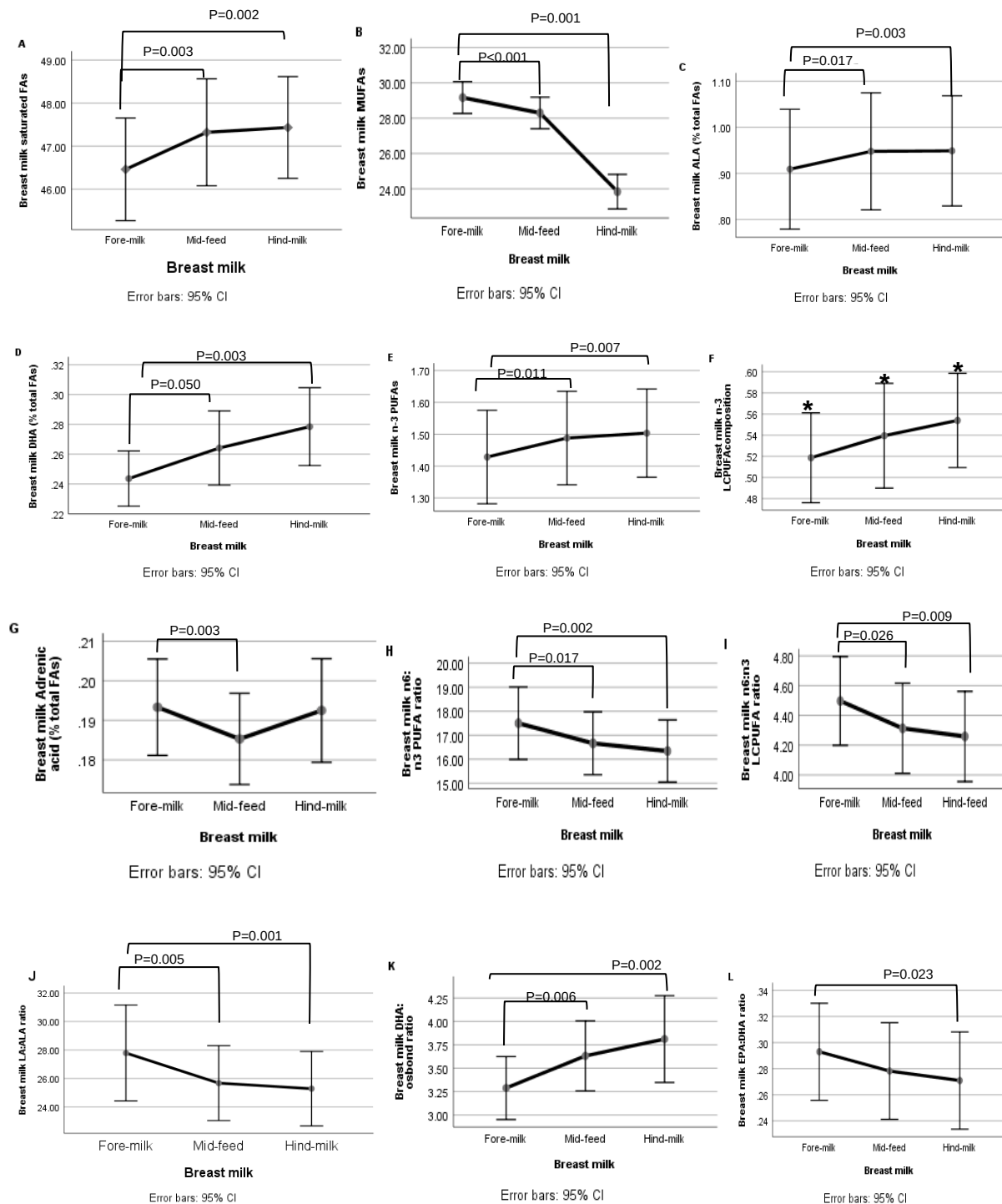


Figure 5-1: Significant within-feed differences of fatty acids (% total FAs) between fore-, mid-feed and hind-milk. GLM repeated measures used to determine differences between fore-, mid-feed and hind-milk samples. Fore-milk: n=80; Mid-feed: n= 85; Hind-milk: n=74. Maternal age, BMI, HIV status, Hb status and number of children were added as covariates. No significant differences were observed in FA contents excluded from the figure. FA- Fatty acids DHA- Docosahexaenoic acid; BM- breast milk. Level of significance, $p<0.05$. * Indicates significant differences between fore-, mid-feed and hind-milk

Table 5-3 shows the results from a linear model analysis of the association between maternal RBC FAs and overall breast milk FAs. The p-values for trends was calculated interpolating the tertiles. Saturated FAs and MUFAs, by tertiles, were positively associated with their respective FA contents in RBCs. Positive associations were observed between maternal RBCs and all n-3 and n-6 PUFAs and in breast milk by tertiles, with the exception of DPA ($p = 0.097$), adrenic acid ($p = 0.844$) and a trend towards significance for AA ($p = 0.060$). While overall breast milk FAs were associated with maternal RBC FA status (Table 5-3), other individual FAs in fore-, mid-feed and hind-milk were not consistently associated with maternal RBC FA status (Table 5-4).

Table 5-3: Linear model analysis of association between maternal red blood cell total phospholipid and overall breast milk fatty acids

PARAMETER	ESTIMATE	95% CI	P
SFA	1.014	0.10, 1.92	0.029
MUFA	0.948	-0.13, 2.03	0.084
PUFA	1.986	0.82, 3.15	0.001
n-9 fatty acids			
C20:3n-9 (Mead)	0.000	0.00, 0.00	0.787
n-3 fatty acids			
C18:3n-3 (ALA)	0.291	0.17, 0.41	<.0001
C20:5n3 (EPA)*	1.501	1.31, 1.72	<.0001
C22:5n3 (DPA)*	1.092	0.98, 1.21	0.097
C22:6n3 (DHA)*	1.216	1.11, 1.33	<.0001
Σn-3 PUFAs*	1.179	1.06, 1.31	0.002
Σn-3 LCPUFAs	0.084	0.01, 0.16	0.035
n-6 fatty acids			
C18:2n6 (LA)	1.908	0.92, 2.90	0.000
C20:4n6 (AA)*	1.051	1.00, 1.11	0.060
C20:3n6 (DGLA)*	1.101	0.93, 1.09	0.003
C22:4n6 (Adrenic)*	1.008	1.12, 1.31	0.844
C22:5n6 (Osbond)*	1.207	0.53, 2.79	<.0001
Σn-6 PUFAs	1.664	0.53, 2.79	0.004
Σn-6 LCPUFAs*	1.132	1.10, 3.02	<.0001
ratios			
n6: n3 PUFA	2.117	0.49, 3.75	0.012
n6: n3 LCPUFA	0.436	0.23, 0.64	<.0001
LA: ALA*	0.913	0.80, 1.03	0.150
DHA: Osbond*	1.397	1.29, 1.51	<.0001
EPUFA status index ¹	0.117	0.06, 0.17	<.0001
Omega 3 index ^{2*}	1.228	1.11, 1.36	0.000

FA – Fatty acid; LA - Linoleic Acid; ALA- α- Linoleic Acid; AA- Arachidonic acid; DGLA- Dihomo-γ-linolenic acid; EPA- Eicosapentaenoic Acid; DPA- Docosapentaenoic acid; DHA- Docosahexaenoic acid; PUFA- Polyunsaturated Fatty Acids; LCPUFA- Long-chain polyunsaturated fatty acids

¹Ratio between all essential PUFAs (the sum of all n-3 and n-6 FAs) and all non-essential unsaturated fatty acids (the sum of all n-7 and n-9 FAs)

²Sum of EPA and DHA

*Maternal RBC values were log transformed and exponential values were calculated and presented

P value for trend was calculated on tertiles and represents the trend for linearity

Maternal RBC total PUFAs were positively associated with total PUFAs in fore-milk ($\beta = 0.425$, $p < 0.001$) and mid-feed milk ($\beta = 0.308$, $p = 0.008$), but the association was not significant in hind-milk ($\beta = 0.228$, $p = 0.059$) (Table 5-4).

Of the n-3 PUFAs, maternal RBC ALA, EPA and DHA positively correlated with their respective FA contents in fore-, mid-feed and hind-milk. The correlations between EPA contents in RBC and breast milk were stronger in hind-milk, while correlations between DHA content in RBC and breast milk were stronger in fore-milk ($\beta = 0.508$) and weaker in mid-feed ($\beta = 0.316$) and hind-milk ($\beta = 0.338$). RBC Total n-3 PUFAs and n-3 LCPUFAs positively correlated with their respective FA

contents in the different breast milk fractions, however, for the n-3 LCPUFA the association was stronger in fore-milk than mid-feed milk and hind-milk.

Of the n-6 PUFAs, maternal RBC DGLA correlated positively with hind-milk DGLA contents ($\beta = 0.247$, $p = 0.044$). Overall total n-6 PUFAs correlated positively with fore-milk ($\beta = 0.393$, $p = 0.001$) and mid-feed ($\beta = 0.298$, $p = 0.010$), but the association was weaker and insignificant with hind-milk ($p = 0.066$). Total n-6 LCPUFAs correlated positively with their respective FA contents in mid-feed but the correlations were insignificant in fore-milk and hind-milk.

