

FATTY ACIDS IN NUTRITION SOURCES FOR PRETERM INFANTS

**FATTY ACIDS IN NUTRITION SOURCES
FOR PRETERM INFANTS**

by

Naomi Fink (BMSc.)

**A thesis submitted in partial fulfillment of the
requirements for the degree
MASTER OF SCIENCE**

Approved:

**Dr. Christoph Fusch
Principal Investigator**

**Dr. Sandeep Raha
Committee Member**

**Dr. Salhab el Helou
Committee Member**

**Dr. Joseph Macri
External Reviewer**

**MCMASTER UNIVERSITY
©Copyright by Naomi Fink
2013**

INSTITUTION: McMaster University, Hamilton, Ontario

**DEGREE: Master of Science (M.Sc.) Medical Sciences: Metabolism and Nutrition
(2013)**

TITLE: Fatty Acids in Nutrition Sources for Preterm Infants

AUTHOR: Naomi Fink, BMSc. (Western University)

SUPERVISOR: Dr. Christoph Fusch

NUMBER OF PAGES: XIII, 89

ABSTRACT

Of the three components of parenteral macronutrient classes (protein, carbohydrate and lipid), the lipid class is the least understood and the fatty acid distribution in lipid emulsion products has the potential to play a critical role in the development of infant morbidities. Breast milk has long been considered the gold standard for infant nutrition, but when infants cannot tolerate enteral feeds, the use of lipid emulsions cannot be avoided despite their adverse side effects.

In this study, fatty acid profiles from five commercially available lipid emulsion products were compared to the profile of breast milk. As well, resulting serum on each of these nutrition sources were compared to the profile of either lipid emulsion (Intralipid) or breast milk. Fatty acid profiles for matched pairs of breast milk from mother and resulting serum from infants were compared as well as the profile of normotriglyceridemic serum samples to hypertriglyceridemic ones. Results indicate that not one lipid emulsion product is like another, nor do their profiles closely resemble breast milk even though they are intended to replace the fat portion of the infant's natural source of nutrition. Serum was not found to directly reflect the fatty acid profile of the nutrition source administered, as was expected based on literature, highlighting that there is a complex web of pathways between nutrition administration and appearance of fatty acids in the serum. Further research is necessary to define the effect of fatty acid chain length and degree of saturation on these metabolic pathways, as the very essence of interrelations such as these can complicate interpretations of results.

This thesis is dedicated to George, Janice, Corby, Shauna-Lynn and Gabrielle Fink
Words cannot truly describe the uplifting influence of your love, encouragement and
support; I could not have asked for more optimistic team by my side.

ACKNOWLEDGEMENTS

Dr. Sandeep Raha and Dr. Salhab el Helou for your basic science and neonatology expertise, respectively, that allowed for suggestions to enhance the depth of the project. The freedom you offered me to decide certain pathways for the thesis was perfectly balanced by your critical appraisals that pushed me to sharpen my analytical skills.

Dr. Joe Macri for opening my eyes to plethora of opportunities available to me as a graduate student, and for going above and beyond the call of duty of a collaborator. I am grateful for your wisdom and mentorship throughout my MSc degree.

Dr. Ken Rosenthal for your guidance as I entered the unfamiliar field of cell culture. Many thanks for the time dedicated to instruct me in the required techniques of culturing, passaging and maintenance.

The attending and staff physicians, fellows, residents, nursing staff, dietary assistants and pharmacy technicians in the NICU and McMaster University's Hamilton Regional Lab Medicine Program personnel for the extra time taken to complete tasks that allowed the study to run smoothly and to completion.

Dr. Christoph Fusch, Dr. Gerhard Fusch and Dr. Niels Rochow for your clinical and laboratory science guidance and David Pogorzelski for your dedication to the recruitment process. A special thank you to Laura Bagnulo; not just for all the time spent ensuring smooth progress leading up to deadlines, but for adding such a positive touch to the research group.

Adam Pocrnic for exemplifying the standard of dedication required for completion of a written thesis and for the demonstration of the work ethic necessary to excel in all other facets of graduate school.

To the "Fusch girls": Tarushika, Jen, Preeya, Asmaa, Anaam, Sau-Young and Arum. Thank you for being the ray of sunshine that made coming to the lab each day something to look forward to. As colleagues and friends, a dynamic existed between us that allowed for a positive, stimulating and supportive environment both inside and outside of the hospital.

Contents

1.	LIST OF TABLES	IX
2.	LIST OF FIGURES	X
3.	ABBREVIATIONS	XI
4.	INTRODUCTION	1
5.	LITERATURE REVIEW	2
5.1	FATTY ACIDS AS A NUTRITION SOURCE	2
5.1.1	Essential fatty acids (EFA).....	2
5.1.2	Medium and long chain fatty acids	4
5.2	LIPID EMULSION PRODUCTS	5
5.3	ADVERSE EFFECTS OF LIPID EMULSION USE IN PRETERM INFANTS	7
5.3.1	Clearance of TGs during lipid emulsion infusion	11
5.4	GOLD STANDARD OF INFANT NUTRITION: BREAST MILK	12
5.5	METABOLISM OF FATTY ACIDS	13
5.6	GAPS IN KNOWLEDGE: IDEAL FATTY ACID PROFILES	16
5.7	PROJECT RATIONALE.....	17
5.7.1	Primary objective and hypothesis	18
5.7.2	Secondary objective and hypothesis	18
5.7.3	Tertiary objective and hypothesis	19
6.	MATERIALS AND METHODS.....	19
6.1	Clinical procedure	20
6.2	Collection of hypertriglyceridemic samples from Hamilton Regional Laboratory Medicine Program (HRLMP)	22
6.3	Preparation of internal standard stock solution	23
6.4	Analytical techniques: Extraction and esterification of fatty acids from milk.....	23
6.5	Analytical techniques: Extraction and esterification of fatty acids from serum	25
6.6	Analytical techniques: Gas chromatography/mass spectrometry.....	26
6.7	Determination of retention time ranges for each FA.....	27
6.8	Development of a calibration curve	27
6.9	Validation experiments	28

6.10	Statistical analysis.....	29
6.10.1	Stratification and nutritional grouping.....	30
6.10.2	Sample size	30
7.	RESULTS	31
7.1	Validation.....	31
7.2	Intra- and inter-individual variation in breast milk.....	33
7.3	Aim 1: Comparison of preterm breast milk to lipid emulsion products.....	36
7.4	Aim 2: Comparison of nutrition source and resulting serum fatty acid profiles	39
7.5	Aim 3: Comparison of fatty acid profiles between normo- and hypertriglyceridemic serum	47
8.	DISCUSSION	49
8.1	Strengths and limitations.....	49
8.1.1	Threat to validity: Under-estimation of variance	49
8.1.2	Sample size calculation	50
8.2	Methodology limitations	50
8.2.1	Absolute quantification	50
8.2.2	Increased sample size.....	51
8.2.3	Lack of longitudinal data	53
8.3	Strengths	53
8.4	Inter- and intra-individual variation in breast milk.....	54
8.5	n-6/n-3 EFA ratio.....	55
8.6	Fatty acid profile of lipid emulsion products compared to breast milk.....	56
8.7	Reflection of dietary fatty acids in serum	57
8.8	Fatty acid levels in serum.....	62
8.9	Normo- versus hypertriglyceridemic fatty acid profile.....	64
8.10	Stability in fatty acid profiles.....	65
9.	CONCLUSION AND RECOMMENDATION.....	66
10.	FUTURE DIRECTION: OPTIMIZED LIPID EMULSION FOR PRETERM INFANTS	68
10.1	METHODOLOGY ESTABLISHED TO DATE.....	71
10.1.1	Plating cells.....	71

10.1.2	Cell Splitting	71
10.1.3	Proposed analysis of media: Lipoprotein identification and quantification.....	72
10.1.4	Preliminary results	72
11.	REFERENCES	75
12.	APPENDIX A: Tables	84
13.	APPENDIX B: Figures	89

1. LIST OF TABLES

Table	Page
1: Molecular formula, weight and retention times of each fatty acid detected in milk, serum and/or lipid emulsions	84
2: Fatty acid proportions of the average local preterm breast milk profile (BM) of 69 mother's milk samples	35
3: Fatty acid proportions of the local average preterm breast milk profile compared to 5 commercially available lipid emulsion products	85
4: Fatty acid proportions of the local average breast milk group (BM) and the resulting serum from infants fed breast milk (BMserum)	86
5: Fatty acid proportions for Intralipid and the resulting serum of infants administered this lipid emulsion	87
6: Comparison of fatty acid proportions in serum of infants fed exclusively breast milk (BMserum) or >80% fat from lipid emulsions (LEserum)	88

2. LIST OF FIGURES

Table	Page
1: Anabolic pathway of EFAs	3
2: Particles of parenteral fat emulsions	11
3: Graphical representation of clinical procedure from the stage of consent to sample analysis	22
4: Internal standard C17: Calibration curve	28
5: Method validation: Intra-day variation (n=10)	32
6: Method validation: Inter-day variation (n=10)	32
7: Coefficient of variation in validation results	33
8: Intra-individual variations in FA composition	34
9: Distribution of FAs in 5 LE products and average BM (n=69)	38
10: Compositional difference between BM (n=69) and BMserum (n=31)	40
11: Compositional difference between LE (Intralipid) and LEserum (n=15)	42
12: Compositional difference between BMserum (n=31) and LEserum (n=15)	43
13: Compositional difference between BM and serum for 35 matched pairs from 21 preterm infants	45
14: Comparison of the FA profiles of a single matched pair of milk (001 BM2) and serum (001 SS3)	46
15: Compositional difference between normoTG (n=113) and hyperTG serum (n=22) in preterm infants	48
16: C16:0 and C18:0 abundance in unused DMEM analyzed by GC/MS	73
17: C16:0 and C18:0 abundance in used DMEM analyzed by GC/MS	73
18: Distribution of FAs in unfortified versus fortified BM	89

3. ABBREVIATIONS

NEC: necrotizing enterocolitis

PN: parenteral nutrition

EN: enteral nutrition

EFA: essential fatty acids

TG: triglyceride

LCPUFA: long chain poly-unsaturated fatty acids

DHA: docosahexanoic acid

EPA: eicosapentanoic acid

LA: linoleic acid

ALA: alpha-linolenic acid

AA: arachidonic acid

SFA: saturated fatty acid

MUFA: mono-unsaturated fatty acid

PUFA: poly-unsaturated fatty acid

LPL: lipoprotein lipase

LCT: long chain TG

MCT: medium chain TG

NICU: neonatal intensive care unit

HIREB: Hamilton Integrated Research Ethics Board

HRLMP: Hamilton Regional Laboratory Medicine Program

FAME: fatty acid methyl ester

BHT: butylated hydroxy toluene

CV: coefficient of variation

PCA: principal component analysis

CCA: canonical correlation analysis

DMEM: Dolbecco's Modified Eagle Media

Declaration of Academic Achievement

Dr. Christoph Fusch, Dr. Gerhard Fusch, Dr. Niels Rochow and myself developed the study design. I was responsible for presenting the project to the Neonatal Research Committee for approval to send to the Hamilton Integrated Research Ethics Board (HIREB). I completed the ethics application and amendment documents submitted to HIREB.

All samples were collected and analyzed by myself, as well as data organization and analysis. Dr. 's Christoph Fusch, Gerhard Fusch, Joseph Macri and Kenneth Rosenthal were involved in the development of the future direction of this project involving Caco-2 cell lines and oil sources.

4. INTRODUCTION

In order to sustain proper growth and development of a fetus in each trimester of a pregnancy, nutrition is provided from the placenta via the umbilical vessels. After birth, the infant is disconnected from the energy supply of the placenta and requires an exogenous source of nutrition. Infants born preterm are known to have an immature digestive system, can develop gastroschisis, necrotizing enterocolitis (NEC) or short-bowel syndrome and therefore require intravenous/parenteral nutrition (PN) in the first few days of life or until oral/enteral nutrition (EN) can provide adequate energy and lipid levels during periods of critical illness (Le 2011; Deshpande 2011). Until gastrointestinal motor function has matured and the digestive system can tolerate EN, the infant is at risk for the development of NEC and subsequent adverse events or even death (Puntis 2006). Avoiding such adverse outcomes while still maintaining nutritional support that allows for growth rates and nutrient accretion comparable to those displayed during fetal development remains a daunting challenge for neonatologists (Puntis 2006).

Survival of high-risk neonates has drastically improved in the last decades and since feed intolerance is a common issue faced by physicians caring for preterm infants, PN is a life-saving therapy for this population (Deshpande 2011). Infants as young as 24 weeks gestation have a 50% chance of survival (Puntis 2006) and among other medical advancements, long-term support on PN has become a crucial part of the treatment process for this high-risk population. Although necessary during critical stages of growth and development, the lipid component of PN does not come without the associated risks of hypertriglyceridemia and lipid peroxidation (Deshpande 2011).

5. LITERATURE REVIEW

5.1 FATTY ACIDS AS A NUTRITION SOURCE

5.1.1 Essential fatty acids (EFA)

Fatty acids are named according to carbon chain length, degree of unsaturation, position of the first double bond and orientation (*cis/trans*) around that bond (AOCS Lipid Library A; Heird 2005). Therefore, oleic acid (C18:1n9c) is an 18 carbon chain with one degree of unsaturation 9 positions from the first carbon and a *cis* orientation around the double bond. Dietary fatty acids are usually stored and carried as triglycerides (TGs) within the body; TGs provide a concentrated energy source and are the major component of dietary fat. They have three, usually different, fatty acids attached to a glycerol molecule (Heird 2005).

Special consideration should be given to the long chain poly-unsaturated fatty acids (LCPUFA), as they play a critical role in infant growth and development and have potential long lasting benefits that extend beyond the effects of dietary insufficiency. There is an increased requirement for these LCPUFAs during the third trimester, specifically those of the n-3 series, docosahexanoic acid (DHA) and eicosapentanoic acid (EPA), because they promote visual and neurodevelopment (Uauy 2000). Infants born preterm (during the third trimester) have a special requirement for EFA accretion in order to sustain rapid visual and neurodevelopment (McCann 2005). Mammals lack the enzyme necessary to introduce double bonds at carbon atoms beyond carbon 9 (C9), but both of the parent EFAs can be synthesized in plants (Uauy 2000). The parent EFAs, linoleic (LA) and alpha-linolenic acid (ALA), must therefore be obtained from dietary sources in order to synthesize downstream EFA's, arachidonic acid (AA), DHA and EPA (Davis-

Bruno 2010). The omega 6 or n-6 fatty acid family precursor is LA and leads to synthesis of downstream fatty acid AA and the omega 3 or n-3 fatty acid family precursor is ALA which leads to synthesis of downstream fatty acids DHA and EPA (AOCS Lipid Library A).

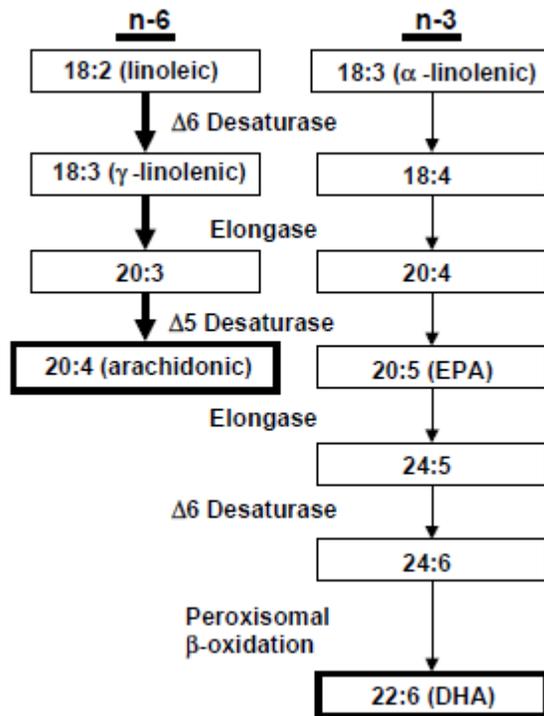


Figure 1. Anabolic pathway of EFAs (Nakamura 2003).

DHA is a neurotrophic factor and is absolutely essential for neural and retinal development, specifically in the photoreceptor membranes of the retina and in cerebral grey matter, cortex and hippocampus (Davis-Bruno 2010). AA is required for proper growth and reproductive function, skin and hair development and neurodevelopment (Davis-Bruno 2010; Kuipers 2011) and breast milk is considered to have the optimal levels of EFAs (Davis-Bruno 2010). Since 1990, infant formula products have been

supplemented with DHA and AA and some studies have shown benefits with respect to visual and neurodevelopment (Heird 2005). Over time, there has been a shift towards increasing dietary n-6 fatty acid levels and a modest reduction in n-3 fatty acid levels due to changes in dietary patterns (Gibson 2011). Elevations in n6 fatty acid levels have the potential to reduce the conversion of ALA to EPA and DHA as there is only one set of desaturase enzymes necessary to convert parent EFAs to downstream target, thus creating a conditional essentiality for increased n-3 fatty acid intake (Gibson 2011; Heird 2005).

Farquharson et al. (1993) state that since breast milk is considered to have optimal levels of EFAs, the concentrations of these fatty acids should be markedly elevated in infant formulas (Farquharson 1993). As previously stated, because there is only one set of desaturase enzymes available, an excess of one of the EFAs will result in inappropriate balances of the downstream fatty acids necessary for infant growth (Farquharson 1993).

5.1.2 Medium and long chain fatty acids

Medium chain fatty acids (C8:0 to C10:0) are absorbed directly into the portal vein and oxidized rapidly in the liver, compared to the long chain fatty acids which must first be absorbed into the lymphatic system and transported via chylomicrons into the systemic circulation (Takeuchi 2008). Since the 1950s, medium chain fatty acids have been used extensively as a nutrition source for those with malabsorption syndrome due to their rapid absorption and a more complete oxidation than long chain fatty acids (Aoyama 2007). In general, fatty acids are transported into the mitochondria for β -oxidation via the carnitine transport system; however, medium chain fatty acids do not require carnitine as

a carrier and are thus preferentially taken up and not dependent on the maintenance of appropriate concentrations of carrier molecules (Reuter 2012; Papamandjaris 1998).

Fatty acids with a chain length longer than 10 carbons are classified into the long chain category, and also have 3 further sub-classifications: saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA) and poly-unsaturated fatty acids (PUFA) (AOCS Lipid Library A). The EFAs and their downstream products fall under the LCPUFA class.

5.2 LIPID EMULSION PRODUCTS

Parenteral nutrition consists of three macronutrient components (protein, carbohydrates and lipids) and micronutrients (electrolytes, vitamins and trace elements). The main energy source in PN is the lipid component, in the form of a lipid emulsion/TG solution (Fusch 2009). Lipid emulsions have been developed not only as a concentrated energy source but also as a source of EFAs, such as LA and ALA that cannot be synthesized endogenously (Valentine 2007). Detrimental effects are observed if there is an essential fatty acid deficiency in a preterm infant during the third trimester such as diminished growth, liver and kidney damage, dermatitis and even death (Trintis 2010). These highly concentrated and isotonic solutions are composed of a vector (the continuous aqueous phase, usually containing glycerol), an active principle (oil or fat source) and an emulsifier (phospholipids) (Lehner 2006; Valentine 2007; Ferezou 1999; Cartwright 2000; Hultin 1995; Mirtallo 2010). Depending on the desired therapeutic outcome, different fat sources or combinations of sources can be included in the active principle compartment. The presence of an emulsifier is required for stability and proper dispersion of the oil. Artificial chylomicrons enclose TGs and other lipid soluble

substances within their hydrophobic core, which is surrounded by a phospholipid monolayer allowing for solubility in the blood once the emulsion has been infused intravenously. Once in the blood, the artificial chylomicrons acquire apolipoproteins and recruit the lipoprotein lipase (LPL) released from the capillary endothelium in order to hydrolyze the TGs and distribute the fatty acids to the infant's tissues (Valentine 2007; Ferezou 1999; Cartwright 2000; Hultin 1995).

Lipid emulsions have been in use for approximately 50 years and the availability of more than one formulation allows for versatility with respect to therapeutic outcome (Deshpande 2011; Driscoll 2006; Hardy 2009; Krohn 2006). Conventional lipid emulsions developed in the 1960s were soybean oil-based and contained predominantly fatty acids of the n-6 category (Deshpande Curr Opin Clin Nutr Metab Care 2011). After some time, the focus shifted away from predominantly soybean oil products and onto the potential for incorporation of other oil sources due to the fact excess n-6 fatty acids can result in pro-inflammatory eicosanoids and are subject to lipid peroxidation (Lehner 2006). Lipidem, Lipofundin and Intralipid are considered conventional, soybean-oil based lipid emulsions (Lehner 2006; Deshpande 2011). Coconut oil (rich in medium-chain fatty acids), fish oil (rich in LCPUFA) and olive oil (source of MUFA) were the alternate oil sources examined for use in lipid emulsion products (Deshpande 2011). Omegaven contains predominantly fish oil; SMOFlipid is a combination of soybean oil, medium chain fatty acids, olive oil and fish oil; and Clinoleic is comprised primarily of olive oil and a small proportion of soybean oil (Deshpande 2011). Clinoleic has shown beneficial

effects with respect to oxidation, as the MUFAs are thought to be more resistant to oxidation (Waitzberg 2006).

Lipid emulsions for pediatric use are adapted from adult formulations and the composition of breast milk is considered to some degree in the design and choice of oils for these products (Hardy 2009). Still though, the lipid emulsions utilized in clinical care do not match the fatty acid profile of the infant's natural source of nutrition, i.e., breast milk (Driscoll 2009). Utilizing combinations of oil sources can bring the fatty acid pattern of the lipid emulsions nearer to that of breast milk, but not identical.

5.3 ADVERSE EFFECTS OF LIPID EMULSION USE IN PRETERM INFANTS

Parenteral nutrition studies in preterm and very low birth weight (VLBW, birth weight <1500g) infants highlight that currently available lipid emulsion formulations are not optimal for neonatal nutrition (Valentine 2007; Amusquivar 2008; Koletzko 2003) and the risk of adverse events is influenced by the dosage and duration of administration (Mirtallo 2010). Formulations with high concentrations of PUFAs, for example soybean oil-based, place the infant at risk for lipid peroxidation and potential liver damage (Carpentier 2006; Haumont 2010). A newborn preterm infant does not immediately reach a stable health status and their fat oxidation is increased as a result of perinatal stress as well as ventilatory support (Koletzko 2003; Carpentier 2006) and added oxidative stress can place the infant at risk for future morbidities. Preterm infants have an immature digestive system and have limited, if any, fat stores (Koletzko 2003; Alemi 1981; Hamosh 1991) therefore an immediate source of nutrition is imperative. These lipid emulsions must be able to sustain growth and development, as well as maintain bodily

functions. Studies also highlight that infants receiving PN can demonstrate higher serum TG levels than those fed enterally with breast milk (Valentine 2007; Rochow 2010). Death is an extreme adverse effect and autopsy results in preterm infants have confirmed the cause to be intravascular or intrapleural fat accumulation as a direct result of lipid emulsion use (Mirtallo 2010).

