

Effects of lipid-based nutrient supplements on the immunity of 6-month old infants: A randomised controlled trial

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PREFACE

Twenty years from now you will be more disappointed by the things you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbour. Catch the trade winds in your sails. Explore. Dream. Discover.

Mark Twain.

“Daai selfde feeling van ontdekking gaan jy weer kry” - 2 September 2014

Prof Johann Jerling

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ABSTRACT

Background: Nutrition has the ability to modulate immune function. Both innate immunity and adaptive immunity may be affected. There are however, very little data available where infants are concerned. In developing countries infants often do not meet their nutritional requirements making them more susceptible to infectious diseases. Therefore there is a global shift towards reducing morbidity and mortality in infants through nutritional interventions such as administering small quantity lipid-based nutrient supplements (SQ-LNS).

Objective: To determine the effect of two SQ-LNS on the immunity of South African peri-urban 6-month-old infants.

Design: In a randomised controlled trial two groups of infants (n = 250 each) were supplemented with two SQ-LNS products for six months in Jouberton, Klerksdorp in the Matlosana Municipality in South Africa. The control group (n = 250) did not receive any supplements during the intervention period, but received a six month long intervention with SQ-LNS A upon completion of the study. Both products comprised of similar micronutrients and essential fatty acids (EFA), but SQ-LNS B also included long-chain polyunsaturated fatty acids (LCPUFA), phytase, lysine and additional vitamin C and trace elements. During weekly surveillance, the incidence and duration of infectious morbidity symptoms were recorded by fieldworkers using a questionnaire. In addition, the effect of the intervention on immune function was assessed by comparing the immunoglobulin G (IgG) response to immunisation against measles between the intervention and control groups.

Results: Both SQ-LNS A and B reduced the overall sick days with fever by 19.2% ($P < 0.001$) and 9.8% ($P = 0.006$); of coughing by 9.2% ($P < 0.001$) and 5.7% ($P = 0.001$) and of wheezing by 78.0% ($P < 0.001$) and 53.1% ($P < 0.001$), respectively. SQ-LNS A reduced the number of incidences of wheezing by 57.4% ($P = 0.006$) and decreased the overall sick days with runny nose by 6.6% ($P = 0.002$), whereas SQ-LNS B increased the overall sick days with runny nose by 4.5% ($P = 0.035$). Both SQ-LNS A and SQ-LNS B increased the overall days of diarrhoea by 29.6% ($P < 0.001$) and 68.4% ($P < 0.001$) and both groups showed an increase in the number of incidences of diarrhoea by 24.6% ($P = 0.002$) and 25.5% ($P = 0.001$), respectively. The SQ-LNS A group had 4.8 times more overall sick days of vomiting ($P < 0.001$) and the SQ-LNS B group had 2.4 times more overall sick days of vomiting compared to the control group ($P < 0.001$). Both intervention groups had 2.4 times more incidences of vomiting compared to the control group ($P < 0.001$). Both SQ-LNS A and B increased the overall days with rash/sores by 13.0% ($P = 0.012$) and 99.6% ($P < 0.001$), and increased the number of incidences of rash/sores by 36.2% ($P = 0.031$).

and 41.9% ($P = 0.013$), respectively. Measles IgG response was significantly reduced by SQ-LNS-B ($B: -608.337$ 95% CI: $-1178.974, -37.699$).

Conclusion: These novel findings are a significant advancement to current knowledge. It is one of the first soy-based SQ-LNS intervention studies in infants. Both SQ-LNS products reduced fever and certain respiratory symptoms. However, there was an increase in diarrhoea, vomiting and rash/sores in both groups, although the symptoms were mostly mild and not serious. Smaller quantities taken more frequently may provide the benefits without the side effects of the products. Nevertheless, the results should be interpreted taking the main outcomes of this study, linear growth and psychomotor development into consideration. This study showed the need not only for more trials to be done on this topic but also to monitor and record morbidity data frequently and accurately.

OPSOMMING

Agtergrond: Voeding het die vermoë om immunfunksie te beïnvloed. Beide aangebore immuniteit en verworwe immuniteit kan gemoduleer word deur nutriënte. Daar is egter baie min data beskikbaar wat babas betref. In ontwikkelende lande word daar dikwels nie voldoen aan babas se voedingsbehoefte nie en dit maak hulle meer vatbaar vir aansteeklike siektes. Daarom is daar 'n wêreldwye neiging om morbiditeit en mortaliteit as gevolg van wanvoeding in babas te probeer verminder deur ingrypings soos klein-hoeveelheid lipied-gebaseerde nutriënt supplemente (KH-LNS) te gebruik.

Doelwit: Om die effek van twee KH-LNSe op die immuniteit van Suid-Afrikaanse buitestedelike 6-maande-oue babas te bepaal.

Ontwerp: Twee groepe babas ($n = 250$ elk) is vir ses maande lank in 'n gerandomiseerde studie gesupplementeer met twee KH-LNS produkte in Jouberton, Klerksdorp in die Matlosana Munisipaliteit in Suid-Afrika. Die kontrole groep ($n = 250$) het nie enige aanvullings ontvang tydens die ingryping nie, maar is vir ses maande lank met KH-LNS A gesupplementeer na afloop van die studie. Beide produkte het bestaan uit soortgelyke mikronutriënte en essensiële vetsure, maar KH-LNS B het lang ketting poli-onversadigde vetsure (LKPOV), fitase, lisien en ekstra vitamien C en sekere spoorelemente ook bevat. Die voorkoms en tydsduur van aansteeklike siekte simptome is tydens weeklikse moniteringsbesoeke deur veldwerkers ingesamel met behulp van vraelyste. Daarbenewens is die effek van die intervensie op immuunfunksie ondersoek deur die immunoglobulien G (IgG) reaksie op die immunisering teen masels tussen die intervensie en kontrole groepe te vergelyk.

Resultate: Beide KH-LNS A en B het die algehele siek dae met koors met 19,2% ($P < 0.001$) en 9,8% ($P = 0.006$); van hoes met 9,2% ($P < 0.001$) en 5,7% ($P = 0.001$); en van hyging met 78,0% ($P < 0.001$) en 53,1% ($P < 0.001$), onderskeidelik, verminder. KH-LNS A het die aantal voorvalle van hyging met 57,4% ($P = 0.006$) en die algehele siek dae met loopneus met 6,6% ($P = 0.002$) verminder, terwyl KH-LNS B die algehele siek dae met loopneus met 4,5% ($P = 0.035$) vermeerder het. Beide KH-LNS A en KH-LNS B het die algehele dae met diarree met 29,6% ($P < 0.001$) en 68,4% ($P < 0.001$), onderskeidelik, laat toeneem. Beide groepe het 'n toename in die aantal voorvalle van diarree van 24,6% ($P = 0.002$) en 25,5% ($P = 0.001$), onderskeidelik, tot gevolg gehad. Die KH-LNS A groep het 4.8 keer meer ($P < 0.001$) en die KH-LNS B groep het 2.4 keer meer algemene siek dae van braking gehad in vergelyking met die kontrole groep ($P < 0.001$). Beide intervensie groepe het 2.4 keer meer voorvalle van braking in vergelyking met die kontrole groep ($P < 0.001$) gehad. Beide KH-LNS A en KH-LNS B die algehele dae van uitslag/sere met 13,0% ($P = 0.012$) en 99,6% ($P < 0.001$), onderskeidelik, vermeerder. Beide die produkte het ook die

aantal voorvalle van uitslag/sere met 36,2% ($P = 0.031$) en 41,9% ($P = 0.013$), onderskeidelik, laat toeneem. Masels IgG reaksie was aansienlik verminder in KH-LNS-B ($B: -608.337$ 95% CI: $-1178.974, -37.699$).

Gevolgtrekking: Hierdie nuwe bevindinge is 'n beduidende vooruitgang op huidige kennis. Hierdie was een van die eerste soja-gebaseerde KH-LNS intervensie studies in babas. Beide KH-LNS produkte het koors en sekere respiratoriese simptome verminder. Daar was egter 'n toename in diarree, braking en uitslag/sere in beide groepe, alhoewel die simptome meestal lig en nie ernstig was nie. Kleiner hoeveelhede wat meer gereeld geneem word kan moontlik die voordele van die produkte voorsien sonder die newe-effekte. Nieteenstaande het KH-LNS B beide groei en psigomotoriese ontwikkeling verbeter. Hierdie studie het nie net getoon daar 'n behoefte vir meer studies oor die onderwerp is nie, maar ook om morbiditeitsdata tydens hierdie studies gereeld en akkuraat in te samel.

LIST OF ABBREVIATIONS

AE	adverse event
AGP	alpha-1-acid glycoprotein
ALA	alpha-linolenic acid
APC	antigen-presenting cells
ARA	arachidonic acid
BMI	body mass index
CF	complementary foods
CRA	clinical research associate
CRF	case report form
CRO	clinical research organization
CRP	c-reactive protein
DHA	docosahexaenoic acid
DMP	data management plan
DOH	Department of Health
DRI	dietary reference intake
EAR	estimated average requirement
EDTA	ethylene-diamine-tetra-acetic
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
FA	fatty acids
FAME	fatty acid methyl ester
Fe	iron
FTU	phytase unit

GAIN	Global Alliance for Improved Nutrition
GCMSMS	gas chromatography tandem mass spectrometry
GMT	geometric mean titres
HACCP	hazard analysis and critical control points
HAZ	height-for-age z scores
Hb	haemoglobin
HIV	human immunodeficiency virus
IL	interleukin
LA	linoleic acid
LAZ	length-for-age z scores
LCPUFA	long-chain polyunsaturated fatty acids
LNS	lipid-based nutrient supplement
MCV	measles-containing vaccine
MDG	Millennium Development Goal
MHC	major histocompatibility complex
MN	micronutrient
MNP	micronutrient powder
MRC	Medical Research Council
MUAC	mid-upper arm circumference
MUFA	monounsaturated fatty acids
NIP	National Immunization Programme
NK	natural killer cells
NWU	North-West University
PAMPS	pattern-associated molecular patterns
PGE ₂	dienoic prostaglandins

PUFA	polyunsaturated fatty acids
RBC	red blood cells
RCT	randomised controlled trial
RNI	recommended nutrient intake
ROS	reactive oxygen species
RSA	Republic of South Africa
SAE	serious adverse event
SDV	source data verification
SMB	safety monitoring board
SQ-LNS	small-quantity lipid-based nutrient supplement
Th1	T-helper 1
Th2	T-helper 2
TNF	tumor necrosis factor
URI	upper respiratory infections
USA	United States of America
WHO	World Health Organization
Zn	zinc

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CHAPTER 1 INTRODUCTION

1.1 Rationale of the study

The purpose of Millennium Development Goal (MDG) 4 was to reduce child mortality by two-thirds between 1990 and 2015, and although great progress has been made (53% reduction), the target was not reached. It was reported that 5.9 million children under the age of five died in 2015 (nearly 16000 per day), and 83% of deaths were attributed to either infectious diseases, neonatal or nutritional causes (WHO, 2015; WHO, 2016a). Among the main contributors to these infectious diseases are respiratory diseases, and gastrointestinal illnesses, which remain the leading cause of child mortality, especially in low socio-economic populations (WHO, 2003b; Liu *et al.*, 2012; Bhutta & Black, 2013).

It is well accepted that nutrition influences the immune system and has the ability to modulate resistance to infection (Albers *et al.*, 2007; De Rosa *et al.*, 2015). There is an increasing body of evidence which suggests that supplementing the diets of infants and children with long-chain polyunsaturated fatty acids (LCPUFAs) improves immune function and therefore has a positive effect on respiratory health (Hageman *et al.*, 2012). Thus, efforts to reduce respiratory diseases include, amongst others, nutritional approaches such as micronutrient (MN) and fatty acid (FA) supplementation making use of different carrier platforms (Birch *et al.*, 2002; Smuts *et al.*, 2005; Pastor *et al.*, 2006; Adu-Afarwuah *et al.*, 2007; Minns *et al.*, 2010; Imhoff-Kunsch *et al.*, 2011).

The first 1 000 days of life, from conception to a child's second birthday, has been shown to offer a critical window of opportunity during which optimal nutrition gives children a healthy start in life (Black *et al.*, 2013). But many vulnerable population groups (such as children under the age of five) suffer from nutritional deficiencies in developing countries. Especially in children, nutrient deficiencies increase infectious morbidity and mortality, and even the ones that survive have an increased risk of not developing to their full potential (Smuts *et al.*, 2005). One of the causes of nutrient deficiencies is the low micronutrient content of complementary foods (CF) found in low socio-economic populations (Adu-Afarwuah *et al.*, 2007). Due to this, it is presumable that these infants will have several, coexisting deficiencies as poor nutrition generally causes multiple deficiencies (Smuts *et al.*, 2005). Most nutrients play an essential role in maintaining an optimal immune response, which means that any nutrient in deficiency, and also certain nutrients in excess, can compromise the immune system and have negative effects, making an infant susceptible to many pathogens (Field *et al.*, 2002).

Exclusive breastfeeding provides adequate nutrition for infants and is encouraged up until six months of age in developing countries, after which the inclusion of nutritious CF is recommended together with continued breastfeeding up until two years of age (WHO, 2003a; Hageman *et al.*, 2012). However, the

danger of nutrient deficiencies is greatly increased when CF are introduced and the infants are weaned onto a household diet (Brown *et al.*, 1998). It has thus been suggested that, in food-insecure populations, if foods which are locally available do not provide sufficient levels of macro- and micronutrients, then in-home fortification of foods (such as micronutrient powders or lipid-based nutrient supplements [LNS]) may be necessary to provide the rest of the nutrients the infants require for optimal growth (Huffman & Schofield, 2013).

Traditionally, recommended intake levels of nutrients were based on preventing deficiencies, but in recent years the approach started shifting towards recommendation levels which are associated with specific disease prevention (WHO, 2004). A relatively low-cost approach to prevent nutrient deficiencies among infants and children is to supplement them with a small-quantity lipid-based nutrient supplement (SQ-LNS), with the aim of improving their overall nutrient status, and in effect, possibly improving growth and development, as well as decreasing infectious morbidity (Adu-Afarwuah *et al.*, 2007; Dalton *et al.*, 2009; Hess *et al.*, 2015).

A number of food supplements have been developed and tested for the purpose of enriching CF in a cost-effective manner to prevent growth faltering. Examples of these are: energy-dense “Nutri-butter” (fat paste), “Sprinkles” (powder), “Nutritabs” (tablets) (Adu-Afarwuah *et al.*, 2007) and a fatty bread spread (Dalton *et al.*, 2009). These had positive results in terms of growth (Adu-Afarwuah *et al.*, 2007) and cognitive function (Dalton *et al.*, 2009). However, more information is needed on the effect of SQ-LNS, on the infectious morbidity of infants and children.

1.2 The study site

The study population consisted of 6-month-old infants from the peri-urban Jouberton area in the Matlosana Municipality, Klerksdorp, South Africa (Figure 1-1).

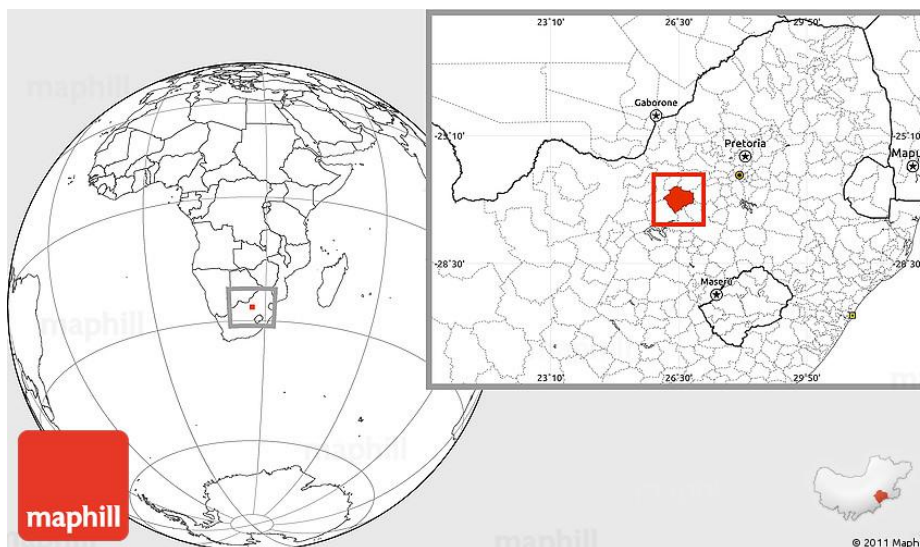


Figure 1-1: The peri-urban Jouberton area in the Matlosana Municipality, Klerksdorp, South Africa. Available at: <http://www.maphill.com/south-africa/north-west/klersdorp/jouberton/location-maps/blank-map/free/>. Accessed: 26/10/2016

1.3 Aim

This study aims to determine the effect of the intake of two SQ-LNS, on the incidence and duration of infectious morbidity symptoms and the response to vaccination in 6-month old infants.

1.4 Objectives

- (1) To determine the effect of SQ-LNS A and SQ-LNS B (each in comparison to the control group) on the incidence and duration of infectious morbidity symptoms in infants between 6-12 months.
- (2) To determine the effects of SQ-LNS A and SQ-LNS B (each in comparison to the control group) on the response to vaccination against measles in infants between 6-12 months.

1.5 Ethical Approval

This randomised controlled trial was registered at clinicaltrials.gov under NCT01845610 and ethical approval was obtained from the Ethical Committees of the North-West University (NWU-00001-14-A1) and the South African Medical Research Council (SAMRC, EC011-03/2012).

1.6 Research team

The members of the research team and their main roles and contributions are listed in Table 1-1.

Table 1-1: Research team and their responsibilities.

Name	Role and contribution to study	Signature
Marius Smuts ¹	Supervisor of this study, principal investigator of the Tswaka study and involved in study design and responsible for overall data collection and quality control, guidance on collection and analysis of biochemical data, academic input, revision of paper and co-author.	
Mieke Faber ²	Co-principal investigator of the Tswaka study involved in training, guidance on data collection, quality control and analysis of feeding practices, academic input and guidance on interpretation of research results, review of article and co-author.	
Salome Kruger ¹	Provided training, guidance, quality control and direction on the interpretation of anthropometry and checking the final data.	
Carl Lombard ¹	Tswaka trial statistician; guidance on statistical analysis and interpretation, revision of paper and co-author.	
Marinel Rothman ¹	One of the study coordinators of the Tswaka trial, supervising field data collection and data quality control of feeding practices. Review of article and co-author.	
Tonderayi Matsungu ¹	Co-study-manager of the Tswaka study, responsible for anthropometry measurements, supervising field data collection and quality control and study product management. Review of article and co-author.	
Linda Malan ¹	Co-supervisor of this study, responsible for immune and morbidity data and study planning; statistical analysis, review of article and co-author.	

Name	Role and contribution to study	Signature
Karen Joosten ¹	MSc student, responsible for morbidity data collection and data capturing, involved in dietary data quality control, biochemical analysis, immune and morbidity data analysis and interpretation of results. Full responsibility of writing mini-dissertation and first author on manuscript.	

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²South African Medical Research Council (SAMRC)

1.7 Structure of this mini-dissertation

The following literature review (Chapter two) is divided into three sections: Nutrition in infants, immunity, and a non-systematic review on nutrition and immunity in infants and children. The first two sections will outline basic concepts and provide a broad understanding of each subject, while the third section provides evidence from previous studies in which the aim is to determine the effects of LNS on the infectious morbidity, mainly respiratory diseases and gastrointestinal illness in infants and children.

For the aim of the non-systematic review, a search was done in the following databases: Google Scholar, ScienceDirect, Pubmed, EbscoHost, and the Cochrane library. Articles which were selected for this review were found by using the following words and phrases, individually and in combination: “lipid-based nutrient supplement (LNS)”, “omega-3/n-3”, “morbidity”, “infants”, “children”, and “micronutrient deficiencies”. Only English articles were included in the search. However, an inclusion and exclusion criteria (Table 1-2) was used for the selection of the articles for the purpose of focusing on the research question. Additional studies were obtained through the “snowball” technique. The intention was to focus only on children under the age of five, but it was decided to also include randomised controlled trials (RCT) in which the test subjects were older than five years because the available evidence is so limited for children under five years of age.

Chapter Three describes the methodology used throughout the intervention study.

Chapter Four is a manuscript with the title “The effect of small-quantity lipid-based nutrient supplements on the immunity of 6-month-old infants: A randomised controlled trial”. It was prepared for the Public Health Nutrition Journal and possible presentation of the results at National and International conferences.

Table 1-2: Inclusion and exclusion criteria for the inclusion of RCT for this non-systematic review.

Inclusion	• Interventions which used lipids as a main supplement • Outcome measurements included either incidence or duration of infectious disease as well as including absenteeism from school (related to infectious disease) as an indicator of immune function • Age: 0-12yrs • Apparently healthy subjects
Exclusion	• Preterm infants • Wasted or undernourished subjects • Children with known positive HIV status

1.8 References

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CHAPTER 2 LITERATURE REVIEW

2.1 Section 1 – Nutrition in infants

2.1.1 Nutrition requirements and actual nutritional intake in South African infants

Exclusive breastfeeding is promoted in the developing world as the nutrition of choice for all infants up until 6 months of age, as there are a host of advantages associated with breastfeeding which include: ideal nutritional composition, it provides enzymatic and immunologic properties, it is economic and convenient, there is a decreased risk for respiratory and gastrointestinal infections, and there is improved cognitive development (WHO, 2003a; Koletzko *et al.*, 2008; Porter & Kaplan, 2011; Mahan & Escott-Stump, 2012; Samour & King, 2012). However, in South Africa, the percentage of mothers who exclusively breastfeed their infants for up to four months and up to six months is 11.8% and 8.3%, respectively (WHO, 2010). These figures are very low when comparing them to other developing African countries such as Rwanda and Malawi, where the exclusive breastfeeding percentages for less than 6 months are 88.4% (WHO, 2005) and 56.7% respectively (WHO, 2006). Furthermore, in a study done by Mamabolo *et al.* (2004) in the Limpopo region, Republic of South Africa (RSA), they found that it was uncommon for mothers to exclusively breastfeed their infants beyond three months of age, where 56% of infants had received some form of complementary food by the end of their first month (Mamabolo *et al.*, 2004). Consequently, the risk for nutrient deficiencies is higher when infants are weaned onto a household diet (Brown *et al.*, 1998), thus contributing to “hidden hunger” at an early age. Hidden hunger is a form of malnutrition where there may be sufficient energy intake, but the essential micronutrient intake or other nutrients, such as essential fatty acid intake, is inadequate without necessarily displaying any major visible outward clinical signs, for example wasting. However this does not only occur when sufficient energy is consumed, micronutrient deficiencies are also often associated with insufficient and excess energy intake (Steyn & Temple, 2008).

2.1.2 Micronutrients

Micronutrients such as vitamins and minerals play significant roles in the human body and healthy infants are expected to consume sufficient amounts when they are exclusively breastfed for the first few months of life. However, from around six months of age, infant’s growth starts to slow down and they are weaned onto a household diet. There does not seem to be a large difference in nutrient needs at this point in time except for certain nutrients such as iron (Fe) and zinc (Zn) which have an increased requirement (Otten *et al.*, 2006).

In developing countries the cereal-based diets usually contain a large amount of phytates (which binds trace elements) and too few animal products (Fe-rich food sources including meat, fish, and poultry which have a high bioavailability as they consist largely of haem Fe), which diminishes Fe and Zn absorption and causes low vitamin intakes (Adu-Afarwuah *et al.*, 2007). Micronutrients work synergistically with one another as they interact with one another. Typical examples of this are the interactions which Fe and Zn share as they compete for the same binding sites. Furthermore, Zn deficiency may negatively affect vitamin A as this deficiency influences the mobilisation of vitamin A from the liver and its transport into the circulation (Otten *et al.*, 2006). Public health interventions are now focusing on alleviating micronutrient deficiencies (especially Fe) so as to reduce the associated risk of infectious disease (WHO, 2003a). However, not only do Fe and Zn affect immune function, a number of other micronutrients are linked to immune function as well including vitamin A, vitamin C, vitamin E, vitamin D and folate. (Calder, 2002; De Rosa *et al.*, 2015).

2.1.3 Iron

Iron deficiency is the most common preventable nutritional deficiency affecting more than half the world's population, and has been identified as one of the largest disease burdens regarding micronutrients (Vist *et al.*, 2011; WHO 2001; WHO 2009). Iron is found *in vivo* in proteins such as enzymes, cytochromes, myoglobin and the majority is found in haemoglobin (Hb). Its main function is to transport oxygen (through Hb) from the environment to the body's tissues to aid in metabolism. Furthermore, it exists in different oxidation states, namely ferrous, ferric, and ferryl. Dietary sources of Fe are shown in Table 2–1 as dietary forms of Fe are classified as either haem or non-haem Fe. When Fe levels are low, less oxygen is transported to the rest of the body, energy metabolism is altered, and this has a great impact on many bodily functions. Iron deficiency may lead to anaemia and the risk for anaemia is especially high in infants as they go through rapid growth and development (Otten *et al.*, 2006).

Haem Fe is absorbed better than non-haem Fe. Table 2–1 shows the different food sources and under which category they fall. As non-haem Fe is not so well absorbed, it is recommended to combine it with Vitamin C-rich food sources to improve the absorption (Otten *et al.*, 2006; Nair & Iyengar, 2009).

