FATTY ACID PROFILES
OF NUTRTION SOURCES AND PLASMA
IN PRETERM INFANTS

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by
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This thesis is dedicated to my supportive family in Korea, my fiancé Peter Lee, my best friend Jihae Lee, and my roomie Kelly Sun.
Without the love, support, and encouragement, it would be very difficult to complete this two-year journey

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Abbreviations

AA: Arachidonic Acid
BHT: Butylated Hydroxytoluene
BM: Breast Milk
BPD: Bronchopulmonary Dysplasia
BSDL: Bile Salt-Dependent Lipase
BSSL: Bile Salt-Stimulated Lipase
CFA: Coefficient Fat Absorbed
CV: Coefficient of Variation
DHA: Docosahexanoic Acid
EPA: Eicosapentaenoic Acid
FAMEs: Fatty Acids Methyl Esters
GL: Gastric Lipase
HDL: High Density Lipoprotein
LC-PUFA: Long Chain-Polyunsaturated Fatty Acid
LDL: Low Density Lipoprotein
LOD: Limit Of Detection
LOQ: Limit Of Quantification
LPL: Lipoprotein Lipase
MCFA: Medium Chain Fatty Acid
MUFA: Monounsaturated Fatty Acid
NICU: Neonatal Intensive Care Unit
PLRP: Pancreatic Lipase-Related Protein
PTL: Pancreatic TG Lipase
TBME: Tert-Butyl Methyl Ether
TG: Triglycerides
VLDL: Very Low Density Lipoprotein

Abstract

Preterm infants are not able to tolerate full enteral feeding of breast milk due to gut immaturity. To fill this nutritional gap, parenteral nutrition is introduced while enteral feeding is gradually increased. Of parenteral nutrition, lipid emulsions supply energy, essential and polyunsaturated fatty acids, which significantly affect short- and long-term health outcomes of growth, visual-and neuro-development for preterm infants. However, elevated plasma triglyceride (TG) levels in preterm infants receiving lipid emulsions have been observed despite less lipid intake compared to breast milk fed infants. We hypothesized that unbalanced fatty acid profiles in lipid emulsion was one factor to cause high plasma TGs for preterm infants. In the multi-center, observational, prospective study, the following samples were analyzed using GC-MS: (1) lipid emulsions (n=5) and breast milk (n=112), (2) plasma (n=294) including normal TG (n=116) and high TG (n=88). Lipoproteins in normal TG (n=18) and high TG (n=24) plasma were measured using gel electrophoresis. Fatty acid profiles in lipid emulsions differed from ones in breast milk. Plasma fatty acid profiles were related to dietary fatty acid intake. Accumulation of all fatty acids except C20:5n3 and relatively high (LDL+VLDL) levels ($p<0.001$) resulted in high TG plasma compared to normal TG plasma. Overall, this study supports the hypothesis that nutrition of lipid emulsions was associated high TG with differences in fatty acid uptake but it is still unclear if the imbalance of fatty acids directly causes high TG. More research is necessary to investigate other factors such as enzyme activity, lipid clearance rate, or different rate of fatty acid metabolism.

CHAPTER 1. Introduction

1.1 Nutrition and Growth during Prenatal Period

Nutrition is one of the important factors that can affect fetal development, growth, and overall health. During the fetal period, nutrition is determined by maternal nutrition, metabolism, endocrinology, and placental perfusion (Sparks et al. 1998). Among the biological building blocks for macronutrients, glucose is the most important nutrient crossing the placenta because it is the primary metabolic fuel for the fetus (Hay 2006). Amino acids are source for protein synthesis and accretion, which are essential for fetal growth and synthesis of fetal tissues (Kalhan 2000).

Until recently, lipids and fatty acids were not traditionally considered in the clinical management of pregnancy because their individual role in nutrient and growth were not as well known. Generally, fatty acids have important roles in membrane formation, are precursors of molecules such as prostaglandins, and constitute a source of energy for the fetus. Intrauterine requirements for (n-3) and (n-6) fatty acids in the last trimester have been calculated to be approximately 50 and 400 mg/kg/d respectively due to the necessity for brain and retina development for the fetus (Hossain et al. 2016). In brain tissues, almost half of the lipid content is comprised of long chain polyunsaturated fatty acids (LC-PUFAs) such as arachidonic acid (AA) and docosahexanoic acid (DHA). Within the retina, DHA is concentrated in the outer part of rod photoreceptors, which have an important role in the visual pigment rhodopsin (Schmitz & Ecker 2008). The percentage of LC-PUFAs in fetal circulation is higher than in the maternal circulation (Innis 2005). This suggests that the placenta is selectively transferring sufficient amount