Rochow et al. (2010) report that an enteral fat intake with breast milk of 7g/kg/day leads to 50% lower serum TG levels compared to previous studies where infants were fed PN amounting to 3g/kg/day (Rochow 2010). The study details that the fat intake of breast milk was more than double that of PN, however the serum TG levels were half the amount. This discrepancy highlights a possible difference in uptake and metabolism of the two sources of fat, as the TGs in PN formulations are not being hydrolyzed properly and distributed to tissues but instead are remaining in the blood for longer periods of time (Rochow 2010). This is not an ideal situation because the infant requires this concentrated energy source to be available and delivered to tissues in order to achieve proper growth and development at such a critical and energy-intensive stage in life. Even though growth of a preterm infant is energy intensive, nutrition strategies cannot be too aggressive with respect to increasing caloric content on the level of protein or carbohydrates in PN; increasing protein content places stress on the kidneys because of high renal solute loads from urea accretion and increasing carbohydrates results in protein accretion (Puntis 2006). Fat content could be increased, yet infants seem unable to tolerate much higher than 3g fat/kg/day intravenously, even though as previously stated they have a tolerance for 7g fat/kg/day in breast milk (Rochow 2010).

Human milk fat is superiorly absorbed compared to other nutrition sources in both term and preterm infants (Haddad 2012). One suggested mechanism for the optimal absorption characteristic of breast milk is the positional distribution of the fatty acids within the TGs, which has an effect on metabolism and incorporation of fatty acids within the tissues (Nutrition Reviews 1987). Fatty acid composition of mother's milk is thought to greatly influence absorption in the infant, and it has been recently stated by Haddad et al. (2012) that continued research into the area of fatty acid composition of infant nutrition sources is warranted (Haddad 2012) despite the multitude of infant nutrition products available. Intestinal absorption of dietary fats can also be influenced by chain length and degree of unsaturation of the fatty acid as well as the supply of lipolytic enzymes at the site of lipolysis or maturity of the digestive system (Nutrition Reviews 1987). Balance of fatty acid classes has also been implicated in the absorption of fatty acids from lipid emulsions. Lehner et al. (2006) demonstrate a differential absorption pattern in fatty acids between long chain TG (LCT) and medium chain TG (MCT)+LCT fat solutions (Lehner 2006). The authors of that study conclude that although the LCT solution contained almost double the amount of C16:0, C18:0, C18:1n9 and C18:2n6 the plasma levels of these fatty acids in both groups were similar, citing that the MCT reduced the oxidation of the long chain fatty acids and they were instead incorporated into plasma lipids (Lehner 2006). Thus, combining oil sources can change not only the profile of fatty acids, but also how they are metabolized and used by the body.

Another proposed reason for the increased serum TGs while on PN is that perhaps not all artificial chylomicrons in the lipid emulsions associate with lipoproteins once they

are administered within the blood. This association of the artificial chylomicron with lipoproteins is essential, as it is the first step in recruiting lipase so that hydrolysis can occur. The mechanism behind this is not known - and has not yet been established - but it may account for the elevated serum TGs in infants on PN. As previously stated, the fatty acid composition within the TGs might influence digestion as there is evidence to suggest that the metabolism of the chylomicron is affected by fatty acid positioning and composition (Nelson 1999).

Lipid emulsions contain excess emulsifier in the form of phospholipids in order to keep the emulsion stable during storage and administration and to promote proper dispersion of the oil (Mirtallo 2010; Ferezou 1999; Rochow 2010). Without proper emulsifiers, the lipids would coalesce and form fat emboli (Mirtallo 2010). Problematic for lipid clearance, the excess phospholipids can aggregate and form hollow spheres called liposomes, which can sequester enzymes such as lipoprotein lipase leading to a reduction in clearance of TG (Mirtallo 2010; Ferezou 1999; Rochow 2010). Preterm infants already have lower levels of LPL than term infants and rendering a portion of the supply of this enzyme useless is not favourable (Aleml 1981; Valentine 2007).

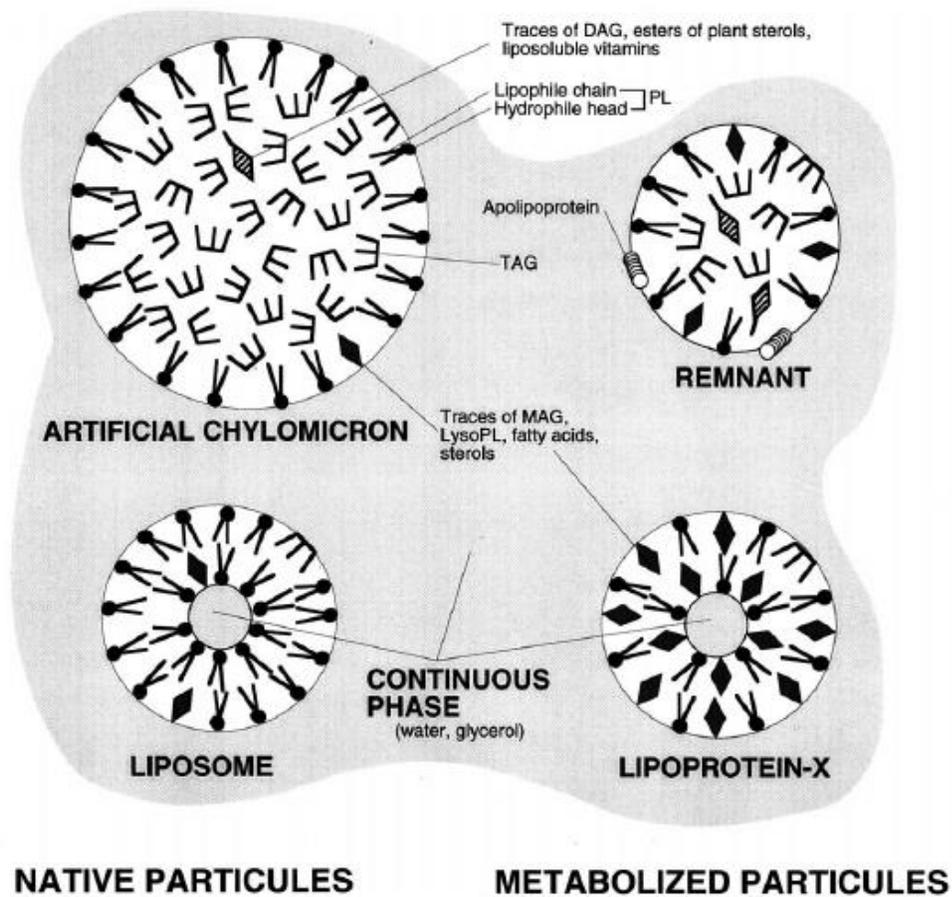


Figure 2. Particles of parenteral fat emulsions (Ferezou 1999).

5.3.1 Clearance of TGs during lipid emulsion infusion

Adverse effects with lipid emulsion can also be a direct result of infusion rates, i.e., the lipid emulsion is administered in excess of energy expenditure. Lipid emulsions can only be cleared at a rate of 3.8g fat/kg/24 hours and beyond this rate the clearance mechanism (LPL) becomes saturated and thus begins the accumulation of serum TGs. This is also known as fat overload syndrome which occurs with large daily doses of fat for extended periods of time and in addition to hyperlipidemia, presents itself via fever, jaundice and spontaneous hemorrhage (Mirtallo 2010). Despite the influence of infusion

rates in the precipitation of adverse events, there are infants that display a hypertriglyceridemic profile without elevations in infusion rates. Regardless of the reason for the elevated serum TGs seen in infants on PN, it is a situation that should be avoided at all costs due to the risk imposed on the infant for pulmonary dysfunction, hemorrhage, liver dysfunction and coagulopathy thus TG levels are monitored closely (Deshpande 2011).

5.4 GOLD STANDARD OF INFANT NUTRITION: BREAST MILK

Early nutrition has a fundamental influence on development via the elicitation of modulatory effects on immune and allergic responses, body composition and the potential for development of nutrition-related chronic diseases (Rombaldi Bernardi 2012; Lapillone 2013; Puntis 2006). Exclusive breastfeeding of term infants early in life is associated with lower blood cholesterol concentrations later in life (Owen 2008) and it has been shown to reduce the risk of obesity later in life by 20% when compared to formula-feeding (Koletzko 2013; Koletzko 2006; Plagemann 2012). Breast milk has long been promoted as optimal nutrition for neonates (Arslanoglu 2009; King 2010) because of its nutrient profile which is perfectly balanced for human infants with respect to fatty acids, amino acids, vitamins and minerals (Haumont 2010; Groh-Wargo 2009; Berenhauser 2012) and its LPL and bile salt-stimulated lipase content (Lapillone 2013). The fat portion of breast milk sustains approximately 50% of the infant's energy requirements (Sala-Vila 2005; Heird 2005). Although the composition of breast milk varies widely between countries, among women and between feedings, it is still recommended as the optimal nutrition source, as it produces more favourable results in the infant than lipid emulsions or infant

formulas (Groh-Wargo 2009; Lapillone 2013) and is postulated to protect against NEC (Puntis 2006). This alludes to the fact that there may also be additional factors contributing to the putative superiority of breast milk, in addition to the fatty acid composition (Hippalgaonkar 2010). Growth stages in a preterm infant are more energy-intensive than a term neonate, and therefore milk from mothers of preterm infants is often fortified to increased caloric and EFA content, among other nutrients (Puntis 2006). The efficiency of fat absorption from human milk compared to lipid emulsions or formula might be attributed to any of the following parameters, or a combination of them: fat globule integrity, inherent lipase activity or any factor in milk responsible for expanding the bile salt pool (Chappell 1986). A study by Chappell et al. (1986) demonstrates that increasing calcium supplementation has the potential to increase fatty acid excretion, an outcome not ideal for a preterm infant in critical stages of growth and development (Chappell 1986).

5.5 METABOLISM OF FATTY ACIDS

Although the optimal balance between short, medium and long chain fatty acids, or SFA, MUFA and PUFA has not been determined, it is evident that the chain length and degree of saturation of the fatty acid has an influence on the human body upon consumption (Ramírez 2001). Despite reported differences in fatty acid profiles of mother's milk, the pattern of fatty acid absorption has not been outlined fully, nor have recommended levels for each fatty acid been established for preterm infants (Chappell 1986; Lepage 2006). It has been reported that increased levels of ALA in the diet can inhibit the absorption of palmitic acid (C16:0) by adipocytes when compared to infants

fed breast milk (Farquharson 1993). This is extremely important to note, as it suggests that elevated amounts of certain fatty acids have the potential to inhibit the metabolism of others can elicit a disruption in equilibrium of fatty acid mobilization and deposition (Farquharson 1993). Friedman et al. (1979) speculate that there may be a competition between fatty acids for esterification and/or storage in tissue lipids (Friedman 1979). Although the exact mechanism was not elucidated, it lends credibility to the idea that some degree of balance in fatty acid profiles must exist in order for an infant to metabolize and/or uptake fatty acids appropriately and the importance of the fine tuning of fatty acid profiles in lipid emulsion and formula products must not be underestimated.

Fatty acids exist in equilibrium between those obtained in nutrition and those released by tissues in the body. Adipocytes are the main storage tissue for fatty acids, and major turnover between absorption and release occurs in this tissue. Circulating dietary fat uptake by adipocytes occurs via two mechanisms in the human body: 1) hydrolysis via LPL from the capillary epithelium and uptake and, 2) direct free fatty acid uptake, in the unbound form, and storage (Ginsberg 1998; Mirtallo 2010). Evidence of fatty acid chain length-dependent absorption exists in humans but the results are inconclusive (Garaulet 2006; Kishino 2011; Koutsari 2008). Ramirez et al. (2001) report that medium chain and unsaturated fatty acid absorption was more efficient than long chain absorption, also citing differences based on stereoisomerism (Ramírez 2001). In support of the same theory, Chappell et al. (1986) state that reconsideration should be given to increased levels of medium chain fatty acids in fatty acid preparations as they demonstrate increased metabolic efficiency (Chappell 1986) and could therefore be utilized rapidly as

an energy source by neonates. Jones et al. (1985) also challenge the idea that dietary fat is metabolized independently of the chain length of fatty acids contained in the TGs, as their results demonstrated differential oxidation levels for stearic (C18:0), oleic (C18:1n9) and linoleic (C18:2n6) acids (Jones 1985). Although only 3 fatty acids were examined, the same study also found increased oxidation rates for MUFA versus SFA.

Evidence also indicates that different fat depots may have an effect on fatty acid uptake, as visceral versus subcutaneous fat depots may have different rate-limiting steps in dietary fatty acid storage (Santosa 2008) or different transport routes of fatty acids (McDonald 1980). This is important to note when considering what an “optimal” fatty acid profile is for preterm infants because in addition to each fatty acid having a potentially different rate of metabolism and uptake, their visceral versus subcutaneous ratio is much different than a term infant’s and the same nutrition sources may have a very different metabolic effect. Subcutaneous adipose tissue has been referred to as a “metabolic sink” where excess free fatty acids are stored (Ibrahim 2010). Results from Farquharson et al. (1993) elucidate that it is not until approximately 4 weeks of life that a preterm infant’s rate of deposition and mobilization of fatty acids reach an equilibrium phase as preterm infants have low amounts of subcutaneous fat at birth (Farquharson 1993). Further, results obtained from adult men demonstrate that visceral and subcutaneous adipose tissues differ in quantity of free fatty acid uptake (Hannukainen 2010). A sense of precariousness is nested within many of these studies as the subcutaneous and visceral fatty acid composition results are potentially tainted by the confounding effect of adipocyte membrane phospholipids (Farquharson 1993).

Nonetheless, these results are important as the influences of fatty acid chain length or saturation on uptake have not been elucidated in preterm infants, nor have the same differences in visceral versus subcutaneous fatty acid uptake been confirmed. Additionally, it has been suggested that the role of fatty acids as signal transducing molecules has opened new doors in the investigations of the nutritional control of adipose tissue development (Amri 1994). Despite the knowledge gleaned from adult and animal models, there still exists a scarce amount of information about whether or not the metabolism of adipocyte fatty acids play a role in the growth stages of a preterm infant, or merely act as a storage depot for excess fatty acids supplied by the diet (Farquharson 1993) and the current findings are not without inconsistency.

5.6 GAPS IN KNOWLEDGE: IDEAL FATTY ACID PROFILES

Currently, very little is known about the optimal growth patterns of preterm neonates. Data on lipid profiles for preterm infants that are defined as “healthy” are limited, and it is unclear at what level serum TGs begin to cause adverse effects in infants (Rochow 2010). Lipid emulsion products are commercially designed to replace the fat content of human milk for preterm infants that are unable to be fed enterally, yet it is not known if they are considered optimal for nutrition, metabolic development and later life fat patterning at such an early age. It is unknown which fatty acids have the potential to inhibit the metabolism of others, and in what amounts. Without an ideal fatty acid profile for nutrition sources or resulting serum, it seems counterintuitive that so many lipid emulsion products are available for use in preterm infants. The fat composition of these products varies widely but the same standard, TG levels, is used to monitor infant safety

regardless of the nutrition type administered. Evidence for a better monitor of safety in infants is lacking and not enough research has been conducted to determine if there might in fact be a more descriptive indicator to monitor lipid balance in infants.

Furthermore, it is not known if elevations in certain fatty acids characteristically cause hypertriglyceridemia in preterm infants. In addition to the infants administered lipid emulsions, those that are fed according to normal feeding regimens can still display elevated TGs and if single fatty acids can be implicated in hypertriglyceridemic situations, consideration can be given to the amounts of these particular fatty acids in infant nutrition formulations.

5.7 PROJECT RATIONALE

Acceptable profiles on an individual fatty acid level have not been established for infant nutrition sources, nor have they been established for serum levels in preterm infants. Examination of the fatty acid profile of breast milk and a further comparison to commercially available lipid emulsion and infant formula products may help to explain variances in the fatty acid profiles and determine if they are associated with differences in the serum profile. Examination of the fatty acid profile of the infant's nutrition source and comparison to the resulting fatty acid profile may help to determine if the infant is metabolizing all fatty acids at similar rates or at least comparable to the rates at which they are supplied. Differences in fatty acid profiles between nutrition and serum may help to elucidate which fatty acids are preferentially taken up and which ones remain circulating in the blood for longer periods of time, potentially contributing to elevated serum TGs. Lastly, since hypertriglyceridemia is an extremely undesirable situation

during the administration of a lipid emulsion, it would be useful to know if there is a characteristic profile of fatty acid absorption during hypertriglyceridemic situations. Hypertriglyceridemia may provide ideal conditions to observe imbalances in lipid metabolism and examine whether these imbalances can affect all fatty acids on the same level. Examination of fatty acid profiles in hypertriglyceridemia has the potential to elucidate any differences in metabolism patterns of fatty acids in a clinical situation.

5.7.1 Primary objective and hypothesis

The primary research objective is to determine if the fatty acid profile of breast milk obtained from mothers of preterm infants differs from the fatty acid profile of several formulations of currently available lipid emulsions for parenteral nutrition. Based on the literature, it is expected that the fatty acid profile between breast milk and each lipid emulsion will vary with respect to the entire profile (ie. medium versus long chain fatty acids) and on an individual fatty acid level.

5.7.2 Secondary objective and hypothesis

The secondary research objective is to determine if the fatty acid profile in the nutrition source correlates with the resulting fatty acid pattern 48-72 hours after administration. Furthermore, the research investigates if the serum fatty acid profile of infants fed via parenteral nutrition differs from the profile of preterm infants that are fed enterally via mother's milk. Free fatty acid profiles in the blood are mainly derived from dietary sources and represent dietary fatty acid intake from the previous days, acting as a sort of biomarker of intake (Hodson 2008); therefore serum profiles of fatty acids are expected to correlate with the profile of the nutrition source. Thus, if the fatty acid

patterns of lipid emulsions are found to differ from breast milk, it is expected that the serum profiles would be different as well.

5.7.3 Tertiary objective and hypothesis

The tertiary objective is to determine if there are certain fatty acids that are consistently elevated in instances of hypertriglyceridemia in preterm infants. Based on the evidence presented on excesses of some fatty acids that inhibit the metabolism of others or that certain fatty acids are preferentially taken up by adipose tissue before others, it is expected that the fatty acid profile of hypertriglyceridemia will differ from that of normotriglyceridemia. It is hypothesized that if certain fatty acids are characteristically elevated in hypertriglyceridemia and not in normotriglyceridemia, these elevations may follow predictable proportions.

6. MATERIALS AND METHODS

This section details the clinical procedure, materials and methods utilized for the single-centre longitudinal observational study “Fatty acids in infant nutrition sources for preterm infants”, completed at McMaster University Hospital Level II and Level III Neonatal Intensive Care Unit (NICU) (see Figure). Samples were collected from the NICU between August 2012 and May 2013 under the approval of the HIREB, project 12-220. This pilot study is exploratory in nature and the objective for sample collection was to analyze the fatty acid profile in mother’s milk, lipid emulsions, formula and infant’s serum.

6.1 Clinical procedure

The study coordinator screened infants using Meditech, the health information management system used for inpatients at McMaster Children's Hospital, and/or during morning rounds with attending physicians. Infants <36 weeks were eligible for the study and all mothers of recruited infants were eligible for the study as long as they were breastfeeding. Infants were only excluded if they had sepsis at time of recruitment, and were ineligible for two days before and one week following treatment. The parent(s) was approached by the study coordinator pre- or antenatally and if interested, a consent form was signed. Once the baby was consented, a standing order was printed and signed by a physician or nurse practitioner on clinical service for that day and placed in the bedside binder. This standing order contained information required by the nurses for proper sample collection and applied to every routine blood draw (on our desired timeline of collection: once in the first 3 days of life, once weekly for one month and once monthly thereafter) for the duration of the infant's time in the NICU. Meditech was used to determine the next scheduled blood draw for the infant. A package containing a pre-labelled microtainer in a small bag was attached to the infant's incubator prior to scheduled blood work. Routine blood work was usually performed on Monday mornings and a 2 mL sample of pooled breast milk sample was obtained from the dietary assistants in the NICU on Friday afternoons (or on the first day of life, if the infant was on enteral feeds and was consented prenatally). Milk samples were prepared by the dietary assistants in the NICU and were frozen in a -20°C freezer until analysis. The milk sample obtained was a fully prepared and fortified (Enfamil human milk fortifier) mother's milk sample. This allowed for the fatty acid profile to be analyzed for the milk sample, and after fatty

acid profiles have reached equilibrium from that nutrition source, the fatty acid profile from the serum 48-72 hours later (ie. Monday) was analyzed. Morris et al. (1998) reports that after 36 hours of fat-free parenteral nutrition, plasma triglycerides declined uniformly and rapidly (Morris 1998), and Hodson et al. (2008) adds that the fatty acid composition of plasma triglycerides represents dietary fatty acid intake from the previous days (Hodson 2008). Based on this information that plasma fatty acid profiles are more reflective of recent diet than red blood cell profiles and at the convenience of blood sampling schedules in the NICU, a period of 2-3 days prior to blood sample was a reasonable timeline on which to collect nutrition samples.

After the blood sample was drawn, nurses stored it in the specimen fridge in the NICU for immediate pick-up. The microtainer was spun down using at 3000 rpm (5°C) and serum was isolated and frozen in an 0.5 mL Eppendorf tube at -80°C until analysis (see Analytical techniques: Extraction and esterification of blood samples). Nurses and/or physicians only drew the extra 200 µL sample if the infant's condition was favourable and a routine blood work event was previously scheduled (we did not request additional bloodwork events). Nutritional information was recorded from flow sheets in the bedside binder or from Sovera, McMaster Children's Hospital's health information management system for previous patients, two days prior, one day prior and the day of the blood sample. Total daily volume of enteral and/or parenteral feeds was recorded for each infant. If a serum triglyceride test was ordered by a physician within 48 hours of any blood sample obtained for the study, the resulting values were recorded from Meditech in order to determine if a particular sample was hypo-, normo- or hypertriglyceridemic.