Table 5-4: Partial correlations between selected maternal red blood cell total phospholipid, fore-, mid-feed and hind-milk fatty acids

FATTY ACID	PARTIAL CORRELATIONS								
	Fore-milk and M-RBC ² (n=75)			Mid-feed and M-RBC ² (n=80)			Hind-milk and M-RBC ² (n=69)		
	β	95% CI	p	β	95% CI	p	β	95% CI	p
SFA	0.082	-8.3, 16.2	0.522	0.093	-8.6, 19.5	0.443	0.111	-8.2, 20.5	0.399
MUFA	0.145	-4.3, 16.6	0.243	0.229	-0.21, 20.1	0.055	0.191	-2.1, 17.8	0.118
PUFA	0.425	7.5, 25.3	<0.001	0.308	3.3, 21.2	0.008	0.228	-0.4, 17.9	0.059
n-9 fatty acids									
C20:3n-9 (Mead)	0.084	0.00, 0.00	0.468	-0.005	0.00, 0.00	0.966	0.06	0.00, 0.00	0.623
n-3 fatty acids									
C18:3n-3 (ALA)	0.431	0.3, 1.1	<0.001	0.435	0.4, 1.2	<0.001	0.426	0.3, 1.1	0.001
C20:5n3 (EPA)	0.419	0.03, 0.09	<0.001	0.463	0.1, 0.2	<0.001	0.473	0.1, 0.2	<0.001
C22:5n3 (DPA)	0.077	-0.1, 1.1	0.540	0.071	-0.1, 0.1	0.559	0.129	-0.1, 0.1	0.322
C22:6n3 (DHA)	0.508	0.1, 0.2	<0.001	0.316	0.1, 0.3	0.005	0.338	0.1, 0.4	0.005
Σ n-3 PUFA	0.457	0.7, 2.0	<0.001	0.432	0.7, 2.1	<0.001	0.476	0.8, 2.3	<0.001
Σ n-3 LCPUFA	0.380	0.1, 0.5	0.001	0.283	0.1, 0.8	0.014	0.299	0.1, 0.9	0.016
n-6 fatty acids									
C18:2n6 (LA)	0.344	3.2, 14.3	0.002	0.272	1.3, 12.3	0.017	0.342	2.9, 13.8	0.003
C20:4n6 (AA)	0.196	-0.04, 0.4	0.106	0.197	-0.03, 0.4	0.094	0.015	-0.2, 0.2	0.909
C20:3n6 (DGLA)	0.039	-0.2, 0.3	0.766	0.158	-0.1, 0.3	0.192	0.247	0.01, 0.4	0.044
C22:4n6 (Adrenic)	-0.005	-0.1, 1.0	0.971	0.069	-0.1, 0.1	0.565	-0.074	-0.1, 0.1	0.575
C22:5n6 (Osbond)	0.578	0.04, 0.1	<0.001	0.523	0.04, 0.1	<0.001	0.493	0.03, 0.1	<0.001
Σ n-6 PUFA	0.393	5.5, 21.3	0.001	0.298	2.6, 18.5	0.010	0.223	-0.5, 15.8	0.066
Σ n-6 LCPUFA	0.211	-0.1, 1.3	0.106	0.255	0.1, 1.4	0.033	0.088	-0.5, 1.0	0.496
Ratios									
n6: n3 PUFA	0.292	1.5, 17.8	0.021	0.414	5.9, 18.9	<0.001	0.498	7.9, 20.9	<0.001
n6: n3 LCPUFA	0.469	1.4, 3.9	<0.001	0.525	2.0, 4.6	<0.001	0.605	2.6, 5.2	<0.001
DHA: Osbond	0.644	2.0, 3.5	<0.001	0.635	2.5, 4.5	<0.001	0.565	2.7, 5.7	<0.001
EPA: DHA	0.457	0.1, 0.2	<0.001	0.413	0.1, 0.3	<0.001	0.532	0.1, 0.3	<0.001
EPUFA status index ⁴	0.554	0.6, 1.4	<0.001	0.472	0.5, 1.3	<0.001	0.196	0.3, 1.1	0.001
Omega 3 index ⁵	0.471	0.1, 0.3	<0.001	0.318	0.1, 0.5	0.005	0.134	0.1, 0.6	0.009

FA – Fatty acid; LA - Linoleic Acid; ALA- α - Linoleic Acid; AA- Arachidonic acid; DGLA- Dihomo- γ -linolenic acid; EPA- Eicosapentaenoic Acid; DPA- Docosapentanoic acid; DHA- Docosahexaenoic acid; SFA- Saturated fatty acids; PUFA- Polyunsaturated Fatty Acids; LCPUFA- Long-chain polyunsaturated fatty acids; EPUFA- Essential PUFA. ¹ FAs were log transformed before all analysis, values presented as Geometric Mean (95% CI), all such values. ² Linear regression model adjusted for maternal age, BMI, Hb status, HIV status and number of children. ³ Ratio between all essential PUFAs (the sum of all n-3 and n-6 FAs) and all non-essential unsaturated fatty acids (the sum of all n-7 and n-9 FAs) ⁴ Sum of EPA and DHA

DISCUSSION

In the present study, we evaluated the FA composition of breast milk sampled at three time points within-feed, and determined associations with RBC total phospholipid FA status, in lactating mothers of 2-4-month-old breastfed infants. To our knowledge, this is the first study in South Africa which provides current data on the breast milk FA composition in South African women and its associations with maternal FA status. We also determined the differences in breast milk FA composition within-feed and separate associations thereof with maternal FA status. Our results in South African lactating mothers indicate that breast milk EFA and LCPUFA composition may change within-feed, with an increase in ALA and DHA within a breastfeeding session, while LA and AA remained unchanged. Furthermore, breast milk DHA was associated with maternal RBC

status, while breast milk AA was not. The association between breast milk DHA and maternal RBC DHA was stronger in fore-milk and became weaker in mid-feed and hind-milk. Total breast milk PUFAs and the intermediate metabolite of the n-6 series, DGLA, were associated with fore-milk and hind-milk, respectively.

The DHA content of three breast milk fractions in this study were lower than the mean content of $0.32 \pm 0.22\%$; while AA was higher than the mean content of $0.47 \pm 0.13\%$ reported by Brenna *et al.* (2007) in a meta-analysis of 65 articles, including breast milk FA composition data from 2474 subjects worldwide. In contrast, the breast milk DHA levels reported in this study are higher while AA levels were lower than the previously reported DHA (0.10%) and AA (1.00%) breast milk concentrations reported in rural South Africa in 1988 (Van der Westhuyzen *et al.*, 1988 in Brenna *et al.*, 2007). We also previously showed a similar median [interquartile range] of breast milk DHA (0.23 [0.19-0.30]) and higher AA (0.76 [0.66-0.87]) (Siziba *et al.*, chapter 3) in lactating women from a similar setting.

In 1988, the breast milk of rural mothers living in South Africa was substantially higher in saturated FAs, while the breast milk of urban mothers was higher in MUFAs and LA. These differences were a result of the diet of rural mothers which was a typical traditional diet, low in animal protein and fat and high in carbohydrate and fibre; while the urban mothers consumed more varied and partially westernized diets (Van der Westhuyzen *et al.*, 1988). We hypothesized that since South Africa is a country that is undergoing the nutrition transition, the breast milk concentrations of lactating women in this study would be inclined towards breast milk concentrations from western countries. However, although they have doubled in amount over the years, the overall saturated FA and MUFA contents of fore-, mid-feed and hind-milk in lactating women in this study was comparable to that of women in Europe. The MUFAs were comparable in breast milk from Germany (Szabo *et al.*, 2010), Italy (Marangoni *et al.*, 2000b) and Brazil (da Cunha *et al.*, 2005) and the predominant breast milk FA was C18:1. Moreover, our results are consistent with data from Asian and African countries which also reported higher breast milk AA levels compared to Western countries (Borschel *et al.*, 1986; Chen *et al.*, 1997; Chulei *et al.*, 1995; Kneebone *et al.*, 1985).

Jensen *et al.* (1996) suggested that the saturated FAs of women consuming a non-western diet, such as women from India, South Africa, and the Dominican Republic, amongst others, account for almost or above 50% of total FAs in the milk. A typical diet in these low-income countries is high in carbohydrate which ultimately results in higher amounts of saturated FAs in the breast milk and circulating lipids. Furthermore, granted that the composition of EFAs in breast milk is greatly influenced by habitual maternal diet (Koletzko, 2016), we propose that the high content of LA observed in the breast milk of women in this population may reflect a high dietary intake of LA.

However, a higher concentration of LA in maternal blood or breast milk has been negatively associated with neurodevelopment in premature infants in three studies (Dijck-Brouwer *et al.*, 2005; Lundqvist-Persson *et al.*, 2010; Sabel *et al.*, 2009).

Despite the lower DHA levels in breast milk, we previously reported higher total plasma phospholipid DHA in breastfed than non-breastfed infants (Siziba *et al.*, chapter 3 and 4). Thus, breast milk supplies the infant with the needed LCPUFAs for development. However, the lactating mother has to sustain her own PUFA requirements and in turn, while providing a continuous supply of LCPUFAs to infant through breast milk (Carnielli *et al.*, 2007). There were no significant associations found between maternal RBC FAs or breast milk DHA levels with fish eating habits of women in this study. Since DHA is sensitive to maternal diet, nutritional intake in these lactating women might not have been optimal. Therefore, regular consumption of ocean fish and seafood, including oily fish by these breastfeeding women, could increase the DHA content in breast milk and thus increase the supply to the breastfed infant (Fidler *et al.*, 2000). Considering that there is a linear relationship between dietary DHA and breast milk DHA, researchers concluded that a regular and daily intake of 200 mg DHA leads to the required breast milk DHA content of 0.3% which will provide a daily supply of 100 mg DHA/d to infants (Fidler *et al.*, 2000).

Results from fore-, mid-feed and hind-milk indicate that breast milk EFAs and LCPUFA composition may be different, with an increase in ALA and DHA, while LA and AA remain unchanged within a feed. Also, for the DHA and ALA that increased within-feed, the correlations observed between DHA and ALA with their respective maternal RBC FAs were stronger in fore-milk and became weaker in mid-feed and hind-milk. We speculate that there is an upregulation of DHA towards the end of the feed and this increase in DHA that is likely independent of maternal status in settings where intake of dietary DHA is low. We therefore attribute these changes in the composition of these n-3 FAs in breast milk to the mother's metabolic adaptation to ensure the n-3 PUFAs are supplied to the infant. The correlations observed further confirm that n-3 PUFAs, particularly DHA seems to be selectively transferred from the mother to the infant through breast milk (Montgomery *et al.*, 2003).

Our results showed positive correlations between maternal RBC DHA and breast milk FAs by tertiles overall, but none were observed for AA. These results remained significant even when the correlations were performed separately for individual FAs with their respective FAs in breast milk; with the exception of dihomo- γ -linolenic acid (DGLA, C20: 3n-6). Similarly, with other studies (Kilari *et al.*, 2009; Kuipers *et al.*, 2005), our results suggest that breast milk AA content may not be dependent on maternal RBC status. However, it is estimated that about 30% of breast milk LA is directly transferred from the diet, while 11% of the LA metabolite DGLA in breast milk and 10% of AA in breast milk originates from direct endogenous conversion of dietary LA (Demmelmair &

Koletzko, 2017). It is possible that a large amount of these FAs passes through large pools with slow transfer such as adipose tissue. However, with the study designs applied in research, it is not possible to determine whether these different pools store the EFAs (precursors), before conversion or store the LCPUFA products, or whether the EFAs and LCPUFAs pass slow turnover pools before their appearance in breast milk (Demmelmair & Koletzko, 2017).