Table 2-1: Food sources categorized by the type of iron they contain

Haem iron	Non-Haem iron
Red meat (Beef, pork, lamb, mutton)	Iron-fortified cereals and bread
Seafood (Shrimp, clams, sardines, oysters)	Vegetables (Spinach, broccoli, silver beet)
Poultry (Chicken, duck, turkey)	Nuts
Liver	Legumes
	Dried fruits
	Eggs

(Otten *et al.*, 2006)

2.1.4 Iron and immunity

The role that Fe plays in resistance to disease is very controversial (Gera & Sachdev, 2002). Supplementation may cause damage to cells facilitated through free radicals (Kadiiska *et al.*, 1995). According to Iannotti *et al.* (2006) Fe supplementation in Fe-replete infants may increase the likelihood of infections and impair growth (Iannotti *et al.*, 2006). Furthermore, Fe deficiency may be beneficial as a defence mechanism as it has been suggested that hypoferraemia may be advantageous in the prevention of bacterial growth (Kochan, 1973). Conversely, data have shown that Fe regulates T-lymphocyte function and a deficiency impairs cell-mediated immunity *in vivo* and *in vitro*, as well as delay the development of cell-mediated immunity (Beard, 2001; Oppenheimer, 2001). More intervention studies are needed to determine the safety of Fe supplementation as trials have shown both negative effects (increase in infectious morbidity) (Murray *et al.*, 1978; Smith *et al.*, 1989; Malan *et al.*, 2015) and beneficial effects (in terms of growth and morbidity) (Chwang *et al.*, 1988; Sazawal *et al.*, 2007; Troesch *et al.*, 2011). A study done by Malan *et al.* (2015) demonstrated that Fe supplementation increased illness incidents and duration in school children, however, when Fe was supplemented in combination with n-3 long-chain polyunsaturated fatty acids (LCPUFAs), namely docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), the increase in illness was attenuated (Malan *et al.*, 2015).

2.1.5 Zinc

Zinc is very important for growth and development as it facilitates many enzymatic processes in carbohydrate, protein and fat metabolism. It is widely distributed in foods but dietary food sources of Zn-rich foods include red meat, certain seafood, whole grains, and some fortified breakfast cereals. As Zn is found mostly in the germ and bran of grains, up to 80% of total Zn is lost through the milling process. Zinc deficiency is difficult to pinpoint as Zn is involved in so many processes in the human body, the signs and symptoms of deficiency are diverse and inconsistent. However, some basic and non-specific signs and symptoms include: sup-optimal growth, hair loss (alopecia), diarrhoea, eye and skin

lesions, and an impaired appetite. There is no evidence to show that excess intake from naturally occurring Zn in foods causes adverse events (Otten *et al.*, 2006). However, home fortifications have shown that supplementation of micronutrients including Zn (Sprinkles) reduces diarrhoea in infants (Sharieff *et al.*, 2006). Zinc plays a significant role in immune function as it is a cofactor for many enzymes and is important in DNA synthesis, cellular growth and differentiation, and has antioxidant properties (Shankar & Prasad, 1998; Calder, 2013a).

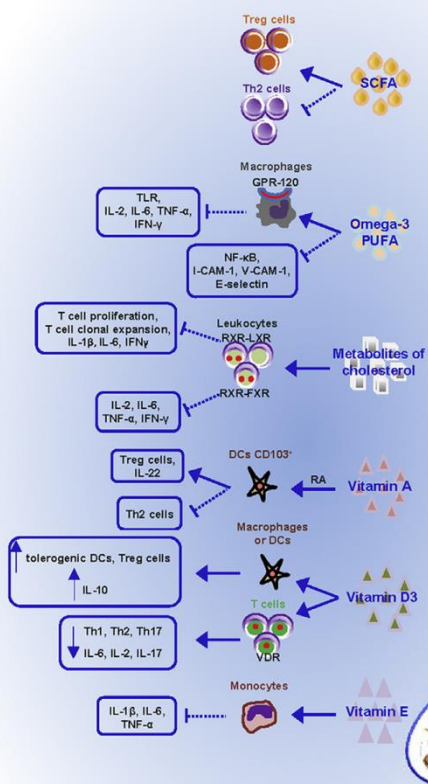
2.1.6 Vitamin A

Vitamin A is one of the micronutrients known to influence immune function. It is a fat-soluble vitamin and it is found in the form of carotenoids (converted to vitamin A in the body) in oils, and various plants including carrots, peppers, and sweet potatoes, and found in the form of retinol (preformed vitamin A) in animal products including milk, livers, eggs, and tuna (Otten *et al.*, 2006; De Rosa *et al.*, 2015). There is a strong correlation between vitamin A deficiency and infectious morbidity, specifically respiratory infections and diarrhoea, and although supplementation with vitamin A may reduce morbidity and mortality due to measles and diarrhoea, it has had little effect on the risk or severity of respiratory infections, except in association with measles (Calder, 2002; Otten *et al.*, 2006). Vitamin A deficiency is linked to impairment of barrier function, an altered immune response, and increased susceptibility to infections (Stephensen, 2001; Villamor & Fawzi, 2005). It is recommended that children who suffer from measles, prolonged diarrhoea, and other acute infections should be treated with vitamin A as it has anti-inflammatory properties (Figure 2–1) (De Rosa *et al.*, 2015). However, high doses of vitamin A are not necessarily beneficial for well-nourished children and may even have adverse consequences (Sempértegui *et al.*, 1999; Fawzi *et al.*, 2000; Otten *et al.*, 2006).

2.1.7 Vitamin C

Vitamin C is a water-soluble nutrient, with several functions including: neutralising free radicals, and acts as a cofactor for several enzymes involved in the biosynthesis of carnitine, collagen, and neurotransmitters. Vitamin C has antioxidant properties whereby it, amongst others, is a scavenger of free radicals in activated leukocytes, lung, and gastric mucosa. Major food sources which contain vitamin C are fruits and vegetables such as citrus fruits and juices, tomatoes, and potatoes. Other sources include cauliflower, broccoli, brussel sprouts, strawberries, cabbage, and spinach. Inadequate intakes and severe deficiency is not a very common occurrence, but is occasionally seen when a diet is lacking in fruits and vegetables (Otten *et al.*, 2006). Vitamin C is found in high concentrations in white blood cells and is utilised very quickly during infection, and low concentrations in the plasma are usually associated with a depressed immune system (Siegel, 1993).

Dietary components with anti-inflammatory properties



Dietary components with pro-inflammatory properties

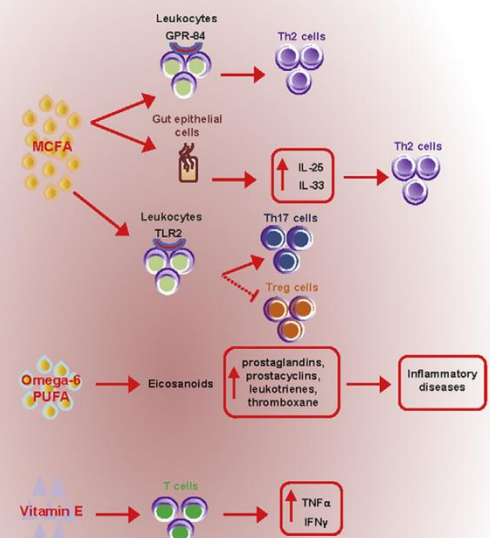


Figure 2-1: The effects of various dietary components on immune cell function (De Rosa et al., 2015).

2.1.8 Vitamin E

Vitamin E is a fat-soluble nutrient with antioxidant properties. It is mostly found in vegetable oils including canola oil, olive oil, wheat-germ oil, sunflower oil, cottonseed oil, safflower oil, palm oil and rice-bran oil. Other dietary sources of vitamin C include unprocessed cereal grains, nuts, fruits, vegetables and the fatty portions of meats. Vitamin E can be found in eight naturally occurring forms: four tocopherols (α -, β -, γ -, δ -tocopherols), and four tocotrienols (α -, β -, γ -, δ -tocotrienols). Yet, unlike other non- α -tocopherol forms of vitamin E, only the natural form of α -tocopherol found in unfortified foods counts toward meeting the required daily allowance (Otten *et al.*, 2006). Free radicals and lipid peroxidation are immunosuppressive and therefore vitamin E, being the most effective chain-breaking antioxidant in cell membranes, plays a significant role in maintaining cell membrane integrity by limiting lipid peroxidation by reactive oxygen species (ROS) and even enhancing the immune response (Figure 2–1) (Calder, 2002; Calder, 2013a).

2.1.9 Vitamin D

Vitamin D (calciferol) is a fat-soluble nutrient and is found in various forms, but the two dietary forms are ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃). Vitamin D is not found in many foods. Foods rich in vitamin D include the flesh of fatty fish, certain fish liver oils, and eggs from vitamin D fed hens. The most common source of vitamin D is obtained from sunlight as it is synthesised in the skin when exposed to ultraviolet B rays (sunlight), and its primary function is to assist in the absorption of calcium and phosphorous in the intestines (Otten *et al.*, 2006). *In vitro* studies show that vitamin D₃ has an overall anti-inflammatory effect (De Rosa *et al.*, 2015). Furthermore, vitamin D and its metabolites is a direct regulator of gene expression in immune cells, and plays a key role in the maturation, differentiation and responsiveness of immune cells (Calder, 2013a).

2.1.10 Fatty acids

Fatty acids (FA) are classified according to the number of carbon chains attached to the lipid backbone, whether the bonds are saturated or not, and the number and position of double bonds. Fatty acids without double bonds are termed “saturated” fats, and the FA with one or more double bonds are unsaturated and are termed “monounsaturated fatty acids” (MUFA) and “polyunsaturated fatty acids” (PUFA), respectively. Saturated FA are known to have a pro-inflammatory effect, while unsaturated FA have either pro- or anti-inflammatory effects (Shi *et al.*, 2006; Wang *et al.*, 2006)(Figure 2–2).

Essential fatty acids, which are the precursors of the n-6 and n-3 LCPUFAs respectively, namely linoleic acid (LA) and alpha-linolenic acid (ALA), are two FA that are vital in infants and children as well. The word ‘essential’ in this context, means that these fats cannot be synthesised endogenously and need to be obtained from the diet or through supplementation (Mahan & Escott-Stump, 2012).

ALA is the parent compound of the n-3 polyunsaturated fatty acids (PUFA) EPA and DHA and one of its main functions is to serve as a precursor for the synthesis of these LCPUFAs. The n-3 PUFA then become incorporated into tissue lipids, used in eicosanoid synthesis, or are oxidised to carbon dioxide and water (Calder *et al.*, 2002; Serhan & Savill, 2005; Otten *et al.*, 2006).

The parent compound of n-6 is LA which can be converted into arachidonic acid (ARA) (Calder, 2002). Arachidonic acid is the precursor to various eicosanoids, namely prostaglandins, thromboxanes, and leukotrienes. These eicosanoids are involved in platelet aggregation, coronary vascular tone and hemodynamics. Similar to n-3 PUFA, the n-6 PUFA are also incorporated into tissue lipids, used in eicosanoid synthesis, or are oxidised to carbon dioxide and water (Calder *et al.*, 2002; Serhan & Savill, 2005; Otten *et al.*, 2006).

As the parent compounds ALA and LA are used to synthesise the LCPUFAs (ARA , EPA and DHA) endogenously through a series of elongation and desaturation steps common to the n-3 and n-6 pathways (Figure 2–2). It is important to keep in mind that this conversion, especially of ALA to DHA is not very efficient in humans, and endogenous production of LCPUFAs may not be sufficient to meet the requirements of many infants during rapid tissue growth and development (Brenna *et al.*, 2009). Another aspect to consider is that the consumption of diets high in LA (>10% total energy) reduces the conversion of ALA to DHA quite dramatically even though the presence of ALA in the diet is higher than the amounts recommended by international bodies. This reduction in conversion is attributed to the fact that there is competition of LA at the desaturase level for the conversion of ALA to EPA and DHA (Makrides *et al.*, 2000; Gibson *et al.*, 2011). Furthermore, according to the findings of Gibson *et al.* (2011), diets high in LA even prevent the incorporation of pre-formed LCPUFAs into tissues (Gibson *et al.*, 2011). This would suggest that infants' whose diet is low in n-3 LCPUFA sources such as fatty fish or seafood, and their diet is rich in n-6 LCPUFA sources such as corn, peanut, cottonseed, safflower, sunflower, and soybean oil, will have a suboptimal n-3 LCPUFA status with potentially negative effects in terms of cognitive development and growth (Briend *et al.*, 2011).

Infants have a unique need for DHA and ARA. These fats are needed for cognitive function and vision as they form important structural components in the central nervous system (Pastor *et al.*, 2006). It has been established that DHA supplementation between 50–100mg in older infants is beneficial for visual function (Authority, 2010). Furthermore, there is a growing body of evidence which suggests that the intake of LCPUFA aids in improving respiratory health early in life, particularly n-3 (Hageman *et al.*, 2012). A study done by Horrobin (1987) sparked an increased interest in LCPUFA and what effect this may have on asthma, as there was speculation that due to their large consumption of fatty fish (containing n-3 FA), Eskimos have lower incidence of asthma (Horrobin, 1987). Similarly, a Japanese study found that school children who consumed more fish had a lower prevalence of asthma (Satomi *et al.*, 1994).

It has become clear in recent years that PUFAs are significant regulators in numerous cellular functions relating to inflammation and immunity. Enough convincing evidence shows that this type of fatty acid has a major impact on immune function and this has formed the basis of using fish oil as an intervention for immune-related studies. The influence of the different fatty acids on the functional responses of cells of the immune system has been examined in many *in vitro* studies as well as in animal feeding models and human intervention studies (Calder, 2002).

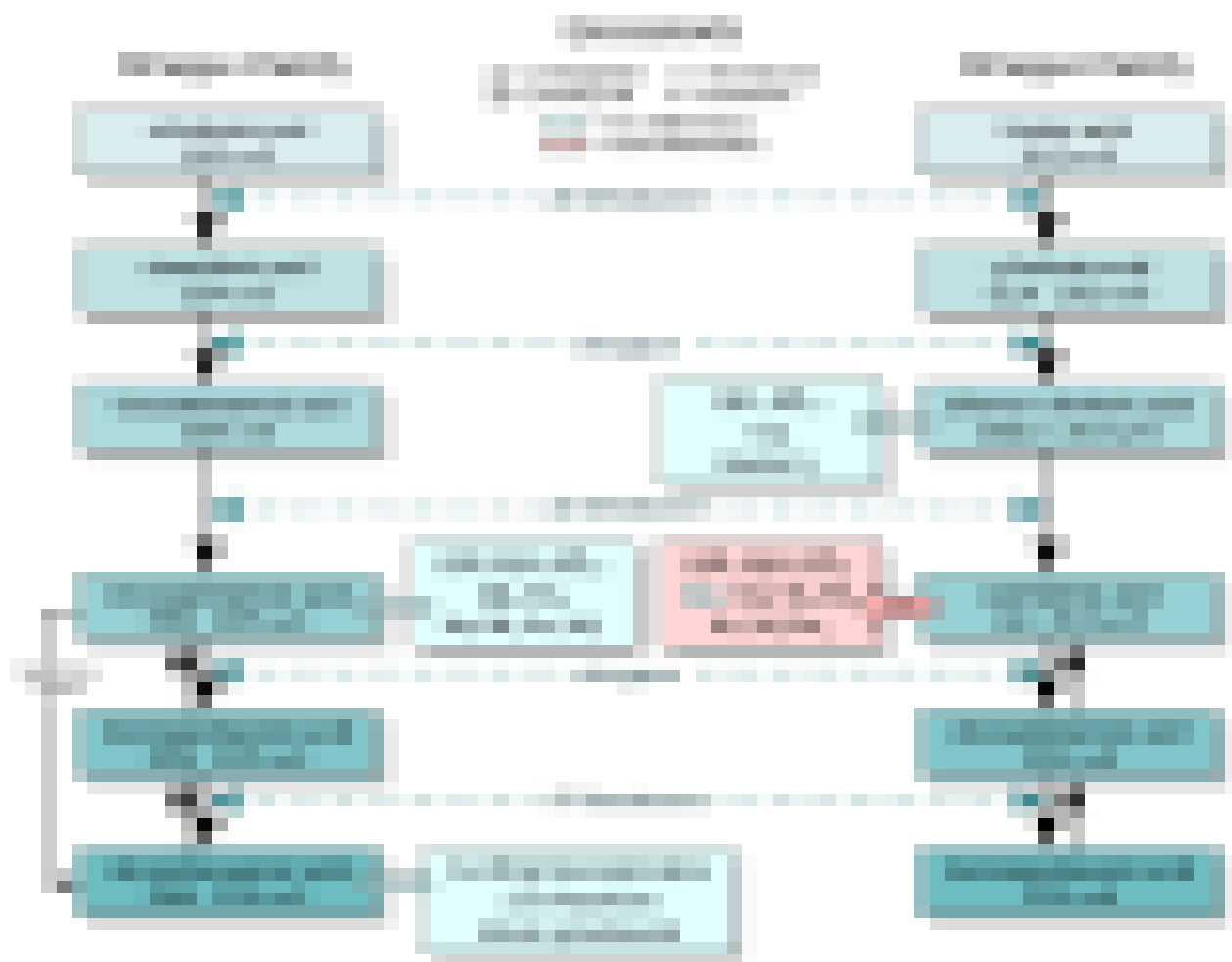


Figure 2-2: Essential fatty acid production and metabolism to form eicosanoids. Available at: https://en.wikipedia.org/wiki/Essential_fatty_acid_interactions. Accessed: 28/08/2016.

2.1.11 Fatty acids and immunity

It is now well accepted that dietary fatty acids have the ability to alter immune function and inflammatory responses, but the full understanding of the mechanisms involved is still lacking. A few mechanisms have been proposed, such as changes in membrane structure and composition, alterations in membrane-mediated functions and signals (involving proteins and eicosanoids), altering gene expression, and affecting the development of the immune system (Calder, 2002; Calder, 2013b).

Eicosanoids are a group of bioactive mediators which are an important link between fatty acids and immune function. Eicosanoids (especially dienoic prostaglandins [PGE_2] and 4-series leukotrienes [LT]) are involved in modulating the intensity and duration of inflammatory and immune responses, and the pro-inflammatory effects of certain eicosanoids (e.g. PGE_2) include inducing fever, increasing vascular permeability and vasodilation, and increasing pain and oedema caused by other agents e.g. histamine (Calder, 2002). n-6 = proinflammatory and n-3 = anti-inflammatory, as seen in Figure 2–2. This may be as

a result of a shift in balance in T-helper 1 (Th1), T-helper 2 (Th2) and T-regulatory cells, compounded by the decrease in synthesis of pro-inflammatory ARA derived eicosanoids when supplementing with specifically DHA and EPA (Calder *et al.*, 2006; Uauy & Dangour, 2009). Supplementation of these nutrients are often necessary as the diets of infants and children in Western and non-Western countries are usually lacking sufficient n-3 PUFA intake (Uauy & Dangour, 2009), as well as other micronutrients such as Fe and Zn (Hotz & Brown, 2004; Zimmermann & Hurrell, 2007). Table 2–2 shows the recommended intake of the n-3 LCPUFAs for each age group (FAO, 2010).

Table 2-2: DHA and EPA recommendations for each age group (FAO, 2010).

	DHA and EPA recommendations
0–6mo	DHA: 0.1–0.18 % TE
6–24mo	DHA: 10–12 mg/kg bw
2–4yr	EPA + DHA: 100–150 mg
4–6yr	EPA + DHA: 150–200 mg
6–10yr	EPA + DHA: 200–250 mg

BW: body weight; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; TE: total energy

Over the last decade there have been significant advances in understanding the mechanisms of action of n-3 LCPUFA, especially mechanisms that are critical in the resolution of inflammation. The composition of the phospholipid bilayer of membranes of immune cells is influenced by the availability of EFA and the LCPUFAs, therefore determining the amounts of monocytes and lymphocytes in the peripheral blood mononuclear cells. This affects cell signalling, the fluidity, and the type and amount of mediators derived from the various FA which are incorporated into the immune cell membranes (Calder, 2013b).

2.1.12 Required nutritional intake in infants

Infants have high nutritional requirements in relation to their body size, and therefore need to consume nutrient-dense foods as they eat foods in small quantities. This presents a problem because as mentioned previously, CF are often inadequate in developing countries (Gibson *et al.*, 1998; Faber, 2005).

In 2005 a study was conducted in KwaZulu-Natal (RSA) in which the nutrient composition of the nutritional intake of 6–12 month old infants was determined. It was discovered that the nutrient density

of the complementary foods within this age group of infants was inadequate to meet their dietary needs, especially for Fe, Zn and calcium (Faber, 2005). Similarly, eleven years later, in a cross-sectional study done by Rothman (2016) in peri-urban North-West, the data revealed that South African infant's complementary diets at 6 months (Table 2–3) was deficient in iron, zinc and calcium for 80% of the infants (Rothman *et al.*, in press).

Table 2-3: Comparison between required nutrient intake* in infants 7–12 months and the actual intake of peri-urban 6-month-old infants based on a cross-sectional study done in South Africa.

Nutrient	Nutrient requirements for infants 7–12 months	Nutrient intake based on a single 24-hr recall of infants at 6 months; n = 720 (Rothman <i>et al.</i> , 2016)
Energy (kJ/d)	2839-3120	2856 (2418, 3364)
CHO (g/d)	95	85.6 (69.7, 106.1)
Protein (g/d)	11	12.7 (9.9, 16.9)
Fat (g/d)	30	32.6 (30.0, 36.2)
Essential fatty acids		
LA (g)	4.6	- [#]
ALA (g)	0.5	-
Long-chain polyunsaturated fatty acids		
DHA (mg)	-	-
ARA (mg)	-	-
Micronutrients		
Vitamin A (RAE)	500	297 (112.5, 614.5)
Vitamin D (µg)	10	2.3 (0.7, 5.5)
Vitamin E (mg)	5	2.3 (0.7, 5.5)
Vitamin K (µg)	2.5	-
Thiamine (mg)	0.3	0.4 (0.3, 0.6)
Riboflavin (mg)	0.4	0.6 (0.4, 0.8)
Niacin (mg)	4	4.8 (2.9, 7.2)
Pantothenate (mg)	1.8	2.2 (1.7, 3.1)
Vitamin B6 (mg)	0.3	0.3 (0.2, 0.6)
Biotin (mg)	6	8.0 (2.3, 16.6)

Nutrient	Nutrient requirements for infants 7–12 months	Nutrient intake based on a single 24-hr recall of infants at 6 months; n = 720 (Rothman <i>et al.</i> , 2016)
Folate (B9) (µg)	80	0.0 (0.0, 42)
Vitamin B12 (µg)	0.5	0.7 (0.4, 1.1)
Vitamin C (mg)	50	61.6 (44.7, 81.8)
Calcium (mg)	260	361.1 (274.7, 468)
Iodine (µg)	130	-
Iron (mg)	11	5.5 (2.3, 9.5)
Zinc (mg)	3	2.7 (1.6, 4.4)
Copper (µg)	220	408 (354, 500)
Selenium (µg)	20	-
Magnesium (mg)	75	40.5 (25.8, 61.3)
Manganese (mg)	0.6	0.2 (0.2, 0.3)
Phosphorus (mg)	275	210.9 (140.7, 296.5)
Potassium (mg)	700	606.6 (450, 808.2)

Arachidonic acid (ARA); Docosahexaenoic acid (DHA); α -linolenic acid (ALA); Linoleic acid (LA); Retinol Activity Equivalent (RAE).

Values are given as median and interquartile range (Q1, Q3.)

*Required nutrient intakes are from combined sources representing Recommended Dietary Allowances (RDAs) in **bold type** and Adequate Intakes (AIs) in regular type.

All such values not given.

Sources: Adapted from the *Dietary Reference Intakes series*, National Academies Press. Copyright 1997, 1998, 2000, 2001, 2002, 2004, 2005, 2011 by the National Academies of Sciences (Whitney & Rolfes, 2013).

From Table 2–3 it is evident that even though most nutrients are consumed in sufficient amounts among 6-month-old infants, certain nutrient intakes are still well below desired amounts. Vitamin A intakes are less than 60% of requirements; dietary vitamin D intakes through food are 23% of requirements. Vitamin E intakes are less than 50% of requirements; folate intakes are less than 5% of requirements, and Fe intakes are 50% of required intake levels (Whitney & Rolfes, 2013; Rothman *et al.*, 2016).

2.1.13 Nutritional interventions

It is common practice in many countries to initiate early introduction to solids or other fluids. However, for the complementary feeds to be adequate and safe, measures need to be taken to ensure that the feeds are nutrient dense and meets the nutritional requirements of that particular age group. This can be done through interventions such as fortifying complementary feeds with inexpensive food products. Nevertheless, these feeds should be prepared, stored and fed in hygienic conditions in order to reduce the risk of infectious disease, in particular diarrhoea. There are a few obstacles to achieving successful fortification in South Africa which may include sustainability, cultural acceptability, social marketing, targeting, and distribution (Steyn & Temple, 2008). Traditionally, recommended intake levels of nutrients were based on preventing deficiencies (Figure 2–3), but in recent years the approach started shifting towards recommendation levels which are associated with specific disease prevention (FAO/WHO, 2005).