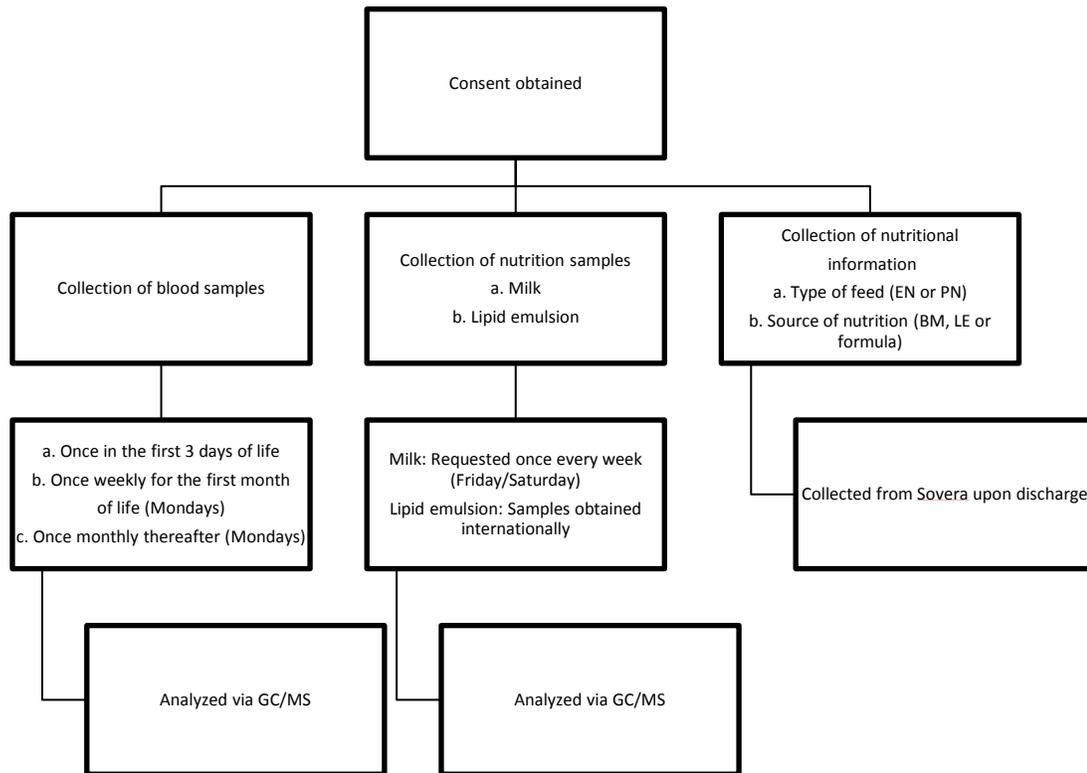


Figure 3. Graphical representation of clinical procedure from the stage of consent to sample analysis.

6.2 Collection of hypertriglyceridemic samples from Hamilton Regional Laboratory Medicine Program (HRLMP)

An HIREB amendment was submitted to receive leftover samples from the NICU that were identified as hypertriglyceridemic. Patient information was anonymized and consent was not required from the parents as the sample would normally be discarded and the hypertriglyceridemic patients could not be identified proactively in order to receive consent. Daily at 1200h a search function was run within the HRLMP system for samples from the NICU that were identified as hypertriglyceridemic. They were collected and assigned a numerical value in order of collection date and for anonymity purposes (i.e., the first sample collected was Sample#1).

6.3 Preparation of internal standard stock solution

The fat content of breast milk (approximately 4.5%; Rochow 2013) was used to estimate the expected absolute concentrations of single fatty acids. This estimation was used to calculate the absolute concentration of internal standards to add to the stock solution. It was decided that this calculated concentration of fatty acid methyl esters (FAMES) would be doubled in order to account for the wide variation in fat content of mother's milk. It was calculated that 7.2 mg of each C17 FAME and C23 FAME were to be added to 10 mL of petroleum ether to create a stock solution of internal standard. During the first extraction step, 50 μ L of petroleum ether was added to the milk or serum sample (see Materials and Methods, 6.4 Analytical techniques: Extraction and esterification of fatty acids from milk), and this allowed for the potential loss of fatty acids in the sample (due to human error) to match the potential loss of internal standard.

C17 and C23 FAMES were chosen as internal standards because they were not expected to appear naturally in either milk or serum and because saturated, straight chain fatty acids make ideal standards (Skartland 2011; Wu 2011). C17 was to be used for quantification, but a calibration curve for C23 was developed as well in order to verify concentration values generated by C17 internal standard.

6.4 Analytical techniques: Extraction and esterification of fatty acids from milk

The isolation of fatty acids from biological material can be accomplished using various extraction techniques and analysis can occur via high performance liquid chromatography (HPLC), gas chromatography (GC) or gas-liquid chromatography (GLC). Methods to isolate and prepare the fatty acids bound in the form of TGs for

chromatographic analysis follow two main procedures: 1) *saponification* using an inorganic or organic basic solution or *acidic hydrolysis* and an addition step for chromatographic analysis; 2) *derivitization* or *direct transesterification* - alcoholysis or methanolysis (AOCS Lipid Library B). The main methods utilized in literature for fat extraction/isolation are the Bligh and Dyer method (Bligh 1959) , Folch method (Folch 1957), followed by preparation for chromatographic analysis via transesterification method. Other methods found in literature as well as the method used in this study are combinations and/or adaptations of the Bligh and Dyer and Folch methods.

For this study, a previously described method was used to extract and esterify fatty acids from mother's milk (Fidler 2000; Fidler 1998) and this method incorporates an ether extraction with a transesterification step to derivitize. Note that the tert-butyl-mether ether used in this extraction instead of chloroform has been shown to allow for a more effective and cleaner lipid recovery and extensive testing has ranked it on par with the "gold-standard" Bligh and Dyer or Folch methods (Matyash 2008).

The milk sample was thawed subsequently homogenized (Sonics Vibra-cell, model CV 18). Potassium oxalate solution (3.5%) (5 μ L), pure ethanol (50 μ L), pure tert-butyl methyl ether (50 μ L), and the petroleum ether internal standard stock solution (50 μ L) were added to the 100 μ L milk sample. The mixture was vortexed and subsequently centrifuged for 5 minutes at 3000 rpm. The organic phase containing the fatty acids was extracted off and 50 μ L each tert-butyl methyl ether and petroleum ether were added to the organic layer for a second round of triglyceride extraction. The organic layer was then dried under a stream of air for approximately 10 minutes (or until dry). Hydrochloric acid

(3N, 50 μ L), methanol (50 μ L) and hexane (25 μ L) were added to the sample vial. The mixture was removed from the plastic Eppendorf vial and transferred to a 300 μ L glass vial and covered with a double layer of Teflon tape. Teflon tape was used instead of a lid or capped vial as the pressure accumulation during heating caused the lids to pop off. The mixture was heated to 90°C for 25 minutes to 1 hour (depending on evaporation times) to transesterify the fatty acids into FAMES. Upon completion of the heating phase, HPLC-grade water (100 μ L) was added to the glass vial, and the fatty acid methyl esters were extracted from the organic phase twice with 100 μ L hexane plus butylated hydroxy toluene (BHT, 1 mg/10mL). The BHT was used as a stabilizing agent for the FAMES. The final volume of the extracted FAME/hexane/BHT mixture is approximately 200 μ L, which does not occupy a large volume of the 1.5 mL Agilent vials used during GC/MS analysis. A 200 μ L glass insert was added to 1.5 mL Agilent GC glass vial to raise the fluid level and allow the syringe to reach the small sample volume. Samples were sealed with Teflon-lined rubber caps stored in the -20°C freezer until analysis.

6.5 Analytical techniques: Extraction and esterification of fatty acids from serum

A similar procedure to the one used here has also been used in literature to extract and esterify fatty acids from serum as well, utilizing similar aspects of this method such as tert-butyl-methyl ether extraction and methanolysis under heated conditions (Ichihara 2011). No noticeable differences were found in composition obtained by GC using this method or conventional chloroform/methanol procedures (Ichihara 2011). Blood collected from bedside in the NICU was allowed to coagulate for 20-40 minutes in the microtainer and subsequently centrifuged at 3000 rpm for 10 minutes at 5°C. Because

only a small sample volume could be obtained given the size of preterm infants, the serum was not further separated and therefore fatty acids analyzed were not only from TGs and free fatty acids, but also from cholesterol esters and phospholipids (Uusitalo 2013). The same extraction and esterification method and volume was used for serum with the exception of the preparation of the frozen sample prior to extraction and esterification, as it was allowed to thaw completely for 20-30 minutes but was not placed in a water bath.

6.6 Analytical techniques: Gas chromatography/mass spectrometry

Samples were analyzed on a gas chromatographer (Agilent 7890) with a Supelco 1L111 GC column attached to a single quad mass detector (Agilent 5795 MSD). A fatty acid standard was purchased from Supelco (1mL- 47885-U) containing 37 FAMES and it was run on the column prior to any samples. This allows for identification of each standard fatty acid in unknown samples by using the retention time and mass/fragmentation pattern. Three pre-washes and 3 post-washes of the syringe with hexane were conducted prior to and after injection. The injection volume was 0.1 μ L and it was run in splitless mode. The oven temperature was held at 130°C for 10 minutes, ramped up 2°C/minute to 240°C and held at 240°C for 2 minutes at the end of the ramping time. This 2 minute hold time was included to ensure all fatty acids and solvent are carried fully through the column and to avoid accumulation of contaminants on the column. Auxillary column temperature was maintained at 250°C. For each FAME, retention times and mass/fragment patterns were matched so that each one could be identified and fatty acid ratios could be calculated. Autotune files were run on a regular

basis (approximately every 20 runs) using the ChemStation software in order to ensure proper calibration and the column was baked out at 260°C after every 100 runs.

6.7 Determination of retention time ranges for each FA

The retention times for each fatty acid were recorded along with the area under the curve (AUC) for 20 milk and serum samples. Mass fragmentation patterns were consulted for each fatty acid in each sample in order to ensure that the same fatty acid continually matched the one expected within a particular retention time. Average retention times \pm SD were calculated for each fatty acid in the FAME standard. Upon determination of the retention time ranges for each fatty acid, they were identified solely based on retention time. For retention times of fatty acids detected in milk, serum and lipid emulsions see Table 1 (Appendix A). Analysis of fatty acids by GC retention time alone is an extremely powerful means of characterization (AOCS Lipid Library B).

6.8 Development of a calibration curve

Palmitic acid (C16:0) is commonly found in both milk and serum samples, and this compound was used to develop a calibration curve for quantification purposes. A stock solution of approximately 3-fold the concentration of the C17:0/C23:0 internal standard solution was created via the addition of 43.2 mg of C16:0 FAME to 10 mL of hexane. To an Agilent GC vial, 50 μ L of the internal standard stock solution was added and the ether was dried off under a stream of air. A calibration curve was developed by creating 8 dilutions ranging from 1.08 mg/mL of C16:0 to 4.2 μ g/mL. The C16:0/C17:0 AUC ratio was plotted against concentration of C16:0 and the R^2 value obtained was 0.9993 (Figure 4).

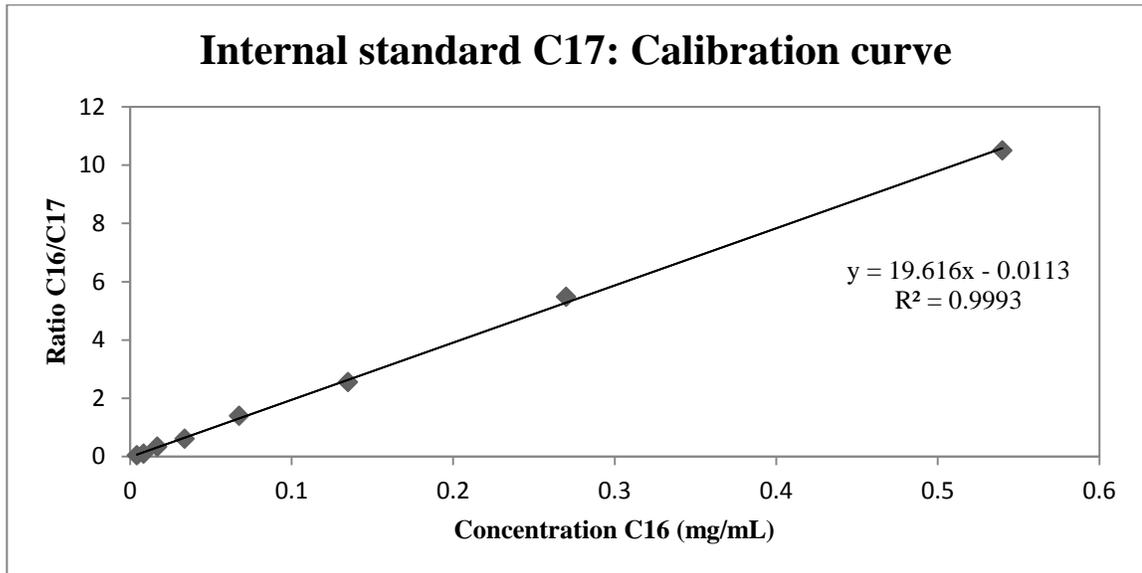


Figure 4. Calibration curve plotting the ratio of C16/C17 against known concentration of C16.

Quantification using this calibration curve did not produce absolute concentrations for fatty acids within the range expected. Based on the fact that we estimate human milk to have a concentration of 4.4g/100mL (Rochow 2013), the addition of concentrations of all fatty acids did not sum to this amount during test runs for breast milk. A bulk of the literature on fatty acid compositions has reported results in the form of percent composition and due to complications with the quantification step, reporting results in absolute concentrations was not included as part of this thesis.

6.9 Validation experiments

Three phases of validation were performed using an average breast milk created for this study by pooling 6 breast milk samples from the NICU (2 mL each from 6 samples obtained from 6 different mothers). The pooled sample was divided into 30 aliquots of 100 μ L each and frozen until analysis. The first phase consisted of intra-day

variation in the extraction/esterification method (n=10, Figure 5). The second phase consisted of inter-day variation in the method (n=10, Figure 6). The third phase involved “limit of detection” experiments using the series of dilutions prepared for the development of the calibration curve. Dilutions were not made below 4.2 µg/mL of pure standard, as fatty acids occurring in concentrations lower than this would not likely affect ratios significantly, and therefore it was determined that the limit of detection for this method is 4.2 µg/mL of pure standard.

6.10 Statistical analysis

Analyses were performed using Graphpad Prism 6 for Windows. The analysis results of patient characteristics and outcome variables were summarized using descriptive summary measures: expressed as a mean (standard deviation) for continuous variables and number (percent) for categorical variables. An independent two-tailed t-test was performed in order to compare means between a. LE and BM; b. nutrition source (LE or BM) and serum; c. serum fatty acid profiles on BM and LE; d. serum fatty acid profile of hypertriglyceridemic and normotriglyceridemic samples. Standard deviations were not assumed to be equal and Welch’s correction factor was applied. For matched pairs of BM and serum, a paired t-test was computed. Results were displayed as bar graphs comparing the percent composition of each fatty acid across the groups studied. As a visual tool to directly compare the lipid composition of two samples, resulting compositional differences (difference of means) were displayed as a bar graph with a 95% confidence interval for the difference of the mean and significance was marked above the bar with (an) asterisk(s).

6.10.1 Stratification and nutritional grouping

Due to the relatively small sample size of this pilot study (n=47), infants were not stratified according to gestational age or sex, nor were these parameters controlled for in the analysis. Serum samples were grouped according to nutrition class administered over the 48 hours prior to and day of blood sampling. Volume of nutrition was used to calculate fat (g) resulting from each source and percent fat was calculated based on all nutrition sources. Fat content used for calculations are as follows: milk = 4.4g/100 mL; Enfamil preterm 24 kcal formula = 4.1g/10 mL; Enfamil preterm 20 kcal formula = 3.4 g/100 mL; Nutramigen A+ formula = 3.6g/100 mL; Lipid emulsion (Intralipid) = 20g/100 mL. The nutrition groups were as follows: *nil per os* (NPO); 100% BM; 100% formula; 100% LE; and mixed nutrition (any combination of BM, formula and LE). Since only one sample belonged to the 100% LE category, this group was expanded to include >50% LE, meaning that greater than 50% of the infant's dietary fat was supplied by LE over the 2 days prior to and day of blood sampling.

6.10.2 Sample size

Given that this was an exploratory pilot study prior knowledge of the differences we intended to detect between individual fatty acid percentages or estimates of the variances between samples were not known. The projected sample size (n=100) at time of study commencement was based on a sample size of convenience, the number of beds in McMaster's NICU and estimated recruitment rate of 50%. The final sample size of this project (n=47) was a reflection of recruitment rate between August 2012 and May 2013.

7. RESULTS

A total of 47 infants were included in the study with a sex distribution of 23 females and 24 males. The average age at birth was 30^{6/7} weeks gestation, with a range from 24^{2/7} to 36^{4/7} weeks gestation. A total of 69 milk samples were collected from 28 mothers of infants enrolled in the study, and 117 serum samples were collected from 47 infants.

7.1 Validation

Intra-day variation (Figure 5) and inter-day variation (Figure 6) in the extraction and esterification method was assessed in 10 identical samples. Variation was negligible based on the coefficients of variation (CV) (Figure 7) and we determined the method to be effective in consistency extracting and esterifying FAs of interest. For the CVs with higher values (i.e., C15:0, C18:3n6 and C20:3n6), the variation is still considered negligible as these FAs occurred in such low values in our samples that the CV values are a reflection of the noise rather than variation in signal.

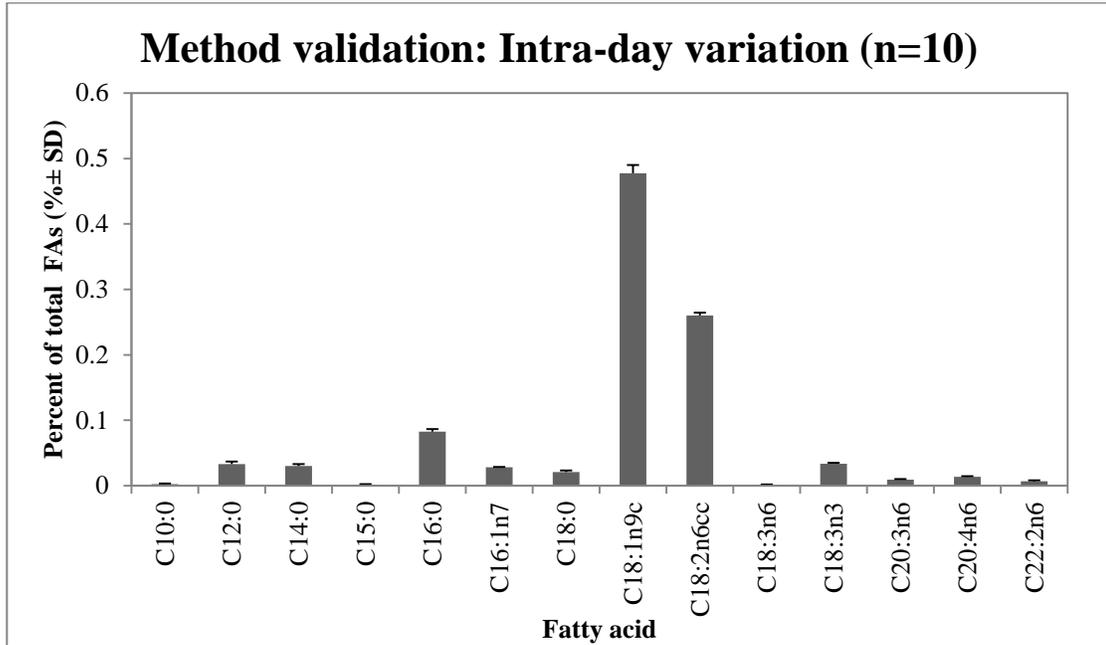


Figure 5. Intra-day variation assessed in 10 aliquots of a pooled milk sample (100µL each).

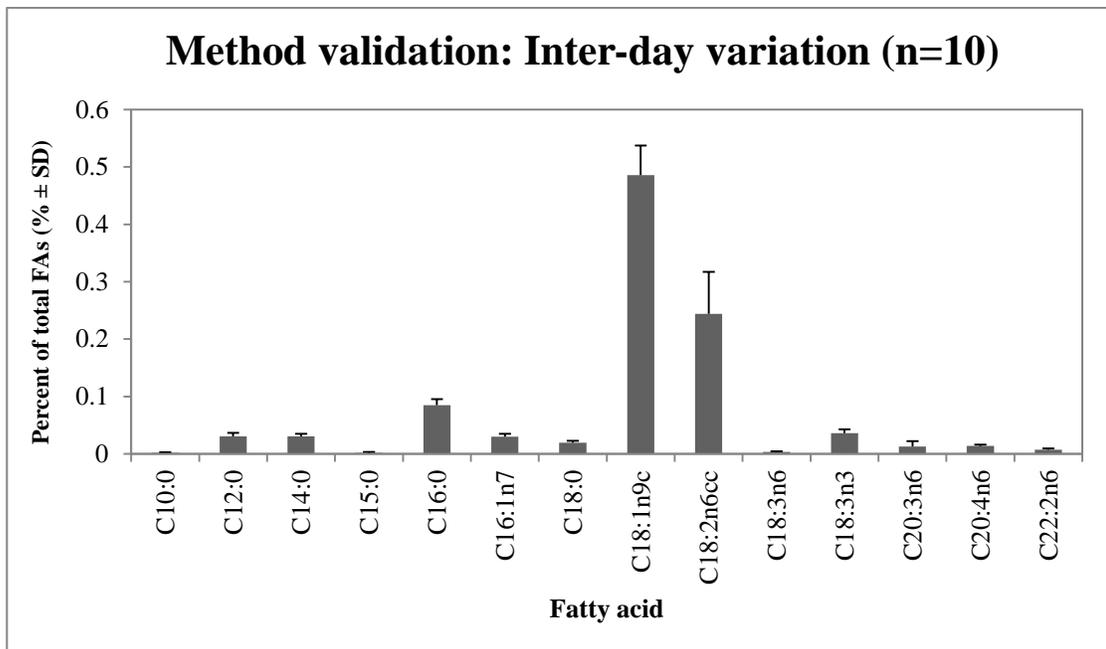


Figure 6. Inter-day variation assessed in 10 aliquots of a pooled milk sample (100 µL each).