A limitation of this study is the small and non-representative sample of this study thus the data cannot be used to generalise information regarding all lactating women in South Africa. However, a strength of this study is the use of RBCs to determine maternal FAs status. While PUFA composition of plasma phospholipids reflects short-term intake of PUFA and is subject to high biological variability, red blood cell (RBC) FA composition appears to be more appropriate to represent an integrative measure of the FA status over the previous few months (Harris & Thomas, 2010). Therefore, although the changes observed in RBCs are not conclusive, selected transfer of LCPUFAs or changes in the metabolism of lactating mothers are expected to be reflected in the FAs composition of RBCs (Jørgensen *et al.*, 2006). However, plasma FA status could have provided additional information on associations with circulating FA. In addition, the within-feed differences reported in this study did not have a batch effect because all breast milk fractions from an individual mother were analysed in the same batch.

Our results suggest that different fractions of breast milk may indicate different phenomena. For instance, it appears the fore-milk fraction is most strongly correlated with maternal FA status while the FA composition of hind-milk is driven by the mother's metabolic adaptations. However, while lactating mothers have ample supply of AA and the transfer of AA to breast milk is fairly stable, breast milk AA content is not associated with maternal RBC AA while DHA is. Therefore, our results confirm the preferential transfer of DHA in breast milk through biomagnification. Furthermore, there is a change of FA composition within-feed, and this change has to be considered when selecting a breast milk fraction to analyse the FA composition in human milk.

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CHAPTER 6: GENERAL SUMMARY AND RECOMMENDATIONS

6.1 INTRODUCTION

Breast milk contains a variety of biological components that are necessary for the development of the growing infant. Thus, during the first six months of life, breast milk is universally recognised as the optimal source of nutrition for infants (Lessen & Kavanagh, 2015). The lipid fraction of breast milk provides the infant with the much needed long-chain polyunsaturated fatty acids (LCPUFAs) docosahexaenoic acid (DHA; C22:6n-3) and arachidonic acid (AA; C20:4n-6) for growth and development of the central nervous system (Lauritzen *et al.*, 2016). In light of this, fatty acids (FAs) in breast milk are mobilised endogenously from maternal FAs stores, synthesised by the liver or breast tissue and sometimes come from the diet (Innis, 2014). However, FAs that have been synthesised endogenously and made available in plasma or red blood cells (RBCs) are influenced by maternal nutrition. Particularly a dietary supply of DHA influences the DHA levels in breast milk, while AA is not mainly from the diet, but from maternal stores (Koletzko, 2016; Peng *et al.*, 2009). Therefore, the breast milk FA composition of mothers will vary across regions and populations depending on their access to food. However, the contribution of breast milk towards the LCPUFA intake and status of the infant depends on the LCPUFA content of breast milk. Thus, maternal plasma FA concentrations are reflected in breast milk which correlate with the levels of certain FAs in infant blood (Lauritzen & Carlson, 2011). Therefore, for infants to be ensured of an adequate LCPUFA supply through breast milk, breastfeeding women are advised to consume preformed DHA and AA during lactation (Carnielli *et al.*, 2007).

The WHO recommends that mothers worldwide should exclusively breastfeed their infants for the first six months to achieve optimal growth, development and health. Thereafter, infants should be introduced to nutritionally adequate and diverse complementary foods with continued breastfeeding for up to two years and beyond (Butte *et al.*, 2002). However, compared with other low-income and middle-income countries, South Africa is among the countries with the lowest breast milk DHA concentrations (Brenna *et al.*, 2007). Furthermore, in both high- (Schwartz *et al.*, 2010) and low-income (Forsyth *et al.*, 2016) countries, complementary foods are most likely to have low DHA and AA, thus their contribution to dietary intakes compared with breast milk is low.

Despite the reported inappropriate feeding practices in South Africa, the LCPUFA status of infants who are breastfed and those who receive complementary foods remains unstudied; yet infants should be assured of an adequate intake of LCPUFAs for optimal neurodevelopment, growth and other positive health outcomes (Colombo *et al.*, 2013). Moreover, breast milk composition is

dynamic and can vary within a feed, diurnally, over the course of lactation, and between mothers and populations (Ballard & Morrow, 2013). In light of this, this PhD research aimed to assess LCPUFA nutrition of South African infants during breastfeeding and complementary feeding.

The specific objectives of this research were:

1. To assess plasma FA patterns of six-month-old South African infants and to determine their association with feeding practices, growth and psychomotor development.
2. To investigate the efficacy of small-quantity lipid-based nutrient supplements (SQ-LNS) containing EFAs with or without LCPUFAs in improving LCPUFA status in South African infants fed complementary foods.
3. To assess the FA composition of breast milk sampled at three time points within-feed, and to determine associations with red blood cell (RBC) FA status in lactating South African mothers of 2-4-month-old breastfed infants.

This chapter provides a summary of the main findings of each of the three manuscripts in chapters 3 to 5. Recommendations for future research and the public health significance of this research are also discussed in this section.

6.2 ASSOCIATIONS OF PLASMA TOTAL PHOSPHOLIPID FATTY ACID PATTERNS WITH FEEDING PRACTICES, GROWTH AND PSYCHOMOTOR DEVELOPMENT IN SIX-MONTH-OLD SOUTH AFRICAN INFANTS

This study provided evidence that the infants (n=353) had an adequate EFA and LCPUFA status overall. Although breast milk DHA (0.23 [0.19-0.30]) levels were lower than world-wide reported averages, breast milk AA levels were higher (0.76 [0.66-0.87]). Also, more than two thirds (66%) of the infants were still receiving breast milk and breastfed infants had higher DHA (4.73 [4.57, 4.90]) and AA (13.08 [12.83, 13.34]), but lower LA (20.4 [20.1, 20.8]) and ALA (0.09 [0.08, 0.09]) compared with non-breastfed infants.

Four major plasma total phospholipid patterns were identified. Infants who received infant formula milk had higher scores, while breastfed infants had lower scores for the “*high EFAs with low DHA and AA*” and “*high MUFA and nervonic acid*” patterns. Infants who received breast milk, semi-solid foods or cow's milk but not infant formula milk had higher scores, while formula-fed infants had lower scores for the “*high n-6 LCPUFA*” pattern. Infants who received breast milk and semi-solids had higher scores for the “*trans-FA*”. The ‘*high MUFA and nervonic acid*’ and ‘*trans-FA*’ patterns were also positively associated with psychomotor development of infants. No associations were found with growth.

Limitations of this study included the low success in drawing blood and the cross-sectional nature of the study. The low success rate of drawing blood reduced the sample size for the FA analyses done in this study. The cross-sectional and observational nature of this study restricts the possibility of drawing conclusions on causation, particularly between FA status and psychomotor development. However, a strength of the study was the use of the principal component analysis (PCA) and factor analysis to determine FA patterns. Our data showed that a combination of FAs may be important in determining the diet of infants.

This study concluded that breastfed infants are likely to have an adequate supply of n-6 LCPUFAs while infants receiving formula milk are at a risk of inadequate DHA and AA intake. Also, it is plausible that breastfeeding women in this population did not consume adequate n-3 LCPUFAs which could have led to the lower DHA levels in breast milk.

6.3 EFFICACY OF NOVEL SMALL-QUANTITY LIPID-BASED NUTRIENT SUPPLEMENTS IN IMPROVING LONG-CHAIN POLYUNSATURATED FATTY ACID STATUS OF SOUTH AFRICAN INFANTS: A RANDOMISED, CONTROLLED TRIAL

In this study, infants who were breastfeeding at six months had higher plasma total phospholipid DHA and AA compared with those infants who were no longer receiving breast milk. The provision of a SQ-LNS containing EFAs and LCPUFAs (SQ-LNS-plus) resulted in higher plasma DHA at 12 months, while maintaining AA. Furthermore, particularly infants who were no longer receiving breast milk benefited from the SQ-LNS, indicated by a stronger effect size in non-breastfed than breastfed infants. This LCPUFA-enriched SQ-LNS resulted in non-breastfed infants having a similar DHA status to the breastfed infants in the control group. On the other hand, the SQ-LNS containing the EFAs LA and ALA did not have an effect on the plasma total phospholipid DHA and AA of infants in this study.

A strength of this study was the use of plasma FAs as a marker of current LCPUFA intake and status in comparison to RBCs and other lipid fractions. However, the limitation was the low success rate in drawing blood at 12 months from infants who had completed the study; which led to fewer infants who had an extra plasma aliquot for plasma FA analysis.

From this study it was concluded that consumption of an LCPUFA-enriched SQ-LNS with continued breastfeeding from the age of six to 12 months could improve DHA status of infants. It is possible that infants who no longer receive breast milk by the age of 12 months may be at a risk of inadequate DHA supply in complementary foods. Therefore, the LCPUFA-enriched SQ-LNS may be more beneficial to these infants.

6.4 BREAST MILK AND ERYTHROCYTE FATTY ACID COMPOSITION OF LACTATING WOMEN RESIDING IN A PERI-URBAN SOUTH AFRICAN TOWNSHIP

This study showed that the breast milk of lactating women (n=100) living in a township in the Potchefstroom area, was high in LA levels, and, the predominant monounsaturated FA was oleic acid. Based on the DHA levels in breast milk which were lower than the world-wide averages, findings from this research suggest that lactating women in this peri-urban township do not consume adequate amounts of DHA. The saturated FAs in breast milk were substantially higher which is indicative of a typical high carbohydrate and low-fat diet in most low-income countries (Jensen, 1996).

In addition, the n-3 FA composition of the different fractions of breast milk indicate that fore-milk is strongly correlated with maternal FA status, while the n-3 FA composition of hind-milk may be driven by the mother's metabolism of endogenous FAs. These variations confirm the mother's metabolic adaptation to ensure that adequate LCPUFAs are transferred through breast milk to the breastfeeding infant. The associations reported between maternal RBC status and breast milk EFAs and DHA further confirm that n-3 LCPUFAs are selectively transferred to the infant through breast milk. Also, the change in FA composition of the different breast milk fractions within a feed needs to be considered when selecting a breast milk fraction for FA analysis.