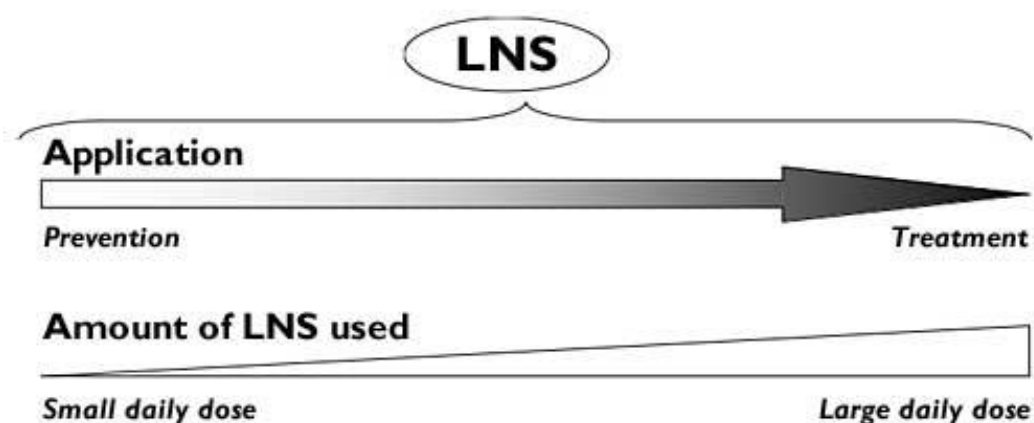


Figure 2-3: LNS application tailored to meet the individuals need depending on the programmatic context (preventative or therapeutic). Available at: <http://ilins.org/resources>. Accessed: 18/09/2016.

According to the findings of Faber (2005), South African mothers are fortifying their infants' porridge with various energy-dense food items, including margarine (66%), peanut butter (42%), sugar (41%), formula milk powder (23%) and eggs (5%). But even with these interventions nutrient deficiencies are present. Figure 2–4 shows that most complimentary foods do not suffice in nutrient density per age category (Faber, 2005).

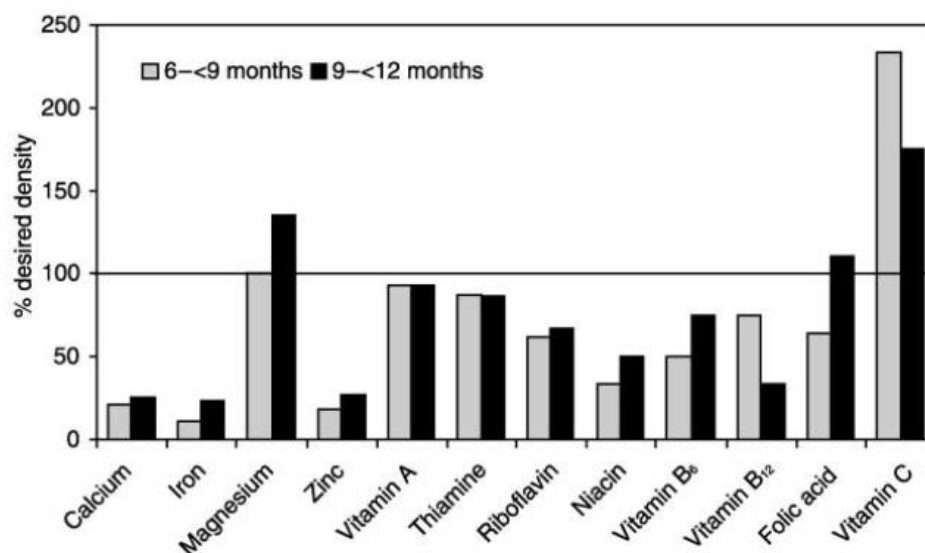


Figure 2-4: Nutrient densities of complementary food expressed as a percentage of the desired nutrient density per age category, (Faber, 2005).

Section 3 of this literature review covers the effect of supplementation as a nutritional intervention on infectious diseases in more detail.

2.2 Section 2 – Immunity

Immunology is defined as the study of the physiological defences by which the body/host recognises itself from non-self/foreign matter (Widmaier *et al.*, 2011), with the purpose of protecting the host against pathogens which cause infectious disease (Kabelitz & Kaufmann, 2010). There are many pathogens, namely: bacteria, parasites, fungi, viruses and helminths, which utilise different tactics of invading the host and avoiding an attack from the immune system. Conversely, the immune system has developed defence mechanisms in such a way that it can protect the host from pathogens in different ways (Hughes, 2002).

There are three physical barriers which act as first line of defence to infection: the skin, pH and cilia. If these physical barriers are breached, it triggers the immune system to initiate an appropriate response. The immune system has two arms, the innate and adaptive immune responses (Figure 2–5). The innate immune response is characterised by the host protecting itself from foreign matter without recognising the substance, whereas the adaptive immune response occurs when the host identifies the substance through recognition (prior exposure) by lymphocytes and consequently exclusively attacks the identified cells (Table 2–4) (Janeway *et al.*, 2005). These two systems work synergistically and independently of one another but regularly require a mutual interaction when protecting the host against the invasion of pathogens (Widmaier *et al.*, 2011).

Table 2-4: Cells and signalling molecules involved in the immune system.

Cells and signalling molecules	Definition
Collectin	Molecules forming part of the innate immune system, containing lectin (carbohydrate-binding) domains and collagen-like domains
Inteferon	A cytokine with anti-viral effects
Cytokine	Soluble molecules used to transmit messages from cell to cell. Inteferons and chemokines are types of cytokines
Dendritic cell	Irregularly shaped cell with many branch-like processes that is critical in antigen capture and presentation to T-cells.

Cells and signalling molecules	Definition
Langerhans cell	Dendritic cells (antigen-presenting cells) which are found in the skin and mucosa.
Interleukin (IL)	Interleukin is an old name for cytokine, the IL abbreviation is used as a naming system for cytokines.
Natural Killer (NK) cells	A lymphocyte able to bind to virus-infected cells and certain tumour cells without the stimulation of antigens, and cause them to die off.
Tumor necrosis factor (TNF)	A cytokine involved in systemic inflammation and one of the cytokines which help make up the acute phase reaction.
Antigen	Molecules specifically recognised by receptors of the adaptive immune system
Th1-cell	T-helper cell that provides help for cytotoxic T-cells, macrophages, and IgG production.
Th2-cell	T-helper cell that provides help for immunoglobulin E secretion and eosinophil activation.

Adopted from Widmaier *et al.* (2011)

2.2.1 The innate immune system

The innate immune response is designed in such a way that it occurs almost immediately after a pathogen enters and infects the host, and it consists of two components: (1) physical, molecular and chemical barriers which recognise and eliminate antigens in a non-specific manner, and (2) cell receptors which recognise pattern-associated molecular patterns (PAMPS) (Mak & Saunders, 2007). Consequently most pathogens are taken care of by the innate immune system. The main cell types constitute of macrophages, dendritic cells and natural killer (NK) cells (Nairn & Helbert, 2007).

Macrophages have the functions of ingesting and eliminating the pathogen, process and present antigens, and help regulate other immune cells in the innate and adaptive immune system. Dendritic cells reside in tissues in an immature or dormant form, and once an infection or injury occurs they

become mature and induce an innate response. Furthermore, mature dendritic cells can travel to the lymphoid organs with antigens on their surfaces and act as antigen-presenting cells (APC). These cells also influence activated T-cells in the adaptive immune system. Natural killer cells have the unique ability to detect infected cells without antibodies, releasing lytic enzymes causing the infected cells to die via apoptosis (Janeway *et al.*, 2005).

2.2.2 The adaptive immune system

The adaptive immune response requires prior exposure to a pathogen, is antigen-specific, and clonally distributed antigen-receptors make specificity possible. This occurs when the innate immune response cannot eliminate or at least neutralise the pathogens, and thus initiates and mediates the adaptive immune response. Once the pathogen is cleared, “memory cells” are created, which causes the immune system to recognise pathogens and initiate the response more quickly in the event that the same pathogen should enter the host (Kabelitz & Kaufmann, 2010).

The main cells involved in the adaptive immune response are B-cells and T-cells. B-cells recognise antigens differently to T-cells as the antibody expressed on the B-cell binds directly to soluble antigens. T-cells recognise antigens only if they are presented in the context of appropriate major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs) (Smith-Garvin *et al.*, 2009).

T-cells are differentiated into three types: helper, regulatory and cytotoxic. Th1 cells promote cell-mediated immunity through cytotoxic T-cells and macrophages, while Th2 cells promote humoral immunity through antibody production via B-cells (Porter & Kaplan, 2011).

The principle of vaccination is the stimulation of the adaptive immune system which, by exposing the host to harmless components of an infectious pathogen or attenuated strains, will create long-lasting memory. Upon a real infection the host is then able to combat the same pathogen with efficiency and prevent outbreak of the disease (Kabelitz & Kaufmann, 2010).

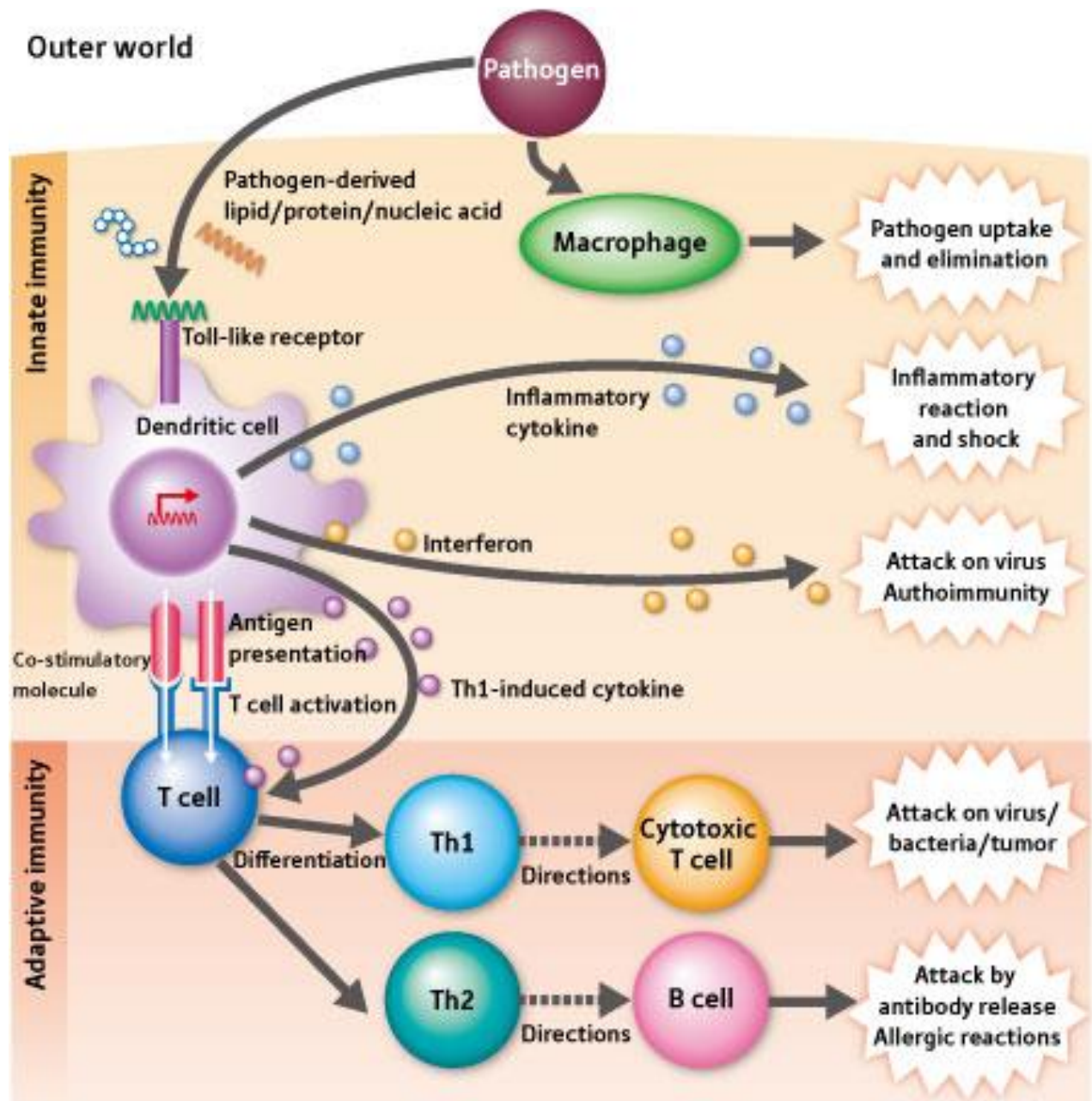


Figure 2-5: The innate and adaptive immune responses. The innate response occurs almost immediately after a pathogen enters the human body, while the adaptive response can take up to a few days to develop. Available at: <https://joellezimprich.wikispaces.com/08-Immunology>. Accessed: 09/02/2016.

Table 2-5: Differences between the innate and adaptive immune systems (adopted from Nairn and Helbert (2007)).

Innate system	Adaptive system
Distinguishes danger from homeostasis	Distinguishes self from non-self
Preformed (constitutive) or rapidly formed components	Relies on genetic events and cellular growth
Responds within minutes to infection	Response develops over days
No specificity: the same molecules and cells responds to a range of pathogens	Very specific; each cell is genetically programmed to respond to a single antigen
Uses pattern-recognition molecules	Uses antigen-recognition molecules
Uses germline genes to produce collectins (mannan-binding lectin, surfactant), complement, C-reactive protein (CRP) and Toll-like receptor	Uses hypervariable regions in immunoglobulin and T-cell receptors and genetic recombination to produce immunoglobulin and T-cell receptors
Probably fewer than 100 receptors exist	Possibility of up to 10^{18} different receptors
Pattern-recognition molecules detect molecules unique to pathogens, for example lipopolysaccharide	Recognises conformational structures (immunoglobulin) or short, peptides bound to MHC molecules (T-cell receptor)
No memory: the response does not change after repeated exposure	Immunological memory; on repeated exposure the response is faster, stronger and qualitatively different
The older system, seen in all members of the animal kingdom	Evolved in early vertebrate kingdom, long after the innate immune system
Can only recognise molecules signalling infection or injury	Cannot distinguish host from pathogens
Rarely malfunctions	Frequently malfunctions and may cause autoimmunity

2.2.3 Immunity in early life

2.2.3.1 Gastrointestinal illness

Gastrointestinal illness is the second leading cause of death worldwide in children under five and it is preventable and treatable. Infection and malnutrition are major causes and about 760 000 lives are claimed every year as a result (WHO, 2013). Diarrhoea by definition is the passage of three or more loose/liquid stools per day or more frequently than what is normal for an individual (Steyn & Temple, 2008). It occurs when the internal milieu of the intestine is not in balance but in favour of net secretion. This may be attributed to decreased absorption from the lumen or increased secretion of water into the lumen, which therefore stimulates peristaltic activity due to increased luminal content (Thapar & Sanderson, 2004). There are a variety of pathogens which may cause diarrhoea in children, especially in developing countries. These would include *Escherichia coli*, *Shigella*, *Vibrio cholera*, *Salmonella* and *Entamoeba histolytica* (Calder, 2002).

The epidemiology of diarrhoea and pneumonia in infants overlap, mainly due to shared risk factors which include under-nutrition, suboptimal breastfeeding and Zn deficiency (Walker *et al.*, 2013). Treatment of diarrhoea is 3-fold as rehydration therapy, Zn supplementation and continued feeding are essential. Rehydration with oral rehydrating solution (ORS; a mixture of water, salt and sugar) replaces water and electrolyte losses; Zn supplementation has proven to reduce the duration of a diarrhoea episode by 25% and correlates to a 30% reduction in stool volume; and by providing nutrient-rich foods, the diarrhoea-malnutrition circle (Figure 2–6) can be broken (Victora *et al.*, 2000; WHO, 2013).

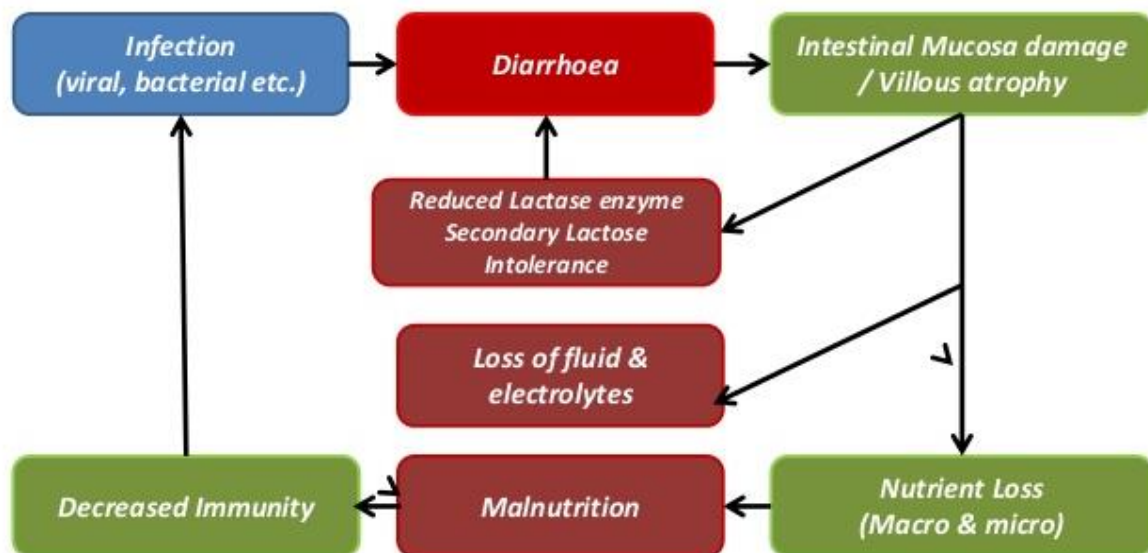


Figure 2-6: The vicious circle of diarrhoea-malnutrition. Available at: https://www.google.co.za/search?q=malnutrition+diarrhoea+cycle&rlz=1C1KMZB_enZA574ZA574&espv=2&biw=1280&bih=855&source=lnms&tbm=isch&sa=X&ved=0ahUKEwj54f6w9eDLAhVBbBoKHTkuATEQ_AUIBigB#imgsrc=6-jW4-kviYxcqM%3A. Accessed: 27/03/16.

2.2.3.2 Respiratory Illness

Respiratory illnesses are one of the most common causes of infectious morbidity in children, and it is found that about 95% of all children under the age of three years suffer from at least one respiratory infection, of which 75% are upper respiratory infections (URI) (Harsten *et al.*, 1990; Birch *et al.*, 2010). It is critical for infants and children to receive adequate nutrition early in life in order to support normal development and functioning of the immune system (Hageman *et al.*, 2012), and it has been suggested that there are some nutrients, including n-3 PUFA which can specifically modulate host defence (Field *et al.*, 2002).

2.2.4 Measuring immune function

There exists a wide range of methodologies in which immune function can be measured and how it is impacted by nutrient consumption. A large spectrum of immune parameters need to be targeted in order to thoroughly assess the immune system status as well as its functionality (Albers *et al.*, 2007). Assessments may be done *ex vivo* on cell functions where isolated cells are studied in short- or long-

term cultures, or *in vivo* assessments can be done by measuring the concentrations of proteins in the bloodstream which are indicators of immune function. Although immune responses are often assessed *ex vivo*, the changes seen do not necessarily predict the same response *in vivo*. Therefore in order to establish the effects of a nutritional intervention, measures reflecting an immune response *in vivo* are superior to *ex vivo* measures. Nevertheless it is recommended that both types of measures be used in combination to better understand the impacts of nutritional interventions on the immune system (Calder & Kew, 2002).

Previous studies looking at the effect of nutritional interventions on immune function have used clinical outcomes to measure immune function which included incidence and duration of infectious disease (in infants and children), and absenteeism from school (in older children) (Dalton, 2006; Pastor *et al.*, 2006; Thienprasert *et al.*, 2009; Birch *et al.*, 2010; Malan *et al.*, 2015). These clinical outcomes are still the best way to measure immune function holistically as there is currently no single marker which can predict the effect of a nutritional intervention on the resistance to infection (Albers *et al.*, 2007).

Vaccines are another way to measure the differences in immune function as they are widely used markers which provide reliable, high quality information on the effect of nutrients on immune responsiveness *in vivo* (Albers *et al.*, 2007). It has been shown that the presence or absence of different micronutrients influence specific immune responses, e.g. Fe and Zn are vital for the development and maintenance of cell-mediated and humoral responses, and FA also affect cell-mediated and humoral responses, however some lipids stimulate the immune response whereas others down-regulate it (van Loveren *et al.*, 2001).

2.2.5 Measles and vaccination

Part of millenium development goal 4 was to reduce child mortality through the eradication of measles by 2015. With the global implementation of routine measles vaccination in infants, a 79% drop in deaths due to measles between 2000 and 2014 has been achieved. This means that 17.1 million deaths were prevented making the measles vaccine one of the best acquisitions in public health. However, the problem is still common in developing countries, especially large parts of Africa and Asia (WHO, 2016b), and it seems to be re-emerging in countries (Europe) with low incidence and two-dose vaccination programmes in place (Muscat *et al.*, 2009).

The measles virus presents itself through fever, characteristic red spots and a retardation of antibody and cell-mediated responses. Therefore opportunistic infections may be fatal. People who survive the measles virus attain natural immunity and thus have life-long protection against re-infection. The measles virus has limited variation in its surface proteins and therefore all current vaccines are based on

live, attenuated viruses, thus being referred to as *measles-containing vaccine* (MCV) (Mak & Saunders, 2007).

Primary immunity against infectious diseases is provided at birth through maternal antibodies. Interestingly, the antibody concentrations are higher in naturally immune mothers compared to vaccinated mothers and these concentrations correlate with neonatal values (Leuridan *et al.*, 2010). Therefore the timing of vaccination should be taken into consideration as these antibodies could compromise the humoral antibody response to vaccination (Glezen, 2003). During the 1989-1991 measles epidemic in the United States of America (USA), the majority of measles cases were found in pre-school children who had not received the measles vaccine at 15 months of age (which was the recommended age at the time), but approximately 20% of these children were less than a year old. The incidence of measles in infants thus demonstrates the transformed epidemiology of measles in the vaccine era (Control & Prevention, 1993).

Leuridan *et al.* (2010) investigated the duration of maternal antibodies to measles in infants and they found that at 6 months more than 99% of infants from vaccinated mothers and 95% of infants from naturally immune mothers had already lost maternal antibodies. This increases their susceptibility at an early age, making the timeliness of the vaccination an important factor in the prevention of measles (Leuridan *et al.*, 2010). Thus immunising infants from the earliest age possible has the potential to reduce morbidity and mortality (Gans *et al.*, 2001). In South Africa, the measles vaccine (Rouvax) is given to infants routinely at 9 and 18 months at all government clinics (Baker, 2010) and 70% of 1-year old children were vaccinated against measles in South Africa in 2014, leaving 30% vulnerable and susceptible (WHO, 2014).

2.2.6 The vaccination-induced immune response

Vaccines activate an immune response *in vivo* without producing the symptoms of the actual disease. Typically the immune response is measured by assessing the concentrations of vaccine-specific antibodies (immunoglobulin G) in serum or plasma. This is usually done through the use of an enzyme-linked immunosorbent assay (ELISA) or pathogen neutralisation assays (Albers *et al.*, 2007).

Very few studies have investigated the vaccine induced immune response. In 2001, Gans *et al.* (2001) performed a cohort by looking at infants' response to measles and mumps vaccines at 6, 9, and 12 months respectively. Vaccine responses were evaluated through T cell proliferation measurements, interferon- γ production, and neutralising antibody titers before and after the vaccinations were given. Equivalent cellular immune responses to both vaccines (with or without passive antibodies) were observed in infants of all age groups. However, 6-month old infants without passive antibodies had low

geometric mean titers (GMT) of antibodies to measles or mumps, and showed low seroconversion rates. These GMT of antibody to measles increased when infants were given another vaccine at 12 months (Gans *et al.*, 2001). As many of the world's children are more than likely to be malnourished when receiving their childhood vaccines, the effect of the co-administration of nutrient supplements needs to be clarified. It has been investigated to a limited extent due to an absence of evidence for most nutrients. Therefore the studies which have investigated the effect of nutrients on the immune response to vaccines, reviewed by Savy *et al.* (2009), focused on protein-energy, vitamin A and vitamin D, Fe and Zn, and all vaccines were considered. In general, the inconsistency of the acquired data made it difficult to draw definite conclusions. However, their analysis indicated that malnutrition had little or no effect on vaccine responses (Savy *et al.*, 2009).

2.3 Section 3 – Nutrition and immunity in infants and children

2.3.1 The effect of LNS on infectious diseases of infants and children

As there are very limited amount of RCT done to date on the effect of LNS on the morbidity of infants and children, only 9 studies were included for the compilation of this review (Table 2–6). Five studies involved infants and children ≤ 5 years (Pastor *et al.*, 2006; Birch *et al.*, 2010; Minns *et al.*, 2010; Huybregts *et al.*, 2012; Bendabenda *et al.*, 2016), and four studies included children between 6–12 years of age (Dalton, 2006; Thienprasert *et al.*, 2009; Thomas *et al.*, 2012; Malan *et al.*, 2015).