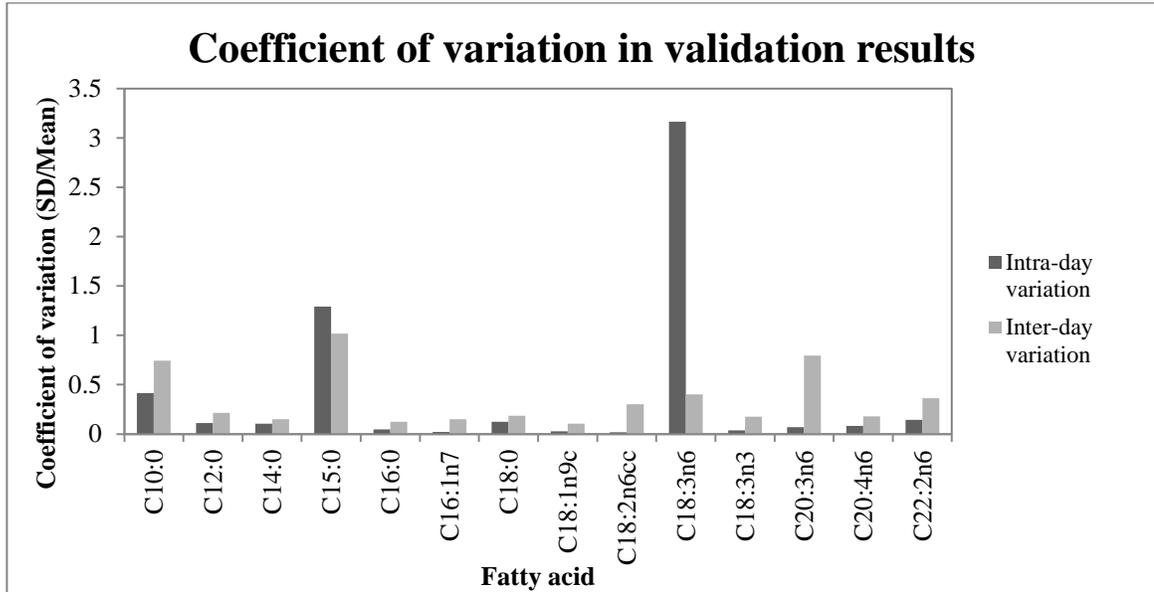


Figure 7. Comparison of CV in intra- versus inter-day variation in extraction and esterification method.

7.2 Intra- and inter-individual variation in breast milk

Intra-individual variation was assessed by analyzing the range in percent composition of each fatty acid between feedings by analyzing milk samples collected from the NICU. Ranges are reported for 6 samples from one mother in the time frame of Jan 29, 2012: 0400h to Jan 30, 2130h (Figure 8).

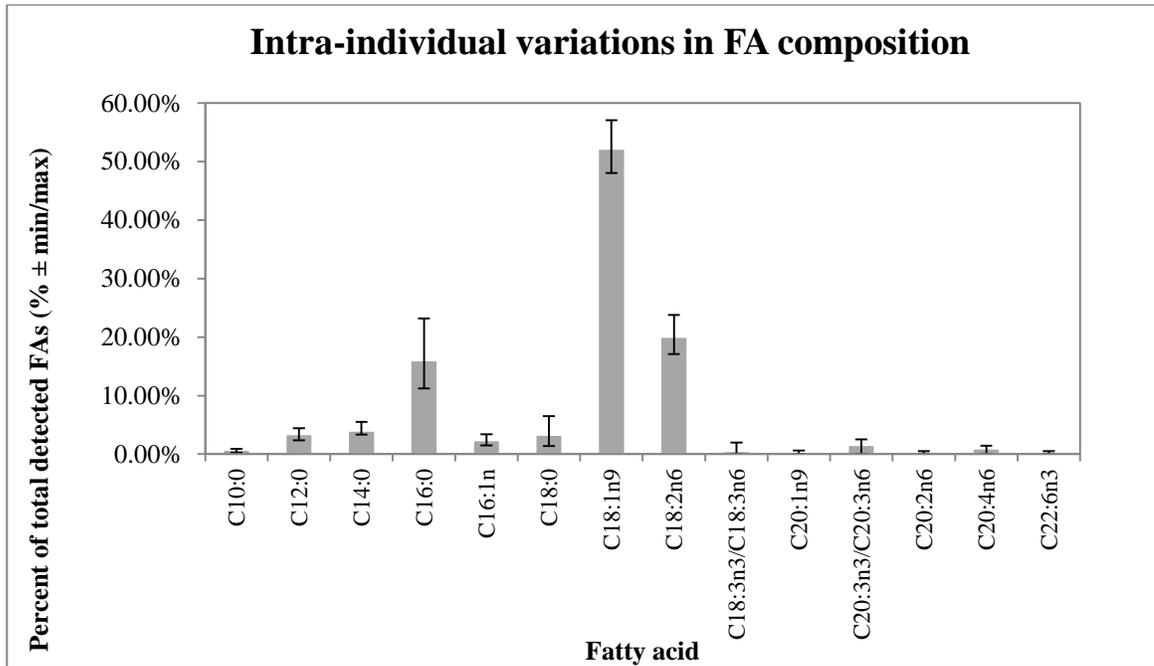


Figure 8. Range of fatty acid (FA) distributions for 6 breast milk (BM) samples from one mother in a 29.5 hour period (Jan 20 2012, 0400h to Jan 21 2012, 2130h).

In the time frame of Jan 30, 0500 to 1930, the percent of C16:0 changed from 11.22% to 23.20%. Other changes were observed in percent of total fatty acids, but the change in C16:0 was of the largest magnitude. This highlights that although there is variation in the fatty acid composition between mothers, there is also variation between pumpings within mothers in a 24 hour period.

Inter-individual variation was assessed through the analysis of all breast milk samples collected as part of the study (n=69). Table 2 depicts the average fatty acid percent compositions, SD and maximum and minimum values for each fatty acid across all 69 samples.

Table 2. Fatty acid proportions of the average local preterm breast milk profile (BM) of 69 mother’s milk samples.

Fatty acid	Mean % of total FAs (%)	SD (%)	Range (min%- max%)
C8:0	2.3	3.3	0-11.9
C10:0	11.1	11.5	0-59.6
C12:0	3.9	3.5	0-20.2
C14:0	3.3	2.5	0-10.8
C15:0	0.1	0.1	0-0.4
C16:0	14.7	5.7	0-31.6
C16:1n7	1.2	1.4	0-4.6
C18:0	5.0	4.9	0-26.0
C18:1n9c	36.6	9.3	14-62.5
C18:1n9t	0.1	0.4	0-1.8
C18:2n6cc	18.5	6.4	0-31.6
C20:0	0.0	0.1	0-0.5
C18:3n6	0.1	0.1	0-0.6
C20:1n9	0.0	0.1	0-0.7
C18:3n3	1.3	1.2	0-4.7
C20:2n6	0.1	0.1	0-0.5
C20:3n6	0.3	0.5	0-2.4
C22:1n9	0.0	0.1	0-0.9
C20:4n6	0.6	1.1	0-7.5
C22:2n6	0.9	5.2	0-41.5

Average values, SD and maximum/minimum values for detected fatty acids in mothers milk samples (n=69).

These results were compiled into an average breast milk or “BM” profile. For the purposes of this thesis, this “BM” average will be considered as a local average of fortified preterm breast milk. It is important to point out that every milk sample received was fortified with Enfamil human milk fortifier, as is standard practice for all preterm breast milk before feeds are administered to preterm infants. The fortifier contains additional DHA and AA, among other vitamins and minerals. This local breast milk average is an approximate reflection of actual preterm breast milk as well and it is a direct

reflection of the milk received by mothers of enrolled preterm infants. An average of 6 unfortified preterm milk samples were compared to the average of all fortified samples included in this study (n=69), and fatty acid distributions were not considered to be markedly different (see Appendix B, Figure 18). Due to the fact that all infants in this study received fortified human milk, as is the practice for preterm breast milk in McMaster's NICU, the fatty acid profiles of fortified samples were not adjusted to account for the fortification.

7.3 Aim 1: Comparison of preterm breast milk to lipid emulsion products

Figure 9 displays the distribution of fatty acids in the five lipid emulsion products analyzed to the average "BM" profile (n=69) and values for means and SD are presented in Table 3 (Appendix A). SMOFlipid and Lipidem contain the highest proportion of MCT compared to all other lipid emulsions and BM, and Omegaven, Intralipid and Clinoleic do not contain any fatty acids in the medium chain class. Omegaven and Lipidem contain the highest proportions of PUFAs compared to the other products and BM has the richest SFA component. Clinoleic is composed primarily of MUFA (C18:1n9) and contains the smallest range of fatty acids, mainly C16:0 to C18:2n6. Intralipid has the highest proportion of the n6 PUFA C18:2n6, followed by Lipidem. The n-3 EFA C18:3n3 is found in the highest proportion in Intralipid, and not at all in SMOFlipid. Omegaven contains a total of 8 fatty acids in proportions higher than 5%; Lipidem, Intralipid and BM a total of five fatty acids; SMOFlipid and Clinoleic a total of four fatty acids. The ratio of n-6 to n-3 EFAs was calculated for each lipid emulsion product and the average

BM profile and is as follows: Omegaven (8:1), SMOFlipid (15:0), Lipidem (9:1), Intralipid (9:1), Clinoleic (12:1) and BM (15:1).

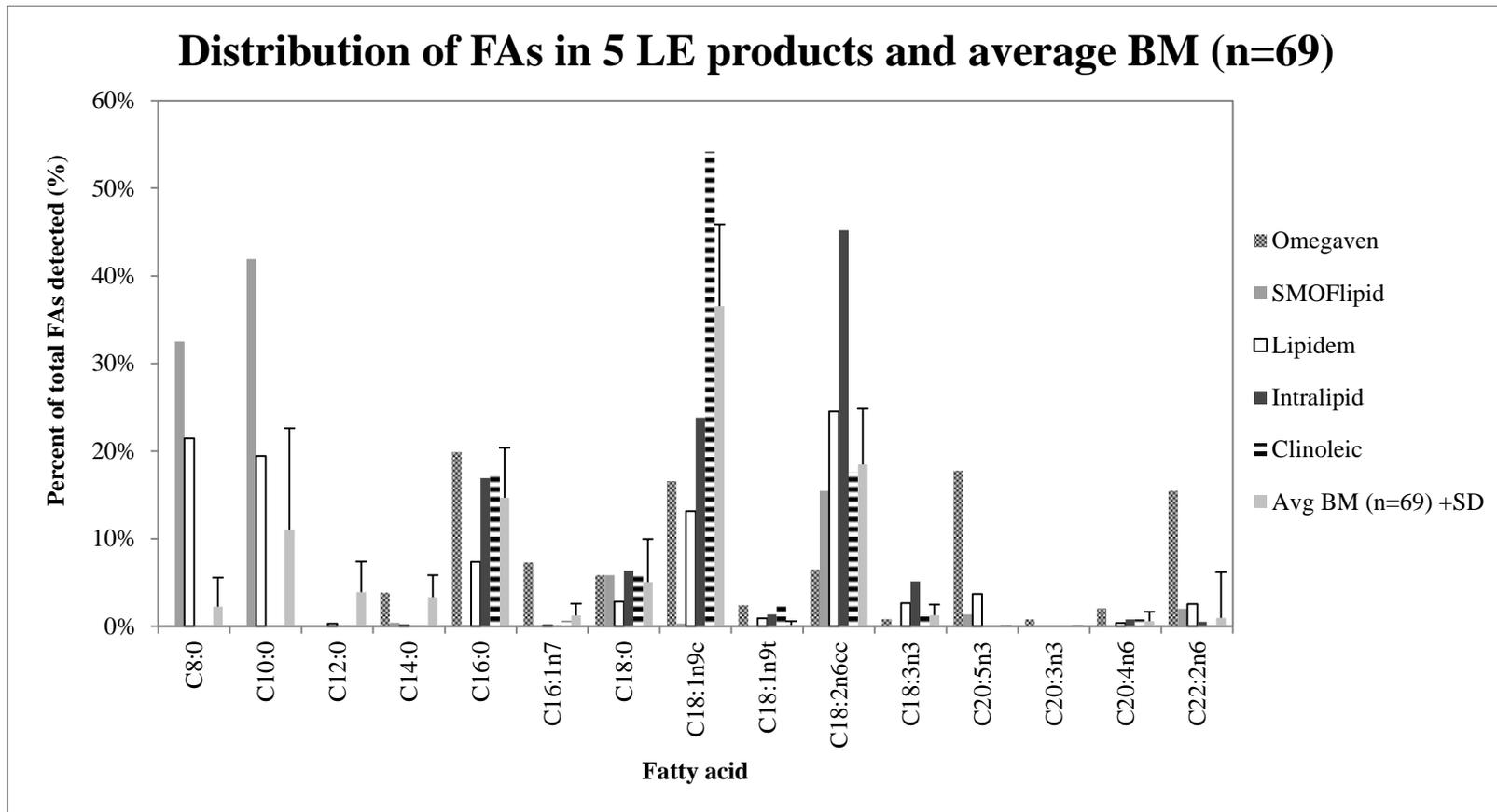


Figure 9: Comparison of the FA profile of 5 commercially available LE products to the average of fortified preterm breast milk (BM) (n=69). Fatty acids that appeared in concentrations <1% in all samples were removed.

7.4 Aim 2: Comparison of nutrition source and resulting serum fatty acid profiles

Infant serum samples that were grouped into the 100% breast milk category were analyzed and an average of each fatty acid was calculated to define the average serum profile on breast milk, “BMserum” group (n=31). This group was compared to the BM profile (n=69) and compositional differences are presented in Figure 10 and values for means, SD, p-value and confidence interval of the differences are presented in Table 4 (Appendix A). Mean percentages were significantly different at $p < 0.0001$ for C8:0; C10; C12:0; C14:0; C15:0; C16:0; C16:1n7; C18:0; C18:1n9c; C18:3n3; and C20:4n6. Mean percentages were significantly different at $p < 0.001$ for C18:3n6 and C20:2n6 and at $p < 0.05$ for C20:0 and C20:1n9. The fatty acids not found to be significantly different and found in proportions less than 5% of total fatty acids were relatively similar between BM and BMserum. The BM group contains more medium chain SFA and the MUFA C18:1n9c while the serum of infants fed BM contains higher proportions of long chain SFAs C16:0 and C18:0 and the PUFAs C18:3n3 and C20:4n6. Of most significance as an entire class of fatty acids, BMserum did not contain any medium chain fatty acids.

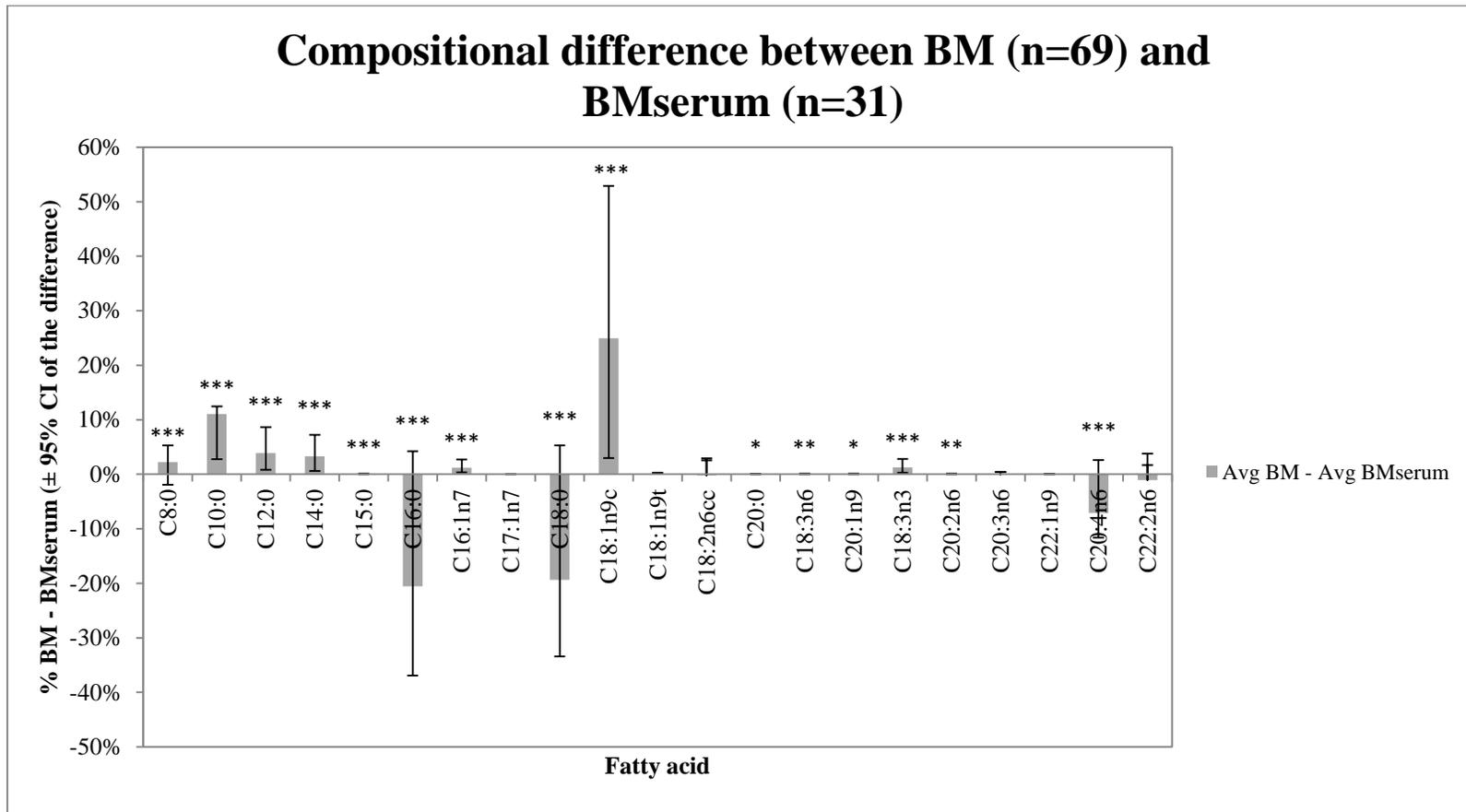


Figure 10: Compositional difference between fatty acids in breast milk (BM) and serum samples from infants fed BM (BMserum). Results are presented as compositional differences in the mean of both groups \pm the 95% confidence interval of the mean. Means were analyzed by an independent samples t-test, unequal SD's assumed and Welch's correction factor was applied. Significantly different means are marked with a “***” ($p < 0.0001$), “**” ($p < 0.001$), “*” ($p < 0.05$).

Infant serum samples that were grouped into the >50% lipid emulsion category were analyzed and an average of each fatty acid was calculated to define the average serum profile on lipid emulsions, “LEserum” group (n=15). This group was compared to the lipid emulsion profile of the product used in McMaster’s NICU: Intralipid “LE” (n=1), and compositional differences are presented in Figure 11 and values for means and SD are presented in Table 5 (Appendix A). The serum of infants on Intralipid contained relatively higher proportions of long chain SFAs C16:0 and C18:0 and the PUFA C20:4n6 compared to Intralipid, which contained relatively higher proportions of the EFAs C18:2n6cc and C18:3n3. Other fatty acids occurring in proportions <5% were relatively similar between the groups.

The average serum profile on BM (BMserum, n=31) was compared to the average serum profile on LE (LEserum, n=15) and means of each fatty acid proportion were compared (Figure 12). Values for means, SD, p-value and confidence interval of the differences are presented in Table 6 (Appendix A). BMserum contained significantly higher proportions of the EFA C18:2n6cc while LEserum contained significantly higher proportion of the MUFA C18:1n9c. The proportions of other fatty acids were not significantly different between the groups.

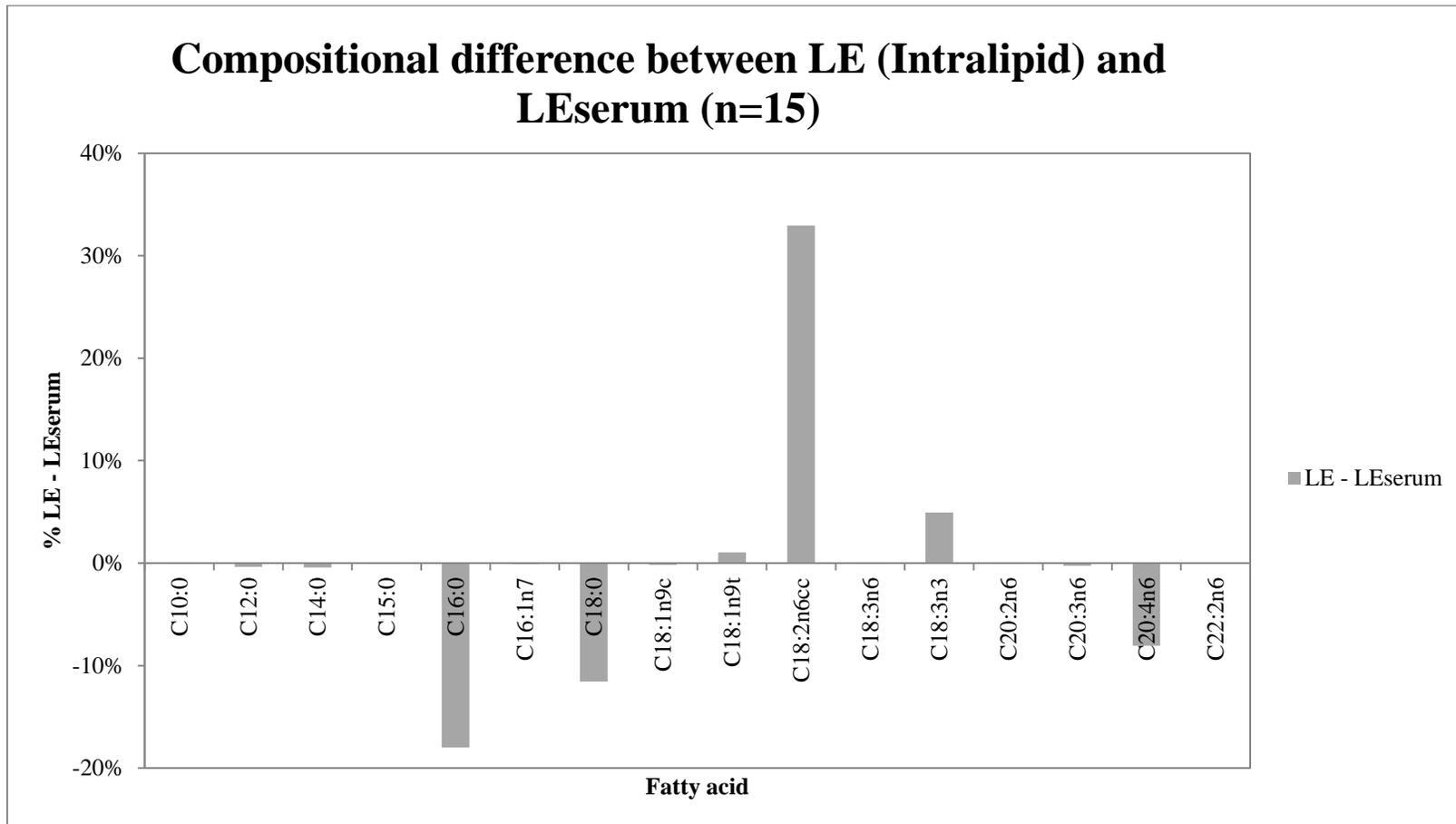


Figure 11: Compositional difference between fatty acids in LE (Intralipid) and serum samples from infants where fat from LE comprised greater than 50% of total fat (LEserum). Results are presented as compositional differences in the mean of both groups.