A limitation of this study is the small and non-representative sample of lactating women in this study. In addition, the cross-sectional nature of this study does not allow one to generalise information regarding all lactating women in South Africa. However, the strength of this study is the analysis of different fractions of breast milk. This gives a clear understanding of the importance of selecting breast milk fractions which give an accurate depiction of the FAs transferred to the infant through breast milk.

Conclusions drawn were, n-3 LCPUFA composition may change within a feed, while LA and AA remain unchanged. The maternal RBC DHA status was associated with breast milk DHA, while AA was not. These findings suggest that DHA is preferentially transferred to breast milk through biomagnification.

6.5 PUBLIC HEALTH PERSPECTIVE

The DHA levels in the breast milk of lactating women included in this study were lower than the world-wide averages. Higher levels of n-3 LCPUFAs and MUFAs were observed in hind-milk compared to the other milk fractions. The higher levels of n-3 LCPUFA and variations in hind-milk confirm that there is a complex mechanism possibly involving hormones, synthesis, infant demand and maternal supply in the regulation of FA transfer through breast milk (da Cunha *et al.*,

2005). This research warrants further exploration of the endogenous and exogenous factors that may be associated with preferential transfer of FAs into breast milk, to ensure that the infant receives an adequate supply of LCPUFAs.

The results from this study indicate that breast milk FA composition differs across populations. The within-feed variations reported in this study indicate the mother's naturally occurring physiological aspects of LCPUFA transfer through breast milk. Interrelationships between breast milk FA composition and maternal RBC FA status could therefore be used for future recommendations (Smit *et al.*, 2003). However, both national and international authorities have based their recommendations for the FA composition of infant formula using breast milk FA composition as the gold standard. Because there is a possible lack of additional information, these "standards" are based on breast milk FA data obtained from lactating mothers of Caucasian ethnicity, typically consuming western diets (Smit *et al.*, 2003). Most importantly, the inclusion of *trans*-FAs in the diet of western countries could influence the current breast milk composition on which infant formula recommendations are based. Therefore, future studies should take into consideration the different factors that influence breast milk FA composition across different populations so as to provide adequate scientific evidence based on the varied breast milk FA compositions.

The results from this study showed that DHA levels in breast milk of lactating women are associated with maternal blood DHA. Also, the LCPUFA status of infants who are breastfed is higher than in infants who do not receive breast milk. We also found that "a high *n*-6 LCPUFA" pattern is positively associated with breastfeeding in six-month-old infants. These research findings confirm that LCPUFAs (DHA) are transferred through the breast milk to the infants, possibly through the process of biomagnification. These data regarding the LCPUFA status of infants and lactating women are particularly relevant because globally, the childhood population may be at risk of LCPUFA deficiency in early life (Forsyth *et al.*, 2017a; Forsyth *et al.*, 2016). With a birth rate that is seven times higher in low-income countries than in high-income countries, it is estimated that nearly 22 million infants are born, and they are at risk of LCPUFA insufficiency (Forsyth *et al.*, 2017b). These findings reinforce the WHO recommendations of exclusive breastfeeding for the first six months of life, and introduction of appropriate foods at six months with continued breastfeeding for up to two years and beyond. However, despite the maternal RBC DHA status that does not reflect a low *n*-3 LCPUFA intake, the findings that DHA levels in breast milk were lower than reported world-wide estimates further suggest that the lactating women in this study may not have been consuming adequate dietary *n*-3 LCPUFAs. Strategies that encourage women to consume adequate amounts of preformed DHA should be employed. Only then, will infants receive an adequate LCPUFA supply through breast milk.

Furthermore, subsequent research has shown that blood levels of DHA and AA are higher in infants who are given infant formula milk enriched with DHA and AA, than in infants who are given an infant formula that is not supplemented with these LCPUFAs (Birch *et al.*, 2005; Forsyth *et al.*, 2003). Consistent with these data, in this current research, receiving infant formula milk was positively associated with a “*high EFAs and low DHA and AA*” pattern in six-month infants. In light of this, it is evident that dietary intake of EFAs during infancy is significantly associated with the FA status on infants. Thus, a continued supply of dietary DHA and AA during this critical period of rapid growth and development is an important nutritional objective. The addition of DHA and AA to infant formulas has been investigated and tested in high-income countries and there, no concerns have been raised (Hadley *et al.*, 2016). For that reason, in a public health approach towards improving DHA and AA intakes during early development within populations and across regions, and particularly in South Africa, the addition of DHA to infant formulas should be mandatory to ensure that all infants, especially those at a risk of inadequate LCPUFA intake, are protected.

Based on current research findings, it is clear that there is a need for a balance between breast milk intake and consumption of nutritionally adequate complementary foods, after the age of six months. The results of this PhD research showed that breastfed infants may benefit from LCPUFA-enriched SQ-LNS, but the effect was stronger in infants who were not receiving breast milk. Thus, even though breast milk is a major source of LCPUFAs during infancy, after the age of six months, the volume of breast milk intake decreases, thereby allowing intake of high-density complementary foods. However, if the complementary foods that are introduced, do not supply the infant with adequate DHA and AA, there will be a concomitant decrease in blood FA levels (Birch *et al.*, 2005; Libuda *et al.*, 2016). In addition to improving or maintain DHA and AA status in infants, the LCPUFA-enriched SQ-LNS had a transient effect on growth and a positive effect on the locomotor outcome in psychomotor development (Smuts *et al.*, 2018). Previous research has also shown other health benefits of LCPUFAs, especially DHA, during early infancy and even later in childhood (Colombo *et al.*, 2013; Matias *et al.*, 2017). Moving forward, in addition to introducing complementary foods at the age of six months, it is important that these foods will provide an adequate supply of DHA and AA.

Public health nutrition interventions should continue focusing on the promotion of optimal feeding practices, especially during infancy, that is, the period of critical growth. Although some infants could be at a risk of inadequate LCPUFA intake in early life, a shorter duration of breastfeeding and the introduction of complementary foods low in DHA and AA, need to be addressed. It is of paramount importance that the researchers communicate their findings clearly in the most understandable way and make their findings available to policy makers. Nonetheless, data from

observational studies can be feasible and appropriate for identifying populations 'at risk' and evaluating public health interventions (Forsyth *et al.*, 2017b).

6.6 RECOMMENDATIONS FOR FUTURE RESEARCH

Further research still needs to be done to determine inter-individual differences and variations of FA composition in breast milk taking into consideration;

- (i) time elapsed since last feed;
- (ii) volume of breast milk emptied at specific feed sampled; and
- (iii) time of feed (morning or evening).

There are currently no programs in South Africa that focus on promoting adequate LCPUFA intake during lactation. Thus, future studies that aim to assess and improve the DHA status of lactating women can be promoted.

Future studies/research could make use of the PCA and factor analysis to determine different FA patterns in the dietary data collected between the period of six to 12 months. This information would enable researcher to determine the quality of the diet of infants fed complementary foods; taking into account the variability and interdependence of FAs.

Further research is also needed to evaluate different formulations of SQ-LNS, possibly with higher amounts of ALA and reduced ratios of LA:ALA, which could be beneficial in further examining the potential of the SQ-LNS to improve both EFA and DHA status.

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ADDENDA

ADDENDUM 1: Guidelines for authors Maternal & Child Nutrition



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The main text should be typed double-spaced, include continuous line numbers and structured as follows: introduction; participants (or materials) and methods (including appropriate subsections, e.g. statistical methods); results; discussion; key messages; references; legends to figures; tables; and figures.

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Dewey K.G. & Begum K. (2011) Long-term consequences of stunting in early life. *Maternal & Child Nutrition* 7, 5–18.

Habicht J.P. (2000) The association between prolonged breastfeeding and poor growth. In: *Short and Long Term Effects of Breast Feeding on Child Health* (eds B. Koletzko, K.F. Michaelsen & O. Hernell), pp 193–200. Kluwer Academic/Plenum Publishers: New York.

Baby Friendly Health Initiative (2012) 10 Steps to Successful Breastfeeding. Available at: <http://www.babyfriendly.org.au/about-bfhi/ten-steps-to-successful-breastfeeding/> (Accessed 23 November 2014).

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ADDENDUM 2: Tswaka baseline questionnaire

BASELINE QUESTIONNAIRE

Baby's code:

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Date of the interview (dd/mm/yyyy):

		/			/	2	0	1	
--	--	---	--	--	---	---	---	---	--

Fieldworker's code:

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SOCIO-ECONOMIC INFORMATION

1. With whom is interview?	Mother of the baby (<i>go to question 3</i>)	1	
	Caregiver of the baby	2	

2. Caregiver's relationship to the child?	Father	1	
	Aunt	2	
	Uncle	3	
	Grandmother	4	
	Grandfather	5	
	Not related caregiver	6	

3.a How old are you? (in years)		
---------------------------------	--	--

3.b What is your birth date?			/			/	1	9		
------------------------------	--	--	---	--	--	---	---	---	--	--

4. Are you married?	Yes, married	1	
	Common-law husband/wife	2	
	Living together	3	
	No, unmarried	4	
	Separated / divorced	5	
	Widowed/widow	6	

5. Did you ever attend school?	Yes	1	
	No (<i>go to question 7</i>)	2	

6. What was the highest standard that you passed at school?	Sub A / Grade 1	01		
	Sub B / Grade 2	02		
	Standard 1 / Grade 3	03		
	Standard 2 / Grade 4	04		
	Standard 3 / Grade 5	05		
	Standard 4 / Grade 6	06		
	Standard 5 / Grade 7	07		
	Standard 6 / Grade 8 / Form I	08		
	Standard 7 / Grade 9 / Form II	09		
	Standard 8 / Grade 10 / Form III / NTC I	10		
	Standard 9 / Grade 11 / Form IV / NTC II	11		
	Standard 10 / Grade 12 / Form V / NTC III	12		
	Higher qualifications	13		

7. How many people live in the house?	a. Total number of people		
	b. Babies and small children		
	c. Primary school children		
	d. High school children		
	e. Adults		
	f. Elderly people		

8.a	How many people in the household are employed and earns a salary or wage (weekly or monthly income)		
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8.b	How many children in the household gets a child grant		
------------	--	--	--

8.c	How many people in the household gets either an old age pension or disability		
------------	--	--	--

9. Where does the household usually get its drinking water from? (Mark only one)	Own tap – inside the house	1	
	Own tap – outside the house	2	
	Neighbour's tap	3	
	Public tap	4	
	Borehole	5	
	Other, specify	6	