In Table 2–6 we can see that the studies are quite recent, ranging from 2006 to 2016. Furthermore, the duration of supplementation ranged from 60 days–12 months across all trials, and the infants and children ≤ 5 years received supplementation either through formula (with added lipids), a ready-to-use supplementary food (RUSF) containing micronutrients and parent-compound lipids (LA and ALA), or LNS with and without milk. The older children received supplementation in various different forms such as fortified foods or milk, capsules, or a fish spread. Most of the trials were performed in regions with subjects of low socio-economic status (Dalton, 2006; Thienprasert *et al.*, 2009; Huybregts *et al.*, 2012; Thomas *et al.*, 2012; Malan *et al.*, 2015; Bendabenda *et al.*, 2016), two trials targeted the middle and upper socio-economic class (Pastor *et al.*, 2006; Minns *et al.*, 2010), and one study does not indicate the socio-economic background of the test subjects (Birch *et al.*, 2010).

The dosage of n-3 LCPUFAs varies across the studies, depending on the age of the different target populations. However, most of the outcomes of the trials were unanimous in improving the immune function of the infants and children.

There were seven studies which showed significant positive effects of the n-3 PUFA supplements on respiratory morbidity. Pastor *et al.* (2006) conducted a multiple-centre, open-label, 12 month observational study in infants by supplementing them with DHA (0.32% of total FA) and ARA (0.64% of total FA), and discovered significantly higher incidences of bronchitis/bronchiolitis in the control group at 5 months (13.9% vs. 6.1%, $P = 0.01$), 7 months (10.8% vs. 5.1%, $P = 0.01$), and 9 months (11.3% versus 5.8%, $P = 0.01$) when comparing it to the DHA/ARA group. Furthermore, they detected a higher percentage of upper airway infection at 12 month in the control group compared to the DHA/ARA group (24.2% versus 16.2%). Similarly, Birch *et al.* (2010) provided the same supplementation regime to infants in 2 original cohorts, in which they found that the DHA/ARA group had significantly lower odds for developing upper respiratory infections (URI) (odds ratio [OR], 0.22; 95% confidence interval [CI], 0.08–0.58) versus the control group. Furthermore, it was discovered that the incidence of contracting at least

one URI within the first 3 years of life was significantly higher in the control group compared to the DHA/ARA group (76% versus 45%) (Birch *et al.*, 2010).

In a randomized, double-blind, controlled study in Thailand, Thienprasert *et al.* (2009) supplemented 9–12 year old school children for six months with relatively high dosages of n-3 LCPUFAs for 5 days per week. In the intervention arm, approximately 10% of children reported fewer episodes of illness, mostly respiratory, than in the control group, and the number of episodes per subject and total days illness per child was also significantly less in the intervention group when compared to the control group (Thienprasert *et al.*, 2009). These findings are similar to a study done in Bangalore, India, where children 6–10 years received foods fortified with either high (100% Required Daily Amount [RDA]) or low (15% RDA) micronutrients, combined with either high (100 mg DHA and 900 mg ALA) or low (140 mg ALA) n-3 LCPUFAs for 12 months. The children who consumed the high n-3 LCPUFAs had significantly fewer episodes and shorter duration of upper respiratory infection per child compared to children who received only ALA (Thomas *et al.*, 2012). Furthermore, a study conducted in the USA in infants 6–36 months, supplemented toddler formula with 130 mg DHA per day for 60 days. Toddlers consuming this formula had fewer adverse events ($P = 0.007$) and a lower incidence of respiratory illness ($P = 0.024$), compared to the group who received unfortified formula (Minns *et al.*, 2010).

A study done in South Africa, supplemented 7–9 year old school children with a 25 g fish spread over the course of 6 months. The outcome measures included absenteeism from school related to infectious morbidity. The intervention group received a daily fish spread with ~190 mg DHA and ~82 mg EPA and the control group received a similar daily spread with only ~36 mg DHA and ~14 mg EPA. The intervention group had significantly fewer days absent from school due to respiratory illness when compared to the control group (14.3% versus 27.7%) (Dalton, 2006). Another study was done in South Africa in 6–11 year old children, where the aim was to determine whether n-3 LCPUFAs and Fe supplementation, alone or in combination, would affect absenteeism and illness in Fe-deficient school children with low fish intake. It was discovered that Fe supplementation increased the number of days absent as well as the odds for being absent with respiratory symptoms, but when given in combination with DHA/EPA, respiratory symptoms were reduced by at least half (Malan *et al.*, 2015).

Table 2-6: Randomised controlled trials of n-3 PUFA intervention in infants and children with morbidity outcomes.

Study	Subjects Age	Type	Dosage of fatty acids					Duration	Immunity	Outcome measures
			DHA	ARA	EPA	ALA	LA			
Pastor <i>et al.</i> (2006)(Spain)	0-12mo	Formula	0.32% TFA (17mg/100kcal)	0.64% TFA (34mg/100kcal)				12mo	Improved	5.8% vs 11.3% bronchitis /bronchiolitis, 16.2% URI vs 24.2 % at 12 mo
(Bendabenda <i>et al.</i> , 2016)(Malawi)	6-18mo	LNS (with and without milk)				290mg 580mg 160mg	2220mg 4440mg 8880mg	12mo	10- and 20g LNS/d did not increase morbidity	No differences in Gastroenteritis or Acute Respiratory Infection between Intervention groups and Control
Huybregts <i>et al.</i> (2012) (Chad)	6-36mo	RUSF				300mg	2000mg	4 mo	Improved	lower risk of self-reported diarrhea by 29.3% and fever by 22.5% in intervention group
Minns <i>et al.</i> (2010) (USA)	18-36mo	Formula	130mg					60 days	Improved	17% vs 46% in controls of participants experiencing one or more respiratory illnesses
Birch <i>et al.</i> (2010) (USA)	3yrs	Formula	0.32%-0.36% TFA (17mg/100kcal)	0.64%-0.72% TFA (34mg/100kcal)				12 mo at ≤5days-12mo age	Improved	Improved all respiratory illnesses (79% vs 84% in control), 45% URI vs 76% in control, and 16% wheezing vs 35% in control
Thomas <i>et al.</i> (2012) (India)	6-10yr	Fortified Fruit flavoured wheat biscuit and milk powder drink	100mg			900mg		12 mo	Improved	Fewer episodes of URTI/child/year (RR=0.88, 95% CI: 0.79, 0.97) and significantly shorter duration/episode of URTI (RR=0.81, 95% CI: 0.78, 0.85), LRTI (RR=0.91, 95% CI: 0.85, 0.97), compared with children who received low n-3 fatty acid intervention

Study	Subjects Age	Type	Dosage of fatty acids					Duration	Immunity	Outcome measures
			DHA	ARA	EPA	ALA	LA			
Malan <i>et al.</i> (2015) (RSA)	6-11yrs	Supplement	420mg		80mg			8.5mo	Improved in combination with Fe suppl	Days ill and days at school with respiratory symptoms were attenuated when Fe was given with DHA/EPA.
Dalton (2006) (RSA)	7-9yr	Fish flour spread (25g per day)	192mg	82mg		335mg		6mo	Improved	14.3% subjects absent with respiratory illness vs 27.7% in control group; EG absent 40d/100d due to Resp illness vs 84d/100d in CG
(Thienprasert <i>et al.</i> (2009)) (Thailand)	9-12yr	Fish oil in milk	1000mg		200mg			6mo	Improved	54.3% ill vs 67.4% in control group; 28.7% ill with fever vs 37.2% in control group (P = 0.086); same episodes of illness; 2 days ill/subject vs 4 days in control group; illness = rhinitis, cold, influenza and diarrhoea;

ALA: alpha-linolenic acid; CG: control group; DHA: docosahexaenoic acid; EG: experimental group; EPA: eicosapentaenoic acid; Fe: iron; LA: linoleic acid; RUSF: ready-to-use supplementary food; TFA: total fatty acids; URI: upper respiratory tract infection

One of the most recent studies done to date was conducted in the south-eastern part of Malawi where 6-month-old infants were randomly assigned to one of six groups as follows: milk-containing LNS 10g/d, 20g/d and 40g/d; and milk-free LNS 20g/d and 40g/d; and a control group which received no supplementation (note that all LNS contained 6mg Fe/d). The supplements were given up until the age of 18 months and outcomes focused on serious adverse events (SAE), non-scheduled visits to a health facility due to malaria, gastroenteritis, acute respiratory infection, or other illnesses. It seemed that long-term provision of 10- and 20g/d LNS did not increase morbidity in the infants and children who were more prone to be Fe deficient and where infectious diseases are common (Bendabenda *et al.*, 2016).

Lastly, in a two-arm cluster-randomised controlled pragmatic intervention study done in Abeche, Chad, children aged 6-36 months were supplemented with a LNS containing 300 mg ALA and 2000 mg LA. After adjustment for age, sex, socio-economic status, and morbidity status at inclusion, they found that children from the LNS group had lower risk of self-reported diarrhea by 29.3% (95% CI: 20.5, 37.2; $P < 0.001$) and fever by 22.5% (95% CI: 14.0, 30.2; $P < 0.001$), compared to the control group. No morbidity related to respiratory illness was reported (Huybregts *et al.*, 2012).

As this is a relatively new research field, the findings of the chosen studies present large variability in the age of participants, dosage and duration of supplementation, and morbidity assessment. However, seven out of the nine studies show the benefits surrounding respiratory morbidity when dosages of DHA ranged between 130–1000 mg, and four out of these seven studies supplemented with additional ARA and one with EPA. Only one study showed a reduction in diarrhoea and fever, while another study showed no benefits.

It has already been established that formulas with the inclusion of LCPUFAs in quantities which are similar to the median worldwide breast milk levels, benefit infants with regards to visual development, cognitive development and growth (Neuringer *et al.*, 1988; Martinez, 1992; Adu-Afarwuah *et al.*, 2007). Furthermore, researchers have discovered the potential for LCPUFAs to be used to treat acute and chronic inflammatory diseases, as well as improve immune function (Nagakura *et al.*, 2000; Mahrshahi *et al.*, 2004; Hageman *et al.*, 2012), however the immune response to infectious disease has received little attention thus far. In a study done by Field *et al.* (2000), it was reported that infants who's formulas were supplemented with n-3 LCPUFAs had an increased proportion of antigen mature CD4+ cells (improved immune response), improved interleukin-10 production (anti-inflammatory), and reduced interleukin-2 production (pro-inflammatory) compared with infants whose formulas were not fortified (Field *et al.*, 2000).

From these results it is clear that supplementing with n-3 LCPUFAs within the first few weeks of life and continuing through to 1 year reduces the incidence of respiratory morbidity and delays the onset of upper respiratory infections as well. This is apparent from the infant study done by Birch *et al.* (2010) in the USA, where the DHA/ARA group had fewer doctor visits within the first 3 years of life. It was suggested that the benefits of DHA/ARA supplementation may be related to viral infections and not bacterial infections (Birch *et al.*, 2010).

According to Calder (2010), n-3 LCPUFAs have anti-inflammatory effects, which in turn affect the immune system's response to pathogens, and it has been suggested that large doses may impair the immune response against infections. This seems to be dose-dependent as Field *et al.* (2002) have shown that when n-3 LCPUFA supplementation in healthy animal and human subjects consists of more than 10% n-3 PUFA (of total fat), the lymphocytic response to mitogen stimulation as well as natural killer cell activity and delayed-type hypersensitive reactions are all suppressed when compared to a diet high in n-6 PUFA. However, when n-3 LCPUFAs are supplemented in moderate amounts such as ~1000 mg DHA and ARA or <1000 mg EPA and DHA in humans, the immune response seems to be enhanced, and certain immune functions such as lymphocyte proliferation/activation and natural killer cell activity, IL-1, IL-2, and TNF- α production after mitogen stimulation, and macrophage activation become more optimal (Robinson *et al.*, 2001; Field *et al.*, 2002). In the study done by Minns *et al.* (2010) it has been suggested that due to the overall poor DHA intake in US infants and their low DHA status, supplementation may benefit immune function.

A study which showed different but interesting results, were the findings of Malan *et al.* (2015) who, by supplementing with Fe in apparently healthy Fe-deficient children (in a malaria-free area), found that this increased their infectious morbidity, especially in respiratory morbidity. But when iron supplementation was given in combination with DHA/EPA, this result was attenuated, and the amount of respiratory-related absenteeism was decreased significantly. They speculated that perhaps the DHA/EPA which was incorporated into the macrophage phospholipid bilayers may have enhanced phagocytosis, and thus improved the uptake of Fe from senescent red blood cells and therefore leaving the Fe which is required for pathogen growth to be less (Malan *et al.*, 2015). However, from a follow-up paper it became evident that this attenuation of morbidity, took place together with simultaneous reduction in inflammation as measured with circulating lipid-mediators. These findings suggest that the resolution of inflammation, facilitated by n-3-PUFA derived lipid mediators, are important for resistance to infectious morbidity (Malan *et al.*, 2016).

A major strength in most of these studies was the large number of participants, ranging from 180 to 7259 participants. The only study which had fewer subjects was Minns *et al.* (2010) with 28/29 subjects per group. However, this study still had 80% power based on an effect size of 0.66% and 1.2% standard deviation (SD). Therefore this is not considered a limitation.

There were limitations in each study which are shown in Table 2–7. However, none of the limitations seemed to influence the results of any of the studies significantly. This review has its own limitations as the original focus was on the effect of LNS on the infectious morbidity of children under the age of five, specifically from a low socio-economic area. However, as current data are so limited, it is difficult to make generalisations based on very little data. Therefore it was necessary to broaden the research question and make room for additional inclusions such as studies from middle and upper socio-economic status and studies with children over the age of five.

Table 2-7: Limitations of each study

Study	Limitations
Pastor 2006 (Spain)	Paediatricians may have been biased towards families of higher SES. Study population represents Spain which is not a country representative of extreme poverty. Infants fed control formula received a heterogeneous group of formulas including two with DHA and ARA, albeit at levels less than that present in the DHA+ formula
Bendabenda 2016 (Malawi)	It is possible that the research assistants may have under-reported morbidity as they were not blinded.
Huybregts 2012 (Chad)	The projected sample size was not fully attained and significantly fewer children from the control group were present at follow-up sessions. The study was not blinded.
Minns 2010 (USA)	A limitation of estimating DHA intake is that parents have been found to over-report the amount of food consumed by their toddlers. However, DHA intakes are likely underestimated by software used to analyze intake
Birch 2010 (USA)	Current outcomes were not part of the original protocol and thus were obtained by chart review. As with any such review, participants lost to follow-up, missing charts, and missing visits are to be expected

Study	Limitations
Thomas 2012 (India)	No limitations were discussed
Malan 2015 (RSA)	Could not identify the source of respiratory morbidity. Could not collect data during holidays or a teachers' strike, which amounted to 34% of the total study time.
Dalton 2009 (RSA)	No limitations were discussed
Thienprasert 2008 (Thailand)	No limitations were discussed

In conclusion, from the results of these studies it is apparent that the inclusion of DHA and ARA in the diet of infants and children 0–12 years of age significantly improves respiratory health. However, as the dosages and duration are so varied for each age group, it is difficult to determine what the right combination of LCPUFA should be for optimal respiratory health. Therefore more RCT are needed to obtain more grounded evidence, especially in infants under the age of five.

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CHAPTER 3 METHODOLOGY

3.1 Study design and population

This study is the immunity study of a randomised controlled trial, called the TSWAKA study, investigating the effects of two soy-based pastes in the form of small-quantity lipid-based nutrient supplements (SQ-LNS) on the growth of 6-month-old infants. The study used a randomised controlled design where infants were randomised to one of three groups: small-quantity lipid-based nutrient supplement A (SQ-LNS A); small-quantity lipid-based nutrient supplement B (SQ-LNS B) and the control group. The SQ-LNS groups received supplements for six months during the intervention period, and the control group received a six month supply of the SQ-LNS A (supplement with essential fatty acids (EFA)) upon completion of the intervention period.

3.2 Subject selection, recruitment and sample size

3.2.1 Selection Criteria

All infants enrolled in the study had to be at least 6 months old (<7 months) and from the Jouberton area in Klerksdorp. However, infants were excluded if:

- They had never received any breast milk previously
- They had severe obvious congenital abnormalities
- They had haemoglobin (Hb) <70 g/L
- They had severe acute malnutrition (Weight-for-height < -3 z-score)
- They had other diseases referred for hospitalization by clinic staff
- The caregivers planned to move out of the study area within the next 7 months
- They were receiving special nutritional supplements as part of feeding programmes
- They were known to be HIV+
- They had not been born as a singleton

3.3 Recruitment of infants

Infants who had the potential to be enrolled in the study were recruited through five clinics as well as door-to-door visits. Mothers were recruited through churches and at the community centre on days when grants were given. Posters were put up in supermarkets, primary schools, crèches, and all the clinics in the Jouberton area. Seven hundred and fifty babies were recruited and they were randomised into 250 babies per group, with a calculated sample size of 186 babies per group on

which provision was made for an expected 25% dropout rate. This meant to give the study 80% power and a type-1 error of 5%.

During recruitment fieldworkers were stationed at each clinic where the nursing staff screened all the infants at their 14-week vaccinations. The designated fieldworker informed mothers/ primary caregivers (hereafter collectively will be referred to as caregiver) about the purpose and nature of the study along with an information sheet, thereby giving them the option to be recruited and allowing their babies to participate in the study. The information sheet (translated into Tswana and English) assisted the fieldworker in explaining the study, and it was read out loud for illiterate caregivers. Hereafter, the caregiver was given the opportunity to ask questions. Furthermore, as it makes provision for both the caregiver and the infant to participate in the study, the infant's details were recorded on the recruitment form.

Once the infant was 6 months old, the caregiver came to the study site and needed to sign an informed consent form in order to be enrolled. Upon enrolment an infant was classified as a participant. Illiterate caregivers used their thumb prints as their signature. The consent form required permission for the following: for the mother/caregiver and child to participate in the study; clarity on the purpose, advantages and disadvantages of the study; allowance for withdrawal during the intervention period; assurance of confidentiality; the caregiver's full participation in adhering to the study rules; permission for blood to be withdrawn, and anthropometrical measurements to be taken of both mother and child. Mothers who were minors (under 18 years) signed assent on the consent form and the caregiver of the mother also needed to sign the consent form.

All participants involved in the study still had normal access to routine health care services including check-ups, vaccinations, and vitamin A supplements. The study did not interfere with any of these. The measles vaccination was given routinely at 9 months according to the National Immunization Programme (NIP).

3.4 Sample size calculation and justification

The sample size of the main study was based on the expected difference in growth achieved during the 6 months of active intervention. The growth indicator selected for the sample size calculation was length-for-age (LAZ). This was based on a study done by Adu-Afarwuah *et al.*, which was conducted in Ghana (Adu-Afarwuah *et al.*, 2007). There was a difference in LAZ observed between the Sprinkles and Nutri-butter groups which were 0.16 LAZ scores with a pooled standard deviation of 0.54. Given this information, this study has been designed to detect a difference of 0.15 LAZ scores at 12 months of age with a pooled standard deviation of 0.54 (i.e. effect size of 0.27) between

each intervention group and the control group with 80% power and a type-1 error of 5%. A sample size of 186 participants was calculated to be give adequate power for the primary study outcomes. An expected drop-out rate of 25% was anticipated and the final sample size per group was adjusted to 250 infants giving a total sample size of 750 infants.

Based on a study done by Malan *et al.*, the number of infants of the main study gave 88% power for the morbidity study to detect a significant difference between the number of children with respiratory illness on EPA/DHA supplementation and controls (Malan *et al.*, 2015). Based on the results of a study by Dalton *et al.* (2009), a sample size of 144 infants per group would give the study 80% power to detect a significant difference ($\alpha = 0.05$) in days absent due to illness (Dalton, 2006). Furthermore, based on Thomas *et al.*, the current study, with 186 infants per group, would have 99% power to detect a significant difference (10%) between infants with upper respiratory infection in an intervention and control group (Thomas *et al.*, 2012).

3.5 Randomisation, blindness and delivery of intervention

3.5.1 Randomisation

The enrolled participants were randomised according to block randomisation 3, 6 and 9 in order to allocate them to one of three groups: SQ-LNS A, SQ-LNS B, and the control group. The randomisation lists were prepared by a trial statistician using NQuery version 7. Each group was colour-coded to blue, green or orange. This facilitated the implementation and monitoring. Each participant's details was recorded on the enrolment form and a colour-coded identification card was given to each participant, where upon it needed to be produced at each project-related home-, clinic-, and research site visit. Caregivers were reimbursed for travel costs to the research site.

3.5.2 Blindness of treatment

There were visible differences in the treatments as each treatment had a colour allocation, and the control group, which received no supplement. Therefore only the people involved in the laboratory, anthropometric, child development and dietary intake assessments, as well as data and dietary analysis, were blinded. However, to eliminate any bias, two members of the research team who were not directly involved with the analysis or initial interpretation of the data, re-coded the colours into three random numerical codes. This was done after all data had been collected and before any data analysis was done. The key to these new codes was a document, held by the director of the Centre of Excellence for Nutrition of the NWU (2011-2015), and another copy was held by the office

of the director of the Nutrition Intervention Research Unit of the MRC. Once the data analyses had been completed, the codes were broken.

3.6 Delivery of intervention

Once the participant was enrolled in the study and baseline measurements were taken, every caregiver received a demonstration on how to mix the SQ-LNS (Table 3-1) into about 20 g of complementary food of own choice (preferably breakfast). It was emphasised that the SQ-LNS consumption was to be for that particular participant only, and should not be shared among other household members. A 2-week supply of individual sachets was given to each caregiver and an assigned fieldworker visited the caregiver within 7 days from enrolment to ensure the correct usage of the product. Hereafter, the fieldworker paid weekly visits to the caregiver to collect data on daily adherence and any illness that may have occurred. Participants in the control group were also visited weekly in order to collect morbidity data. The caregivers were encouraged on a regular basis to continue their breastfeeding practices to prevent changes due to the introduction of the SQ-LNS. All three groups were treated in a similar fashion.

3.6.1 Products

Two newly developed small quantity soy-based pastes in the form of lipid-based nutrient supplements (SQ-LNS A and SQ-LNS B) were used in the trial as intervention products (Table 3-1). SQ-LNS A provided essential fatty acids and SQ-LNS B provided both essential fatty acids and docosahexaenoic acid (DHA; fish oil powder), arachidonic acid (ARA; microbial powder), microbial phytase and lysine. The phytase (150 FTU) in SQ-LNS B was included to improve iron and zinc bioavailability (Troesch *et al.*, 2011). The study was conducted in a study population that is exposed to higher linoleic acid (LA) diets due to the inclusion of mostly high LA-containing sunflower oil during food preparation. The products should contribute towards restoring the balance between LA and alpha-linolenic acid (ALA) intake. Furthermore, because soy is cheaper than peanuts (USDA, 2010, Index Mundi, 2010) and because peanuts are not used for child feeding in many parts of the world, it is important to test whether soy pastes will be effective.

Table 3-1: The nutrient content of the intervention products

	Fortified fat-based paste with essential FA (SQ-LNS A)	Fortified fat-based paste containing DHA/ARA and phytase (SQ-LNS B)
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
n-6/n-3 ratio	5.7	5.0
Long-chain polyunsaturated fatty acids		
Docosahexaenoic acid (DHA) (mg)	-	75
Arachidonic acid (ARA) (mg)	-	75
Micronutrients		
Vitamin A (μ g)	200	200
Vitamin D (μ g)	2.5	2.5
Vitamin E (mg)	2.5	2.5
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 (mg)	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

	Fortified fat-based paste with essential FA (SQ-LNS A)	Fortified fat-based paste containing DHA/ARA and phytase (SQ-LNS B)
Iron (mg)	5.8	5.8
Zinc (mg)	6.2	6.2
Copper (mg)	0.28	0.28
Selenium (mg)	8.5	8.5
Magnesium (mg)	-	30
Manganese (mg)	-	0.6
Phosphorus (mg)	-	230
Potassium (mg)	-	257
Lecithin (Choline) (mg)	2.0	7.8
Other		
L-Lysine (mg)	-	160
Phytase (FTU)	-	200

3.6.2 Safety and management of products

According to Global Alliance for Improved Nutrition (GAIN), the product was safe and acceptable within this population. The product had an acceptable shelf life between 23–35°C. A sufficient number of different batches of products were delivered to the main laboratory in Potchefstroom at the NWU always considering the expiry date of such a batch. The products were delivered approximately every three to six months and stock was checked to see how much and in what condition it was received in. It was stored at the NWU and was distributed to the field research site on a first-in-first-out (FIFO) basis which was grounded on the Hazard Analysis and Critical Control Points (HACCP) principles.

The field research site received the stock every week from the NWU and held 2 weeks' worth of stock in order to minimise potential theft and damages. All stock received and issued to caregivers were recorded in the *Field Laboratory Stock Control Inventory*. There was also a separate compliance sheet where additional details were recorded such as: Amount of stock issued to a caregiver, the amount left in the container and the number of empty packages returned. These compliance sheets were returned to the field laboratory for data entry into the participant's paper register and for the electronic database. After this they were taken back to the main laboratory every week for safe keeping.