LC-PUFAs to support the needs of the developing fetus. Due to the lack of enzyme activity such as desaturases and elongases, essential fatty acids (linoleic and α-linolenic acids) cannot be converted to AA or DHA throughout biological pathways in the fetus. The placenta must transfer these fatty acids from the maternal circulation and deliver them to the fetus. This highlights that providing optimal nutrients to the fetus through adequate maternal nutrition is only one aspect of improving fetal growth. Moreover, impaired growth and development in utero affects fetal programming and hence predisposes individuals to cardiovascular, metabolic, and endocrine diseases later in adult life considering the developmental origins of the health and disease (DOHaD) hypothesis (Godfrey & Barker 2000).

1.2 Nutrition and Growth during Postnatal Period for Preterm Newborns

Preterm infants are born during the last trimester of pregnancy, in which optimal nutrition for rapid organ growth and development is needed. Preterm infants need about 20% higher nutritional requirements on a per kilogram basis than term infants. Preterm infants need about 130 kcal/kg/d compared to term infants who require about 90 kcal/kg/d (Agostoni et al. 2010; Hay 2013). In addition, preterm infants need more nutritional intake compared to a fetus since they require more energy for thermogenesis, breathing, organ functions, and physical activity.

Breast milk is recommended for preterm infants due to various health benefits including decreased rates of late-onset sepsis, necrotizing enterocolitis, retinopathy of prematurity and improved neurodevelopmental outcomes (Ashraf et al. 1991; Herrmann & Carroll 2014; Underwood 2013; Anderson et al. 1999). Additionally, preterm infants

who received breast milk have shown long-term benefits in later life demonstrating lower rates of metabolic syndrome, lower blood pressure (Singhal et al. 2001), and lower ratios of cholesterol lipoproteins (Singhal et al. 2004), which is associated with cardiovascular diseases.

In spite of tremendous potential benefits of breast milk, providing full enteral feeds of breast milk to preterm infants is a challenge due to their gastrointestinal immaturity. As a result, parenteral nutrition is needed to bridge the time after birth until the infants are on full enteral feedings. The parenteral nutrition consists of mixtures of macronutrients (glucose, amino acids, and lipids) and micronutrients (vitamins, electrolytes, trace elements, phosphate, and calcium) (Valentine & Puthoff 2007). During the parenteral nutrition infusion, enteral feedings of breast milk are gradually increased in a step-wise manner until preterm infants can tolerate full enteral feedings. Since preterm infants grow about 2.5 times faster than term infants (growth rate: 17 g/kg/d for preterm infants vs 7 g/kg/d for term infants) (Fenton et al. 2013), one concern in solely providing breast milk to preterm infants is growth restriction. They need more concentrated energy than term infants. Due to the fact that preterm infants cannot tolerate double volume of breast milk, breast milk is usually fortified with human milk fortifier to maintain the high growth rate (Kuschel & Harding 2009; Uhing & Das 2009). Fortification of breast milk with human milk fortifier leads to improved growth in weight, length, and head circumference compared to infants fed only breast milk (Kuschel & Harding 2009).

Although survival of preterm infants has significantly improved with the parenteral nutrition, it is crucial to provide optimal nutrition at a critical stage of

development. Total energy intake from fortified breast milk is 130-135 kcal/kg/d, which is recommended for preterm infants from the ESPGHAN guideline (Koletzko et al. 2005). However, total energy intake from the parenteral nutrition is 90-98 kcal/kg/d, which is about 30% less energy intake than the recommendation. It indicates that the parenteral nutrition is unable to meet the full needs of rapidly growing preterm infants. Among macronutrients in the parenteral nutrition, the deviation in energy intake primarily comes from fat. The energy intake from fat is about 60 kcal/kg/d from the fortified breast milk but only 22-27 kcal/kg/d from the parenteral nutrition. The lack of fat intake under the parenteral nutrition contributes to postnatal growth restriction and magnifies detrimental effects on rapid organ growth, especially for brain growth. Moreover, preterm infants have immature digestive systems with low levels of intestinal lipases that reduce fat retention: 50-90% of dietary fat absorbed depends on fat sources used for their feedings (Manson & Weaver 1997).