10. What type of toilet does the household have?	Flush toilet	1	
	Pit toilet	2	
	None	3	
	Other; specify	4	

11. Do you have electricity available inside your home?	Yes	1	
	No (<i>go to question 13</i>)	2	

12. During the last 4 weeks, were there times that you did not use electricity because you had not money to pay it?	Yes	1	
	No	2	

INFANT FEEDING

13. Are you currently breastfeeding your baby/Is the baby currently breastfed?	Yes (<i>go to question 15</i>)	1	
	No	2	
	Don't know (<i>go to question 15</i>)	3	

14. How old was the baby when breastfeeding was stopped? (<i>in months</i>)	
---	--

15. What was the <u>first</u> drink other than breast milk that your baby was ever given to drink?	
---	--

16. How old was your baby when you gave this drink for the first time? (<i>in months</i>)	
---	--

17. At the moment, does your baby get any <u>milk feeds</u> other than breast milk?	Yes	1	
	No (<i>go to question 20</i>)	2	
	Don't know (<i>go to question 20</i>)	3	

18. What type of milk, other than breast milk is your baby getting?	Cow's milk (full strength)	1	
	Cow's milk (diluted)	2	
	Klim / Nespray	3	
	Infant formula	4	
	Give name:		
	Other, specify:	5	

19. How old was your baby when he/she was given this <u>milk feed</u> for the first time? (<i>in months</i>)	
--	--

20. At the moment, does your baby get any semi-solid or solid food (with a spoon)?	Yes	1	
	No (<i>go to question 24</i>)	2	
	Don't know (<i>go to question 24</i>)	3	

21. What was the <u>first</u> semi-solid or solid food (with a spoon) that your baby ate?	
--	--

22. How old was your baby when he/she ate semi-solid or solid food (with a spoon) for the first time? (<i>in months</i>)	
--	--

23a. How many times did your baby eat solid, semi-solid or soft foods (with a spoon) other than liquids yesterday during the day?	
---	--

23b. How many times did your baby eat solid, semi-solid or soft foods (with a spoon) other than liquids yesterday during the night?	
---	--

24. Did your baby drink anything from a bottle with a teat yesterday during the day and/or the night?	Yes	1	
	No	2	
	Don't know	3	

25. Does your baby get any dietary supplements (e.g. vitamin syrup, vitamin tablets)?	Yes, specify:	1	
	No	2	
	Don't know	3	

MORBIDITY

M1. During the past week (7 days), did your baby have diarrhoea (at least three watery stools per day)	1	Yes *	
	2	no (go to question M2)	
	3	don't know (go to question M2)	

M1a. For how many days did he/she have diarrhoea?	
---	--

When did your baby's diarrhoea start?	Date: ____/____/ 20____
When did your baby's diarrhoea stop?	Date: ____/____/ 20____ Or is it carrying on? _____

* If marked "yes": Fieldworkers please collate information for study nurse to complete AE form

M2. During the past week (7 days), did your baby vomit?	1	Yes *	
	2	no (go to question M3)	
	3	don't know (go to question M3)	

M2a. For how many days did he/she vomit?	
--	--

When did your baby's vomiting start?	Date: ____/____/ 20____
When did your baby's vomiting stop?	Date: ____/____/ 20____ Or it is carrying on? _____

* If marked "yes": Fieldworkers please collate information for study nurse to complete AE form

M3. During the past week (7 days), did your baby have a continuous cough (at least for one whole day)?	1	Yes *	
	2	no (go to question M4)	
	3	don't know (go to question M4)	

M3a. For how many days did he/she cough continuously?	
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M3b. Was the cough wet or dry?	1	Wet	
	2	Dry	

When did your baby's coughing start?	Date: ____/____/ 20____
--------------------------------------	-------------------------

When did your baby's coughing stop?	Date: ____/____/20____ Or is it carrying on? _____
-------------------------------------	---

* If marked "yes": Fieldworkers please collate information for study nurse to complete AE form

M4. During the past week (7 days), did your baby have wheezing or difficult breathing?	1	Yes *
	2	no (go to question M5)
	3	don't know (go to question M5)

M4a. For how many days did he/she have wheezing or difficult breath?	
--	--

When did your baby's wheezing / difficulty in breathing start?	Date: ____/____/20____
When did your baby's wheezing / difficulty in breathing stop?	Date: ____/____/20____ Or is it carrying on? _____

* If marked "yes": Fieldworkers please collate information for study nurse to complete AE form

M5. During the past week (7 days), did your baby have a hot body?	1	Yes *
	2	no (go to question M6)
	3	don't know (go to question M6)

M5a. For how many days did he/she have a hot body?	
--	--

When did you notice that your baby first have a hot body?	Date: ____/____/20____
When did you notice that your baby's body is no longer hot?	Date: ____/____/20____ Or is it carrying on? _____

* If marked "yes": Fieldworkers please collate information for study nurse to complete AE form

M6. During the past week (7 days), did your baby have any sores on the skin or rash?	1	Yes *
	2	no (go to question M7)
	3	don't know (go to question M7)

M6a. For how many days did he/she have the sores or rash?	
---	--

When did your baby first have a sore or rash?	Date: ____/____/20____
When did your baby's sore or rash stop?	Date: ____/____/20____

	Or is it carrying on? _____
--	-----------------------------

* If marked "yes": Fieldworkers please collate information for study nurse to complete AE form

M7 During the past week (7 days), did your baby have a continuous runny nose/ blocked nose?	1	Yes *	
	2	no (go to question M8)	
	3	don't know (go to question M8)	

M7a. For how many days did he/she have a runny nose/blocked nose?	
---	--

When did your baby's runny nose / blocked nose start?	Date: ____/____/ 20____
When did your baby's runny nose / blocked nose stop?	Date: ____/____/ 20____ Or is it carrying on? _____

* If marked "yes": Fieldworkers please collate information for study nurse to complete AE form

M8. During the past week (7 days), did your baby have any other illness?	1	Yes *	
	2	no (go to question M9)	
	3	don't know (go to question M9)	

M8a. Which illness did your baby have?
.....
.....

When did your baby's illness start?	Date: ____/____/ 20____
When did your baby's illness stop?	Date: ____/____/ 20____ Or is it carrying on? _____

* If marked "yes": Fieldworkers please collate information for study nurse to complete AE form

M9. Did your baby visit any health service during the last week (7 days) because he/she was ill?	1	Yes	
	2	no (end of questionnaire)	
	3	don't know (end of questionnaire)	

M10.	Which health services did your baby go to during the last week?		
a. Clinic	yes = 1	no = 2	
b. Private physician / doctor	yes = 1	no = 2	

c. Hospital	yes = 1	no = 2	
d. Traditional healer	yes = 1	no = 2	
e. Other (specify):	yes = 1	no = 2	

M11. Did your baby receive any medicine prescribed by a nurse or doctor for his/her illness during the last week (7 days)	1	yes	
	2	no (go to question M12)	

M11. What type of medicine?

M11b. For how many days did he/she receive medicine?	
--	--

M12. Was your baby officially diagnosed with any illness during the last week (7 days)?	1	yes	
	2	no (go to question M14)	

M13. With what illness was your baby diagnosed?

M14. Was your baby hospitalised during the last week (7 days)	1	Yes *	
	2	no (end of questionnaire)	

M15. How long was your baby in hospital? (days)	
---	--

When did your baby's stay in hospital start?	Date: ____/____/ 20____
When did your baby's stay in hospital stop?	Date: ____/____/ 20____ Or is it carrying on?_____

* If marked "yes": Fieldworkers please collate information for study nurse to complete AE form

M16. Why was your baby hospitalised?

ADDENDUM 3: Tswaka food frequency questionnaire**FOOD FREQUENCY****Baby's code:**

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Ask for each food item, one at a time, how often the child usually eats the specific food item. The last week (or last seven days) should be taken as guideline, therefore the frequency that the child ate the food item during the last week. Make a cross on the option that describes the mother's answer the best). The options are as follows: Every day

Most days: not every day, but at least 4 times per week

Once a week: less than 4 times per week, but at least once per week

Never

Food item	Frequency of intake during the last week			
	Baseline	Month 2	Month 4	Month 6 (end)
Date (dd/mm/yyyy):				
Fieldworker's code:				
Breast milk	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Formula milk <i>If formula milk was used, please give name of the formula milk:</i>	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Cow's milk / amasi / maas Milk powder e.g. Klim, Nespray	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Yoghurt / danone	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Baby foods in a jar e.g. Purity	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Infant cereals or infant porridge e.g. Nestum, Cerelac, Cream of Maize, Baby Mabele	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never

Porridge made with maize meal (soft, stiff or crumbly)	1__ Every day 2__ Most days 3__ Once a week	1__ Every day 2__ Most days 3__ Once a week	1__ Every day 2__ Most days 3__ Once a week	1__ Every day 2__ Most days 3__ Once a week
---	---	---	---	---

Food item	Frequency of intake during the last week			
	Baseline	Month 2	Month 4	Month 6 (end)
	4__ Never	4__ Never	4__ Never	4__ Never
Cooked porridge, other than maize meal porridge e.g. oats, mabele	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Instant porridge, e.g. instant Maize, Mabele	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Bread	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Rice	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Potatoes	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Vegetables, any type (NOT potatoes)	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
<i>If vegetables were eaten, please name the type of vegetables eaten mostly:</i>	1 2 3	1 2 3	1 2 3	1 2 3
Fruit juice (includes juice squeezed from the fruit)	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never

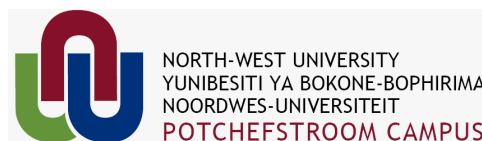
Fresh fruit (any type)	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
<i>If fruit were eaten, please name the type of fruit eaten mostly:</i>	1 2 3	1 2 3	1 2 3	1 2 3
Eggs	1__ Every day 2__ Most days 3__ Once a week	1__ Every day 2__ Most days 3__ Once a week	1__ Every day 2__ Most days 3__ Once a week	1__ Every day 2__ Most days 3__ Once a week

Food item	Frequency of intake during the last week			
	Baseline	Month 2	Month 4	Month 6 (end)
	4__ Never	4__ Never	4__ Never	4__ Never
Red meat (beef, pork, mutton) / stew / sausage / mince meat	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Chicken / poultry	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Liver (e.g. chicken liver, beef liver, sheep liver etc)	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Fish (fresh or canned)	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Sweets / Chocolates	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Chips / Cheese curls / Niknaks	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Fizzy cold drink e.g. Coke	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never