3.6.3 Data collection methodologies and plan

There are five clinics in the Jouberton area where recruitment took place, as well as door-to-door recruitment and at community level (posters at supermarkets, crèches, etc.). The centrally established field centre was where baseline (participants at 6 months) and exit (participants at 12 months) measurements and blood samples were taken.

Table 3–2 summarises the study activities carried out during the 26 week intervention period. A monthly refresher training session was held for most of the study duration. Protocols were put into place for each operating procedure.

Table 3-2: Study activities along the 26 weeks.

Visit	Day 1 Baseline	Weeks 1 to 8	Month 2 Week 8	Weeks 9 to 17	Month 4 Week 17	Weeks 18 to 25	Month 6
Window period allowed		weekly	+/- 2 days	weekly	+/- 2 days	weekly	+/- 2 days
Informed consent (Appendix A)	x						
Registration	x						
Eligibility Criteria & Assess potential allergy *1	x						
Complete Enrolment Form	x						
Haemoglobin concentration *2, 6	x						x *5
Other Blood sample draw	x						x
Baseline questionnaire (Appendix B) *3	x						
Hand out supplement	x						
Provide instructions on the use of the supplement	x						
Do education on breast feeding and complimentary feeding *4	x		x		x		x
Record Morbidity (Appendix E)		x		x		x	
Monitor and record Adherence (Appendix F)		x		x		x	

Visit	Day 1 Baseline	Weeks 1 to 8	Month 2 Week 8	Weeks 9 to 17	Month 4 Week 17	Weeks 18 to 25	Month 6
Window period allowed		weekly	+/- 2 days	weekly	+/- 2 days	weekly	+/- 2 days
Collect empty containers and issue supplement		x		x		x	
Check for severe malnutrition *5			x		x		
Exit questionnaire (Appendix C) *7							x
Hand out supplement and provide instructions to control group							x
Confirm status of baby							
Document Adverse Events	x	x	x	x	x	x	x

- (1) Ensure that child is apparently healthy; screen the baby according to the eligibility criteria: check severe congenital abnormalities and chronic diseases; ask if mother plans to move out of study area within the next 7 months; measure Hb (if Hb < 70 g/L exclude from study and refer for treatment); measure length and weight (if weight-for-height z-score < -3 exclude from study and refer for treatment)
- (2) Finger pricks will be the last option as that will only provide enough blood for the Hemocue blood test for haemoglobin status
- (3) Socio demographics, morbidity
- (4) Always use the education file to ensure that all mothers receive the same information
- (5) Weight-for-height z-score < -3 exclude from study and/or refer for treatment
- (6) If Hb < 70 g/L refer for treatment.
- (7) Product acceptance and use, and morbidity.

3.6.4 Morbidity

Information on morbidity was recorded weekly by the caregivers using a wellness/illness diary (Appendix D). Fieldworkers also paid weekly visits to caregivers and obtained morbidity data by using questionnaires (Appendix E). All morbidity included diarrhoea, respiratory illnesses, vomiting, and episodes of fever and rash/sores. These questionnaires were captured together as to eliminate errors and to validate the accuracy of the morbidity reports by the fieldworker. The data was captured in Epidata (version 3.1), due to its ease of use and so that errors were minimised. There were only two data capturers to ensure uniform entry of data and a 100% double-entry check was done as a quality control measure.

3.6.5 Socio-economic information

Socio-economic and demographic information was required from the caregiver at baseline in order to put the study into context (Appendix B).

3.6.6 Blood samples

A 4 ml blood sample was taken at 6 months (baseline) and 12 months (exit). The blood sampling was done by a nurse with paediatric experience and she had access to a paediatrician when necessary. The blood sampling was done in preparation of ethylene-diamine-tetra-acetic (EDTA) plasma and was obtained from the antecubital venipuncture of the arm area or dorsal area of the hand. If the paediatric nurse failed to obtain a blood sample from these areas then a sample was taken by means of a finger prick.

3.6.7 Red blood cell total phospholipid fatty acid composition

Red blood cell (RBC) total phospholipid fatty acid composition was analysed in a subsample. Laboratory analysis was performed with gas chromatography tandem mass spectrometry (GCMSMS) using multiple reaction monitoring and ammonia chemical ionisation. Phospholipids were extracted from plasma with chloroform: methanol (2:1 vol: vol; containing 0.01% butylated hydroxytoluene) using a modification of the method of Folch *et al.* (1957). The resulting fatty acid methyl esters (FAMES) were extracted with water and hexane and analysed using GCMSMS on an Agilent Technologies 7890A Gas Chromatograph system equipped with an Agilent Technologies 7000GC/MS triple quad mass selective detector (Agilent Technologies, Santa Clara, US). The gas chromatography separation of FAMES were carried out on a HP88 capillary column (100 m x 0.25 mm x 0.20 µm; Agilent) and mass spectrometry with 70-eV electron ionization was carried out in multiple reaction

monitoring mode, with at least two transitions per compound. Quantification of FAME was performed with Masshunter (B.06.00) and fatty acid results reported as a percentage of the total concentration of all fatty acids identified in the sample.

3.6.8 Anthropometric measurements

To describe the study population's baseline characteristics, anthropometric measurements were included. All anthropometric measurements were done by two fieldworkers who received training according to the International Society for Advancement of Kinanthropometry (IZAK) procedures. The WHO training course on Child Growth Assessment (Version 1, November 2006) was the basis for the length and weight measurements, and the WHO's anthropometry training video was the basis on which the weight, length, head- and mid-upper arm circumference (MUAC) measurements were done (available at <http://www.who.int/childgrowth/training/en/>). Assessors were standardised by comparison with measurements done by Prof HS Kruger (who supervised the anthropometric measurements in the 2005 National Food Consumption Survey Fortification Baseline). This was done in order to minimise weight differences to at least 0.05 kg and height differences by 0.5 cm for both caregiver and participant. The MUAC and head circumference did not have a difference of more than 0.3 cm.

Length and weight of participants were measured at two month intervals at 6- (baseline), 8-, 10- and 12 (exit) months by using a length measuring board, a mechanical infantometer (Seca 416, Germany) and the weight was taken with a calibrated digital scale (Seca 354, Germany). Head circumference and MUAC were taken at baseline and exit by using a Lufkin metal tape and all measurements were duplicated to ensure accuracy. If duplicate weights differed by more than 0.05 kg, a third measurement was taken. Similarly, if the head measurements differed by 0.3 cm, and the two length or MUAC measurements differed by >0.2 cm, then a third measurement was taken and the two closest values were recorded. The WHO growth standards (Onis, 2006) were used for nutritional assessment by using the weight-for height z-scores for age and gender. The weight and height of the caregiver were also required at baseline and this was used to calculate Body Mass Index (BMI).

3.7 Laboratory Analysis

3.7.1 Blood

During routine screening at baseline, the infant's Hb levels were measured immediately (using the HemoCue® Hb 201+ System ; Angelholm, Sweden), whereby it was determined whether the infant was eligible for enrolment in the study if the Hb was > 70 g/L. Hereafter, a 4ml sample of blood was

collected at baseline (6 months) and another one at exit (12 months). Blood samples were centrifuged for 10 minutes at 2000 g and 100 µL plasma aliquoted into 500 µL microfuge tubes and frozen at –20°C. Red blood cells were washed with normal saline and also stored at –80°C. Twice a week these samples were transported from the study site to the main laboratory at the Potchefstroom campus of the NWU for storage at –80°C until being assayed. Storage temperature was monitored and logged for the duration of the study. When all assays had been done, the left-over plasma was discarded according to standard procedures. All blood will be stored for at least 10 years or as ethical procedures require.

Effects of the intervention on immune function were assessed by measuring response to the immunisation against measles in plasma with the Measles IgG enzyme linked immunosorbent assay (ELISA) kit from Human Diagnostics. Ferritin was measured with sandwich ELISA (Erhardt *et al.*, 2004) at VitMin Laboratory in Munich, Germany. Haemoglobin was measured immediately with HemoCue® Hb 201⁺ System (Angelholm, Sweden), (Mathee *et al.*, 2014).

For this study, red blood cells were analysed with liquid chromatography tandem mass spectrometry (LCMSMS) using multiple reaction monitoring and ammonia chemical ionisation. Phospholipids were extracted from plasma with chloroform: methanol (2:1 vol: vol; containing 0.01% butylated hydroxytoluene) by using a modification of the method of Folch *et al.* (1957). Lipid extracts were concentrated, and the neutral lipids were separated from the phospholipids by using thin-layer chromatography (silica gel 60 plates, 10x 20 cm; Merck) and eluted with diethyl ether: petroleum, ether: acetic acid (30:90:1 vol: vol: vol). The lipid band that contained phospholipids was removed from the thin-layer chromatography plate and trans- methylated with methanol: sulphuric acid (95:5 vol: vol) at 70 °C for 2h to yield fatty acid methyl esters (FAMES). The resulting FAMES were extracted with water and hexane. The organic layer evaporated, re-dissolved in hexane, was analysed by using quadrupole gas chromatography–electron impact–mass spectrometry on an Agilent Technologies 7890A Gas Chromatograph system equipped with an Agilent Technologies 7000GC/MS triple quad mass selective detector (Agilent Technologies). The gas chromatography separation of FAMES were carried out on a HP88 capillary column (100 m x 0.25 mm x 0.20 µm; Agilent) by using helium as the carrier gas at a flow rate of 2.2 mL/min. The gas chromatography injector was held at a temperature of 270 °C, and the mass spectrometry source at 250°C. The injection volume of the sample solution was 1 µL by using a split ratio of 1:20. The oven temperature was programmed from 50 °C to 170 °C at 30 °C/min, then from 170 °C to 215 °C at 2 °C/min, and it rose at 4 °C/min to 230 °C. After that the temperature was held isothermally at 230 °C for 7 min. The total analysis time lasted 38.25 min. Mass spectrometry with 70 eV electron ionization was carried out in multiple reaction monitoring

mode, with at least two transitions per compound. Quantification of FAME was done with Masshunter (B.06.00). 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 fatty acids were calculated by taking the concentration of a given fatty acid derivative as a percentage of the total concentration of all fatty acids identified in the sample.

3.7.2 Immune function assessment

Effects of the intervention on immune function were assessed by measuring response to the immunisation against measles in plasma (using exit blood) with the Measles IgG ELISA kit from Human Diagnostics (Hamburg, Germany). Measles immunisation is performed routinely by the National Expanded Programme for Immunisation (EPI) at 9 months of age. This is done at all clinics and captured in the Road to Health Chart of the baby. Therefore, the baseline measles IgG levels were measured to confirm that the participants had not yet contracted measles, if so; they were excluded from this assay. Endpoint measles IgG levels were measured at 12 months at exit from the study, thus three months after immunisation. Since this is a primary immunisation, it increased accuracy for this method to determine differences in immune function between groups.

3.7.3 Iron and inflammation status

Iron status and acute and chronic inflammation were measured to describe the population's baseline characteristics, by using the following:

- (1) Haemoglobin using the HemoCue® Hb 201⁺ System (Angelholm, Sweden)
- (2) Soluble serum transferrin receptor, ferritin (both iron status) and high sensitivity CRP (measuring acute inflammation) as well as alpha-1-acid glycoprotein (AGP, measuring chronic inflammation) were measured by a sandwich ELISA method at the laboratories of Jürgen Erhardt (Erhardt *et al.* 2004). In the case of falsely elevated ferritin levels, these markers of inflammation eliminated this possibility.

3.8 Compliance monitoring and quality control

Every mother was routinely supplied with two weeks' worth of supplements at a time, in the event that a fieldworker fell ill or did not visit the mother within seven days after the previous visit. This eliminated the risk of participants not having enough sachets until the next supply arrived. There were different ways in which adherence was monitored:

A quantification method: During weekly home visits, the fieldworker counted and collected the empty sachets and left-over product.

A qualitative method: Every week the fieldworker used an adherence questionnaire by asking the caregiver whether the SQ-LNS was given daily, how much was given and how much was consumed (Appendix F).

Caregivers were required to return all empty packaging. A questionnaire/calendar, which was provided weekly in advance, was used by the caregiver to indicate how much SQ-LNS the participant consumed on a daily basis. This was a basic and user-friendly questionnaire where the amounts consumed were visually depicted (Appendix D). Adherence information, which was provided by the caregiver, was confirmed by the fieldworker on the retrospective questionnaire as well as observation during weekly visits.

The waste was determined by using the adherence questionnaire which the caregiver used to indicate the amount of SQ-LNS consumed and this was verified weekly by the fieldworker (Appendix F). All compliance and observation data was captured and entered into an electronic database.

3.9 Remuneration

All participants were remunerated for their trips to the study site and back.

3.10 Data analysis plan

3.10.1 Data management

Every week data was collected from the fieldworkers and these source data were given a date, participant number and data collector code. Source data were stored in individual coded folders. All data entered by hand was then entered into an electronic database. All data were routinely backed-up. All documentation specific to the participants, including laboratory samples, source data and Case Report Forms (CRF), were given codes for identification purposes and these were used on hard-copies as well as in the database. Names were only to be used on the informed consent forms and a separate coding list. All data were confidential, therefore no clinical information was given out without written permission by the caregiver, except as necessary for monitoring. All data will be protected (hardware is locked in steel cupboards and software is password protected) for ten years at the NWU. The principle investigator has access to this data.

3.10.2 Morbidity data double checks

All morbidity data was recorded daily by the caregiver on the daily form, and once a week a fieldworker visited the caregiver to verify dates of adverse events (AE) and to fill out the morbidity form. These two forms were compared to check that they corresponded during data entry. If the data on both forms did not correspond, a Standard Operating Procedure (SOP) (Figure 3–1) was set up in order to take appropriate steps to make all data uniform upon electronic entry.

3.10.3 Data Cleaning

The following steps were followed for data cleaning:

- 1 Check that each baby's code is correct by scanning for irregularities.
- 2 Custom filter all data according to baby code (smallest to largest).
- 3 Cross-check each baby code with week numbers 0-26 & exit.
- 4 If week numbers end before 26 & exit, confirm that the baby is a withdrawal.
- 5 Additional columns were created with formulae to check that number of days ill corresponded with the dates stated.
- 6 Only weeks 1–26 were included in the data analysis. Baseline and exit were excluded as these dates were somewhat varied among the infants.

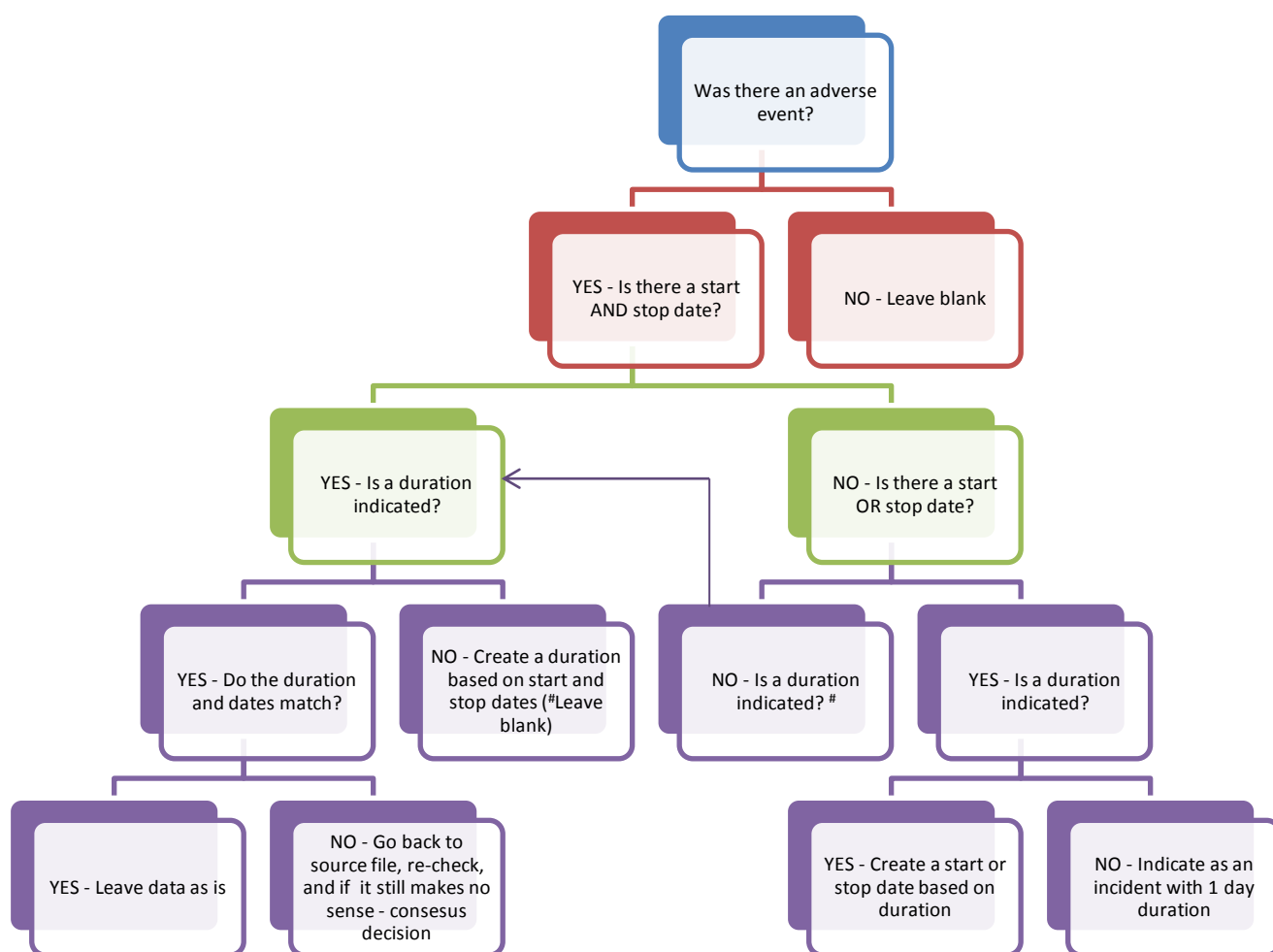


Figure 3-1: Flowchart to standardise checking and cleaning of morbidity data*.

* Electronic checks were done after cross-referencing with source files

3.11 Statistical analysis

The sample size of 250 participants per group was based on growth outcomes (Adu-Afarwuah *et al.*, 2007). However, based on a n-3 long-chain polyunsaturated fatty acid (LCPUFA) supplementation study done by Malan *et al.* (2015), the number of infants in this study had 88% power for detecting a significant difference between respiratory illness symptoms among the supplemented and control groups (Malan *et al.*, 2015). All captured data (except morbidity data) were checked for the presence of outliers (± 3 SD from the mean). Statistical analyses were performed using IBM SPSS Statistics (version 23; IBM Corporation) as well as Stata v13. Description statistics presented included medians with lower and upper quartile values, as well as means and standard deviations.

The study was not powered to compare the two SQ-LNSs to each other, therefore each SQ-LNS was compared independently to the control group. Differences in baseline characteristics between the subgroups were examined by using independent t-tests for continuous variables and by using the chi-square test for categorical variables. For intervention effects, an intent to treat analysis was followed as all children who were enrolled were included in the data analysis, except for the response to measles immunisation and fatty acid analysis. Intervention effects of SQ-LNS A and SQ-LNS B on morbidity symptoms were analysed by using Poisson regression with a log linear link adjusted for sex and correcting for exposure time in the study, using a modified intent to treat analysis augmented for the participants that had no data after enrolment. The intervention effect estimates from this model was reported as incidence rate ratios with 95% confidence intervals. Intervention effects on anaemia and ferritin were estimated using median quantile regression based on imputed data and adjusted for baseline values. Intervention effects on fatty acid status and measles IgG were estimated on subsamples with median quantile regression on endpoint data. Only successfully obtained and analysed endpoint samples were used for estimation of intervention effects (n=46, 51 and 54 in SQ-LNS A, SQ-LNS B and control group, respectively), since there was little overlap between successfully analysed endpoint and baseline samples. We considered P-values ≤ 0.05 to be significant.

3.12 Ethical clearance process and considerations

Ethical approval was obtained for the TSWAKA study from the ethics committees of NWU (NWU-00001-11-S1) and the Medical Research Council (MRC; EC011-03/2012). Hereafter the project was reviewed by the Department of Health and Social Development and registered with the Directorate for Policy, Planning and Research. The community of Jouberton was also consulted, and approval for the study was granted at sub-district level. The sub-study (immunity) also received ethical approval from the NWU ethics committee (NWU-00001-14-A1).

We proposed to utilize a “no-supplement” control group that underwent the same measurements and monitoring as the intervention groups. Children in the control group however received a 6-month supply of the SQ-LNS A with EFA after completion of the intervention period. After much deliberation we were of the opinion that the use of such a group is in line with the intention of the Declaration of Helsinki (October 2008) and in particular with paragraph 32 which states:

“The benefits, risks, burdens and effectiveness of a new intervention must be tested against those of the best current proven intervention, except in the following circumstances:

The use of placebo, or no treatment, is acceptable in studies where no current proven intervention exists; or

Where for compelling and scientifically sound methodological reasons the use of placebo is necessary to determine the efficacy or safety of an intervention and the patients who receive placebo or no treatment will not be subject to any risk of serious or irreversible harm. Extreme care must be taken to avoid abuse of this option.”

According to the Declaration of Helsinki, in order to assess the effectiveness of a new intervention, it is important that the new intervention is tested against a well-established comparator (active comparator or no-supplement control group). When no comparator is included in a study, no clear conclusions can be drawn regarding the effectiveness of the product and therefore is considered to be unethical. As there is no current proven intervention available with a sound proven efficacy, the use of a no-supplement control group in the current study was considered to be justified and in line with the Declaration of Helsinki. The risk/balance for the participants in the no-supplement control group was considered to be positive, since these children received a delayed intervention and there is no evidence that getting the product during the 12-18 months period is worse as compared to the 6-12 month period.

Parents or legal guardians had the right to withdraw their child from the study at any time for any reason, without being obliged to give reasons and without penalty or loss of benefits they were entitled to. The investigator also had the right to withdraw any participant from the study if this was in the best interest of the participant.

Any of the following conditions lead to premature withdrawal:

- Request by participant to discontinue for any reason during the study (withdrawal of consent)
- Erroneously included or erroneously randomised participant
- An adverse event or inter-current illness that in the opinion of the investigator warranted the participant's withdrawal from supplementation
- Volunteers who did not follow the requirements of the investigator, especially those concerning safety and/or if the participant after his entry was uncooperative or not willing to comply with the protocol (non-compliant)
- Failure to return (lost to follow-up)
- request by regulatory agencies for termination of supplementation of an individual patient or all patients under this protocol

The circumstances of any discontinuation had to be documented in detail in the corresponding study termination form in the case report file. The data of the withdrawn participants were documented in the clinical study report. Participants who withdrew prematurely from the study were not replaced.

The trial was registered with clinicaltrials.gov registry (NCT01845610).

A Safety Monitoring Board (SMB) was established to ensure that adverse events were monitored.

3.13 Safety

3.13.1 Definitions and standards:

Adverse events (AE): If a participant involved in a research project, where an investigational product is administered, has any untoward medical occurrence.

Any unfavourable and unintended sign (including abnormal laboratory findings), symptom, or disease, temporarily associated with the use of the product is considered an AE. Accidents, whether or not related to the product or study-related procedure which is reported by the participant's caregiver or observed by the investigator is also classified as an AE.

Adverse reaction (AR): All noxious and unintended responses to a product related to any dose should be considered as adverse reactions.

Serious Adverse Event (SAE): Any adverse event that at any dose fulfils at least one of the following criteria:

- Is fatal (results in death)
(note: death is an outcome, not an event)
- is life-threatening
(note: the term "life-threatening" refers to an event in which the participant was at risk of death at the time of the event; it does not refer to an event which could hypothetically have caused death had it been more severe)
- requires inpatient hospitalization or prolongation of existing hospitalization
(note: "inpatient hospitalization" refers to an unplanned, overnight hospitalization)
- results in persistent or significant disability/incapacity
(note: the term means substantial disruption of one's ability to conduct normal life function)
- is a congenital anomaly/birth defect

(note: congenital anomaly/birth defect in offspring of subject taking the product regardless of time to diagnosis)

- is medically significant

(note: Medical and scientific judgement should be exercised in deciding whether expedited reporting is appropriate in other situations, such as important medical events that may not be immediately life-threatening or result in death or hospitalisation but may jeopardise the participant or may require intervention to prevent one of the other outcomes listed in the definition above).

Suspected Unexpected Serious Adverse Reaction (SUSAR): All suspected adverse reactions related to a product (the tested product or active comparators) that are both unexpected and serious.

Unexpected Adverse Reaction: An adverse reaction, the nature, or severity or incidence of which is not consistent with the applicable product information.

Expected (S)AEs: The study was performed in a rural area where morbidity is very common and infant mortality is high. As occurs in any developing country, the infants mainly die as a result of perinatal complications and common infectious diseases such as diarrhoea and severe respiratory infections.

It has been reported by UNICEF (2011) that the South African infant mortality rate is 18/1000 live births. However our study population was within the age range of 6-12 months, and the expected mortality rate (according to our calculations) for this age group was 12.5/1000 live births. As there were 750 participants in this study, we expected up to 9 participant deaths during the study period. However, the participants in the study were closely monitored and taken better care of than usual (i.e. referred to healthcare services when needed); therefore so many deaths were unlikely, and the total number of deaths was five.