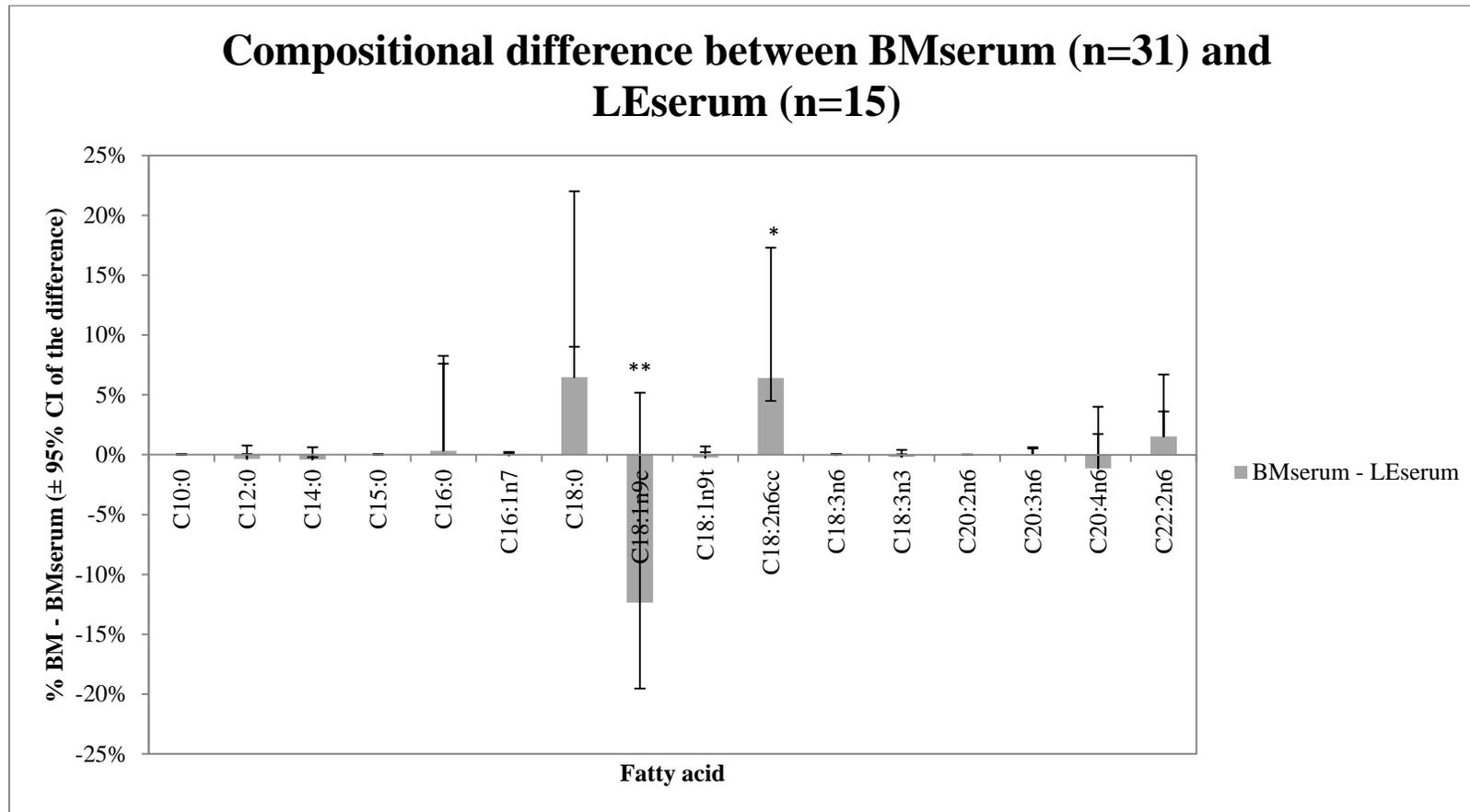


Figure 12: Compositional difference between fatty acids in serum samples from infants fed BM (BMserum) and serum samples from infants fed LE (LEserum). Results are presented as compositional differences in the mean of both groups \pm the 95% confidence interval of the mean. Means were analyzed by an independent samples t-test, unequal SD's assumed and Welch's correction factor was applied. Significantly different means are marked with a "***" ($p < 0.0001$), "*" ($p < 0.01$).

A subset of the population, 35 pairs of milk and serum samples from 21 infants, were included in the matched pair analysis (Figure 13). The milk sample was obtained 48-72 hours prior to the blood sample and the fatty acid profiles were compared. Proportions were significantly different for all fatty acids compared except C18:2n6cc, C20:3n6 and C22:6n3. BM contained significantly higher proportions of the medium chain fatty acids and the MUFA C18:1n9c. The resulting serum displayed significantly higher percentage values of the long chain SFA C16:0 and C18:0 as well as the PUFA C20:4n6.

A single matched pair was chosen from infant 001 and the fatty acid profiles of both were displayed visually (Figure 14). The milk sample obtained on Friday (001 EBM2) and the serum sample obtained on Monday morning (001 SS3) displayed similar trends seen in the overall matched pair analysis in Figure 13. The SFAs C16:0 and C18:0 and the PUFA C20:4n6 are found in higher proportions in serum compared to milk and the medium chain fatty acid C10:0, as well as C12:0 and C14:0 are detected in mother's milk but not in infant's serum.

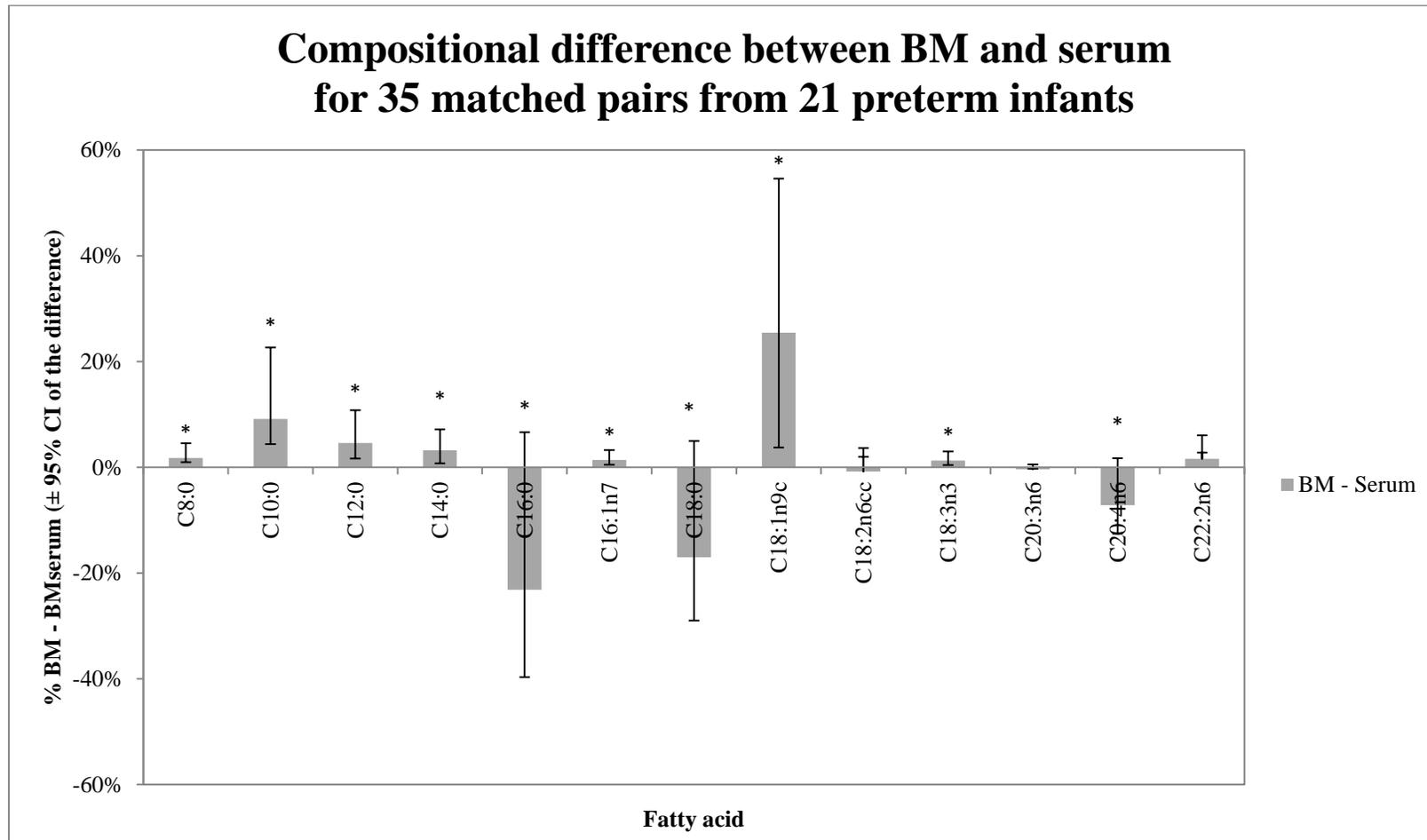


Figure 13: Compositional difference between fatty acids from matched pairs of breast milk (BM) and serum samples obtained from infants 48-72 after milk sample was obtained from mothers. Results are presented as compositional differences in the mean of both groups \pm the 95% confidence interval of the mean. Means were analyzed by a paired t-test. Significantly different means are marked with a “*” ($p < 0.0001$).

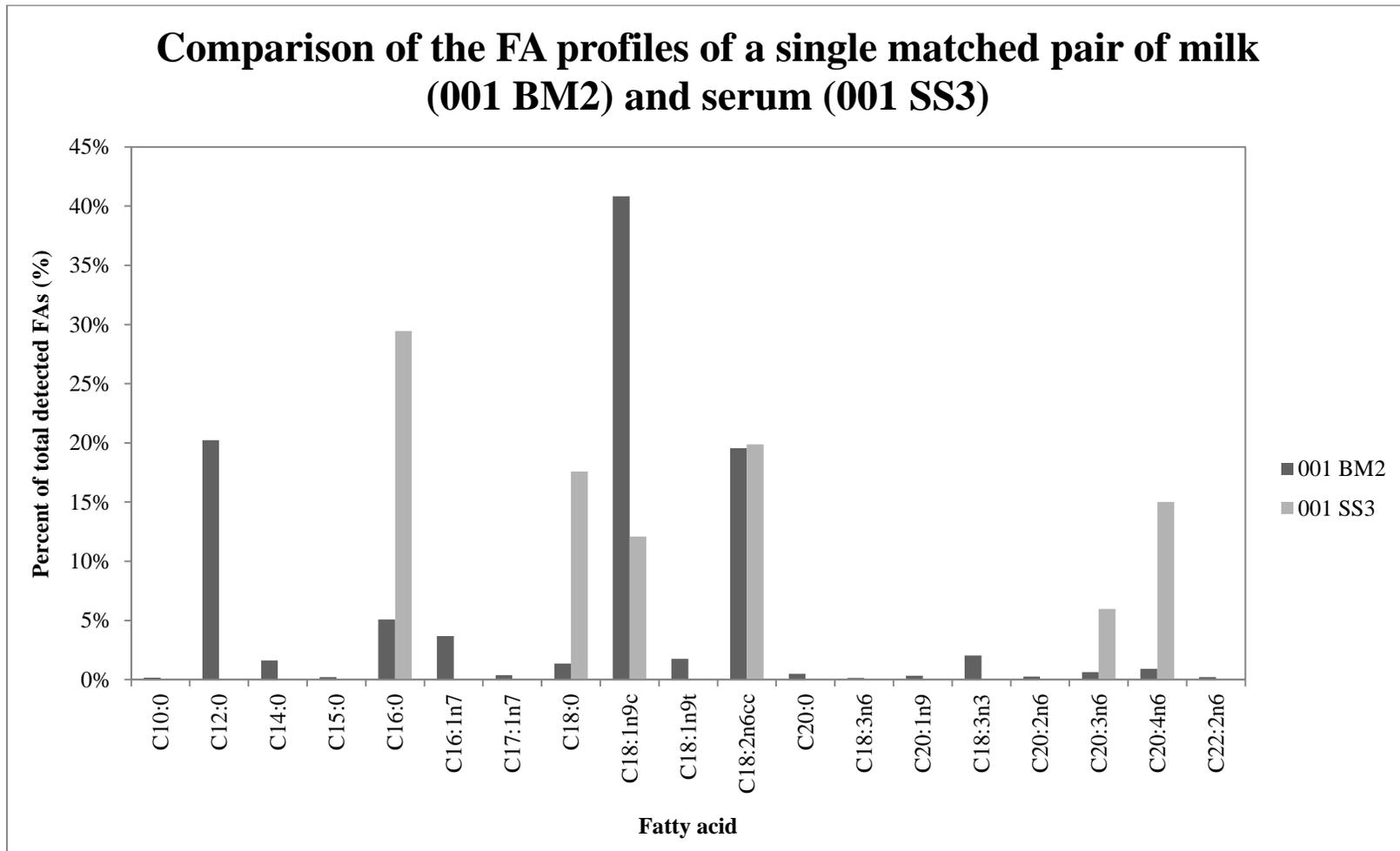


Figure 14: Comparison of fatty acid profiles in a single matched pair of milk (001 EBM2; infant 001, breast milk sample 2) and serum (001 SS3; infant 001, serum sample 3).

7.5 Aim 3: Comparison of fatty acid profiles between normo- and hypertriglyceridemic serum

Meditech was used to identify all samples in the study population with a TG test within 48 hours of a sample received for the study. Samples with TG values exceeding 1.7 mmol/L were grouped with the leftover hypertriglyceridemic samples collected from HRLMP. A total of 113 normotriglyceridemic samples were compared to 22 hypertriglyceridemic samples, and mean percentages of fatty acids were significantly different for C18:0 ($p<0.01$); C18:1n9c ($p<0.0001$); C18:2n6cc ($p<0.01$); C20:3n6 ($p<0.0001$); C20:4n6 ($p<0.01$); and C22:2n6 ($p<0.05$) (Figure 15). The normotriglyceridemic samples had significantly higher proportions of the SFA C18:0 and the PUFAs C18:2n6; C20:3n6; C20:4n6; and C22:2n6 than the hypertriglyceridemic samples, which only had significantly elevated levels of the MUFA C18:1n9.

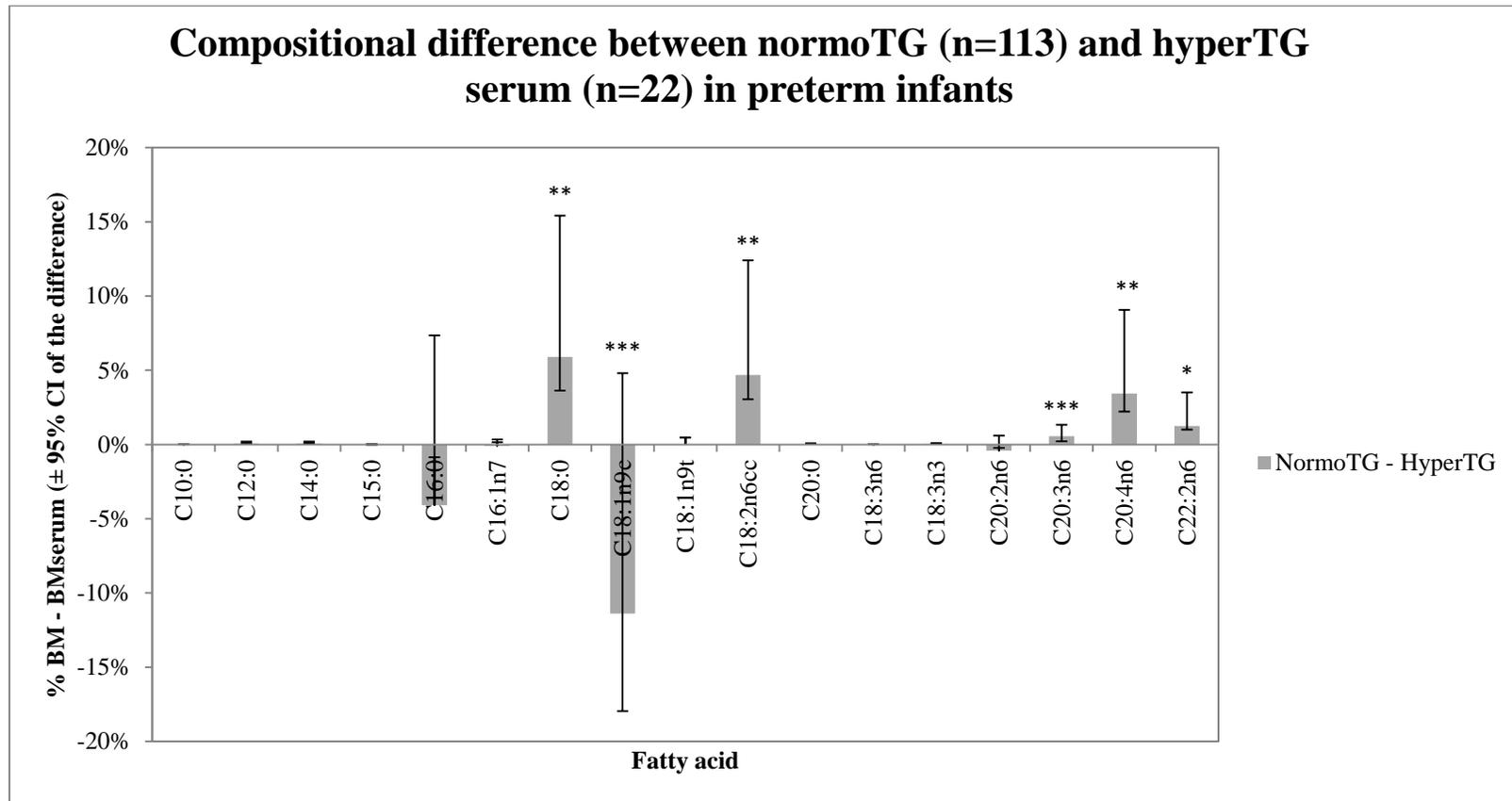


Figure 15. Compositional difference between fatty acids in serum samples from infants with serum triglycerides in the normal range (normoTG) and those with hypertriglyceridemia (hyperTG). Results are presented as compositional differences in the mean of both groups \pm the 95% confidence interval of the mean. Means were analyzed by an independent samples t-test, unequal SD's assumed and Welch's correction factor was applied. Significantly different means are marked with a “****” ($p < 0.0001$), “***” ($p < 0.01$) and “**” ($p < 0.05$).

8. DISCUSSION

8.1 Strengths and limitations

8.1.1 Threat to validity: Under-estimation of variance

With data analysis and interpretation of a sample of this nature, threats to validity must first be addressed. The sample population contained seven sets of twins and two sets of triplets. Although separate milk samples were requested for each of the infants in a twin/triplet set, on some occasions the milk could have been expressed by the same mother on the same day. This situation of repeated samples may contribute to a reduction in the estimate of variance and therefore it is more likely a significant difference would be found. Despite this knowledge, the under-estimation of variance could not be controlled for as the sample size was not large enough; removing the cases of duplicates in breast milk would further reduce the sample size as well as number of matched milk/serum pairs. As well, over-representation of some infants would have occurred in analysis, as some study participants had as many as six serum samples and others only one.

For the BM/BMserum analysis, there may exist a serum sample in the BMserum group with that same infant's mother's milk sample in the BM group. Technically this would warrant the use of a matched pair analysis, however, it is also true that an infant's serum sample may be in the BMserum group without a milk sample from the mother in the BM group. For this reason, an independent t-test was chosen for all analyses outside of the matched pairs comparison. An independent samples t-test allows for a more conservative identification of significant differences compared to a matched pairs analysis, as the variance is not under-estimated to the same degree.

8.1.2 Sample size calculation

The purpose of this pilot study was to inform further research and give an estimate of the fatty acid levels in milk, serum and lipid emulsion products and the differences or similarities expected. There were no *a priori* target ranges of differences in fatty acid levels between sample types that were expected nor was the variance around these differences estimated. This did not allow for sample size calculations to be done in advance, and the study simply included all infants where consent was obtained. The results of this study could be used as an indicator of the desired differences to be detected as well as the variance around these if the same study were to be launched on a larger scale.

8.2 Methodology limitations

8.2.1 Absolute quantification

Values are expressed as percent of total detected fatty acids; therefore, an increase or decrease in one single fatty acid would produce a shift in all other percentage values for fatty acids without a fluctuation in their absolute amounts. Comparing ratios can only be used as an estimate of change in fatty acid profile, and the calculation of absolute amounts of each fatty in a sample would strengthen the conclusions.

The calculation of absolute concentrations of fatty acids would allow for ANOVA's between groups to be computed. ANOVAs were not used for this analysis, as the groups are related across columns (ie. BM versus BMserum) and rows (percent fatty acids of total). If the fatty acid values were expressed as absolute values, it would remove the relation of variables across rows. Absolute quantification was not achieved within the time frame of this thesis as it was suspected that recovery values were much lower than

expected. Because the fatty acids that were expected to appear based on basic dietary fatty acids were detected, and fatty acids that were not expected to appear were not detected, we are confident that the ratios of each fatty acid are appropriate but that the recovery was low. This phenomenon has not been reported widely across the literature and whether that is because it is not treated as a widespread problem across all methods of extraction and esterification or because it is overlooked, expressing values as a percent of total avoids the low recovery effect on final values. Based on knowledge of the method and comparison to other methods reported in the literature, it is reasonable to assume that the low recovery could be attributed to the transesterification step. The solvent may evaporate more quickly than transesterification proceeds, and because the hydrochloric acid used during this step is so volatile, this is a reasonable assumption. Once the liquid phase has evaporated, the reaction ceases to proceed and unesterified fatty acids remain. Despite the realization of low recoveries, the lower intra- and inter-day variations and low coefficients of variation in fatty acid ratios from the method validation stage lend a high degree of credibility to the fatty acid ratios obtained and used during analysis. A possible method for estimating loss would be to utilize an external standard of known concentration not naturally occurring in breast milk and compare it to the absolute concentration obtained after extraction/esterification, utilizing an internal standard added at the beginning of the method to quantify.

8.2.2 Increased sample size

An increased sample size would allow for the computation of more sophisticated statistical analysis methods such as principal component analysis (PCA) or canonical

correlation analysis (CCA). PCA is mostly used as a tool in exploratory data analysis as it allows for the conversion of a set of observations of possibly correlated variables to a set of values of linearly uncorrelated variables (principal components) (Ullah 2013). Simply stated, it is a non-parametric method for extracting relevant information from complex data sets (Schlens 2005). This method of analysis is ideal for profile analysis (i.e., fatty acid profile comparison) but it requires approximately five replicates of each sample. Further method optimization could be achieved in order to extract and esterify samples at 20% of the volume obtained from the NICU, and five aliquots could be prepared from one sample. Working with the 100 μL volume is difficult enough during separation of the organic and aqueous phases, therefore a smaller vial insert would have to be used in order to separate layers beginning with a 20 μL volume.