Juice concentrate, mix with water e.g. Oros	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Rooibos	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Tea, normal	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never
Sugar (any type), eaten as such, in drinks (e.g. tea) or added to food	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never	1__ Every day 2__ Most days 3__ Once a week 4__ Never

Food item	<i>Frequency of intake during the last week</i>			
	Baseline	Month 2	Month 4	Month 6 (end)
Salt (added to food)	1__Every day 2__Most days 3__Once a week 4__Never	1__Every day 2__Most days 3__Once a week 4__Never	1__Every day 2__Most days 3__Once a week 4__Never	1__Every day 2__Most days 3__Once a week 4__Never
How often did you use oil when preparing the <u>baby's food</u> ?	1__Every day 2__Most days 3__Once a week 4__Never	1__Every day 2__Most days 3__Once a week 4__Never	1__Every day 2__Most days 3__Once a week 4__Never	1__Every day 2__Most days 3__Once a week 4__Never
How often did you use margarine when preparing the <u>baby's food</u> ? [any type of margarine]	1__Every day 2__Most days 3__Once a week 4__Never	1__Every day 2__Most days 3__Once a week 4__Never	1__Every day 2__Most days 3__Once a week 4__Never	1__Every day 2__Most days 3__Once a week 4__Never
How often did you use peanut butter when preparing the <u>baby's food</u> ?	1__Every day 2__Most days 3__Once a week 4__Never	1__Every day 2__Most days 3__Once a week 4__Never	1__Every day 2__Most days 3__Once a week 4__Never	1__Every day 2__Most days 3__Once a week 4__Never

ADDENDUM 4: Tswaka informed consent form



INFORMATION SHEET

STUDY: Randomized controlled trial in South Africa comparing the impact of complementary food products on child growth

Principal investigator: Prof Marius Smuts
Co-principal investigator: Prof Mieke Faber

Dear Parent / Legal Guardian

Who are we?

We are from the North West University (PUKKE), Potchefstroom and the Medical Research Council, Cape Town. We are studying the effect of newly-developed nutritional supplements on the growth and development of babies. The supplements are in the form of a paste and can be mixed with the food babies normally eat. We invite you and your baby to participate in this important study.

Why are we doing this?

Iron and fatty acids in foods are important for the growth and development of babies. For optimal growth and development of babies, the different types of fatty acids in food need to be present in the right amounts. The aim of this study is to test two types of nutritional supplements in order to see if these supplements can improve the growth and development of babies. This will be done by measuring nutritional status, growth and development of babies after they have eaten the supplement mixed with their usual food every day for six months.

What do we expect from participants during the study?

Seven hundred and fifty (750) babies and their mothers (or primary caregiver) will be recruited through the local clinics. All babies will be 6 months old at the start of the study and will be followed up until they are 12 months old. Only mothers who planned to stay in the Jouberton area for at least the next 7 months can take part in the study. Only apparently healthy babies with no known allergy to soy, peanut, milk/lactose and fish, who are currently breastfeeding or have previously breastfed, will be included in the study. The products contain allergens from soy, milk, and fish, and may also contain traces of peanuts. Twins cannot participate in the study.

Participants will be divided into three groups. The first group will receive a fortified fat-based paste that contains essential fatty acids with other added fatty acids as well as a substance that may improve the absorption of iron. The second group will receive a paste that contains

essential fatty acids. Both products contain soy. The third group will not receive any supplement during the 6-month study period, but they will receive a 6-month supply of the fat-based paste when the baby is 12 months old. Each child has an equal chance to be in any of the three groups. The amount of nutrients (e.g. iron and fatty acids) used in the two supplements is safe and no side effects such as nausea or diarrhoea is expected. The supplement will be provided free of charge to all study participants. Mothers will be asked to mix a certain amount of the supplement with the child's usual food daily for 6 months.

If you agree to participate in the study, we will ask the following from you:

When your baby is 6 and 12 months old, you will be asked to go with your baby to the research site which is at the Baptist Church. During these two visits, we will ask the following from you:

- You will be asked questions about your household (at the start of the project), the foods that your baby eats and drinks, any illnesses that your baby had during the previous weeks, and development that your baby has achieved.
- You will be asked to recall the foods and drinks that your baby consumed the day before.
- Your baby's weight, length, head circumference and upper arm circumference will be measured.
- Your weight and height will be measured (at the start of the project).
- You will be maybe asked to express a small amount of breast milk (approximately $\frac{1}{4}$ cup). We will measure the fatty acids and iodine in the breast milk. After the fatty acids and iodine have been measured, the remaining breast milk will be discarded according to standard procedures.
- A nursing sister will take a 4 mL (less than one teaspoon) blood sample from the vein in your baby's arm to measure the levels of nutrients in blood. We will also use this blood to measure your baby's response to the measles immunization routinely given at the clinic to babies when they are 9 months old. If the nursing sister cannot get blood from your baby's arm, she will take blood from the vein in top of your baby's hand or by a finger prick. If your baby is showing too much resistance during this process, blood will not be taken from him/her. The procedure is completely safe.
- You will be maybe asked to provide a small amount of urine (at the start and end of the project). We will measure the iodine in the urine.
- We will collect a small amount of urine from your baby using a special nappy. We will measure the iodine in the urine.

You and your baby will be asked to further visit the research site when the baby is 8 and 10 months old. During these two visits you will be asked questions about the foods that your baby eats and drinks, and your baby's weight and length will be measured.

You will also be asked to record daily how much product your baby consumed and to report illness.

A field worker will visit you at home once a week. During these visits the fieldworker will ask you questions on the usage of the supplement, illnesses that your baby may experience and developmental milestones that your baby has achieved.

Payment, Expenses and Costs

You will not have to pay for any costs that are directly related to the research study, for example blood tests. Your taxi-fare from your home in Jouberton to the research site at the Baptist Church will be refunded on the day that you visit the research site.

Who will have access to my child's information?

All information collected about your baby will be treated as confidential (will not be given to or discussed with anybody) and only the researchers and the ethics committees at the Medical research Council and North West University will have access to it. No abnormal finding is expected, but should anything abnormal be found we will refer the baby to the local clinic or a

medical doctor for the necessary treatment. You will be kept informed in this regard and are welcome to discuss any concerns that you may have with us.

What will the benefit be for my child who participates?

Your child may not benefit from the study, but children may in future benefit from the results. Your child will be monitored for the 6-month period and, should anything abnormal be found, be referred to the local clinic or a medical doctor for the necessary treatment. You will also gain information on your child's nutritional status and development.

What will the risks be for my child who participates?

The nutritional supplement is safe and should not harm your child or make your child sick. Your child may experience some discomfort when the blood samples are taken or when the weight and length are taken. This discomfort will be minimised as the staff taking these measurements will be experienced. If your child does not want to cooperate, the procedures will be stopped.

Must I participate?

Participation in this study is completely voluntary (your own choice). Whether you do, or do not, give your permission will not influence your baby's access to health care in any way.

May I change my mind?

Certainly, you may do this at any time without having to give a reason. The study is completely voluntary and it will not be kept against you in any way should you decide to withdraw from the study.

Who can you contact if there are any queries?

For more information on the study you may contact Prof Marius Smuts at 018-299 4670 or 082 451 0486 **OR** Prof Mieke Faber at 021-938 0404 or 0824602946 during office hours.

The study has been approved by the Ethics Committee of North West University (NWU), the Ethics Committee of the Medical Research Council, as well as the Department of Health. If you have any queries or problems regarding the study, you can contact either Prof Amanda Lourens the chairperson of the NWU Ethics Committee at (018) 2992606 or Prof Danie du Toit who is the chairperson of the MRC Ethics Committee at (021) 9380341 or you can send an e-mail to adri.labuschagne@mrc.ac.za.

If you are happy for you and your child to take part in the study, please read and sign the consent form.

Thank you!

ADDENDUM 5: Tswaka exit questionnaire

EXIT QUESTIONNAIRE

Baby's code:

--	--	--	--

Date of the interview (dd/mm/yyyy):

		/			/	2	0	1	
--	--	---	--	--	---	---	---	---	--

Fieldworker code:

--	--

INFANT FEEDING

1. Are you currently breastfeeding your baby / Is the baby currently breastfed?	Yes (<i>go to question 3</i>)	1	
	No	2	
	Don't know (<i>go to question 3</i>)	3	
2. How old was the baby when breastfeeding was stopped? (<i>in months</i>)			
3. At the moment, does your baby get any <u>milk feeds</u> other than breast milk?	Yes	1	
	No (<i>go to question 5</i>)	2	
	Don't know (<i>go to question 5</i>)	3	
4. What type of milk, other than breast milk is your baby getting?	Cow's milk (full strength)	1	
	Cow's milk (diluted)	2	
	Klim / Nespray	3	
	Infant formula	4	
	Give name:		
	Other, specify:	5	
5.a. How many times did your baby eat solid, semi-solid or soft foods (with a spoon) other than liquids yesterday during the day?			
5.b. How many times did your baby eat solid, semi-solid or soft foods (with a spoon) other than liquids yesterday during the night?			
6. Did your baby drink anything from a bottle with a teat yesterday during the day and/or night?	Yes	1	
	No	2	
	Don't know	3	
7. Does your baby get any dietary supplements (e.g. vitamin syrup, vitamin tablets)?	Yes, specify:	1	
	No	2	
	Don't know	3	

INFORMATION ON THE PASTE

8.	In general, how do feel about the paste?
	
	
	
	Why do you feel this way?
	