3.13.2 Adverse event assessment

Expectedness: An unexpected AE is an event of which the nature or severity of the condition is not consistent with the applicable product information.

Casualty Assessment: The casualty assessment of an AE to the investigational product was rated as follows:

- *No (Not related):* The temporal relationship of the clinical event to product administration makes a causal relationship unlikely, or other drugs, therapeutic interventions or underlying conditions provide a sufficient explanation for the observed event.
- *Probable* A reasonable time relationship to product administration and which cannot be explained by concurrent disease or other drugs
- *Possible* A reasonable time relationship to product administration but which can be explained by concurrent disease or other drug
- *Yes (Related):* The temporal relationship of the clinical event to product administration makes a causal relationship possible, and other drugs, therapeutic interventions or underlying conditions do not provide a sufficient explanation for the observed event.

Severity/Intensity: The severity / intensity of adverse events were graded on a three-point scale:

- Mild or Grade 1: Discomfort noted, but no disruption to normal daily activities.
- Moderate or Grade 2: Discomfort sufficient to reduce or affect normal daily activities.
- Severe or Grade 3: Inability to work or perform normal daily activities.

3.13.3 Outcome of event:

The outcome of an event was classified as follows:

- Recovered
- Recovered with sequelae
- On-going
- Fatal
- Unknown / Lost to follow-up

3.13.4 Safety parameters

The children were very closely monitored to avoid any negative health consequences due to the intervention product or the doses provided. This was carried out by fieldworkers who visited each candidate weekly, as well as the bi-monthly visits of the participants to the study site.

Any SAEs which occurred during the study period needed to be reported within 24 hours by the principal investigator to the Safety Monitoring Board (SMB).

The SMB comprised of three independent scientists who have experience in intervention studies. They reviewed all (S)AEs every 2–3 months until one month post intervention. As mentioned before, they were informed of any SAEs within 24 hours, which they reviewed and took necessary action.

3.14 Reporting procedures

There is a section in the CRF where AEs were reported by the investigator.

SAEs were recorded on the SAE report form and the investigator needed to complete it and sent it to the SMB within the next 24 hours of becoming aware of an SAE. The sponsors were informed as well.

All original documentation was retained at the study site with the other participant specific study documentation.

All SUSARs were also reported and processed according to the applicable laws and regulatory requirements governing the biomedical research projects involving human subjects.

If a participant was withdrawn from the study as a result of a SAE the participant was followed up until the SAE outcome was determined or the condition was stabilised.

If a SAE occurred more than 28 days after the last dose of investigational product had been administered, it was only reported if it was suspected to be related to the product.

3.14.1 Declaration of Helsinki (DoH)

The study was conducted in accordance with the principles of the “Declaration of Helsinki” and/or the South African laws and regulations, whichever provides greater protection for the individual.

3.14.2 Good Clinical Practice (GCP)

As this was a biomedical research project, the investigators ensured that it was conducted in conformance with the principles of GCP, outlined as the following:

Biomedical research projects involving human subjects should be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki, and that are consistent with the principles of GCP (where applicable) and the applicable laws and regulatory requirements governing the conduct of human studies;

Foreseeable risks and inconveniences should be weighed against the anticipated benefit for the individual trial subject and the society;

The rights, safety, and well-being of the subjects involved in biomedical research are the most important considerations and should prevail over interests of science and society;

Biomedical research projects involving human subjects should be scientifically sound, and described in a clear, detailed protocol;

Biomedical research projects involving human subjects should be conducted in compliance with the protocol that has received prior institutional review board (IRB)/independent ethics committee (IEC) approval/favourable opinion;

The medical care given to, and medical decisions made on behalf of, subjects involved in biomedical research should always be the responsibility of a qualified physician;

Each individual involved in conducting biomedical research projects involving human subjects should be qualified by education, training, and experience to perform his or her respective task(s);

Freely given written informed consent should be obtained from every subject prior to participation in a biomedical research project;

All information from biomedical research projects involving human subjects should be recorded, handled, and stored in a way that allows its accurate reporting, interpretation and verification;

The confidentiality of records that could identify subjects should be protected, respecting the privacy and confidentiality rules in accordance with the applicable laws and regulatory requirement(s);

Investigational products should be manufactured, handled, and stored in accordance with the highest quality standards, or if applicable, good manufacturing practice (GMP); investigational products should be used in accordance with the approved protocol;

Systems with processes and procedures (such as SOPs) that assure the quality of every aspect of biomedical research projects involving human subjects should be implemented.

3.15 Participant confidentiality

The investigator(s) will ensure that the participant's anonymity towards the sponsors and other third parties will be maintained. On all documents that are to be submitted to the client, participants will be identified only by an identification code – not by their names or hospital/clinical number. The investigator(s) will keep a separate Participant Identification Code List which matches identifying

codes with the participant's names. Documents that are not for submission to the client will be maintained by the investigator in strict confidence.

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CHAPTER 4 MANUSCRIPT

EFFECT OF SMALL-QUANTITY LIPID-BASED NUTRIENT SUPPLEMENTS ON THE IMMUNITY OF 6-MONTH-OLD INFANTS: A RANDOMISED CONTROLLED TRIAL.

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Key words: Infants; morbidity; immunity, lipid-based nutrient supplement; micronutrients; essential fatty acids.

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Conflict of Interest: CMS and TMM have received speaking honoraria from Unilever and DSM, respectively. The researchers had no conflict of interest. None of the study funders had any involvement in the study design, analysis, or interpretation of data; the writing of the report; or the decision to submit the article for publication.

Authorship: K.J. involved in conceptualization, conducted the study and collection of morbidity data, performed data analyses and interpretation and wrote the manuscript; L.M. conceptualisation, data analyses and interpretation, revision of paper; C.L. data analysis and interpretation; M.R. and T.M.M. conceptualised and conducted the study; C.M.S. and M.F. initiated, conceptualised and conducted the study; C.M.S. interpretation and revision of paper. All authors read and approved the final version of the paper.

Ethics of human subject participation: This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethical Committees of the North-West University (NWU-00001-14-A1) and the South African Medical Research Council (SAMRC, EC011-03/2012). Written informed consent was obtained from all subjects' mothers/caregivers. This randomised controlled trial was registered at clinicaltrials.gov under NCT01845610.

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Abstract

Objective: To determine the effect of two small-quantity lipid-nutrient supplements (SQ-LNS) on the immunity of peri-urban South African 6-month-old infants.

Design: In a randomised controlled trial two groups of infants ($n = 250$ each) were supplemented with two soy SQ-LNS products for 6 months. The control group ($n = 250$) did not receive any supplements during the 6-month intervention period. Both products had similar compositions and SQ-LNS B included additional long chain polyunsaturated fatty acids (LCPUFA), phytase, and lysine. During weekly morbidity surveillance, the incidence and duration of infectious morbidity symptoms were recorded by fieldworkers using a questionnaire. In addition, the effect of the intervention on immune function was assessed by measuring response to immunisation against measles.

Setting: Peri-urban Jouberton area in the Matlosana Municipality, Klerksdorp, South Africa.

Subjects: Seven hundred and fifty 6-month old infants.

Results: Both SQ-LNS A and SQ-LNS B reduced the overall sick days of fever by 19.2% ($P < 0.001$) and 9.8% ($P = 0.006$), respectively; as well as the overall sick days of wheezing by 78.0% ($P < 0.001$) and 53.1% ($P < 0.001$). Coughing was also reduced in both groups ($P < 0.05$). Both SQ-LNS A and SQ-LNS B increased the overall days of diarrhoea by 29.6% ($P < 0.001$) and 68.4% ($P < 0.001$) respectively. The SQ-LNS A group had 4.8 and the SQ-LNS B group 2.4 times more overall sick days of vomiting than the control group (both $P < 0.001$). Both products increased the overall days with rash/sores; SQ-LNS A by 13% ($P = 0.012$) and the days with rash/sores almost doubled in the SQ-LNS B group ($P < 0.001$). Measles IgG response was significantly reduced by SQ-LNS-B ($B: -608.337$ 95% CI: $-1178.974, -37.699$).

Conclusion: These novel findings are a significant advancement to current knowledge. It is one of the first soy-based SQ-LNS intervention studies in infants. Both SQ-LNS products reduced fever and certain respiratory symptoms. However, there was an increase in diarrhoea, vomiting and rash/sores in both groups, although mostly mild and not serious. Smaller quantities taken more frequently may provide the benefits without the side effects

of the products. Additionally, immunity should also be monitored for a period after the intervention, when nutritional status has been improved and when there will be no interference from side effects as while supplementing. Nevertheless, the results should be interpreted taking the main outcomes of this study, namely, linear growth and psychomotor development into consideration. This study showed the need not only for more trials to be done on this topic but also to monitor and record morbidity data frequently and accurately.

Introduction

Nutrition's impact on immunity and its ability to modulate resistance to infection have received growing attention over the past few decades ^(1, 2). Diets of poor nutritional quality during infancy lead to insufficient nutritional intakes, and this combined with frequent infections during infancy and early childhood results in increased mortality ⁽³⁾. Therefore, in an effort to reduce child mortality, as stated in Millennium Development Goal 4, there has been a global shift towards implementing strategic effective and affordable interventions through the improvement of infant and child nutrition, especially within the first 1000 days of life ⁽⁴⁾. A relatively low-cost approach to improve nutritional status in infants is to provide them with small-quantity lipid-based nutrient supplements (SQ-LNS). Several studies have used this approach, focusing on growth and cognitive function as the primary outcome ⁽⁵⁻⁸⁾. However, very few studies with a detailed investigation of the effects on morbidity and immune function have been conducted ^(5, 6, 9, 10).

The lipid-based nutrient supplements (LNS) used to date generally contain essential fatty acids (EFA), and/or long-chain polyunsaturated fatty acids (LCPUFAs), and a mixture of micronutrients, often including iron and zinc ⁽¹⁰⁻¹⁶⁾. Most of these studies have shown beneficial effects of LNS on infectious morbidity, and a study by Malan *et al.* ⁽¹⁵⁾ showed that iron supplementation on its own increased infectious morbidity, but in combination with LCPUFA, this increase was attenuated ⁽¹⁵⁾.

There are various ways to measure immune function, including clinical outcomes such as recording the incidence and duration of illness ^(11-13, 16, 17). Clinical outcomes are still considered one of the best markers of immune function as there is currently no single measure which can predict the immunological impact of a nutritional intervention. However, response to vaccines are another way to measure the differences in immune function as they are widely used markers which provide reliable information on the effect of nutrients on immune responsiveness *in vivo* ⁽²⁾.

Therefore, this study aimed to investigate the effect of two SQ-LNSs on the incidence and duration of infectious morbidity symptoms of 6-month-old infants and on their response to measles immunisation. Here we present a comprehensive set of frequently monitored morbidity symptoms obtained with one of the first soy-based SQ-LNS studies in infants.

Methods

Study site, design and subjects

The primary objective of the main study (TSWAKA) was growth; and psychomotor development was also assessed. These were reported elsewhere (unpublished results). In this secondary study, we used a randomised, controlled, single-blind study design to determine the effects of two SQ-LNSs on the immunity of the infants. The study took place in the peri-urban area of Jouberton in the Matlosana Municipality, Klerksdorp, South Africa. We enrolled 750 6-month-old infants from 998 screened, of whom 514 completed the study. All infants enrolled in the study had to be at least six months old (less than seven months) and from the Jouberton area. However, infants were excluded if 1) they had never received any breast milk previously, 2) they had severe obvious congenital abnormalities, 3) they had haemoglobin (Hb) < 70 g/L, 4) they had severe acute malnutrition (weight-for-height < -3 z-score), 5) they had other diseases and were referred for hospitalisation by clinic staff, 6) the caregivers planned to move out of the study area within the next seven months, 7) they were receiving special nutritional supplements as part of feeding programmes, 8) they were known to be HIV+, 9) they were found to be allergic to peanuts, soy, cow's milk protein, and fish, and 10) they had not been born as a singleton.

Randomisation and Blinding

Block randomisation of sizes 3, 6 and 9 was used to randomly allocate each infant to one of three groups (which were colour-coded), i.e. SQ-LNS A (n = 250), SQ-LNS B (n = 250) and the control group (n = 250) (Figure1). Group randomisation lists were prepared using NQuery version 7 (Statistical Solutions Ltd, Cork, Ireland) and at the baseline visit the next sequential baby and group code was allocated to the next infant enrolled. Each infant from the SQ-LNS A and SQ-LNS B group was required to consume 20 g of the supplement daily for 6 months (adherence was reported daily by mothers/caregivers and monitored weekly through home-visits by fieldworkers). The control group did not receive any supplements during the 6-month intervention period, but received a 6-months' intervention with SQ-LNS A upon completion of the study.

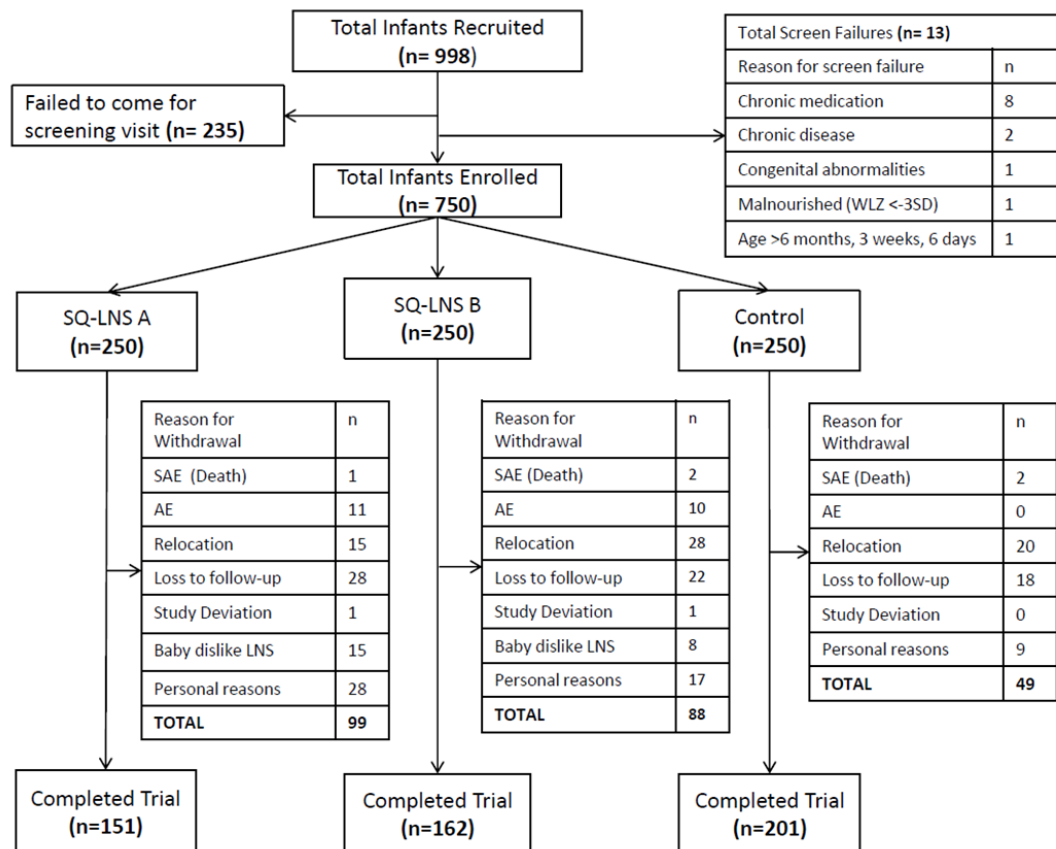


Figure 1: Flow diagram of participant progression through the randomised controlled trial (RCT). A total of 998 infants were screened and 750 were randomly assigned to one of the three groups; 514 (68.5%) infants completed the trial.

There were visible differences in the treatments as each had a colour allocation, and the control group, which received no supplement. Therefore only the people involved in the laboratory, anthropometric, child development and dietary intake assessments, as well as data and dietary analysis, were blinded. However, to eliminate any bias, two members of the research team who were not directly involved with the analysis or initial interpretation of the data, re-coded the colours into three random numerical codes. This was done after all data had been collected and before any data analysis. The key to these new codes was a document, held by two independent senior researchers. Once the data analyses had been completed, the codes were broken.

Intervention

Two SQ-LNS products were used, one with EFA (SQ-LNS A) and the other contained EFA with added LCPUFA, phytase, lysine and trace elements (SQ-LNS B) as shown in Table 1. The products were designed to contain approximately 50% of the reference nutrient intake (RNI) for most nutrients. Upon enrolment, the mothers/caregivers (hereafter collectively referred to as caregivers) were shown how to use the product by mixing the full sachet (20 g) as a complementary food supplement in the mornings to an equal amount of the infants' porridge. Table 1 shows the composition of both SQ-LNS products. Fieldworkers paid weekly visits to the caregivers, provided them with a weekly stock of the supplement and advised them to provide one sachet a day to the infant. They also collected data on daily adherence. The caregivers from all three groups were encouraged on a regular basis to continue their breastfeeding practices to prevent changes due to the introduction of the SQ-LNS. All three groups were treated in a similar fashion. Adherence was monitored by using a separate compliance sheet, carried by the fieldworkers, who recorded the stock issued to a caregiver, the number of unopened sachets in the container and the number of used packages returned.

Table 1: SQ-LNS¹ nutritional profile for infants 6–12 months old²

Nutrient	SQ-LNS A	SQ-LNS B ³
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
n-6/n-3 ratio	5.7	5.0
Long-chain polyunsaturated fatty acids		
Docosahexaenoic acid (DHA) (mg)	-	75
Arachidonic acid (ARA) (mg)	-	75
Micronutrients		
Vitamin A (μ g)	200	200
Vitamin D (μ g)	2.5	2.5
Vitamin E (mg)	2.5	2.5
Vitamin K (μ g)	7.5	7.5
Thiamin (mg)	0.25	0.25
Riboflavin (mg)	0.25	0.25

Nutrient	SQ-LNS A	SQ-LNS B ³
Niacin (mg)	3	3
Pantothenate (mg)	1.0	1.0
Vitamin B6 (mg)	0.25	0.25
Biotin(µg)	4.0	4.0
Folate (B9) (µg)	80	80
Vitamin B12 (µg)	0.45	0.45
Vitamin C (mg)	23.3	103
Calcium (mg)	250	396
Iodine (µg)	45	45
Iron (mg)	5.8	5.8
Zinc (mg)	6.2	6.2
Copper (mg)	0.28	0.28
Selenium (µg)	8.5	8.5
Magnesium (mg)	-	30
Manganese (mg)	-	0.6
Phosphorus (mg)	-	230
Potassium (mg)	-	257
Lecithin (Choline) (mg)	2.0	7.8
Other		
L-Lysine (mg)	-	160
Phytase (FTU)	-	200

¹ Small quantity lipid-based nutrient supplement (SQ-LNS)

² SQ-LNS supplementation (20 g/d) provided 108 kcal, protein, fat, and 19 vitamins and minerals.

³ SQ-LNS B contained additional powdered milk.

Data Collection

Morbidity

Fieldworkers paid weekly visits to caregivers and obtained morbidity data by using questionnaires. Morbidity symptoms comprised diarrhoea (three or more loose stools within 24 hours), vomiting, runny nose, coughing, wheezing, fever and rash/sores. Information on morbidity was also recorded daily by the caregivers using a wellness/illness diary. The questionnaires were captured together with reference to the illness diary so as to improve the accuracy of the data. Incidence and cumulative (overall) days of morbidity symptoms were collected, until the infant finished the study (26 weeks) or withdrew (varied at different weeks).

Morbidity symptoms were classified as adverse events (AEs) which included the same symptoms captured with the questionnaires. Classification of seriousness of symptoms (mild, moderate or severe) were reviewed and signed off by the study paediatrician. Any AE which was considered severe (death, a life-threatening event, required extra care such as

hospitalisation, resulted in persistent or significant disability/incapacity, a congenital anomaly, or was medically significant) was classified as a serious adverse event (SAE). Adverse events and SAEs not captured on the questionnaire, e.g. when the infant was hospitalised long term, were incorporated in the morbidity data. All SAE and AE data were quarterly reviewed by an independent safety monitoring board (SMB) consisting of a paediatrician, biostatistician and nutrition scientist.

An independent clinical research organization (CRO) (OnQ Research Pvt Ltd), represented by a clinical research associate (CRA), conducted bi-weekly visits to the study site in order to monitor the quality of the data collection and perform source data verification (SDV). Every participant had an individual folder identified by a participant number and a code of the data collector. All documentation regarding the participants, including the laboratory samples, source data and the case report forms (CRF), were identified with appropriate participant codes, both on paper and electronically. The names were only used on informed consent forms and a separate coding list.

The data obtained from the caregivers and fieldworkers were entered electronically into the clinical study database and validated under the direction of the data manager. Any missing, impossible or inconsistent recordings were referred back to the investigator. All entries to the study database were available in an audit trail. The data were validated as defined in the data management plan (DMP). Quality control procedures were applied to each stage of data handling to ensure that all data were reliable and had been processed correctly. Morbidity data were captured into EpiData (version 3.1) and exported to Microsoft Excel 2010, where they were cleaned and double checked.

Biochemical measurements

A 4 ml sample of blood was collected at baseline (6 months) and at exit (12 months). Blood samples were centrifuged for 10 minutes at 2000 g, separated and frozen on site at -20°C . Red blood cells were washed twice with normal saline and also stored at -20°C . These samples were transported daily from the study site to the main laboratory for storage at -80°C until assayed. Storage temperature was monitored and logged for the duration of the

study. If a blood sample could not be obtained, a finger-prick sample was taken to determine the Hb concentration.

Immune response to measles vaccine

Effects of the intervention on immune function were assessed by measuring response to the immunisation against measles in plasma with the Measles IgG ELISA kit from Human Diagnostics (Hamburg, Germany) ⁽¹⁸⁾. IgG analyses were performed in duplicate on baseline (6-month-old) and endpoint (12-month-old) samples. In South Africa, infants were immunised against measles at 9 months of age as part of the national immunisation programme at the time this study was performed.

Anaemia, iron and inflammation status

Anaemia, iron status and acute and chronic inflammation were measured by using the following: haemoglobin (measured with HemoCue[®] Hb 201⁺ System; Angelholm, Sweden), ferritin (iron status) and high sensitivity C-reactive protein (CRP, measuring acute inflammation) as well as alpha-1-acid glycoprotein (AGP, measuring chronic inflammation) were measured by a sandwich ELISA method at the VitMin Laboratory in Munich, Germany ⁽¹⁹⁾. Falsely elevated ferritin levels were excluded, by removing participants with CRP > 5 mg/L and/or AGP > 1 g/L for iron status analysis. Haemoglobin concentrations were measured on all infants and other parameters only on those infants from whom blood was obtained via venepuncture.

Red blood cell total phospholipid fatty acid composition

For this study, red blood cell (RBC) total phospholipid fatty acid composition was analysed in a subsample. Laboratory analysis was performed with gas chromatography tandem mass spectrometry (GCMSMS) using multiple reaction monitoring and ammonia chemical ionisation. Phospholipids were extracted from plasma with chloroform: methanol (2:1 vol: vol; containing 0.01% butylated hydroxytoluene) using a modification of the method of Folch *et al.* ⁽²⁰⁾. The resulting fatty acid methyl esters (FAMES) were extracted with water and hexane and analysed using GCMSMS on an Agilent Technologies 7890A Gas Chromatograph system equipped with an Agilent Technologies 7000GC/MS triple quad mass

selective detector (Agilent Technologies, Santa Clara, US). The gas chromatography separation of FAMES were carried out on a HP88 capillary column (100 m x 0.25 mm x 0.20 µm; Agilent) and mass spectrometry with 70-eV electron ionization was carried out in multiple reaction monitoring mode, with at least two transitions per compound. Quantification of FAME was performed with Masshunter (B.06.00) and fatty acid results reported as a percentage of the total concentration of all fatty acids identified in the sample.

Anthropometry

To describe the study population's baseline characteristics, anthropometric measurements were included. All such measurements were performed by two fieldworkers, who received training according to the International Society for Advancement of Kinanthropometry (ISAK) procedures. All infants' length was measured using a length measuring board, a mechanical infantometer (Seca 416, Germany) and weight with a calibrated digital scale (Seca 354, Germany). The length and weight of participants were measured at two-month intervals at 6 (baseline), 8, 10 and 12 (exit) months. At baseline and exit, head circumference and middle upper arm circumference (MUAC) were measured using a Lufkin metal tape. The WHO growth standards were used to calculate z-scores for weight, length, MUAC and head circumference for age and gender ⁽²¹⁾.

Statistical analysis

The sample size of 250 participants per group was based on growth outcomes ⁽⁵⁾. However, based on a n-3 LCPUFA and iron supplementation study conducted by Malan *et al.* ⁽¹⁵⁾, the number of infants in this study had 88% power for detecting a significant difference between respiratory illness symptoms among the supplemented and control groups ⁽¹⁵⁾. All captured data (except morbidity data) were checked for the presence of outliers (± 3 SD from the mean). Statistical analyses were performed using IBM SPSS Statistics (version 23; IBM Corporation) as well as Stata v13. Descriptive statistics presented include medians with lower and upper quartile values, as well as means and standard deviations.