CCA has not been widely employed in nutritional research but proves to be a useful technique for expressing relationships between two sets of variables (Uusitalo 2013). It provides a comprehensive summary of the linear relationships between members of the sets of data that were measured on the same sampling unit. In contrast to PCA, which aims to identify principle sources of variation in data, CCA is effective in finding the association structures in variables between datasets (Uusitalo 2013). The only evidence of this method of analysis that could be found in literature used for comparison of fatty acid profiles utilized datasets with sample sizes greater than 100 (Uusitalo 2013). This method would be a useful starting point to tackle the confounding effect of interdependency of fatty acids on statistical analysis given a larger sample size than the one used in this study.

8.2.3 Lack of longitudinal data

Longitudinal measurements would allow for fatty acid metabolism patterns to be compared over time. Although one serum and one milk sample was requested every week for each infant, they were not always received on a regular basis. Sometimes the mother did not pump enough (or any milk) or the infant's condition was not favourable enough for the extra blood sample. Lastly, infants that were enrolled in the study but discharged a few days later to another Level II facility did not leave a long enough time period for multiple sample collections. Some infants have multiple samples spread out over a period of several weeks or months, but they were not obtained on a consistent longitudinal schedule, nor do the time points correspond to other infants' samples. Therefore, although the data follows a longitudinal time course, it cannot be analyzed longitudinally at this stage of the pilot study.

8.3 Strengths

The comparison of resulting serum to the actual nutrition sample received by the infants is a major strength of this study, as many previous studies of this nature have simply used food journals to estimate intake of fatty acid and compared this to resulting serum (Uusitalo 2013; Nikkari 1995). As well, preterm infants in this study did not receive lipids outside of the nutrition source obtained as their only exogenous fat came from one or a combination of breast milk, formula and lipid emulsions. The mixed diet of a toddler or child does not allow for the same conclusions to be drawn and therefore such direct nutrition to serum comparisons are not possible. Many adult nutrition-to-serum comparison studies on fatty acids have been conducted in the past to the point where

empirical calculations relating dietary to serum fatty acids have been derived, but explorations to the same degree are alarmingly scarce in the pediatric and neonatal population (Uusitalo 2013).

The calculation of fat intake by nutrition source allowed for infants to be grouped almost exclusively within the group of nutrition that was administered (ie. 100% breast milk, >80% lipid emulsion). Results are therefore more conservative than if the study would have grouped the infants' serum on >50% breast milk into the "BMserum". This allows for control against alteration of fatty acid profiles from relatively similar volumes/fat content in other nutrition sources. This exclusive grouping also had the effect of reducing the sample size, as many of the serum samples fell into the mixed nutrition group, however, it allowed for a pure comparison of a single nutrition source to the resulting serum.

8.4 Inter- and intra-individual variation in breast milk

Results of breast milk sample analysis displayed wide inter- and intra-individual variation, which falls within the range of variation reported in literature (Kuipers 2011; Smithers 2010; Genzel-Boroviczény 1997; Berenhauser 2012). Berenhauser et al. (2012) states that if higher proportions of C16:0, C18:1n9c and C18:2n6 are found in mother's milk, it is consistent with a diet high in consumption of soybean and olive oils as well as margarine. The average milk profile of mothers in this study produce values that support this dietary habit, which is expected as these oil types are common in a western diet (Berenhauser 2012) and have the potential to affect the n-6/n-3 EFA ratio.

8.5 n-6/n-3 EFA ratio

The n-6/n-3 ratio of breast milk was 15:1; consistent with a Western diet which has reported ratios anywhere between 10:1 and 50:1 (Berenhauser 2012). A balance in this ratio is important for eicosanoid production, as those synthesized from n-6 precursors have pro-inflammatory and pro-aggregatory effects (Berenhauser 2012; Gibson 2011; Heird 2005). It is interesting to note that next to SMOFlipid, the average local breast milk profile has the highest n-6/n-3 EFA ratio compared to other LE products while Omegaven has the lowest. Strict dietary habits and knowledge about ratios of EFAs may not have been a part of each mother's breast milk "preparation", however this is a fact that should be considered during the design of a lipid emulsion product. Ratios are in the 1:1 and 2:1 range when a diet is high in fish and vegetables (Berenhauser 2012), and while a mother's design of her own breast milk is not feasible, the design of a lipid emulsion product would allow for the fine tuning of the n-6/n-3 ratio. The results of this study suggest that regardless of whether an infant is receiving breast milk or lipid emulsions, the n-6/n-3 EFA ratio in their nutrition source may be placing them at risk for increased pro-inflammatory eicosanoid production.

The wide variation in breast milk in the study sample is supported by literature and depicts a potential reason for the variation in the fatty acid profile of lipid emulsion products. If these products are designed to replace the fat portion of breast milk when an infant cannot tolerate enteral feeds, it is reasonable to assume that they might be designed to mimic the fatty acid profile of breast milk. Such high variation in milk fatty acid composition presents a challenge for lipid emulsion oil source choices and perhaps might

explain why the lipid emulsion products do not resemble the fatty acid profile of the other products or even breast milk.

8.6 Fatty acid profile of lipid emulsion products compared to breast milk

Based on Figure 9, it is evident that not one of the lipid emulsion products closely resembles one another, nor are they very similar in nature to breast milk. The most comparable lipid emulsion product to breast milk is Lipidem and the least similar products are SMOFlipid and Clinoleic, each based on high proportions of medium chain fatty acids and oleic acid (C18:1n9c) respectively. The range of fatty acids in Intralipid is not nearly as wide as that in breast milk; the bulk of fatty acids occur between C16:0 and C18:2n6. However, the proportions of this small range of fatty acids in Intralipid are comparable to those in breast milk. There is no question that breast milk is the gold standard for infant nutrition; so these products are not expected to be superior to human milk, however, the results are inconclusive as to which lipid emulsion products are superior to each other.

Lipidem, SMOFlipid and Omegaven are used across Europe, and Intralipid is used widely in North America and Australia (Deshpande 2009; Deshpande 2011). A study by Deshpande et al. (2009) compares the use of Intralipid to Clinoleic in a randomized controlled trial in an NICU and finds that both lipid emulsions are safe and well tolerated by both groups and they did not find any evidence of increased inflammation as measured by F2-isoprostanes (Deshpande 2009). The authors attributed the lack of significant difference in inflammation due to the short-term nature of the trial and quoted results of a long-term intervention from another study that did find evidence of a reduction in

inflammatory markers on Clinoleic versus Intralipid (Deshpande 2009). The anti-inflammatory properties of Clinoleic are expected to arise due to the high MUFA content and their increased resistance to peroxidation (Deshpande 2009). Fish oil emulsions (ie. Omegaven) have been compared to soybean oil products (Intralipid) and in one study, Le et al. (2011) determines that the infants on a fish oil emulsion displayed a reduction in serum triglycerides versus a soybean oil emulsion group (Le 2011). The authors suggest that the increase in n-3 fatty acids plays a role in reducing hepatic triglyceride synthesis, indicated by the reduction of C16:0, C18:0 and C18:1n9, considered markers of de novo lipogenesis (Le 2011). Although a multitude of literature has compared lipid emulsion products, the study parameters examined are not always consistent and one lipid emulsion has not yet been proven to be a “gold standard”.

8.7 Reflection of dietary fatty acids in serum

Total lipid stores in the blood are comprised of cholesterol esters, triglycerides, and phospholipids across three compartments; total plasma lipids, platelets and erythrocytes (Hodson 2008). Although free fatty acids in blood are mainly derived from dietary sources and have thus been used as biomarkers of dietary intake, they are more reflective of recent diet as opposed to the equilibrium state that adipose tissue fatty acid composition would reflect (Hodson 2008; Morris 1998). Adipose tissue fatty acid composition is considered the gold standard to reflect long-term dietary fatty acid intake patterns, as the turnover time in weight stable individuals is low (Hodson 2008).

Almost all fatty acids proportions detected in breast milk were found to be significantly different from the serum proportions of the infant fed only breast milk. This

suggests that serum fatty acids do not directly reflect nutrition and that there is more at play than simple metabolism, i.e., fatty acid elevations in nutrition do not produce the same elevations in resulting serum. This is indicative of the body handling fatty acid classes differently (i.e., SFA vs. MUFA, or medium chain versus long chain).

Similar trends of fatty acid differences across all nutrition versus serum groups were observed. In the BM to BMserum analysis as well in the matched pair analysis, a significant elevation in the SFAs C16:0 and C18:0 were observed in the serum as well as the PUFA C20:4n6. A similar trend was reported for a study comparing nutrition (estimated via food journals) to serum samples obtained from toddlers (Uusitalo 2013). The same study concluded that there was a correlation for increased serum levels of C16:0 and low levels of MUFA C18:1n9c when infants at 1 year of age were fed fatty milk (i.e., whole milk versus skim milk) (Uusitalo 2013). Our results do not support the trend that this phenomenon is a direct result of milk feeding though, as the same elevation in SFAs and the PUFA C20:4n6 was observed for the LE versus LEserum analysis.

Conversely, from the nutritional side, elevations in the MUFA C18:1n9c did not seem to reflect an accumulation of this fatty acid in the serum, and this was true for the BM/BMserum, LE/LEserum and matched pair analysis. Uusitalo et al. (2013) report that C18:1n9c does not correlate well with diet, tends to change with time, it is correlated positively with vegetable oil consumption at 2 years of age and negatively correlated with fatty milk intake at 3 years of age (Uusitalo 2013). It is not known what the expected trend is for preterm infants, as a toddler's metabolism of fatty acids may well be very different than a neonate's, so this was not taken as a full explanation for the discrepancy

in C18:1n9c proportions between nutrition and serum displayed in this thesis. As well, when the nutrition source contained medium chain fatty acids and C12:0 and C14:0, these fatty acids were not detected in the serum. Because a direct relationship between dietary and serum fatty acids cannot be assumed, only hypotheses as to why these relationships consistently appeared in each analysis group can be drawn. First, the administration of medium chain fatty acids and C12:0 and C14:0 in the nutrition but the failure to detect them in the blood supports the idea that these fatty acids are more readily metabolized and do not remain in the serum long enough to accumulate to detectable levels. As previously stated, C8:0 and C10:0 do not require the carnitine transport pathway in order to enter the mitochondria, and can also be absorbed directly through the stomach instead of through the intestine (Takeuchi 2008; Reuter 2012; Papamandjaris 1998). The absence of C12:0 and C14:0 in the serum, even though this compartment is expected to closely reflect dietary fatty acids, supports the notion that they are also preferentially metabolized over longer chain SFA, MUFA and PUFAs, as was suggested by literature (see Introduction, section 5.5: Metabolism of Fatty Acids). Although the SFA, MUFA and PUFAs do not follow the same metabolic pathway as the medium chain fatty acids, it is evident that they too might have some advantage over the other fatty acids during metabolism and uptake which could drastically affect the appearance of that fatty acid in the serum. For infants with intestinal complications such as NEC or short-bowel syndrome, lipid emulsions are necessary and a rapidly metabolized group of fatty acids in addition to the medium chain class would be ideal components of a lipid emulsion product.

The consistently elevated proportions of long chain SFAs C16:0 and C18:0 in serum are indicative of a slower metabolic time course. SFAs are absorbed less efficiently than their unsaturated counterparts, and as the chain length increases, a higher proportion is absorbed by the lymphatic pathway, and less by portal venous blood (Ramírez 2001). This is a possible explanation for the consistent elevations of C16:0 and C18:0 in the serum when they are not administered in proportionally high ratios in the nutrition, as they are the longest chain SFA detected in the samples. Furthermore, MUFAs have demonstrated more rapid oxidation than SFA (C18:1n9 versus C18:0) (Jones 1985), which may also help to explain why despite the high proportions of the MUFA C18:1n9c found in the average milk profile from this study, elevated proportions of this fatty acid are not found in resulting serum samples collected as part of this study. This is possibly because the increased proportions in dietary sources are offset by the rapid oxidation and utilization of the MUFA.

Endogenous synthesis of even numbered SFA could also in part explain the observed elevations in C16:0 and C18:0 (Uusitalo 2013), however this same trend was not seen for any other SFAs in serum collected from infants at McMaster Children's Hospital. MUFA endogenous synthesis also occurs in the human body, and this fatty acid was not found in high proportions in resulting serum, even when it was introduced in feeds in high amounts (Uusitalo 2013). It is unlikely then that endogenous synthesis of fatty acids can fully explain the accumulation of SFAs in infant serum.

In the BM/BMserum and matched pair analysis, the proportions of the EFA linoleic acid (C18:2n6cc) appear to be in a state of equilibrium between breast milk and

serum. This also supports the observation from other studies that unsaturated fatty acids are absorbed more efficiently compared to their saturated counterparts (Jones 1985; Ramírez 2001). Because it is a precursor to the inflammatory pathway, an accumulation of C18:2n6cc might lead to excess pro-inflammatory eicosanoids, so the results that demonstrate an equilibrium-like situation are favourable.

For all nutrition versus resulting serum analyses, it was found that AA occurred in higher proportions in the serum than in administered nutrition source (significantly different for BM/BMserum, matched pair analysis and different for the LE/LEserum analysis). AA is absolutely essential during the third trimester for visual and neurodevelopment and accretion rates are high during this time (Heird 2005, Gibson 2011). It is interesting then, that AA would still be circulating in excess in the serum if it was required within the central nervous system. Perhaps infants require high quantities of this fatty acid but only in small doses, as there may be some sort of saturation in accretion rate. This is only a hypothesis though, and radiolabelling of AA would be an ideal way to examine such a phenomena.

Overall, it was determined that the fatty acid profile of lipid emulsion products was not similar to that of breast milk. The resulting serum fatty acid profile on each of these nutrition sources was, for the most part, found to be different from the feeds administered. Lastly, the resulting serum fatty acid profile was found to be significantly different for most fatty acids between infants fed breast milk versus lipid emulsions. This reflects a very complex interplay of factors outside of a simple nutrition and serum relationship, because despite some similarities in fatty acid proportions between any of

the groups compared, the resulting consistency in the difference in fatty acid proportions, i.e., increased SFAs in serum versus nutrition and increase MUFA in nutrition versus serum, reflect some underlying trend in fatty acid metabolism not fully elucidated by literature or in the current study.

8.8 Fatty acid levels in serum

Results from a meta-analysis presented by Hodson et al. (2008) compiled data on plasma triglyceride fatty acid ratios from 5 studies, comprising 236 men and 116 women and demonstrated that the 3 fatty acids that occur in the highest proportion in the plasma are C18:1n9 (37.8%); C16:0 (29.5%) and C18:2n6 (15.0%) (Hodson 2008). The LCPUFAs were found in miniscule amounts; DHA (0.4%), EPA (0.1%) and AA (0.8%) (Hodson 2008). Based on the results of this meta-analysis, we conclude that the fatty acid profiles results obtained from our analysis are indicative of adult human data; therefore, the distribution is a real feature and not an instrumental artifact. It has not been found in literature whether the plasma fatty acid profile in preterm infants should be significantly different from that of adults, but we are confident that our results are within the acceptable range for humans based on the results from the Hodson et al. (2008) and Innis et al. (1990) studies. It is interesting to note that the same study from Hodson et al. (2008) also compiled data on adipose tissue fatty acid ratios from 19 studies comprising 4258 men and 3096 women and the ratios are similar to serum (Hodson 2008). The fatty acids occurring in the highest proportions in adipose tissue are C18:1n9 (43.5%), C16:0 (21.5%) and C18:2n6 (13.9%). The LCPUFAs occur in relatively the same ratios as presented for the plasma TG profiles, indicating that even though adipose tissue is

proposed to be the most reflective of long term dietary fatty acid intake, the plasma fatty acid profile seems to correlate with this. It could be surmised that the serum FA profile of the infants included in Hodson et al.'s (2008) study could be used as an estimate to the fatty acid composition of their adipose tissue.

This study did not examine or control for the ratio of visceral to subcutaneous fat stores in preterm infants, so associations between this parameter and possible differences in uptake cannot be examined. Nonetheless, the discrepancy in fatty acid proportions between nutrition and serum cannot be fully attributed to the small sample size and are suggestive of the fact that there are many factors influencing fatty acid metabolism. Also, visceral versus subcutaneous adipose tissue fatty acid stores in a preterm infant have great potential to explain some of the fatty acid proportion discrepancies in resulting serum. Further, parameters such as circulating levels of lipoprotein lipase, ratios of fatty acids contained in chylomicrons versus free fatty acids in the serum and concentrations of LPX if the infant was on lipid emulsions could also explain the discrepancy in fatty acid proportions between nutrition and serum, however, these parameters were not within the scope of this study. Additionally, fatty acids have many roles outside of functioning as an energy substrate (Kremmyda 2011). Involvement of fatty acids in the body extends to modulation of gene transcription, roles in protein acylation, cell membrane structure, 2nd messengers, receptor interactions and signal transduction (Kremmyda 2011). Perhaps it is such that an ideal fatty acid profile exists for optimal function of each pathway, or maybe one single fatty acid profile satisfies all pathways. Regardless, a complex web of factors

is at play and one cannot simply view serum fatty acids as a reflection of diet without considering the other roles of these energy substrates in the body.

8.9 Normo- versus hypertriglyceridemic fatty acid profile

In the same way that one cannot solely rely on serum fatty acid profile to estimate dietary fatty acids, it is evident from results obtained in this study that hypertriglyceridemia does not simply result from elevations in certain fatty acids. There was no trend for particular disproportions in SFA, MUFA or PUFAs or for medium versus long chain fatty acids between normo- and hypertriglyceridemic samples. As previously stated, the results of the nutrition versus serum analysis show elevated proportions of the MUFA C18:1n9c that are not reflected by accumulations in the serum, indicating that it is easily metabolized, even in high amounts. Since the only fatty acid occurring in an elevated proportion in the average hypertriglyceridemia profile was C18:1n9, it does not seem reasonable to assume that an accumulation of this fatty acid is causing elevated serum TGs. This does not rule out the original hypothesis that accumulations of certain fatty acids can block metabolism of others, thus causing elevated lipid levels, but the results from this small sample size of 22 hypertriglyceridemic samples is not supportive of this trend and a larger sample size is necessary to explore other mechanisms. As well, results of this analysis should be interpreted conservatively because the blood samples were left over after analysis by HRLMP and nutritional information could not be obtained. It is possible that several samples could have come from one infant being fed with breast milk that had high proportions of oleic acid (C18:1n9c). This is not likely, but it is obvious that further investigation is necessary. The

trend of higher proportions of C18:0, C18:2n6cc, C20:3n6, C20:4n6 and C22:2n6 in samples with normal TG levels leads one to deduce that perhaps a more balanced serum fatty acid profile promotes optimal metabolism of fatty acids across all classes. It might be such that elevations in a single fatty acid or two can reach saturation in metabolism faster than fatty acids of varying saturation and/or chain length. Combined with the significantly higher proportion in only C18:1n9 in hypertriglyceridemic samples, this lends support to the original hypothesis that single fatty acid accumulations may play a role in elevated serum lipid levels. Again, concrete conclusions cannot be drawn from such a small sample size and only suggestions can be made in order to further test the hypothesis.

8.10 Stability in fatty acid profiles

Imbalances in postnatal fatty acid supply have the potential to affect metabolic and neuroendocrine pathways early in life (Innis 2011) but evidence to define a favourable fatty acid profile is lacking. The results of this study were not able to delineate ideal from non-ideal fatty acid profiles in nutrition sources, however, it is clear that there are drastic differences between and within nutrition types (i.e., variation in breast milk, five types of lipid emulsion products). If it is so that just one of these nutrition types has an “ideal” fatty acid profile, it is clear that feeding the other sources could result in a post-natal imbalance of fatty acids.

Although the protocol did not permit blood sampling on a daily basis while on lipid emulsion products, it would be interesting to examine just how stable the serum fatty acid profile of an infant is while on a single, constant source of lipids. Infants born

extremely preterm (<28 weeks) and very preterm (28-31 weeks) may remain on lipid emulsions for weeks (Moutquin 2003). Even if enteral feeds are introduced, the infant is still receiving the same constant fatty acid proportions from the lipid emulsion. Further research is necessary to define what is “ideal” and at what level certain fatty acids are considered “imbalanced” if preterm infants will continue to be fed with the artificial lipid patterns in lipid emulsions in neonatal care.

9. CONCLUSION AND RECOMMENDATION

Results from this pilot study are consistent with the trend in literature that fatty acids are metabolized differently according to chain length and degree of saturation. Serum samples consistently displayed higher proportions of the SFAs C16:0 and C18:0 and the PUFA C20:4n6 compared to their nutritional counterparts. Medium chain fatty acids as well as C12:0 and C14:0 provided by nutritional sources did not result in a detection of these fatty acids in their corresponding serum samples. The hypothesis that serum fatty acid profiles should match that of their nutritional counterparts was not proven true, nor was the hypothesis that elevations in particular fatty acid proportions were associated with hypertriglyceridemia. Lipid emulsion fatty acid profiles were found to be different from the profile of fortified preterm breast milk, indicating some discord in fatty acid supplementation for infants not on enteral feeds. This information can be used as a platform for further studies to examine whether levels of the fatty acids that are metabolized less efficiently are appropriate in nutrition sources such as formula and lipid emulsions. As well, if for example, the SFAs are found to accumulate in the serum of infants upon consumption, mother’s diet can be steered away from sources of these SFAs

and instead supplemented with more easily metabolized fatty acids, such as C18:1n9c. It is clear from the results of this study that a direct reflection of dietary fatty acids is not found in the serum, as supported by literature, and the large number of fatty acids and their complex interrelationships poses an extra challenge to the interpretation of results. The suspected interdependency in metabolism between fatty acids and the role played by tissue stores of fatty acids leads to the conclusion that the analysis of this data and of the studies conducted previously is relatively underexploited. Not enough focus has been directed towards accounting for this in interpretation of results.

An “ideal” fatty acid profile cannot be gleaned from the results of this study due to the variance in the breast milk samples, and the fact that the serum fatty acid profile does not reflect that of breast milk. Nevertheless, this paves the way for a future examination of ideal profiles as the results of this study can be used to inform further research investigating breast milk and serum fatty acid profiles and the variances expected. Under normal physiologic conditions during the third trimester, infants are in the womb and receive their fatty acid supply via the placenta. Assuming that the infant is carried to term, the fatty acid profile delivered to the infant via the umbilical cord vessels during this time period could be considered “ideal” for growth and development. Infants that remain in utero until term are never exposed to breast milk and its “gold standard” status may only apply to a post-natal term birth. It is plausible that there exists a very different gold standard fatty acid profile for pre-natal conditions. Examination of cord blood fatty acid profile has been previously conducted, but the application of the results

to nutritional products such as infant formula, lipid emulsions and breast milk fortifiers is yet to be explored.