	
	
	

9.	During the past week (7 days), how often did you give the paste to your baby?	Every day	1	
		4-6 days per week	2	
		1-3 days per week	3	
		Never	4	

10.	Was the paste acceptable for you?	Yes	1	
		No	2	
		Don't know / No answer	3	

11.	Was the paste acceptable for your baby?	Yes	1	
		No	2	
		Don't know / No answer	3	

12.	With what foods did you <u>usually</u> mix the paste with?
	
	
	

13.	With what foods did you mix the paste with <u>yesterday</u> ?
	
	
	

14. Please use the five faces, and tick the number under the face that best describes how you feel about each of the following statements

						
	Disagree	Tend to disagree	Un-decided	Tend to agree	Agree	
a. The baby liked the paste	1	2	3	4	5	
b. You liked giving the paste to the baby	1	2	3	4	5	
c. It was easy to mix the paste with the baby's food	1	2	3	4	5	
d. You had problems feeding the baby the paste	1	2	3	4	5	
e. You are interested in using the paste in future if for sale	1	2	3	4	5	
f. You will buy the paste if available	1	2	3	4	5	
g. You sometimes gave the paste with foods other than porridge	1	2	3	4	5	
h. You sometimes gave the daily dose of the paste in small portions throughout the day	1	2	3	4	5	

MORBIDITY

M1. During the past week (7 days), did your baby have diarrhoea (at least three watery stools per day)	1	Yes *	
	2	no (<i>go to question M2</i>)	
	3	don't know (<i>go to question M2</i>)	

M1a. For how many days did he/she have diarrhoea?	
---	--

When did your baby's diarrhoea start?	Date: ____/____/ 20____
When did your baby's diarrhoea stop?	Date: ____/____/ 20____ Or is it carrying on? _____

* If marked "yes": Fieldworkers please collate information for study nurse to complete AE form

M2. During the past week (7 days), did your baby vomit?	1	Yes *	
	2	no (<i>go to question M3</i>)	
	3	don't know (<i>go to question M3</i>)	

M2a. For how many days did he/she vomit?		
When did your baby's vomiting start?	Date: ____/____/ 20____	
When did your baby's vomiting stop?	Date: ____/____/ 20____ Or it is carrying on? _____	

** If marked "yes": Fieldworkers please collate information for study nurse to complete AE form*

M3. During the past week (7 days), did your baby have a continuous cough (at least for one whole day)?	1	Yes *	
	2	no (go to question M4)	
	3	don't know (go to question M4)	

M3a. For how many days did he/she cough continuously?	
---	--

M3b. Was the cough wet or dry?	1	Wet	
	2	Dry	

When did your baby's coughing start?	Date: ____/____/ 20____
When did your baby's coughing stop?	Date: ____/____/ 20____ Or is it carrying on? _____

** If marked "yes": Fieldworkers please collate information for study nurse to complete AE form*

M4. During the past week (7 days), did your baby have wheezing or difficult breathing?	1	Yes *	
	2	no (go to question M5)	
	3	don't know (go to question M5)	

M4a. For how many days did he/she have wheezing or difficult breath?	
--	--

When did your baby's wheezing / difficulty in breathing start?	Date: ____/____/ 20____
When did your baby's wheezing / difficulty in breathing stop?	Date: ____/____/ 20____ Or is it carrying on? _____

** If marked "yes": Fieldworkers please collate information for study nurse to complete AE form*

M5. During the past week (7 days), did your baby have a hot body?	1	Yes *	
	2	no (go to question M6)	
	3	don't know (go to question M6)	

M5a. For how many days did he/she have a hot body?	
--	--

When did you notice that your baby first have a hot body?	Date: ____/____/ 20____
When did you notice that your baby's body is no longer hot?	Date: ____/____/ 20____ Or is it carrying on? _____

*** If marked "yes" : Fieldworkers please collate information for study nurse to complete AE form**

M6. During the past week (7 days), did your baby have any sores on the skin or rash?	1	Yes *	
	2	no (go to question M7)	
	3	don't know (go to question M7)	

M6a. For how many days did he/she have the sores or rash?	
---	--

When did your baby first have a sore or rash?	Date: ____/____/ 20____
When did your baby's sore or rash stop?	Date: ____/____/ 20____ Or is it carrying on? _____

*** If marked "yes": Fieldworkers please collate information for study nurse to complete AE form**

M7. During the past week (7 days), did your baby have a continuous runny nose/ blocked nose?	1	Yes *	
	2	no (go to question M8)	
	3	don't know (go to question M8)	

M7a. For how many days did he/she have a runny nose/blocked nose?	
---	--

When did your baby's runny nose / blocked nose start?	Date: ____/____/ 20____
When did your baby's runny nose / blocked nose stop?	Date: ____/____/ 20____ Or is it carrying on? _____

*** If marked "yes": Fieldworkers please collate information for study nurse to complete AE form**

M8. During the past week (7 days), did your baby have any other illness?	1	Yes *	
	2	no (go to question M9)	
	3	don't know (go to question M9)	

M8a. Which illness did your baby have?

.....

When did your baby's illness start?	Date: ____ / ____ / 20__
When did your baby's illness stop?	Date: ____ / ____ / 20__ Or is it carrying on? _____

* If marked "yes": Fieldworkers please collate information for study nurse to complete AE form

M9. Did your baby visit any health service during the last week (7 days) because he/she was ill?	1	Yes	
	2	no (end of questionnaire)	
	3	don't know (end of questionnaire)	

M10.	Which health services did your baby go to during the last week?			
	a. Clinic	yes = 1	no = 2	
	b. Private physician / doctor	yes = 1	no = 2	
	c. Hospital	yes = 1	no = 2	
	d. Traditional healer	yes = 1	no = 2	
	e. Other (specify):	yes = 1	no = 2	

M11. Did your baby receive any medicine prescribed by a nurse or doctor for his/her illness during the last week (7 days)	1	yes	
	2	no (go to question M12)	

M11. What type of medicine?
.....
.....

M11b. For how many days did he/she receive medicine?	
--	--

M12. Was your baby officially diagnosed with any illness during the last week (7 days)?	1	yes	
	2	no (go to question M14)	

M13. With what illness was your baby diagnosed?
.....
.....

M14. Was your baby hospitalised during the last week (7 days)	1	Yes *	
	2	no (end of questionnaire)	

M15. How long was your baby in hospital? (days)	
---	--

When did your baby's stay in hospital start?	Date: ____/____/ 20____
When did your baby's stay in hospital stop?	Date: ____/____/ 20____ Or is it carrying on? _____

*** If marked "yes": Fieldworkers please collate information for study nurse to complete AE form**

M16. Why was your baby hospitalised?

ADDENDUM 6: Cross-sectional study - informed consent form



INFORMATION SHEET

Study title: Assessment of breast milk iodine and n-3 fatty acid concentrations, iodine, iron and n-3 fatty acid status and thyroid function of lactating women and their infants

Go kgethisa masi a letsele gore a na le iodine, n-3 fatty acids go go kanakang le modiro wa kgeleswa ya thyroid.

Project leader/ Moeteledi pele:

Prof Marius Smuts

Co-project leader/ Motlatsa-moeteledi pele:

Dr. Jeannine Baumgartner

Dear Parent / Legal Guardian/

Motsadi/motlhokomedi

Who are we?

Re bo mang?

We are from the North West University, Potchefstroom. We are testing the breast milk of mothers who are mainly breast feeding their babies, for iodine and n-3 fatty acid concentrations. We are also testing the iodine, iron and n-3 fatty acid status and thyroid function of lactating mothers and their babies. We invite you to participate in this study.

Re tswa ko Yunibesiting ya bokone-bophirima, Potchefstroom. Re tlathloba mashi a letsele a bo mme ba ba amusang masea a bone gore a na le iodine the n3-fatty acids go le kanakang. Gape re tlathloba modiro wa kgeleswa ya thyroid mo maseeng le bo mme ba ba antshang masea. Re go mema go tsaya karolo.

Why do we want to do the research?

Lebaka la tlathlobe e, ke lefe?

Micronutrients such as iron and iodine, as well as n-3 fatty acids are needed for proper brain development and functioning. The micronutrient and n-3 fatty acid status of breast fed babies depends on the micronutrient and n-3 fatty acid concentrations in the breast milk, which they get from their mothers. Currently there is no available data on breast milk iodine and n-3 fatty acid concentrations of lactating mothers in South Africa. There is also no existing data on the iodine and n-3 fatty acid status and thyroid function of lactating South African women and their breast fed babies. This information is needed to optimise the nutritional status of lactating mothers and their developing infants.

Dikotla tse di tshwanang le iodine, iron le n2-fatty acids di tlhokagala mo mebeleng go thusa book go dira le go gola. Masea a ikanyetse go bo mme go ba neya dikotla tse go tswa mo masing a letsele. A go di tokomane tse di tlhalosang maemo a dikotla tse mo bommemg ba Afrika borwa le masea a bona, ke ka moo re batlang go itse gore re kgone go fa bomme le masea a bone dikotla tse di lekanetseng.

What do we expect from the participants in the study?

Re solofela eng go tswa go bomme ba ba tsayang karolo?

If you agree to participate in this study, we will ask you and your baby to visit our metabolic unit at the North West University (Pukke) for a day where you will be asked to do the following:

Ga o dumela go tsaya karolo, re tlo kopa o tle kwa tliniking ya rona ko Yunibesiting ya bokone-bophirima letsatsi le le nngwe fela go re go dire tse di latelang:

We will ask you some questions on food sources that provide iodine as well as on infant feeding practices.

Re tlo go go botsa ka mefuta ya dijo tse o dijang le tse o dineelang lesea la gago.

Your weight and height will be taken. The weight, length and head circumference of your baby will also be taken.

Re tlo go tsaya bo kete le bo telle bag ago le lesea la gago

Urine (40ml for mother; 5ml for baby) and blood (4 ml for mother; 2 ml for baby) samples will be taken from both you and your baby.

Re tlo kopa go tsaya moroto le madi go wean le lesea la gago.

Urine from your baby will be collected using a special urine collection nappy.

Moroto wa lesea la gago o tla tsewa go tswa mo leiring le tle tlo mo apesang lona.

You will be asked to provide a 15 ml breast milk sample. This can be expressed manually or by using an electric breast pump.

Re tlo go kopa go re neya masi a letsele la gago.

All the information collected is confidential and will not be given to anybody else; only the researchers will have access to it.

Polelo yotlhe e o re neyang yona e tlo ba sephiri magareng a ron ale wean fela.

Must I participate?

Ke tshwanetse go tsaya karolo?

Participation in this study is completely voluntary (your own choice). Whether you do, or do not, give your permission will not influence you or your child's access to health care in any way.

Go tsaya karolo go tswa go wena, ga se kgapeletso. Go se tseye karolo go ka se ame tokelo ya gago le ngwana wa gago mo dipetleleng le ditliniki.

May I change my mind?

A nka fetola mogopolo?

You may change your mind at any time without having to give a reason. The study is completely voluntary and it will not be kept against you in any way should you decide to withdraw from the study.