The study was not powered to compare the two SQ-LNSs with each other, therefore each LNS group was compared independently with the control group. Differences in baseline

characteristics between the subgroups were examined by using independent t-tests for continuous variables and by using the chi-square test for categorical variables. For intervention effects, an intent to treat analysis was followed as all children who were enrolled were included in the data analysis, except for the response to measles immunisation and fatty acid analysis. The intervention effects of the SQ-LNS A and SQ-LNS B products on morbidity symptoms were analysed by using Poisson regression with a log linear link adjusted for sex and correcting for exposure time in the study, using a modified intent to treat analysis augmented for the participants that had no data after enrolment. The intervention effect estimates from this model were reported as incidence rate ratios (IRR) with 95% confidence intervals. Intervention effects on anaemia and ferritin were estimated using median quantile regression based on imputed data and adjusted for baseline values. Intervention effects on fatty acid status and measles IgG were estimated on subsamples with median quantile regression on endpoint data. Only successfully obtained and analysed endpoint samples were used to estimate intervention effects (n = 46, 51 and 54 in SQ-LNS A, SQ-LNS B and control group, respectively), since there was little overlap between successfully analysed endpoint and baseline samples. We considered P-values ≤ 0.05 to be significant.

Results

We conducted this study between September 2013 and July 2015 (1 year and 10 months). Out of the 998 infants who were screened, 750 were enrolled to participate in the study and 514 completed the 6-month intervention trial. Figure 1 depicts the group allocation and reasons for withdrawals (withdrawal rate of 31.5%). We found no differences in baseline characteristics (Table 1) between the SQ-LNS A or SQ-LNS B groups and the control group, respectively.

Table 2: Baseline characteristics of all the infants and subgroups of the LNS intervention groups and the control group^a.

Baseline characteristics	All Infants	Control	SQ-LNS A	SQ-LNS B
Total number of participants	750	250	250	250
Male : Female (n)	387 : 363	131 : 119	113 : 137	143 : 107
Male : Female (%)	51.6 : 48.4	52.4 : 47.6	45.2 : 54.8	57.2 : 42.8
Height (m)	63.85 ± 2.49 [†]	64.08 ± 2.5	63.71 ± 2.49	63.77 ± 2.49
Weight (kg)	7.20 (6.52, 7.95) [‡]	7.11 (6.60, 7.99)	7.13 (6.37, 7.85)	7.31 (6.56, 8.05)
Birth weight (kg)	3.00 (2.70, 3.30)	3.00 (2.79, 3.30)	3.00 (2.66, 3.30)	3.00 (2.70, 3.30)
Anthropometric Indices (n = 750)				
Stunted ¹ n (%)	220 (29.3)	71 (28.4)	71 (28.4)	78 (31.2)
Wasted n (%)	10 (1.3)	4 (1.6)	4 (1.6)	2 (0.8)
Overweight ² n (%)	76 (10.1)	28 (11.2)	18 (7.2)	30 (12.0)
Anaemia status (n = 750)				
Haemoglobin (Hb) (g/dl)	11.3 (10.5, 12.2)	11.3 (10.5, 12.1)	11.2 (10.5, 12.1)	11.5 (10.5, 12.3)
Anaemic (Hb < 11 g/dl) n (%)	274 (36.5)	90 (36.0)	98 (39.2)	86 (34.4)
Iron status (n = 485)				
	(n=485)	(n=155)	(n=168)	(n=162)
Serum ferritin (µg/l) ³	25.2 (15.9, 40.2)	25.4 (15.3, 39.8)	24.9 (16.0, 40.6)	25.3 (16.4, 40.2)
C-reactive protein (CRP) > 5 mg/l n (%)	72 (14.8)	20 (12.9)	29 (17.3)	23 (14.2)
Alpha-1 glycoprotein (AGP) > 1 g/l n (%)	156 (32.2)	52 (33.5)	51 (30.4)	53 (32.7)
Iron deficiency (n = 485) (%)				
Serum ferritin < 12 µg/l n (%) ⁴	77 (15.9)	23 (14.9)	23 (13.9)	31 (19.3)
Hb < 11 g/dl+serum ferritin < 12 µg/l n (%) ⁵	50 (10.3)	15 (9.7)	18 (10.9)	17 (10.6)

¹ Length-for-age z-score < -2; ² weight-for-length z-score > 2; ³ after adjustment for inflammation (CRP > 5); ⁴ ID; depleted iron stores ⁵ IDA.

^aDifferences between the three subgroups were examined by using independent *t* tests for continuous variables and by using the chi-square test for categorical variables.

[†]Mean ± SD (all such values).

[‡]Median (25th, 75th) (all such values).

Adherence and compliance

Based on the duration of the trial (182 days), and the numbers of supplements issued and empty sachets collected during the weekly home visits for accountability monitoring, the estimated mean adherence to the intervention (proportion of days when the supplements were consumed) was 94.1% and 94.4% in the SQ-LNS A and SQ-LNS B groups, respectively. The reported average weekly consumption showed that the majority of the infants consumed all of the weekly supply of the supplements in 78.8% and 78.2% of infants in the SQ-LNS A and SQ-LNS B groups, respectively.

Anaemia and iron parameters

Baseline Hb was positively associated with final Hb. The overall median Hb at baseline was 11.3 g/dL. Both SQ-LNS A and SQ-LNS B had a significant positive impact on Hb in comparison to the control ($P = 0.018$ and 0.006 , respectively), as seen in Table 3. The relative improvement in Hb was 3.4% for SQ-LNS A and 4.2% for SQ-LNS B group. Plasma ferritin was increased in the SQ-LNS B group compared to the control group ($P = 0.013$). The proportion of infants who were anaemic was decreased by both SQ-LNS A and SQ-LNS B ($P = 0.021$ and 0.044 , respectively). The proportion of infants who were iron deficient was decreased by both SQ-LNS A and SQ-LNS B ($P = 0.004$ and < 0.001 , respectively).

Table 3: Intervention effects on iron parameters

	Control (n = 250)	SQ-LNS A (n = 250)	Intervention effect between SQ-LNS A and Control	SQ-LNS B (n = 250)	Intervention effect between SQ-LNS B and Control
Haemoglobin ¹ (g/L)					
Baseline	11.3 (10.5, 12.1) ²	11.2 (10.5, 12.1)		11.5 (10.5, 12.3)	
Endpoint	11.5 (10.6, 12.5)	11.8 (11.1, 12.7)	3.936 (0.685, .188) ³	12.0 (10.9, 12.9)	4.808 (1.400, 8.216) ¹
Ferritin ⁴ (µg/L)					
Baseline	25.4 (15.3, 39.8) /	24.9 (16.0, 40.6)		25.3 (16.4, 40.2)	
Endpoint	17.3 (9.4, 27.9)	21.1 (13.7, 32.0)	3.277 (-1.159, .613)	23.8 (15.4, 33.1)	5.371 (1.130, 9.614)
Prevalence n (%)					
Anaemia ⁵ (Hb < 11.5 g/dL)					
Baseline	90 (36.0)	98 (39.2)		86 (34.4)	
Endpoint	70 (34.8)	36 (23.8)	0.559 (0.341, .916) ⁶	41 (25.5)	0.612 (0.379, 0.988)
Iron deficiency ⁷ (Fer < 12 µg/L)					
Baseline	23 (14.9)	23 (13.9)		31 (19.3)	
Endpoint	53 (36.8)	19 (19.6)	0.412 (0.223, 0.758)	15 (13.8)	0.261 (0.137, 0.500)

¹Intervention effect based on intention to treat analysis and imputed endpoint Hb data, assessed with quantile regression and adjusted for baseline Hb.

²Median (25th, 75th) (all such values).

³B-value, 95% CI in parenthesis (all such values).

⁴Intervention effect based on intention to treat analysis and imputed endpoint ferritin data, assessed with quantile regression and adjusted for baseline ferritin; infants with CRP > 5 and AGP > 1 g/L were excluded.

⁵Intervention effect assessed with Pearson's chi-square test adjusted for baseline Hb status

⁶Odds ratio

⁷Intervention effect assessed with Pearson's chi-square test adjusted for baseline iron deficiency status based on ferritin status; excluding CRP > 5 and AGP > 1g/L.

Red blood cell (RBC) fatty acid composition

SQ-LNS B increased the RBC total phospholipid eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), total n-3 PUFA and total n-3 LCPUFA compositions (all $P < 0.001$, except EPA, $P = 0.025$), all indicating good compliance. Furthermore, SQ-LNS B decreased the ratios of total n-6 PUFAs to total n-3 PUFA, and n-6 LCPUFA to total n-3 LCPUFA (both $P = 0.002$), respectively. SQ-LNS A had no effect on the RBC total

phospholipid fatty acid composition. The amount of subsamples for SQ-LNS A, SQ-LNS B and the control group for baseline and endpoint consisted of 68, 61, 67 and 46, 51, 54, respectively. The paired samples (8, 8 and 11, respectively) were insufficient and therefore only endpoint samples were used without baseline samples as a covariate.

Infectious morbidity

Table 4 shows the intervention effects of both SQ-LNSs on the infectious morbidity of 6–12-month-old infants, based on intention to treat analysis, and taking the exposure (days in study) into account. Both SQ-LNS A and SQ-LNS B reduced the overall sick days of fever by 19.2% ($P < 0.001$) and 9.8% ($P = 0.006$), respectively. Both SQ-LNS A and SQ-LNS B increased the overall days of diarrhoea by 29.6% ($P < 0.001$) and 68.4% ($P < 0.001$), respectively; both groups showed an increase in the number of incidences of diarrhoea by 24.6% ($P = 0.002$) and 25.5% ($P = 0.001$), respectively. The SQ-LNS A group had 4.8 times more overall sick days of vomiting compared to the control group ($P < 0.001$) and the SQ-LNS B group had 2.4 times more overall sick days of vomiting compared to the control group ($P < 0.001$). The SQ-LNS A group had 2.4 times more incidences of vomiting compared to the control group ($P < 0.001$), and the SQ-LNS B group had 2.4 times more incidences of vomiting compared to the control group ($P < 0.001$).

Both SQ-LNS A and SQ-LNS B reduced the overall sick days of coughing by 9.2% ($P < 0.001$) and 5.7% ($P = 0.001$), respectively. Both SQ-LNS A and SQ-LNS B reduced the overall sick days of wheezing by 78.0% ($P < 0.001$) and 53.1% ($P < 0.001$), respectively; SQ-LNS A reduced the number of incidences of wheezing by 57.4% ($P = 0.006$). SQ-LNS A decreased the overall sick days of runny nose by 6.6% ($P = 0.002$) and SQ-LNS B increased the overall sick days of runny nose by 4.5% ($P = 0.035$). Both SQ-LNS A and SQ-LNS B increased the overall days of rash/sores by 13.0% ($P = 0.012$) and 99.6% ($P < 0.001$) respectively, and both SQ-LNS A and SQ-LNS B increased the number of incidences of rash/sores by 36.2% ($P = 0.031$) and 41.9% ($P = 0.013$), respectively.

Table 4: Effects of intervention with SQ-LNS on the infectious morbidity of 6-month-old infants¹

	Control n = 250	SQ-LNS A n = 250	Estimated intervention effect (between SQ-LNS A and Control)	SQ-LNS B n = 250	Estimated intervention effect (between SQ-LNS B and Control)
Fever					
Overall sick days	5 (2, 11) ²	4 (2, 8)	0.818 (0.757, 0.883) ⁴⁺	5 (2, 10)	0.902 (0.837, 0.971)*
No of incidences	2 (1, 3)	2 (1, 3)	1.003 (0.875, 1.150)	2 (1, 4)	1.076 (0.942, 1.228)
Duration per incident	1 (1, 4)	1 (1, 3)		1 (1, 3)	
	n = 236	n = 232		n = 237	
Diarrhoea					
Overall sick days	6 (2, 11.5) ³	6 (3, 12)	1.296 (1.202, 1.396) ⁺	7 (3, 14)	1.684 (1.570, 1.806) ⁺
No of incidences	2 (1, 3)	2 (1, 3)	1.246 (1.083, 1.433)*	2 (1, 4)	1.255 (1.093, 1.441) ⁺
Duration per incident	2 (1, 4)	2 (1, 5)		2 (1, 4)	
	n = 148	n = 154		n = 149	
Vomiting					
Overall sick days	2 (1, 6)	3 (2, 10)	4.809 (4.029, 5.741) ⁺	2 (1, 5)	2.382 (1.935, 2.887) ⁺
No of incidences	1 (1, 2)	1 (1, 2)	2.400 (2.397, 4.434) ⁺	1 (1, 2)	2.443 (1.776, 3.360)*
Duration per incident	1 (1, 4)	1.5 (1, 4)		1 (1, 3)	
	n = 39	n = 86		n = 76	
Coughing					
Overall sick days	20 (9, 42)	17 (7, 30)	0.908 (0.876, 0.942) ⁺	19 (8, 38)	0.943 (0.909, 0.977)*
No of incidences	3 (2, 5)	3 (2, 5)	1.054 (0.951, 1.168)	3 (2, 5)	1.080 (0.976, 1.195)
Duration per incident	5 (2, 11)	3 (1, 8)		5 (2, 9)	
	n = 214	n = 203		n = 197	
Wheezing					
Overall sick days	7 (4, 10)	4 (1, 7)	0.220 (0.163, 0.298) ⁺	4 (2, 7)	0.469 (0.376, 0.585) ⁺
No of incidences	1 (1, 2)	1 (1, 1)	0.426 (0.231, 0.787)*	1 (1, 1)	0.672 (0.401, 1.128)
Duration per incident	5 (2, 7)	3.5 (1, 5)		3 (1, 7)	
	n = 169	n = 144		n = 161	
Runny Nose					
Overall sick days	14.5 (6, 40)	14 (6, 32)	0.934 (0.895, 0.975)*	17 (5.5, 35)	1.045 (1.003, 1.089)*
No of incidences	3 (1, 5)	2 (1, 4)	0.968 (0.860, 1.090)	2 (1, 5)	1.001 (0.892, 1.125)
Duration per incident	6 (2, 9)	4 (11, 10)		5 (2, 11)	

	Control n = 250	SQ-LNS A n = 250	Estimated intervention effect (between SQ-LNS A and Control)	SQ-LNS B n = 250	Estimated intervention effect (between SQ-LNS B and Control)
	n = 180	n = 161		n = 172	
Rash/Sores					
Overall sick days	8 (3, 13)	6 (3, 13)	1.130 (1.027, 1.243)*	7 (4, 13)	1.996 (1.836, 2.170) ⁺
No of incidences	1 (1, 2)	1 (1, 2)	1.362 (1.029, 1.804)*	1 (1, 2)	1.419 (1.077, 1.870)*
Duration per incident	6 (2, 10)	5 (2, 8)		6 (3, 9)	
	n = 61	n = 77		n = 77	

¹ SQ-LNS A (EFA); SQ-LNS B (DHA, ARA, phytase, lysine).

² Intervention effects for overall sick days and no of incidences were estimated by using Poisson regression with a log linear link, with a modified intent to treat analysis, excluding children with no data after enrolment and taking exposure (days in study) into account. Number of subjects ill with each symptom indicated below each section.

³ Median (25th, 75th percentile; all such values).

⁴ Incidence rate ratio (IRR), 95% CI in parenthesis (all such values).

+ P < 0.001

* P < 0.05

In Figures 2–4 the incidences (depicted by intensity of the plots) and durations (depicted on the y-axis) of the specific morbidity symptoms over the 180 days of the study are portrayed for each intervention group and the control group. The incidences for diarrhoea and vomiting (Figure 2) throughout the study were higher in both intervention groups compared to the control group, corresponding with the values in Table 4. There were 12 incidences of vomiting in the SQ-LNS A group with a duration longer than 10 days, as compared to only two of these in the control group. There is slight clustering shown in the vomiting symptoms in SQ-LNS B, where, after approximately 110 days, the infants vomited for longer but less frequently.

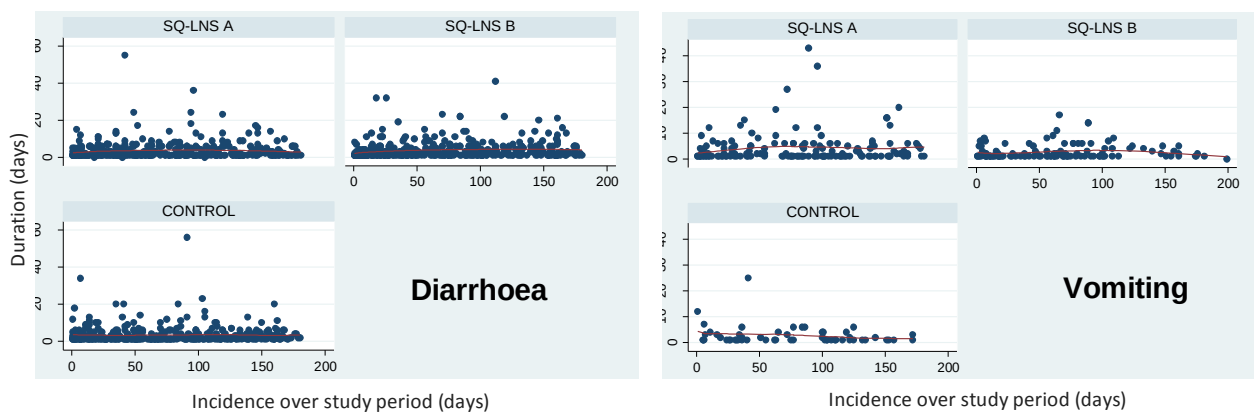


Figure 2: Duration of diarrhoea (left) and vomiting (right) throughout the study.

The duration of coughing (Figure 3) in all three groups was similar being longer in the first half of the study, which later decreased. Incidences of wheezing (Figure 3) were reduced in the SQ-LNS A group compared to the control group. Runny nose durations (Figure 3) show outliers in SQ-LNS A and the control group. There was an increase in durations for SQ-LNS B up until around 100 days, after which the durations were reduced. The number of incidences was higher in both intervention groups for rash/sores (Figure 4) compared to the control group, but there is a break in incidences in SQ-LNS A between approximately 150 days into the study and the end of the study. The rash/sores durations show an increase in both intervention groups compared to the control group.

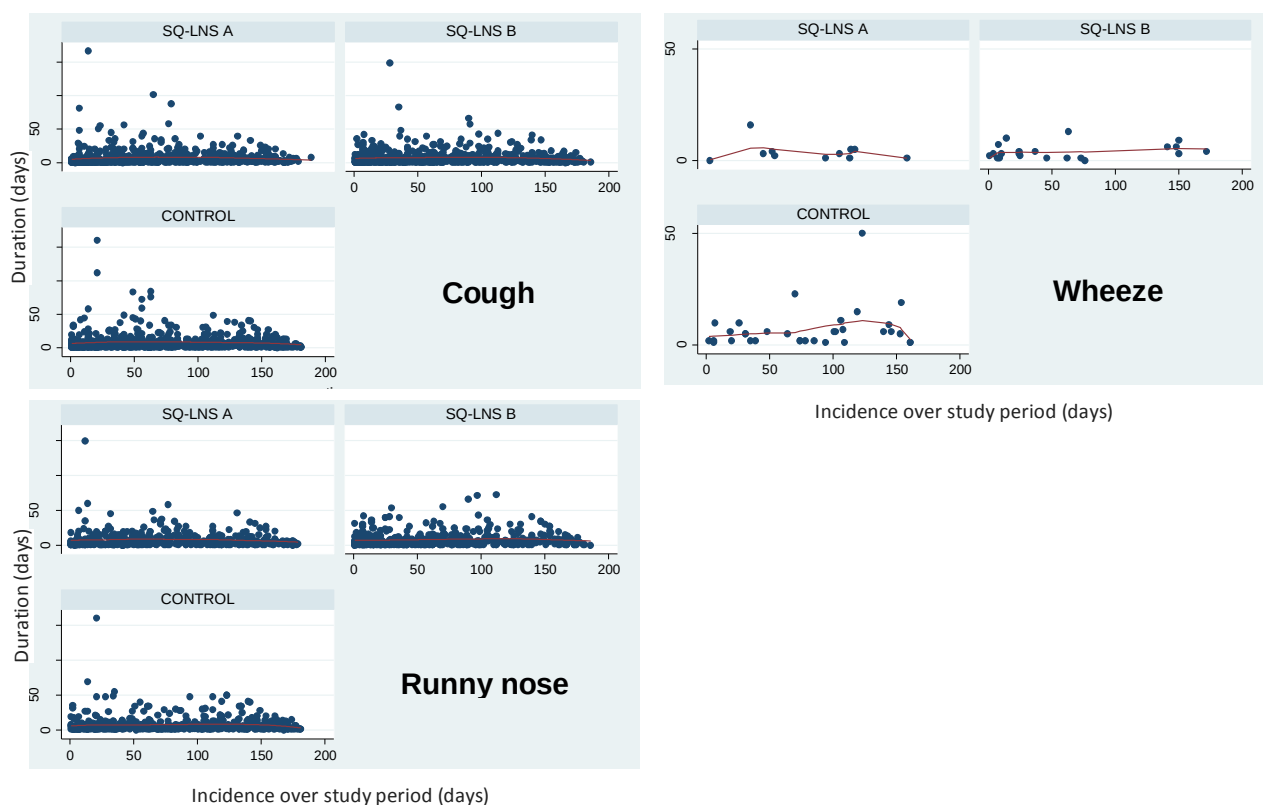


Figure 3: Duration of cough (top left), wheeze (top right) and runny nose (bottom left) throughout the study

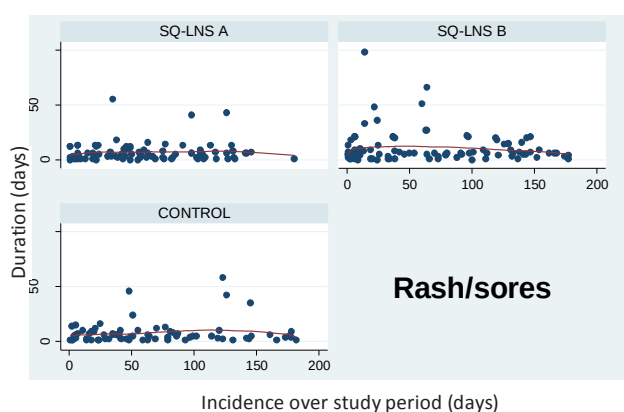


Figure 4: Duration of rash/sores throughout the study

The intensity and seriousness for all AEs combined are summarised in Tables 5 and 6, respectively. More than 99% of AEs in each of the three groups were classified as mild; < 1% of all AEs was classified as serious. The intensity was mild for > 97% for all individual AEs (diarrhea, vomiting, coughing, wheezing, fever, rash/sores and runny nose), with the majority clustering around 99 to 100%. The category of “other” included ear pain, eye disorders, infections and musculoskeletal injuries.

Table 5: The intensity of the adverse events

		Control	SQ-LNS A	SQ-LNS B
		n ¹ (%)	n (%)	n (%)
All symptoms	<i>Intensity</i>			
	Mild	2,016 (99.3)	1,986 (99.4)	2,085 (99.1)
	Moderate	7 (0.3)	8 (0.4)	7 (0.3)
	Severe	8 (0.4)	4 (0.2)	13 (0.6)
Fever	Mild	381 (100)	326 (100)	363 (100)
	Moderate	-	-	-
	Severe	-	-	-
Diarrhoea	Mild	335 (100)	376 (100)	391 (99.2)
	Moderate	-	-	-
	Severe	-	-	3 (0.8)
Vomiting	Mild	49 (98.0)	133 (100)	99 (98.0)
	Moderate	1 (2.0)	-	-
	Severe	-	-	2 (2.0)
Cough	Mild	657 (100)	628 (100)	656 (100)
	Moderate	-	-	-
	Severe	-	-	-
Wheeze	Mild	28 (100)	11 (100)	18 (100)
	Moderate	-	-	-
	Severe	-	-	-
Runny Nose	Mild	482 (100)	417 (100)	450 (100)
	Moderate	-	-	-
	Severe	-	-	-
Rash/sores	Mild	66 (98.5)	78 (98.7)	93 (98.0)
	Moderate	1 (1.5)	1 (1.3)	1 (1.1)
	Severe	-	-	1 (1.1)
Other	Mild	18 (58.1)	17 (60.7)	15 (53.6)
	Moderate	5 (16.1)	7 (25.0)	6 (21.4)
	Severe	8 (25.8)	4 (14.3)	7 (25.0)

Small quantity lipid-based nutrient supplement (SQ-LNS)

¹Number of incidences**Table 6:** The seriousness of the adverse events.

		Control	SQ-LNS A	SQ-LNS B
		n ¹ (%)	n (%)	n (%)
All symptoms	<i>Serious</i>			
	No	2,014 (99.1)	1,987 (99.3)	2,087 (99.1)
	Yes	18 (0.9)	14 (0.7)	20 (1.0)

Small quantity lipid-based nutrient supplement (SQ-LNS)

¹Number of incidences

Immune response to measles vaccine

Measles IgG response was reduced by SQ-LNS B ($P = 0.037$). There was no difference in baseline measles IgG between the two LNS groups and the control group.