10. FUTURE DIRECTION: OPTIMIZED LIPID EMULSION FOR PRETERM INFANTS

In addition to fatty acid composition, the adverse effects of lipid emulsion use in preterm infants could be a result of their physical structure. It may be that the artificial chylomicron does not mimic biological transport in the blood closely enough to be effective in delivering the concentrated energy source to the infant's tissues. Excess phospholipids (PL) are added to lipid emulsions in order to stabilize the artificial chylomicrons (Mirtallo 2010; Ferezou 1999; Rochow 2010). These excess PL can decrease the plasma clearance of the artificial chylomicrons because they aggregate to form a structure called liposomes or lipoprotein-X (LPX) (Ferezou 1999). These moieties are capable of capturing and sequestering lipoprotein lipase, the enzyme required to hydrolyze the artificial chylomicrons (Ferezou 1999). Without enough lipase available in the circulation, the TGs remain in the circulation and can result in increased serum TG in the infant. Developing a TG solution with a fatty acid profile that models that of breast milk, or a combination of lipid sources, and packaging it in chylomicrons produced by a human enterocyte model has potential to overcome the adverse effects seen with currently available lipid emulsions.

Caco-2 cells are human colon carcinoma-derived cells that spontaneously differentiate when grown on microporous membranes (Trotter 1991; van Greevenbroek 1998). They synthesise substantial amounts of (apo)B48 (Trotter 1991), the major

structural protein found exclusively on chylomicrons (van Greevenbroek 1998; Morillas 1992), which is responsible for directing the initial assembly and secretion of chylomicrons (Ginsberg 1998). When the Caco-2 cells are bathed in fatty acid-BSA complexes or mixed micelles, fatty acids are efficiently absorbed and assembled into TG-rich (apo)B48 containing lipoproteins at chylomicron density (van Greevenbroek 1998). The Caco-2 cells then secrete these TG-rich chylomicrons.

Caco-2 cells have been used extensively to study intestinal cell secretions, lipoprotein metabolism and drug transport (Lo 2001; Gundogdu 2011; Buyukozturk 2010). A study by Yasunaga et al. (2007) reveals that chylomicrons could be isolated by ultracentrifugation at 40,000 rpm for 30 min at 4°C from plasma (Yasunaga 2007). Although their aim was to compare metabolism of diacylglycerol and triacylglycerol solutions, they demonstrate that a bolus injection of chylomicrons into the femoral vein could be successfully metabolized by mice. A study examining fatty acid trafficking and metabolism, Levin et al. (1992) demonstrates that Caco-2 cell line uptakes oleic acid (C18:1n9) when it is bound to bovine serum albumin both at the apical and basolateral membranes (Levin 1992). Regardless of whether it was introduced at the apical or basolateral membrane, the majority of the supplied oleic acid was found to be incorporated into TGs within chylomicrons.

Intravenously administered lipid emulsions acquire the apolipoproteins required for hydrolysis by lipoprotein lipase and are then taken up, mainly by the liver, after hydrolysis. Chylomicrons containing the necessary apoB48 acquire apolipoproteins in the apoA, C and E classes from high density lipoproteins. Some apolipoproteins act as

cofactors for plasma lipid-modifying enzymes, and acquiring the necessary ones allow the chylomicrons to bind with lipoprotein lipase so that TGs can be hydrolyzed and taken up by tissues such as the liver (Ginsberg 1998). For example, apoC-I blocks hepatic uptake of lipoproteins, apoC-II is an activator of lipoprotein lipase and apoC-III inhibits the action of lipoprotein lipase (Ginsberg 1998). Hepatic lipase also contributes to the hydrolysis of TG's into free fatty acids and is thought to augment chylomicron uptake by the liver (Ginsberg 1998). Synthesized in adipose tissue and muscle, lipoprotein lipase converts TGs into free fatty acids and mono- and diglycerides, thereby allowing uptake of fatty acids by peripheral tissues (Ginsberg 1998).

With respect to long term outcomes, there is accumulating evidence to suggest that ratios of apolipoproteins are a better predictor for adverse metabolic events than traditional lipid parameters used in the typical clinical diagnostic regimen (Huang 2013). Understanding the lipoprotein metabolism in preterm infants has the potential to provide useful information for the development of more personalized lipid products and improvement in long-term outcomes (Nagano 2012). If it can be determined that Caco-2 cells package and secrete lipids in chylomicrons, evaluation of the apolipoprotein composition is warranted as it may in fact contain a varied apolipoprotein ratio that might enhance absorption and incorporation of fatty acids within the body of a preterm infant.

This data supports the exploration of the use of Caco-2 cells as a bio-production method for lipid emulsions.

10.1 METHODOLOGY ESTABLISHED TO DATE

10.1.1 Plating cells

Caco-2 cell source was obtained from cells frozen in the Rosenthal lab on February 17, 2011. Frozen cells were thawed quickly at 37°C, constantly agitating vial while in the water bath. Approximately 9-10 mL of Dolbecco's Modified Eagle Media (DMEM) was drawn up and transferred into sterile 15 mL tube with cells. Tube was centrifuged at 1500 rpm, 9-10 mins, 20°C to pellet cells and after pouring off aqueous cells were resuspended in DMEM. This wash was repeated 3 times to remove the dimethylsulfoxide (DMSO). Approximately 25 mL of DMEM was drawn up (10 mL of this is used to re-suspend cells) and transferred to T-75 flask. Cells were labeled as P1 (passage 1) and so on. Using a cell counting plate and trypan blue dye, the cell count was recorded for each passage. Once cells reach confluence, DMEM was changed every 3-5 days.

10.1.2 Cell Splitting

Approximately 10 mL of trypsin/EDTA in 1x Ca⁺²/Mg⁺²-free fetal bovine serum (FBS) was prepared, and DMEM was dumped from T-75 flask. Approximately 5-6 mL of trypsin/EDTA was added to flask to wash cells and then poured off. Another 5-6 mL of trypsin/EDTA was added and cells are incubated at 37°C for 5-10 minutes. Approximately 5 mL of DMEM was added to each of 2, 10 mL sterile tubes and the trypsin/EDTA were pipetted off from the flask and transferred to the first tube of DMEM. Again, 5-6 mL of trypsin/EDTA was added to the T-75 flask, incubated for 5-10 minutes and liquid was transferred to the second sterile tube of DMEM. Tubes were centrifuged, liquid waste poured off and the pellet was agitated before the cells were re-suspended in

DMEM. This wash was repeated once more. Approximately 20 mL of DMEM was placed into the desired number of T-75 flasks and approximately 2 mL of cell/DMEM mixture was added to each flask and incubated at 37°C.

10.1.3 Proposed analysis of media: Lipoprotein identification and quantification

Baseline levels of markers of secretion of lipoproteins will first be examined in order to generate reference data to characterize increases or decreases in chylomicron secretion. Chylomicron levels in the absence of lipid supply will constitute a baseline level of secretion, and changes in chylomicron secretion and apolipoprotein distribution with varying lipid levels in the media will be examined.

In order to identify and quantify particles at chylomicron density that contain apoB48, media will be centrifuged in Eppendorf tubes designed to separate based on density, and the chylomicron density portion will be isolated and prepared for agarose gel electrophoresis. ApoB48 is the lipoprotein expressed on the surface of chylomicrons, and therefore the gels will be probed with a polyclonal anti-apoB antibody during immunoblotting. Quantification will occur via densitometry.

Fatty acid uptake and incorporation into chylomicrons will be determined in this manner; the higher the fatty acid concentration of test solution, the higher the output of chylomicrons expected, relative to baseline. Binding agent effects (albumin versus albumin-free conditions) will be explored

10.1.4 Preliminary results

A 2 mL sample of DMEM used to maintain the cells was collected, as well as a 2 mL sample of DMEM that had been incubated with the cells for 4 days. These samples

were extracted and esterified using the same method for milk and serum samples, and run on the GC/MS with the same parameters. Two fatty acids, C16 and C18, are expected to occur in the unused media. The chromatogram for the analysis of the unused DMEM (Figure 16) displays these 2 fatty acids; however, the same fatty acids are identified and detected in the DMEM that was incubated along with the cells at greatly reduced abundances (Figure 17) by the ChemStation software.

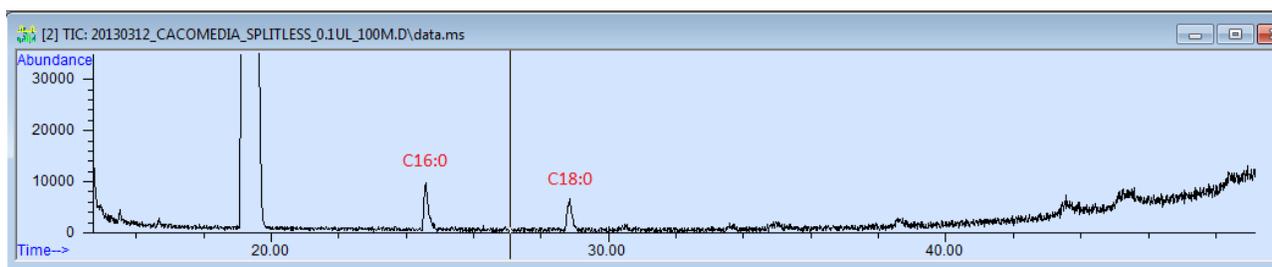


Figure 16. C16:0 and C18:0 abundance in unused DMEM analyzed by GC/MS.

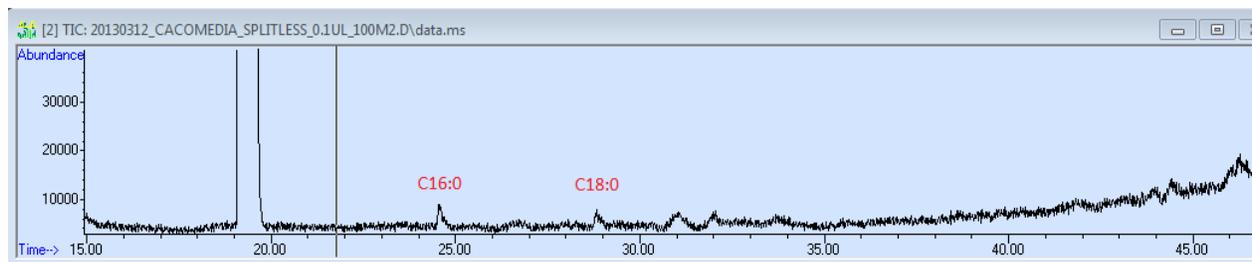


Figure 17. C16:0 and C18:0 abundance in used DMEM analyzed by GC/MS.

This preliminary data confirms that the Caco-2 cells indeed do take up free fatty acids. It cannot be confirmed if they are taken up and used for cellular mechanisms, as precursors to other compounds, for membrane integrity or packaged and secreted in chylomicrons. However, this data supports the use of electrophoresis and immunoblotting

to determine whether or not the Caco-2 cells can uptake fatty acids and secrete them in chylomicron-density particles.

The next step for this project will be to explore the utilization of the fatty acid profiles obtained from breast milk from this thesis in order to design test solutions comprised of various oil sources and/or breast milk for uptake by Caco-2 cells. A preterm infant's hepatic and intestinal systems are immature, therefore the exploration of a more natural packaging mechanism to deliver fat is warranted since the artificial packaging could be causing problems with triglyceride delivery. Since the Caco-2 cell line is a human cell line, the chylomicrons are not expected to be recognized as unnatural by the preterm infant's body and the composition of the chylomicron may be exactly the same as the infant's own or very different (with respect to ratio of cholesterol esters, cholesterol and membrane proteins). Even slight differences in these ratios might change properties such as buoyancy of the chylomicron, and any changes could have the potential to increase efficiency in triglyceride delivery to the preterm infant.

11. REFERENCES

- Alemi B, Hamosh M, Scanlon JW, Salzman-Mann C, Hamosh P. Fat digestion in very low-birth-weight infants: effect of addition of human milk to low-birth-weight formula. *Pediatrics*. 1981; 68: 484.
- Amri EZ, Ailhaud G, Grimaldi PA. Fatty acids as signal transducing molecules: involvement in the differentiation of preadipose to adipose cells. *J Lipid Res*. 1994; 35:930-7.
- Amusquivar E, Sánchez M, Hyde MJ, Laws J, Clarke L, Herrera E. Influence of fatty acid profile of total parenteral nutrition emulsions on the fatty acid composition of different tissues of piglets. *Lipids*. 2008; 43: 713-22.
- AOCS Lipid Library (A). http://lipidlibrary.aocs.org/lipids/fa_poly/index.htm
- AOCS Lipid Library (B). http://lipidlibrary.aocs.org/gc_lipid/04_deriv/index.htm
- Aoyama T, Nosaka N, Kasai M. Research on the nutritional characteristics of medium-chain fatty acids. *J Med Invest*. 2007; 4:385-8.
- Arslanoglu S, Moro GE, Ziegler EE. Preterm infants fed fortified human milk receive less protein than they need. *J Perinatol*. 2009; 29: 489-492.
- Berenhauser AC, Pinheiro do Prado AC, da Silva RC, Gioielli LA, Block JM. Fatty acid composition in preterm and term breast milk. *Int J Food Sci Nutr*. 2012; 63:318-25.
- Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*. 1959; 37:911-7.
- Buyukozturk F, Benneyan JC, Carrier RL. Impact of emulsion-based drug delivery systems on intestinal permeability and drug release kinetics. *J Control Release*. 2010; 142; 22-30.
- Carpentier YA, Hacquebard M. Intravenous lipid emulsions to deliver omega 3 fatty acids. *Prostaglandins Leukot Essent Fatty Acids*. 2006; 75:145-8.
- Cartwright IJ, Plonné D, Higgins JA. Intracellular events in the assembly of chylomicrons in rabbit enterocytes. *J Lipid Res*. 2000; 41; 1728-1739.
- Chappell JE, Clandinin MT, Kearney-Volpe C, Reichman B, Swyer PW. Fatty acid balance studies in premature infants fed human milk or formula: effect of calcium supplementation. *J Pediatr*. 1986;108:439-47.

- Davis-Bruno K, Tassinari MS. Essential fatty acid supplementation of DHA and ARA and effects on neurodevelopment across animal species: a review of the literature. *Birth Defects Res B Dev Reprod Toxicol.* 2010; 92:240-50.
- Deshpande G, Simmer K. Lipids for parenteral nutrition in neonates. *Curr Opin Clin Nutr Metab Care.* 2011; 14: 145-50.
- Deshpande GC, Simmer K, Mori T, Croft K. Parenteral lipid emulsions based on olive oil compared with soybean oil in preterm (<28 weeks' gestation) neonates: a randomised controlled trial. *J Pediatr Gastroenterol Nutr.* 2009; 49:619-25.
- Driscoll DF, Ling PR, Bistrrian BR. Pharmacopeial compliance of fish oil-containing parenteral lipid emulsion mixtures: Globule size distribution (GSD) and fatty acid analyses. *Int J Pharm.* 2009; 379: 125-130.
- Driscoll DF. Invited Review Lipid Injectable Emulsions: 2006. *Nutr Clin Pract.* 2006; 21:381-6.
- Farquharson J, Cockburn F, Patrick WA, Jamieson EC, Logan RW. Effect of diet on infant subcutaneous tissue triglyceride fatty acids. *Arch Dis Child.* 1993; 69:589-93.
- Ferezou J, Bach AC. Structure and metabolic fate of triacylglycerol- and phospholipid-rich particles of commercial parenteral fat emulsions. *Nutrition.* 1999; 15: 44-50.
- Fidler N, Sauerwald TU, Koletzko B, Demmelmair H. Effects of human milk pasteurization and sterilization on available fat content and fatty acid composition. *J Pediatr Gastroenterol Nutr.* 1998; 27:317-22.
- Fidler N, Sauerwald T, Pohl A, Demmelmair H, Koletzko B. Docosahexaenoic acid transfer into human milk after dietary supplementation: a randomized clinical trial. *J Lipid Res.* 2000; 41:1376-83.
- Folch J, Lees M, Sloane SGH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem.* 1957; 226:497-509.
- Friedman Z, Frolich JC. Essential fatty acids and the major urinary metabolites of the E prostaglandins in thriving neonates and in infants receiving parenteral fat emulsions. *Pediatr Res.* 1979; 13:932-6.

- Fusch C, Bauer K, Bohles HJ, et al. Neonatology/Paediatrics- Guidelines on parenteral nutrition, Chapter 13. *Ger Med Sci.* 2009; 7:Doc15
- Garaulet M, Hernandez-Morante JJ, Lujan J, et al. Relationship between fat cell size and number and fatty acid composition in adipose tissue from different fat depots in overweight/obese humans. *Int J Obes.* 2006; 30: 899-905.
- Genzel-Boroviczény O, Wahle J, Koletzko B. Fatty acid composition of human milk during the 1st month after term and preterm delivery. *Eur J Pediatr.* 1997; 156:142-7.
- Gibson RA, Muhlhausler B, Makrides M. Conversion of linoleic acid and alpha-linolenic acid to long-chain polyunsaturated fatty acids (LCPUFAs), with a focus on pregnancy, lactation and the first 2 years of life. *Matern Child Nutr.* 2011;7(Suppl 2):17-26.
- Ginsberg HN. Lipoprotein physiology. *Endocrinol Metab Clin North Am.* 1998; 27:503-519.
- Groh-Wargo S, Sapsford A. Enteral nutrition support of the preterm infant in the neonatal intensive care unit. *Nutr Clin Pract.* 2009; 24:363-376.
- Gundogdu E, Alvarez IG, Karasulu E. Improvement of effect of water-in-oil microemulsion as an oral delivery system for fexofenadine: in vitro and in vivo studies. *Int J Nanomedicine.* 2011; 6:631-640.
- Haddad I, Mozzon M, Frega NG. Trends in fatty acid positional distribution in human colostrum, transitional and mature milk. *Eur Food Res Technol.* 2012; 235:325-332.
- Hamosh M, Mehta NR, Fink CS, et al. Fat absorption in premature infants: medium-chain triglycerides and long-chain triglycerides are absorbed from formula at similar rates. *J Pediatr Gastroenterol Nutr.* 1991; 13:143-149.
- Hannukainen JC, Kalliokoski KK, Borra RJ, et al. Higher free fatty acid uptake in visceral than in abdominal subcutaneous fat tissue in men. *Obesity (Silver Spring).* 2010; 18:261-5.
- Hardy G, Puzovic M. Formulation, stability and administration of parenteral nutrition with new lipid emulsions. *Nutr Clin Pract.* 2009; 24:616-25.
- Haumont D. Lipid infusion and intravenous access in newborn infants. *Chin Med J.* 2010; 123:2766-2768.

- Heird WC, Lapillonne A. The role of essential fatty acids in development. *Annu Rev Nutr.* 2005;25:549-71.
- Hippalgaonkar K, Majumdar S, Kansara V. Injectable lipid emulsions-advancements, opportunities and challenges. *AAPS PharmSciTech.* 2010; 11: 1526-40.
- Hodson L, Skeaff CM, Fielding BA. Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. *Prog Lipid Res.* 2008; 47:348-80.
- Huang F, Yang Z, Xu B, Bi Y, Xu M, Xu Y, Lu J, Liu Y, Dai M, Zhou W, Wang W, Chen Y. Both serum apolipoprotein B and the apolipoprotein B/apolipoprotein A-I ratio are associated with carotid intima-media thickness. *PLoS One.* 2013; 8:e54628.
- Hultin M, Carneheim C, Rosenqvist K, Olivecrona T. Intravenous lipid emulsions: removal mechanisms as compared to chylomicrons. *J Lipid Res.* 1995; 36:2174-2184.
- Ibrahim MM. Subcutaneous and visceral adipose tissue: structural and functional differences. *Obes Rev.* 2010; 11:11-8.
- Ichihara K, Yoneda K, Takahashi A, Hoshino N, Matsuda M. Improved methods for the fatty acid analysis of blood lipid classes. *Lipids.* 2011; 46:297-306.
- Innis SM, Foote KD, MacKinnon MJ, King DJ. Plasma and red blood cell fatty acids of low-birth-weight infants fed their mother's expressed breast milk or preterm-infant formula. *Am J Clin Nutr.* 1990; 51:994-1000.
- Innis SM. Metabolic programming of long-term outcomes due to fatty acid nutrition in early life. *Matern Child Nutr.* 2011; 7(Suppl 2):112-23.
- Jones PJ, Pencharz PB, Clandinin MT. Whole body oxidation of dietary fatty acids: implications for energy utilization. *Am J Clin Nutr.* 1985; 42:769-77.
- King C. What's new in enterally feeding the preterm infant? *Arch Dis Child Fetal Neonatal Ed.* 2010; 95: F304-F308.
- Kishino T, Ohnishi H, Ohtsuka K, et al. Subcutaneous rather than visceral adipose tissue thickness of the abdomen correlates with alterations in serum fatty acid profile in Japanese women. *Clin Chim Acta.* 2011; 412:1893-4.

- Koletzko B, Beyer J, Brands B, Demmelmair H, Grote V, Haile G, Gruszfeld D, Rzehak P, Socha P, Weber M; European Childhood Obesity Trial Study Group. Early influences of nutrition on postnatal growth. *Nestle Nutr Inst Workshop Ser.* 2013; 71:11-27.
- Koletzko B, Sauerwald U, Keicher U, Saule H, Wawatschek S, Böhles H, Bervoets K, Fleith M, Crozier-Willi G. Fatty acid profiles, antioxidant status, and growth of preterm infants fed diets without or with long-chain polyunsaturated fatty acids A randomized clinical trial. *Eur J Nutr* 2003; 253: 243-253.
- Koletzko B. Long-term consequences of early feeding on later obesity risk. *Nestle Nutr Workshop Ser Pediatr Program.* 2006; 58:1-18.
- Koutsari C, Dumesic DA, Patterson BW, Votruba SB, Jensen MD. Plasma free fatty acid storage in subcutaneous and visceral adipose tissue in postabsorptive women. *Diabetes.* 2008; 57:1186-94.
- Kremmyda LS, Tvrzicka E, Stankova B, Zak A. Fatty acids as biocompounds: their role in human metabolism, health and disease: a review. Part 2: fatty acid physiological roles and applications in human health and disease. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub.* 2011; 155:195-218.
- Krohn K, Koletzko B. Parenteral lipid emulsions in paediatrics. *Curr Opin Clin Nutr Metab Care.* 2006; 9: 319-23.
- Kuipers RS, Luxwolda MF, Janneke Dijck-Brouwer DA, Muskiet FA. Intrauterine, postpartum and adult relationships between arachidonic acid (AA) and docosahexaenoic acid (DHA). *Prostaglandins Leukot Essent Fatty Acids.* 2011; 85:245-52.
- Lapillonne A, Groh-Wargo S, Gonzalez CH, Uauy R. Lipid needs of preterm infants: updated recommendations. *J Pediatr.* 2013; 162(3 Suppl):S37-47.
- Le HD, de Meijer VE, Robinson EM, Zurakowski D, Potemkin AK, Arsenault DA, Fallon EM, Malkan A, Bistrrian BR, Gura KM, Puder M. Parenteral fish-oil – based lipid emulsion improves fatty acid profiles and lipids in parenteral nutrition – dependent children 1 – 3. *Am J Clin Nutr.* 2011; 6:749-758.
- Lehner F, Demmelmair H, Röschinger W, Decsi T, Szász M, Adamovich K, Arnecke R, Koletzko B. Metabolic effects of intravenous LCT or MCT/LCT lipid emulsions in preterm infants. *J Lipid Res.* 2006; 47:404-11.