O ka fetola mogopolo nako nngwe le nngwe le ga o sa re fe lebaka. Go tsaya karolo go tswa go wena gape o na le tokelo ya go tswa.

What happens to the collected data?

Go diriwang ka tsotlhe tse di buiwang le tse di tsewang go nna le ngwana wa me?

All the data collected during the study will be made anonymous, handled with strict confidentiality and used only for scientific purposes. We will keep all biological samples (blood, urine) frozen at the study site for 5 years. After these 5 years all samples will be destroyed and discarded. The obtained data will be stored safely and reported only in an anonymous form. Only the responsible investigators and the members of the ethical committee have access to the original data under strict confidentiality.

Tsotlhe tse di diriwang le tse di buiwang magareng a rona le wena di tlo itsiwe ke rona fela. Re tliile go boloka di rekhoto tsotlhe go fitlhela me nwgaga e le methano, morage ga moo di tlo go latlhiwa.

Do I get a reimbursement for participating in the study?

A ke a lebogiwa ga ke tseya karolo?

You will receive a hamper with products for your baby as a token of appreciation and as a compensation for the time you are spending at the metabolic unit (half day).

Ga o tsaya karolo o tlo go fiwa hamper ya dimpho tsa ngwana.

Who can you contact if there are any queries?**Nka ikgolaganya le mang ga ken a le dipotso**

For more information on the study you may contact Prof Marius Smuts at 018-299 2086 or 082 451 0486 **OR** Dr. Jeannine Baumgartner at 018-299 4011 or 0764364439 during office hours.

Ga o na le dipotso o ka letsetsa Prof Marius Smuts go 018-299 2086 kgotsa 082 451 0486 kgotsa Dr. Jeannine Baumgartner go 018-299 4011 or 0764364439

The study has been approved by the Ethics Committee of the North West University. If you have any queries or problems regarding the study, you can contact Prof Amanda Lourens the chairperson of the NWU Ethics Committee at (018) 299 2606.

Ga o na le ditsetlebo mapi le se dirwang, o ka letsetsa Prof Amanda Lourens (Modula setilo wa NWU Ethics Committee) go (018) 299 2606.

If you are happy for you and your child to take part in the study, please read and sign the consent form.

Ga o batla go tsaya karolo wena le lesea la gago, buisa tsebe e latelang ga o fetsa, o e tshwae.

ADDENDUM 7: Cross-sectional study participant consent form



PARTICIPANT CONSENT FORM

Study title: Assessment of breast milk iodine and n-3 fatty acid concentrations, iodine, iron and n-3 fatty acid status and thyroid function of lactating women and their infants.

Go kgethisa masi a letsele gore a na le iodine, n-3 fatty acids tse di kanakang le modiro wa kgeleswa ya thyroid mo basading ba amusing masea le mo maseeng a bone.

	YES/Eya	NO/Nnya
I voluntarily agree to take part in the study. Ke dumela go tsaya karolo		
I have been informed about and understand the purpose of the study. Ke bolelletswe tsotlhe e bile ke tlhaloganya mosola wa teng.		
I understand that I can withdraw my consent at any time and it will not affect my routine health care service received at any health care center. Ke tlhaloganya gore kena le tokelo ya go tlogela go tsaya karolo ga ke batla.		
I have been informed that all information will be treated as private and confidential. Ke bolelletswe gore tsotlhe di tla ba sephiri sa me le ba batlisisi ba Yunibesiti ya Bokone-Bophirima		
I understand that the following will be expected from me: (i) I and my child will be assessed for weight, height and circumferences at the beginning of the study. Nna le lesea la me re tlike go sekese kiwa bokete, bo llele le bo phara ba tlhogo kwa thsimologong. (ii) Blood and urine samples will be collected from me and my baby. Go tlo tseiwa madi le moroto go nna le lesea la me. (iii) I am required to express a sample of my breast milk. Ke tlhoka go ga molola bonnye ba masi a letsele la me.		
I have been given an opportunity to ask any questions regarding the study Kefilwe sebaka sa go botsa dipotso ga ke sa tlhaloganye.		

Would you be willing to be approached for other studies?

Yes ☐ No ☐

A oka rata go ka lalediwa ka di patlisiso tse dingwe gape?

If I have any further queries or problems regarding the study, I can contact Prof Amanda Lourens the chairperson of the NWU Ethics Committee at (018) 2992606.

Ga ke na le dipotso tse dingwe ke filwe tetla ya go letsetsa Prof Amanda Lourens go (018) 2992606.

Name of mother/Lebitso la mme:

Name of baby/Lebitso la ngwana:

Participant code/Nomoro ya karolo:

Phone number/Nomoro ya mogala:

I hereby give consent that I and my baby may participate in the study.

Ke dumela gore nna le lesea la me re tseye karolo.

Signature/Letshwao:

Date/Letatsi:

Signed at/Tshailwe ko:

Witness/Paki:

I hereby give consent that my weight, height and circumferences and that of my baby can be measured.

Ke dumela gore nna le lesea la me re tseye karolo, go kadiwa boima le bo telele.

Signature/Letshwao:

Date/Letsatsi:

Signed at/Tshwailwe ko:

Witness/Paki:

I hereby give consent that my blood sample (4 ml) can be taken.

Ke dumela gore madi (4 ml) a tseiwe go nna.

Signature/Letshwao:

Date/Letsatsi:

Signed at/Tshwailwe ko:

Witness/Paki:

I hereby give consent that the blood (2 ml) of my baby can be taken.

Ke dumela gore madi (2 ml) a tseiwe go lesea la me.

Signature/Letshwao:

Date/Letsatsi:

Signed at/Tshwailwe ko:

Witness/Paki:

I hereby give consent that my breast milk (15 ml) and urine (40 ml) samples, and urine (5 ml) samples of my baby can be taken. Ke dumela gore moroto (40 ml) le masi a letsele (15 ml) a tseiwe go nna le lesea la me.

Signature/Letshwao: Date/Letsatsi:

Signed at/Tshwailwe ko: Witness/Paki:

Fieldworker who informed the mother/Motlhalosi:

Researcher/Motlhotlhomisi:

Thank you for your participation!
Re lebogela go tseya karolo ga gago!

ADDENDUM 8: Cross-sectional study demographic questionnaire



Cross-sectional study on assessment of breast milk iodine concentrations and iodine status and thyroid function of lactating women and their infants

Participant number:

C	S			
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Date of Interview (DD/MM/YYYY):/...../.....

Fieldworker's name:

Section A: Socio-demographic and economic data

Baby

- 1) Gender: M ☐ F ☐
- 2) Age (in weeks):
- 3) Date of birth (DD/MM/YYYY):/...../.....
- 4) Birth weight (grams):

Mother

- 5) Age (in years):
- 6) Date of birth (DD/MM/YYYY):/...../.....
- 7) Ethnicity:
 - ☐ Black
 - ☐ Coloured
 - ☐ Indian or Asian
 - ☐ White
- 8) Marital status:

- ☐ Married
☐ Single
☐ Living together
☐ Divorced/ separated
☐ Widowed

9) Educational level:

- ☐ Primary (grade 1-7)
☐ Secondary (grade 8-12)
☐ Tertiary (college/ university)
☐ None
☐ Other:

10) How many children do you have?

11) Are you currently employed? Yes ☐ No ☐

12) Do you smoke at present? Yes ☐ No ☐

13) Did you smoke before pregnancy? Yes ☐ No ☐

Section B: Information on breast feeding, infant feeding practices and on diet

- 1) Are you currently breastfeeding your baby? Yes ☐ No ☐
 2) How long do you plan to breast feed your baby?
 3) Do you exclusively breastfeed your baby (incl. bottled breast milk)? Yes ☐ No ☐
 4) If yes, how many times per day (24h) do you breastfeed?
 5) If no, how many times per day (24h) do you breastfeed?
 6) If no, please describe everything that the baby ate and/or drank yesterday during the day and night:

Breakfast	Midmorning	Lunch	Mid afternoon	Supper	After supper

- 7) Is this diet recall typical of what your baby eats on a daily basis? Yes ☐ No ☐
 8) What breast milk substitute do you use?

Type	Yes / No	Brand / Ingredients (for home-made substitute)
Commercial infant cereal		
Homemade porridge/cereal		

Commercial cereal/porridge (not specifically made for infants)		
Cow's milk		
Tea		
Juice		
Water		
other		

9) What is the reason for using a breast milk substitute?

Diet of mother

Did you take any supplements during pregnancy? Yes ☐ No ☐

If yes, what type/brand of supplements?

Do you take any supplements at the moment? Yes ☐ No ☐

If yes, what type/brand of supplements?

10) Where do you buy your salt (choose an option)?

☐ Supermarket

☐ Spaza shop

☐ Other:

11) Do you add table salt to your food before eating (or tasting)?

Never 1	Rarely 2	Sometimes 3	Often 4	Always 5
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12) In the food you eat at home, is table salt added during cooking?

Never 1	Rarely 2	Sometimes 3	Often 4	Always 5
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13) Do you consume seafood (apart from fish)?

Never 1	Rarely 2	Sometimes 3	Often 4	Always 5
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If yes, what types of seafood do you consume?

.....
.....

14) Do you consume fish in your household?

Never 1	Rarely 2	Sometimes 3	Often 4	Always 5
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If yes, what type of fish do you consume?

☐ Tinned fish specify:

- ☐ Fresh fish: salt water fish specify: ☐
- Fresh water fish specify: ☐ Dried
- fish specify: ☐ Other
- specify:

15) Do you consume cow's milk?

Never 1	Rarely 2	Sometimes 3	Often 4	Always 5
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16) Do you eat eggs?

Never 1	Rarely 2	Sometimes 3	Often 4	Always 5
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17) Do you eat meat? (Beef, poultry, pork)

Never 1	Rarely 2	Sometimes 3	Often 4	Always 5
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18) What type of meat do you consume the most (indicate with 1) and second most (indicate with 2) etc.?

- ☐ Chicken
- ☐ Liver (from any animal)
- ☐ Beef
- ☐ Lamb
- ☐ Goat
- ☐ Pork
- ☐ Sausages (incl. polony, viennas and boerewors)

19) What type and brand of margarine/oil do you use for food preparation and on bread?

Food preparation:

Bread:

22) How often do you eat away from home (at fast food outlets or take-aways)?

Never 1	Rarely 2	Sometimes 3	Often 4	Always 5
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Thank you for your participation!!