Table 7: The intervention effects on immune response to measles vaccine¹

	Control (BL n = 100 EP n = 143)	SQ-LNS A (BL n = 75 EP n = 99)	Intervention effect between SQ-LNS A and Control	SQ-LNS B (BL n = 87 EP n = 114)	Intervention effect between SQ-LNS B and Control
Measles IgG (IU/mL)					
Baseline	21.27 (-11.14, 71.54) ²	24.85 (-14.98, 88.72)		23.96 (-14.85, 90.90)	
Endpoint	2808.67 (1447.32, 3965.85)	2926.71 (1510.29, 3740)	118.034 (-476, 712.239) ³	2225.86 (708.29, 3408.92)	-608.337 (-1178.974, -37.699)

Baseline (BL); endpoint (EP); small quantity lipid-based nutrient supplement (SQ-LNS)

¹Intervention effect estimated with median quantile regression, excluding subjects without endpoint data.

²Median (25th, 75th) (all such values).

³Estimated median differences, 95% CI in parenthesis (all such values).

Discussion

Our study, which was one of the first to investigate the use of soya-based SQ-LNSs in infants, demonstrated promise for the use of these supplements to reduce fever and respiratory symptoms including wheezing, coughing and runny nose (the last only in the SQ-LNS A group). But our findings were not so favourable towards diarrhoea, vomiting and rash/sores. The immune response to measles vaccination may possibly be indicative of slightly reduced immune function in the SQ-LNS B group, although this is inconclusive and needs further investigation. The findings on immunity should, however, be interpreted with caution and considered in view of the fact that growth and psychomotor development were improved by the SQ-LNS B product (unpublished results).

Fever is a reliable indicator of infectious morbidity ⁽²²⁾, and we found that fever symptoms were improved in both SQ-LNS groups in terms of overall sick days. There are currently relatively few data available on infectious morbidity in infants, especially with regard to fever. We found only one other study, which showed similar results in infants ⁽²³⁾, and another which tended to reduce fever in older children ⁽¹⁶⁾. In a two-arm, cluster-randomised controlled pragmatic intervention study conducted in Chad, children aged 6–36 months were supplemented with a ready-to-use supplementary food containing PUFA, namely 300 mg ALA and 2000 mg LA, and other micronutrients including iron. After adjustment for age, sex, socio-economic status, and morbidity status at inclusion, Huybregts *et al.* ⁽²³⁾ found that children from the intervention group had a lower risk of fever by 22.5% ⁽²³⁾. Furthermore, in a placebo-controlled intervention in Thailand, school children (9–12 years) were given milk containing fish oil (200 mg EPA and 1000 mg DHA); 28.7% of the children were ill with fever compared to 37.2% in the control group. However, there were no additional micronutrients in the intervention product ⁽¹⁶⁾.

The improvement in respiratory morbidity symptoms is consistent with similar findings from previous studies. These symptoms were reduced through supplementation of LNS or (LC)PUFA including both n-3 and n-6 moieties ^(6, 12-14, 23, 24). The supplementation of DHA (and sometimes ARA) as well as micronutrients and trace minerals to the diet of infants and children (0–12 years of age) generally improved respiratory health. Particularly upper respiratory tract infections, bronchitis/bronchiolitis, and wheezing were improved.

The reduction we saw in the coughing symptoms in both groups was significant but relatively small (9.2% and 6.7% in SQ-LNS A and SQ-LNS B, respectively). Wheezing in infants is also a reliable

indicator of respiratory illness in infants. This symptom leans towards being less affected by infant components such as teething and crying, which produce excess salivation, coughing and runny nose⁽²⁵⁻²⁷⁾. Wheezing in infants may be caused by respiratory infectious diseases or allergy⁽²⁸⁾ and frequent wheezing before the age of three years is a key risk factor contributing to severe asthma later in life^(29, 30). In the current study we found that wheezing was reduced by almost 80% in the SQ-LNS A group and more than 50% in the SQ-LNS B group. In agreement, Birch, Khoury⁽¹²⁾, supplemented infants with DHA and ARA in infant formulae, and found a reduction in all respiratory illnesses including wheezing (16% versus 35% in the control group)⁽¹²⁾.

Contrary to previous RCTs^(16, 23), an unanticipated outcome of this study was the increase in morbidity with regard to diarrhoea, vomiting and rash/sores in both SQ-LNS groups. These findings have to our knowledge not been extensively reported in previous studies and it is difficult to point out what the exact underlying causes were. In the current study caregivers continued to supplement their infants when diarrhoea and vomiting episodes occurred, which could have contributed towards the negative findings on these two outcomes. This needs to be further investigated.

We also recognise that both SQ-LNS products increased the incidence and duration of diarrhoea. This evidence lends support to previous studies where iron increased diarrhoea in infants when supplemented with micronutrient powders⁽³¹⁾. A systematic review of RCTs, focusing on iron supplementation and fortification, revealed that provision of iron increased the risk for diarrhoea by 11% ($P = 0.04$)⁽¹⁷⁾. There are other factors in our study which support the theory that iron might have played a vital role in increasing diarrhoea. Only the SQ-LNS B product had added phytase and vitamin C (both of which enhance iron absorption), and as a result was the only SQ-LNS to improve ferritin status. This improvement in iron status coincided with a reduced immune response to measles, almost 70% increase in diarrhoea, 2.4 times increase in vomiting and an almost 2 times increase in rash/sores. Furthermore, the only severe cases of diarrhoea, vomiting and rash were in the SQ-LNS B group (Table 5). The need to deliver iron supplementation more safely is well-recognised and is the subject of much research^(15, 31). Nevertheless, only SQ-LNS B produced the very positive outcomes of improved growth and psychomotor development (unpublished results).

There was an element of bias involved as one of the main reasons for AE dropout or withdrawal was vomiting, which was almost 5 times higher in the SQ-LNS A and 2.4 times higher in SQ-LNS B group, respectively. However, most of the morbidity symptoms were classified as mild and not

serious, indicating that it may have been more a case of gastroesophageal reflux (GER). The proportion of serious cases consisted of 3.0% in SQ-LNS B (of which 2.0% was severe), and 2.0% in the control group, with no serious or severe cases reported in the SQ-LNS A group (Tables 6 and 7, seriousness data not shown for each symptom).

It is unclear why the infants in the intervention groups had a higher rate of vomiting than the control group; this is a new finding. But a mechanism which may explain this phenomenon is the transient relaxation of the lower oesophageal sphincter (LES) as high fat foods tend to lower the muscle tone of the LES, causing GER ^(32, 33). The recommended DRI for fat intake in infants between one and three years of age is in the range 30–40% total energy ^(34, 35). According to two studies, South African infants' diets at 6 and 12 months consisted of > 40% ^(36, 37) and > 35% fat of total energy ⁽³⁷⁾, respectively. This was based on single 24-hour dietary recalls with estimated age-specific breast milk intakes, excluding the SQ-LNS ^(36, 37), and one of the studies was performed in this specific population. As SQ-LNS A and SQ-LNS B consist of 63.0% and 70.0% fat of total energy, respectively, this additional fat bolus to an already high fat diet may have triggered a regurgitation/vomiting response. Moreover, complementary feeding procedures generally require cautious introduction to new foods in small quantities ⁽³²⁾. These infants were introduced to 20 g of a new product with a high fat content; possibly contributing to this reaction. Perhaps if the product is provided in smaller quantities and split over two meals (e.g. 10 g per meal), this response may be attenuated or even prevented.

The increase in rash/sores in our study was a surprisingly new outcome as previous trials, particularly those involving n-3 LCPUFAs only, have shown improvements ^(38, 39). The products in our study contained a combination of lipids and a host of other micronutrients and trace elements, making it difficult to compare results. Interestingly, the SQ-LNS B group had rashes/sores for longer periods at a time in the first two months of the study (Figure 4). Severe diaper rash may accompany chronic diarrhoea ^(40, 41) and our results are concurrent for both these symptoms in both groups. Therefore it is an avenue to be explored in future research. The possibility that allergy could be contributing to increased rash/sores is not excluded, even though we tested the infants for acute allergy reactions upon initial ingestion of the products and only one infant presented with a possible allergy. Thus the definite cause of the increased rash/sores is unknown.

Despite the increase in diarrhoea, vomiting and rash/sores in both intervention groups, the severity of morbidity for all AEs was mostly mild as less than one percent of AEs were classified as serious. Although it should not be dismissed, we do not believe that these symptoms were high risk or life-threatening as the symptoms were mostly mild and the product was well accepted by the caregivers and infants. The acceptability of the products confirms the findings of Rothman, Berti ⁽⁴²⁾, who found that both SQ-LNS products were well accepted in terms of sensory characteristics as well as for practical home use ⁽⁴²⁾. We also had strict safety control measures in place for both products.

One of the main limitations of this study was that vomiting was loosely defined as its interpretation could have ranged from projectile vomiting to oesophageal reflux to spitting, which may have increased the perception of the incidence rate of vomiting. The type of rash/sores was also not specified and could be a side-effect of increased diarrhoea, presented as a diaper rash. Therefore we recommend for future research that symptoms be clearly defined in order to prevent any uncertainty. Another limitation was that measles vaccination results had insufficient pairing of IgG samples for baseline and endpoint. As a result we used only endpoint data without baseline samples as a covariate as it is preferable to analyse based on the largest sample size. Nonetheless, there were no differences in baseline IgG among the groups.

This study had the merits of randomisation of participants, a good sample size, blinding of the investigators involved in data analysis, as well as extensive and frequent morbidity monitoring.

Conclusion

In conclusion, the profound results obtained from this study makes a very significant contribution to what has been done to date as it is one of the first soy-based SQ-LNS intervention trials in infants. Both SQ-LNS products improved fever symptoms as well as certain respiratory illness manifestations. Even though there was an increase in vomiting, diarrhoea and rash/sores in both groups, these symptoms were mostly mild and not serious. Furthermore, the SQ-LNS B products have improved growth and psychomotor development. In future, more in-depth research needs to be conducted on SQ-LNS products in infants, using a holistic approach, taking safety into account and allowing for this type of product to be consumed in smaller quantities more frequently. This may potentially provide the benefits without the side effects of the products. Furthermore, this study also demonstrated the need not only for more trials to be done on this topic but also to monitor and record morbidity data frequently and accurately from a public health perspective.

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CHAPTER 5 CONCLUSION AND RECOMMENDATIONS

4.1 Conclusion

The aim of this mini-dissertation was to determine the effect of the intake of two SQ-LNSs, with and without LCPUFA, phytase, lysine and certain trace-minerals, on the incidence and duration of infectious morbidity and the response to measles vaccination in 6-month-old infants. Our objectives were: 1) To determine the effect of SQ-LNS A and SQ-LNS B (each in comparison to the control group) on the incidence and duration of infectious morbidity in 6-month-old infants; and 2) To determine the effects of SQ-LNS A and SQ-LNS B (each in comparison to the control group) on the response to vaccination against measles in the corresponding population.

This study, which was one of the first to investigate the use of soya-based SQ-LNSs in infants, demonstrated promise for the use of these supplements to reduce fever and respiratory symptoms including wheezing, coughing and runny nose (the last only in the SQ-LNS A group). But our findings were undesirable towards diarrhoea, vomiting and rash/sores. The immune response to measles vaccination may possibly be indicative of slightly reduced immune function in the SQ-LNS B group, although this is inconclusive and needs further investigation. The findings on immunity should, however, be interpreted with caution and considered in view of the fact that growth and psychomotor development were improved by the SQ-LNS B product (unpublished results).

Researchers have discovered the potential for LCPUFAs to be used to treat acute and chronic inflammatory diseases, as well as improve immune function (Nagakura *et al.*, 2000; Mahrshahi *et al.*, 2004; Hageman *et al.*, 2012), however the immune response to infectious disease in infants has received little attention thus far. It has been reported that supplementation with n-3 LCPUFAs in infants increased proportion of antigen mature CD4+ cells (improved immune response), improved interleukin-10 production (anti-inflammatory), and reduced interleukin-2 production (pro-inflammatory) (Field *et al.*, 2000). These markers of immune function are focused on the cellular level. In the current study, we focused more on the clinical endpoints. They reflect the overall balance between pathogen exposure and the integrated host defences which provide the most relevant indication of the ability to cope with common pathogens (Albers *et al.*, 2007). We found that fever symptoms were improved in both SQ-LNS groups but as there are currently relatively few data available on infectious morbidity in infants with regard to fever, we recommend more intervention trials to be conducted in this field.

The improvement in respiratory morbidity symptoms is consistent with similar findings from previous studies. These symptoms were reduced through supplementation of LNS or (LC)PUFAs including both n-3 and n-6 moieties. The supplementation of DHA (and sometimes ARA) as well as micronutrients and trace

minerals to the diet of infants and children (0–12 years of age) generally seem to improve respiratory health, in particular wheezing and coughing (Satomi *et al.*, 1994; Pastor *et al.*, 2006; Dalton *et al.*, 2009; Birch *et al.*, 2010; Minns *et al.*, 2010; Huybregts *et al.*, 2012).

Wheezing seems to be a reliable indicator of respiratory illness in infants. This symptom leans towards being less affected by infant components such as teething and crying, which produce excess salivation, coughing and runny nose (Barlow *et al.*, 2002; Ige & Olubukola, 2013; Azevedo *et al.*, 2015). Wheezing in infants may be caused by respiratory infectious diseases or allergy, and frequent episodes before the age of three years is a key risk factor contributing to severe asthma later in life (Sly, 1999; Taussig *et al.*, 2003). In the current study we found a marked improvement in wheezing in both intervention groups, thus contributing to its clinical significance in possibly preventing or reducing the incidence of asthma later in life.

Unexpected outcomes of this study were the increase in morbidity with regard to diarrhoea, vomiting and rash/sores in both SQ-LNS groups. These findings have to our knowledge not been extensively reported in previous studies and it is therefore difficult to identify the exact underlying causes. We suggested the following thoughts to provide possible explanations for these outcomes. There were factors in our study which support the theory that iron might have played a vital role in increasing diarrhoea. Only the SQ-LNS B product had phytase and additional vitamin C (both of which enhance iron absorption), and as a result was the only group to improve ferritin status. This improvement in iron status coincided with a reduced immune response to measles, almost 70% increase in diarrhoea, 2.4 times increase in vomiting and an almost 2 times increase in rash/sores. Furthermore, the only severe cases of diarrhoea, vomiting and rash were in the SQ-LNS B group. The need to deliver iron supplementation more safely is well-recognised and is the subject of much research. Nevertheless, only SQ-LNS B improved growth and psychomotor development.

The reason for increased vomiting is still unclear, but we speculated that due to a high fat percentage in the infants' diets compared to overall energy intake, the infants' guts did not tolerate the product well and thus displayed symptoms of gastroesophageal reflux (GER) (Mahan & Escott-Stump, 2012; Czinn & Blanchard, 2013). Complementary feeding procedures generally require cautious introduction to new foods in small quantities (Mahan & Escott-Stump, 2012). However, these infants were introduced to 20 g of a new product with a high fat content; possibly contributing to this reaction.

The increase in rash/sores in our study was a surprisingly new outcome as previous trials, particularly those involving n-3 LCPUFAs only, have shown improvements (Palmer *et al.*, 2012; A Miles & C Calder, 2014). The products in our study contained a combination of lipids and a host of other micronutrients and trace elements, making it difficult to compare results. Severe diaper rash may accompany chronic diarrhoea (Walker-Smith, 1980; Fleisher, 2014) and our results are concurrent for both these symptoms in both groups. The possibility that allergy could be contributing to increased rash/sores is not excluded, even

though we tested the infants for acute allergy reactions upon initial ingestion of the products and only one infant presented with a possible allergy. Thus the definite cause of the increased rash/sores is unknown.

Yet, despite the increase in diarrhoea, vomiting and rash/sores in both intervention groups, the severity of morbidity for all AEs were mostly mild as less than one percent of AEs were classified as serious. Although it should not be dismissed, we do not believe that these symptoms are high risk or life-threatening as the symptoms were mild and the product was well accepted by the caregivers and infants.

The SQ-LNS A group had an enhanced immune response compared to the control group (more positive results), as presented by the response to the measles vaccine. Conversely, the increase in overall sick days for runny nose in the SQ-LNS B group, relates to the results shown by the measles assay as well which showed a slightly reduced immune response.

4.2 Recommendations

Our study provides a good foundation for future RCTs focusing on the immunity effects of SQ-LNS interventions in infants due to our extensive and frequent morbidity monitoring. Some of our main limitations of this study were driven by the subjective nature of each morbidity symptom where definitions were open-ended. Therefore we recommend for future research that symptoms be clearly defined in order to prevent any uncertainty.

In an attempt to avoid vomiting in these infants, future research could focus on using the same, or similar SQ-LNSs but in divided doses such as 10 g with food in the morning, and 10 g in the afternoon with lunch. Smaller quantities may make the supplement more tolerable and easier to digest. Furthermore, the addition of probiotics may attenuate the diarrhoeal effect of the supplement, as probiotics have been demonstrated to improve diarrhoea in infants (Guandalini, 2011).

To evaluate the true effect on immunity, infectious morbidity symptoms should also be monitored closely after the intervention. By this time the nutritional status of the infants may be markedly improved and side-effects will not be present as during the supplementation phase.

The profound results obtained from this study makes a very significant contribution to what has been done to date as it is one of the first soy-based SQ-LNS intervention trials in infants. Both SQ-LNS products improved fever symptoms as well as certain respiratory illness manifestations including wheezing, coughing and runny nose. Even though there was an increase in vomiting, diarrhoea and rash/sores in both groups, these symptoms were mostly mild and not serious. Furthermore, SQ-LNS B improved both growth and psychomotor development. In future, more in-depth research needs to be conducted on SQ-LNS products in infants, using a holistic approach, taking safety into account and allowing for this type of product to be consumed in smaller quantities more frequently. This may potentially provide the benefits without the side

effects of the products. Furthermore, this study also demonstrated the need not only for more trials to be done on this topic but also to monitor and record morbidity data frequently and accurately from a public health perspective. Lastly, in-depth focus group discussions with the caregivers after the study could have contributed towards better interpretation of especially the findings on diarrhoea and vomiting. This is also strongly recommended for future studies.

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APPENDIX A

INFORMATION SHEET

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

Project leader: Prof Marius Smuts

Co-project leader: 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 research 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 one of the fat-based pastes 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, preferably the first meal of the day.

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. 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.

Confidentiality

All information collected about your baby will be treated as confidential (will not be given to or discussed with anybody) and only the researchers 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 at no additional cost to you. You will be kept informed in this regard and are welcome to discuss any concerns that you may have with us.

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

What will the benefit be for my child who participates?

Your child will receive the nutritional supplement, free of charge, for 6 months. 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.

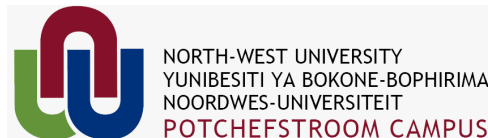
Who can you contact if there are any queries?

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.

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!



CONSENT FORM

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

I voluntarily agree to take part in the study.

I have been informed about and understand the purpose of the study.

I have been informed about and understand the advantages and possible side-effects that may result from procedures.

I understand that I can withdraw my consent at any time without being penalised as far as my routine health care is concerned.

I have been informed that all information will be treated as confidential.

I understand that my baby can be in any of the three groups. If my baby is in either group 1 or group 2, I will receive the nutritional supplement free of charge for six months (starting when my baby is 6 months old) and must mix one portion of the supplement with my baby's normal food every day. If my baby is in group 3, I will receive a 6-month supply of the supplement when my baby is 12 months old.

I understand that, regardless of in which group my baby is, my baby and I need to go to the research site when my baby is 6 and 12 months old for the following:

-I will be asked questions about my household (at the start of the project), the foods that my baby eats and drinks, any illnesses that my baby had during the previous weeks, and development milestones that my baby has achieved.

- I will be asked to recall the foods and drinks that my baby consumed the day before.
- My baby's weight, length, head circumference and upper arm circumference will be measured.
- My weight and height will be measured (at the start of the project).
- I will be asked to express a small amount of breast milk (approximately $\frac{1}{4}$ cup).
- I will be asked to provide a small amount of urine (at the start and end of the project).
- A small amount of urine will be collected from my baby by using a special nappy.
- A nursing sister will take 4 mL (less than one teaspoon) blood from the vein in my baby's arm.

I understand that my baby and I also need to go to the research site when my baby is 8 and 10 months old. During these two visits I will be asked questions about the foods that my baby eats and drinks, and my baby's weight and length will be measured.

I understand that a field worker will visit me at home once a week. During these visits the fieldworker will ask me questions on the usage of the supplement, illnesses that my baby had and developmental milestones that my baby has achieved.

Any abnormal findings will be attended to and referred, where necessary.

If I have any queries or problems regarding the study, I 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 I can send an e-mail to adri.labuschagne@mrc.ac.za.

Baby's code:

--	--	--	--

Recruitment number:

Mother/guardian's name & surname:

Baby's name & surname:

Address:

.....

.....

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

Signature: Date:

Signed at:

Witness:

2. I hereby give consent that my weight and height can be measured, and some urine can be collected.

Signature: Date:

Signed at:

Witness:

3. I hereby give consent that some expressed breast milk can be collected.

Signature: Date:

Signed at:

Witness:

Fieldworker who informed the mother / guardian:

Researcher's signature:

APPENDIX B

BASELINE QUESTIONNAIRE

Baby's code:

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

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

Fieldworker's
code:

--	--

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)		
---	--	--

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 grant		
---	--	--

9. Where does the household usually get its drinking water from? (<i>Mark only one</i>)	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 first 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 milk feeds 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 Give name:	4	
	Other, specify:	5	

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

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

21. What was the first 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? (in months)	
---	--

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)	1	Yes *
	2	no (go to question M2)

per day)	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?	
---	--

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)	

IM1	What type of medicine?
a.	
	
	
	

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?
.....

APPENDIX C

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 (go to question 3)	1	
	No	2	
	Don't know (go to question 3)	3	

2. How old was the baby when breastfeeding was stopped? (in months)		
---	--	--

3. At the moment, does your baby get any milk feeds other than breast milk?	Yes	1	
	No (go to question 5)	2	
	Don't know (go to question 5)	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 Give name:	4	
	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 usually mix the paste with?
.	
	
	
	
	

13	With what foods did you mix the paste with yesterday?
.	
	
	
	
	

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 (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?

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)

IM1 What type of medicine?
a.
.....
.....
.....

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	Date: ____ / ____ / 20____
---------------------------------------	----------------------------

start?	
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?

APPENDIX D:

DAILY FORM to be completed by the MOTHER/CAREGIVER

Baby's code:

--	--	--	--

Week number:

--	--

 Date (dd/mm/yyyy) from

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

 to

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

 Fieldworker's code:

--	--

1. How much of the mixture was left in the baby's feeding bowl?

Day	a. Monday	b. Tuesday	c. Wednesday	d. Thursday	e. Friday	f. Saturday	g. Sunday							
 No left	<table border="1"><tr><td>1</td></tr></table>	1	<table border="1"><tr><td>1</td></tr></table>	1	<table border="1"><tr><td>1</td></tr></table>	1	<table border="1"><tr><td>1</td></tr></table>	1	<table border="1"><tr><td>1</td></tr></table>	1	<table border="1"><tr><td>1</td></tr></table>	1	<table border="1"><tr><td>1</td></tr></table>	1
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2. How is your baby's health?

 Healthy		1		1		1		1		1		1		1
 Gastro		2		2		2		2		2		2		2
 Cold/Flu		3		3		3		3		3		3		3
 Hot Body		4		4		4		4		4		4		4

APPENDIX E

MORBIDITY

Baby's code:

--	--	--	--

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

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

Week
number:

--	--

Fieldworker's
code:

--	--

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?	
---	--

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)

IM1	What type of medicine?
a.	

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?
--

APPENDIX F:

WEEKLY ADHERENCE for the FIELDWORKER

Baby's code:

--	--	--	--

Week	Fieldworker code	Date of delivery and visit (dd/mm/yyyy)	No. of empty sachets collected (Do counting)	No. of sachets used (Ask caregiver)	No. of full sachets (Do counting)	No. of sachets issued (By fieldworker)	Fieldworker SIGNATURE	Mother SIGNATURE
		/ /201						
		Comment						
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ANNEXURE A: ETHICAL APPROVAL

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South Africa 2520

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Web: <http://www.nwu.ac.za>

Faculty of Health Sciences
Tel: 018-299 2092
Fax: 018-299 2088
Email: Minrie.Greeff@nwu.ac.za

16 July 2015

Prof CM Smuts
Nutrition

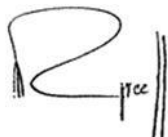
Dear Prof Smuts

ETHICS APPLICATION: NWU-00001-11-A1 (CM SMUTS-K JOOSTE) "EFFECTS OF LIPID-BASED NUTRIENT SUPPLEMENTS (LNS) ON IMMUNITY OF 6 MONTH-OLD INFANTS: A RANDOMISED CONTROLLED TRIAL"

Thank you for amending your sub-study application. All ethical concerns have now been addressed and ethical approval is granted until 31/12/2016.

Please note that any changes to the approved application must be submitted to the Health Research Ethics Committee for approval before implementation.

Yours sincerely



Prof Minrie Greeff
Health Research Ethics Committee Chairperson