- Lepage N, Li D, Kavsak PA, Bamforth F, Callahan J, Dooley K, Potter M. Incomplete pediatric reference intervals for the management of patients with inborn errors of metabolism. *Clin Biochem.* 2006; 39:595-9.
- Levin MS, Talkad VD, Gordon JI, Stenson WF. Trafficking of exogenous fatty acids within Caco-2 cells. *J Lipid Res.* 1992; 33:9-19.
- Lo Y, Liu F, Cherng J. Effect of PSC 833 liposomes and Intralipid on the transport of epirubicin in Caco-2 cells and rat intestines. *J Control Release.* 2001; 76:1-10.
- Matyash V, Liebisch G, Kurzchalia TV, Shevchenko A, Schwudke D. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *J Lipid Res.* 2008; 49:1137-46.
- McCann JC, Ames BN. Is docosahexaenoic acid, an n-3 long-chain polyunsaturated fatty acid, required for development of normal brain function? An overview of evidence from cognitive and behavioral tests in humans and animals. *Am J Clin Nutr.* 2005; 82:281-95.
- McDonald GB, Saunders DR, Weidman M, Fisher L. Portal venous transport of long-chain fatty acids absorbed from rat intestine. *Am J Physiol.* 1980; 239:G141-50.
- Mirtallo JM, Dasta JF, Kleinschmidt KC, Varon J. State of the art review: Intravenous fat emulsions: Current applications, safety profile, and clinical implications. *Ann Pharmacother.* 2010; 44:688-700.
- Morillas JM, Moltó L, Robles R, Gil A, Sánchez-Pozo A. Lipoproteins in preterm and small-for-gestational-age infants during the first week of life. *Acta Paediatr.* 1992; 81:774-8.
- Morris S, Simmer K, Gibson R. Characterization of fatty acid clearance in premature neonates during intralipid infusion. *Pediatr Res.* 1998; 43:245-9.
- Moutquin JM. Classification and Heterogeneity of preterm birth. *BJOG.* 2003; 110:30-33.
- Nagano N, Okada T, Yonezawa R, Yoshikawa K, Fujita H, Usukura Y, Makimoto M, Hosono S, Fujita Y, Takahashi S, Mugishima H, Yamamoto T. Early postnatal changes of lipoprotein subclass profile in late preterm infants. *Clin Chim Acta.* 2012; 413:109-12.

- Nakamura MT, Nara TY. Essential fatty acid synthesis and its regulation in mammals. *Prostaglandins Leukot Essent Fatty Acids*. 2003; 68:145-50.
- Nelson CM, Innis SM. Plasma lipoprotein fatty acids are altered by the positional distribution of fatty acids in infant formula triacylglycerols and human milk. *Am J Clin Nutr*. 1999; 70:62-9.
- Nikkari T, Luukkainen P, Pietinen P, Puska P. Fatty acid composition of serum lipid fractions in relation to gender and quality of dietary fat. *Ann Med*. 1995; 27:491-8.
- Nutrition Reviews. Fatty acid absorption by preterm infants. *Nutr Rev*. 1987; 45:199-200.
- Owen CG, Whincup PH, Kaye SJ, Martin RM, Davey Smith G, Cook DG, Bergstrom E, Black S, Wadsworth ME, Fall CH, Freudenheim JL, Nie J, Huxley RR, Kolacek S, Leeson CP, Pearce MS, Raitakari OT, Lisinen I, Viikari JS, Ravelli AC, Rudnicka AR, Strachan DP, Williams SM. Does initial breastfeeding lead to lower blood cholesterol in adult life? A quantitative review of the evidence. *Am J Clin Nutr*. 2008; 88:305-14.
- Papamandjaris AA, MacDougall DE, Jones PJ. Medium chain fatty acid metabolism and energy expenditure: obesity treatment implications. *Life Sci*. 1998; 62:1203-15.
- Plagemann A, Harder T, Schellong K, Schulz S, Stupin JH. Early postnatal life as a critical time window for determination of long-term metabolic health. *Best Pract Res Clin Endocrinol Metab*. 2012; 26:641-53.
- Puntis JW. Nutritional support in the premature newborn. *Postgrad Med J*. 2006; 82:192-8.
- Ramírez M, Amate L, Gil A. Absorption and distribution of dietary fatty acids from different sources. *Early Hum Dev*. 2001; 65(Suppl):S95-S101.
- Reuter SE, Evans AM. Carnitine and acylcarnitines: pharmacokinetic, pharmacological and clinical aspects. *Clin Pharmacokinet*. 2012; 51:553-72.
- Rochow N, Fusch G, Choi A, Chessell L, Elliott L, McDonald K, Kuiper E, Purcha M, Turner S, Chan E, Xia MY, Fusch C. Target Fortification of Breast Milk with Fat, Protein, and Carbohydrates for Preterm Infants. *J Pediatr*. 2013 Jun

12. pii: S0022-3476(13)00517-9. doi: 10.1016/j.jpeds.2013.04.052. [Epub ahead of print]
- Rochow N, Möller S, Fusch G, Drogies T, Fusch C. Levels of lipids in preterm infants fed breast milk. *Clin Nutr.* 2010; 29:94-9.
- Rombaldi Bernardi J, de Souza Escobar R, Ferreira CF, Pelufo Silveira P. Fetal and neonatal levels of omega-3: effects on neurodevelopment, nutrition, and growth. *Scientific World Journal.* 2012; 2012:202473. doi: 10.1100/2012/202473. Epub 2012 Oct 17.
- Sala-Vila A, Castellote AI, Rodriguez-Palmero M, Campoy C, López-Sabater MC. Lipid composition in human breast milk from Granada (Spain): changes during lactation. *Nutrition.* 2005; 21:467-73.
- Santosa S, Jensen MD. Why are we shaped differently, and why does it matter? *Am J Physiol Endocrinol Metab.* 2008; 295:E531-5.
- Schlens J. A Tutorial on Principal Component Analysis. New York University. 2005.
- Skartland LK, Mjøs SA, Grung B. Experimental designs for modeling retention patterns and separation efficiency in analysis of fatty acid methyl esters by gas chromatography-mass spectrometry. *J Chromatogr A.* 2011; 1218:6823-31.
- Smithers LG, Markrides M, Gibson RA. Human milk fatty acids from lactating mothers of preterm infants: a study revealing wide intra- and inter-individual variation. *Prostaglandins Leukot Essent Fatty Acids.* 2010; 83:9-13.
- Takeuchi H, Sekine S, Kojima K, Aoyama T. The application of medium-chain fatty acids: edible oil with a suppressing effect on body fat accumulation. *Asia Pac J Clin Nutr.* 2008; 17(Suppl 1):320-3.
- Trintis J, Donohue P, Aucott S. Outcomes of early parenteral nutrition for premature infants. *J Perinatol.* 2010; 30: 403-407.
- Trotter PJ, Storch J. Fatty acid uptake and metabolism in a human intestinal cell line (Caco-2): Comparison of apical and basolateral incubation. *J Lipid Res.* 1991; 32:293-304.
- Uauy R, Mena P, Rojas C. Essential fatty acids in early life: structural and functional role. *Proc Nutr Soc.* 2000; 59:3-15.

- Ullah S, Finch CF. Applications of functional data analysis: A systematic review. *BMC Med Res Methodol.* 2013; 19:13:43.
- Uusitalo L, Nevalainen J, Salminen I, Ovaskainen ML, Kronberg-Kippilä C, Ahonen S, Niinistö S, Alfthan G, Simell O, Ilonen J, Veijola R, Knip M, Virtanen SM. Fatty acids in serum and diet - a canonical correlation analysis among toddlers. *Matern Child Nutr.* 2013; 9:381-395.
- Valentine CJ, Puthoff TD. Enhancing parenteral nutrition therapy for the neonate. *Nutr Clin Pract.* 2007; 22:183-97.
- van Greevenbroek MM, d Bruin TW. Chylomicron synthesis by intestinal cells in vitro and in vivo. *Atherosclerosis.* 1998; 141(Suppl 1); S9-16.
- Waitzberg DL, Torrinhas RS, Jacintho TM. New parenteral lipid emulsions for clinical use. *JPEN J Parenter Enteral Nutr.* 2006; 30:351-67.
- Wells JC, Chomtho S, Fewtrell MS. Programming of body composition by early growth and nutrition. *Proc Nutr Soc.* 2007; 66:423-34.
- Wu X, Tong Y, Shankar K, Baumgardner JN, Kang J, Badeaux J, Badger TM, Ronis MJ. Lipid fatty acid profile analyses in liver and serum in rats with nonalcoholic steatohepatitis using improved gas chromatography-mass spectrometry methodology. *J Agric Food Chem.* 2011; 59:747-54.
- Yasunaga K, Saito S, Zhang YL, et al. Effects of triacylglycerol and diacylglycerol oils on blood clearance, tissue uptake, and hepatic apolipoprotein B secretion in mice. *J Lipid Res.* 2007; 48:1108-1121.

12. APPENDIX A: Tables

Table 1. Molecular formula, weight and retention times of each fatty acid detected in milk, serum and/or lipid emulsions.

Fatty Acid	Molecular formula	Molecular Weight (g/mol)	Retention time ± SD (min)
Caprylic (C8:0)	C ₈ H ₁₆ O ₂	158.24	15.386 ± 0.0622
Capric (C10:0)	C ₁₀ H ₂₀ O ₂	186.29	16.518 ± 0.0533
Lauric (C12:0)	C ₁₂ H ₂₄ O ₂	214.34	18.330 ± 0.0586
Myristic (C14:0)	C ₁₄ H ₂₈ O ₂	242.40	20.965 ± 0.0586
Pentadecanoic (C15:0)	C ₁₅ H ₃₀ O ₂	256.42	22.695 ± 0.0766
Palmitic (C16:0)	C ₁₆ H ₃₂ O ₂	270.45	24.467 ± 0.0432
Stearic (C18:0)	C ₁₈ H ₃₆ O ₂	298.50	28.772 ± 0.0615
Arachidic (C20:0)	C ₂₀ H ₄₀ O ₂	326.56	33.654 ± 0.0837
Palmitoleic (C16:1n7)	C ₁₆ H ₃₀ O ₂	268.43	26.261 ± 0.0482
Cis-10-heptadecanoic (C17:1)	C ₁₇ H ₃₄ O ₂	282.46	28.323 ± 0.000
Elaidic (trans-C18:1n9)	C ₁₈ H ₃₄ O ₂	296.49	30.717 ± 0.0961
Oleic (cis-C18:1n9)	C ₁₈ H ₃₄ O ₂	296.49	30.400 ± 0.0837
Linoleic (cis, cis-C18:2n6)	C ₁₈ H ₃₂ O ₂	294.47	32.961 ± 0.0546
γ-linolenic (C18:3n6)	C ₁₈ H ₃₀ O ₂	292.46	34.805 ± 0.415
Eicosenoic (C20:1n9)	C ₂₀ H ₃₈ O ₂	324.54	35.130 ± 0.0565
α-Linolenic (C18:3n3)	C ₁₈ H ₃₀ O ₂	292.46	35.979 ± 0.0423
Eicosadienoic (C20:2n6)	C ₂₀ H ₃₆ O ₂	322.53	37.62 ± 0.0509
Cis-8,11,14-Eicosatrienoic (C20:3n6)	C ₂₀ H ₃₄ O ₂	320.51	39.441 ± 0.0360
Erucic (C22:1n9)	C ₂₂ H ₄₂ O ₂	352.59	39.41 ± 0.000
Arachidonic (C20:4n6)	C ₂₀ H ₃₂ O ₂	318.49	40.622 ± 0.0307
Docosohexaenoic (C22:6n3)	C ₂₂ H ₃₂ O ₂	342.51	49.318 ± 0.00141

Table 3. Fatty acid proportions of the local average preterm breast milk profile compared to 5 commercially available lipid emulsion products.

Fatty Acid	Omegaven	SMOFlipid	Lipidem	Intralipid	Clinoleic	BM ± SD (%)
C8:0	0	32	21	0	0	2 ± 3
C10:0	0	42	19	0	0	11 ± 12
C12:0	0	0	0	0	0	4 ± 3
C14:0	4	0	0	0	0	3 ± 3
C16:0	20	0	7	17	17	15 ± 6
C16:1n7	7	0	0	0	1	1 ± 1
C18:0	6	6	3	6	6	5 ± 5
C18:1n9c	17	0	13	24	54	37 ± 9
C18:1n9t	2	0	1	1	2	0 ± 0
C18:2n6cc	6	15	25	45	18	18 ± 6
C18:3n3	1	0	3	5	1	1 ± 1
C20:5n3	18	1	4	0	0	0 ± 0
C20:3n3	1	0	0	0	0	0 ± 0
C20:4n6	2	0	0	1	1	1 ± 1
C22:2n6	15	2	3	0	0	1 ± 5

Mean proportion ± SD (%) for the local average breast milk fatty acid profile (BM, n=69) and fatty acid proportions (%) of 5 commercially available lipid emulsion products.

Table 4. Fatty acid proportions of the local average breast milk group (BM) and the resulting serum from infants fed breast milk (BMserum).

Fatty acid	Mean BM ± SD (%)	Mean BMserum ± SD (%)	p-value	Confidence interval of the difference (%)
C8:0	2.3 ± 3.3	0.0 ± 0.0	p<0.0001	-3.0 to -4.2
C10:0	11.1 ± 11.5	0.0 ± 0.0	p<0.0001	-1.4 to -8.3
C12:0	3.9 ± 3.5	0.0 ± 0.0	p<0.0001	-4.7 to -3.1
C14:0	3.3 ± 2.5	0.0 ± 0.0	p<0.0001	-3.9 to -2.7
C16:0	14.7 ± 5.7	35.2 ± 10.9	p<0.0001	16.4 to 24.8
C16:1n7	1.2 ± 1.4	0.0 ± 0.2	p<0.0001	-1.5 to -0.9
C18:0	5.0 ± 4.9	24.4 ± 14.1	p<0.0001	14.0 to 24.7
C18:1n9c	36.6 ± 9.3	11.6 ± 5.6	p<0.0001	-27.9 to -22.0
C18:1n9t	0.1 ± 0.4	0.1 ± 0.4	p=0.5405	-0.2 to 0.1
C18:2n6cc	18.5 ± 6.4	18.6 ± 6.9	p=0.8968	-2.7 to 3.1
C18:3n3	1.3 ± 1.2	0.0 ± 0.0	p<0.0001	-1.6 to -1.0
C20:3n6	0.3 ± 0.5	0.3 ± 1.2	p=0.9190	-0.4 to 0.5
C20:4n6	0.6 ± 1.1	7.7 ± 7.1	p<0.0001	4.5 to 9.7
C22:2n6	0.9 ± 5.2	2.0 ± 9.8	p=0.5751	-2.7 to 4.8

Mean proportion ± SD (%) for the local average breast milk fatty acid profile (BM, n=69) and resulting serum profile of infants fed BM (BMserum, n=31). P-values and 95% confidence interval of the difference were obtained via unpaired t-test, SD's assumed to be unequal and Welch's correction factor applied. Fatty acids occurring in proportions less than 0.1% in both groups were not included.

Table 5. Fatty acid proportions for Intralipid and the resulting serum of infants administered this lipid emulsion.

Fatty acid	LE (%)	Mean LEserum \pm SD (%)
C12:0	0.0	0.4 \pm 1.4
C14:0	0.0	0.4 \pm 1.1
C16:0	16.9	34.9 \pm 12.1
C16:1n7	0.0	0.1 \pm 0.4
C18:0	6.3	17.9 \pm 14.0
C18:1n9c	23.8	24.0 \pm 8.8
C18:1n9t	1.3	0.3 \pm 1.2
C18:2n6cc	45.2	12.2 \pm 7.0
C18:3n3	5.1	0.2 \pm 0.7
C20:3n6	0.0	0.3 \pm 0.6
C20:4n6	0.8	8.8 \pm 5.8
C22:2n6	0.5	0.5 \pm 0.9

Fatty acid proportions (%) for LE (Intralipid) profile (LE, n=1) and mean proportion \pm SD (%) for resulting serum profile of infants fed LE (LEserum, n=15). Fatty acids occurring in proportions less than 0.1% in both groups were not included.

Table 6. Comparison of fatty acid proportions in serum of infants fed exclusively breast milk (BMserum) or >80% fat from lipid emulsions (LEserum).

Fatty acid	Mean BMserum ± SD (%)	Mean LEserum ± SD (%)	p-value	Confidence interval of the difference (%)
C10:0	0.0 ± 0.0	0.0 ± 0.1	p=0.3342	0.0 to 0.1
C12:0	0.0 ± 0.0	0.4 ± 1.4	p=0.3343	-0.4 to 1.1
C14:0	0.0 ± 0.0	0.4 ± 1.1	p=0.1758	-0.2 to 1.0
C16:0	35.2 ± 10.9	34.9 ± 12.1	p=0.9293	-7.9 to 7.3
C16:1n7	0.0 ± 0.2	0.1 ± 0.4	p=0.5739	-0.2 to 0.3
C18:0	24.4 ± 14.1	17.9 ± 14.0	p=0.1522	-15.5 to 2.5
C18:1n9c	11.6 ± 5.6	24.0 ± 8.8	p<0.0001	7.2 to 17.5
C18:1n9t	0.1 ± 0.4	0.3 ± 1.2	p=0.4750	-0.5 to 0.9
C18:2n6cc	18.6 ± 6.9	12.2 ± 7.0	p<0.01	-10.9 to -1.9
C18:3n3	0.0 ± 0.0	0.2 ± 0.7	p=0.3343	-0.2 to 0.6
C20:3n6	0.3 ± 1.2	0.3 ± 0.6	p=0.8660	-0.6 to 0.5
C20:4n6	7.7 ± 7.1	8.8 ± 5.8	p=0.5675	-2.9 to 5.1
C22:2n6	2.0 ± 9.8	0.5 ± 0.9	p=0.3928	-5.2 to 2.1

Mean proportion ± SD (%) for the resulting serum profile of infants fed BM (BMserum, n=31) and LE (LEserum, n=15). P-values and 95% confidence interval of the difference were obtained via unpaired t-test, SD's assumed to be unequal and Welch's correction factor applied. Fatty acids occurring in proportions less than 0.1% in both groups were not included.

13. APPENDIX B: Figures

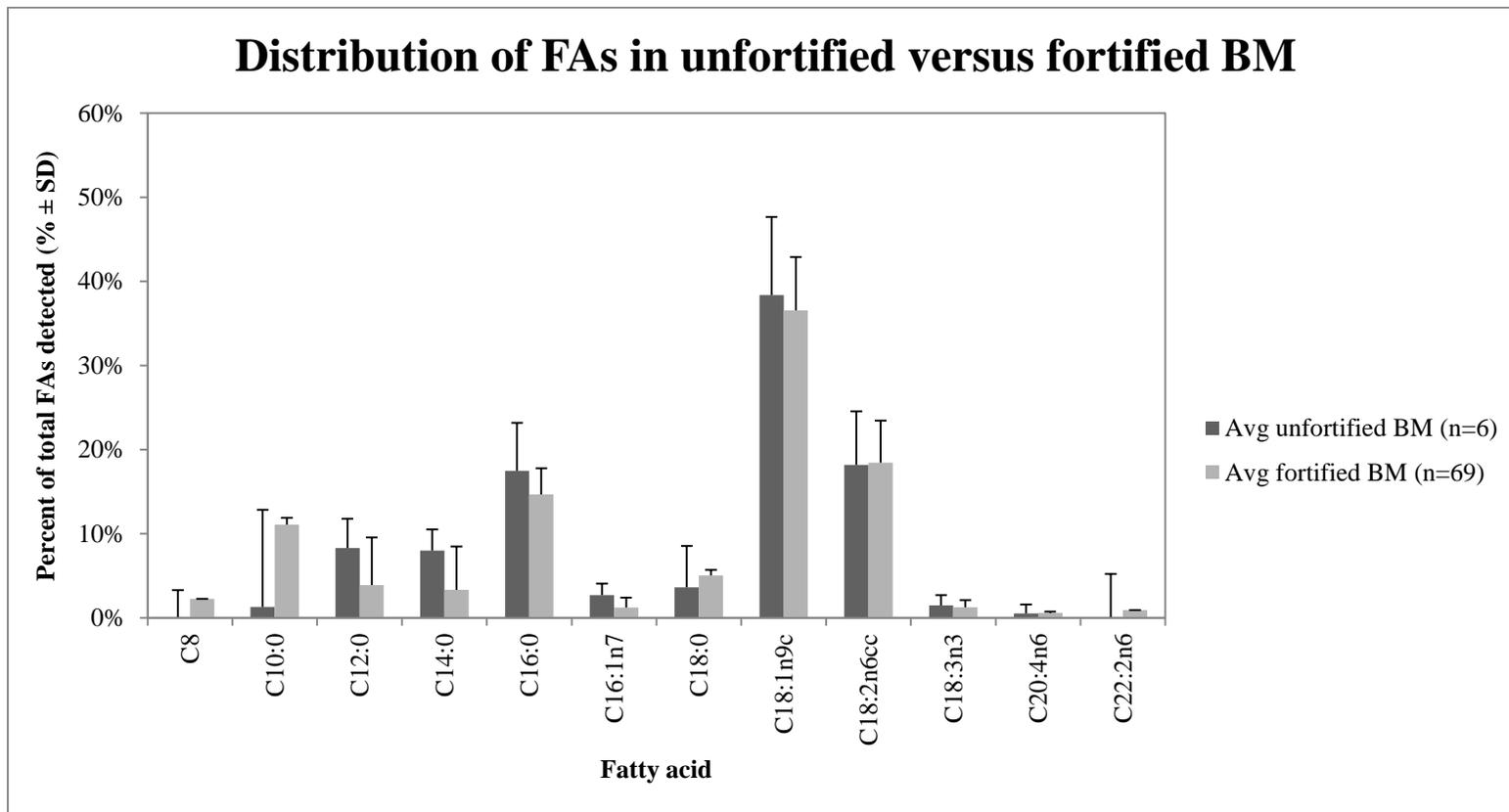


Figure 18: Comparison of the FA profile of average unfortified preterm BM (n=6) to the average fortified preterm BM (n=69). Fatty acids that appeared in concentrations <1% in all samples were removed.