Overall, optimal nutrition during the neonatal period is critical for promotion of proper physiological growth, body composition, and positive health outcomes after discharge and for later life.

1.3 Lipids

Lipids are one of the main important sources of energy for physiological growth and development for newborn infants. In addition to providing about 50% of the total calories and energy, lipids provide essential fatty acids, which the body cannot endogenously synthesize. The essential PUFAs are important for brain and retina development (Innis 2007).

The major lipid classes are triglycerides (TGs), cholesterols, phospholipids, and free fatty acids. Each lipid has unique functions in the biological system. TGs are the most abundant lipids in animal tissues and are composed of one glycerol backbone and three fatty acids. Their main function is to store energy. Cholesterols are important precursor of hormones, vitamin D, and bile production as well as constituents of cell membranes. Phospholipids are composed of two fatty acids and one glycerol compound. Their functions include adding fluidity in cell membranes, acting as cell signal transducers or second messengers between cells. Lastly, but most importantly, fatty acids are the major energy source yielding large quantities of ATP. Depending on chain length and (un)saturation of fatty acids, some fatty acids have distinct roles. These are further discussed in section 1.4

1.3.1 Lipids in Breast Milk

Breast milk contains an average 4.0 g/dL of lipids but the content and composition of lipids vary depending on maternal diet and the stage of lactation. Lipid content in breast milk increases during lactation from 2.0 g/dL in colostrum to 4.9 g/dL in mature milk in order to adapt to the needs of the growing infants (Manson & Weaver 1997).

The lipids in breast milk are contained within membrane-enclosed milk fat globules. The core of the globules is composed of mainly TGs in 98% of total milk lipids, whereas the globule membrane consists of phospholipids and cholesterol (Jensen et al. 1980). Total lipid content increases gradually from in colostrum to mature milk. However, cholesterol and phospholipids have an opposite trend. They are high in colostrum but decrease in mature milk (Hamosh et al. 1985).

Moreover, breast milk produced for preterm infants has about 30% more lipids than the breast milk for term infants (6.8 ± 0.3 g/dL vs 4.9 ± 0.2 g/dL for preterm and term milk at 8 weeks of lactation) (Bauer & Gerss 2011). The high lipid content is responsible for the high energy density of preterm milk, indicating that preterm infants need high energy to maintain a faster growth rate compared to term infants. However, due to high variations of lipid content in breast milk, some preterm infants still experience suboptimal weight gain and nutritional deficits (Hay 2013).

1.3.2 Lipid Emulsions

Lipid emulsion is one of the components used for the parenteral nutrition providing preterm infants the high energy they need and essential fatty acids such as linoleic and α-linolenic acids that cannot be endogenously synthesized. These essential fatty acids are crucial for brain and eye development and detrimental effects are observed upon deficiency in preterm infants (Valentine & Puthoff 2007).

Commercially available lipid emulsion products are composed of a variety of fat sources (olive, coconut, fish or soybean oils) or combinations of different fat sources (Table 1). Each lipid emulsion includes different classes of fatty acids such as omega-3,6,9 fatty acids and medium chain fatty acids depending on the fat source being used (Table 2).

The 100% soybean oil based lipid emulsion has been widely used for several decades; this is the only lipid emulsion with FDA approval. However, 100% soybean oil contains mainly omega 6 fatty acids. The excess of (n-6) fatty acids can result in pro-inflammatory effects (Schmitz & Ecker 2008; Fleith & Clandinin 2005). Moreover,

preterm infants are often under ventilator support, LC-PUFAs are highly susceptible to oxidation modification leading to lipid peroxidation (Helbock et al. 1993). Other lipid emulsions were investigated using different formulations of lipid sources. Thus, depending on the composition of fatty acids and their respective ratio to each other, different lipid emulsions are selected for different desired therapeutic outcomes.

Lipid emulsions are composed of a vector (glycerol), an active principle (oil source) and emulsifiers (phospholipids) (Ferezou & Bach 1999). The presence of excess artificial emulsifiers in the form of phospholipids in lipid emulsions keeps the emulsion stable during storage and administration (Rochow et al. 2010). Artificial chylomicrons enclose TGs and other lipid soluble substances in the core, which are surrounded by phospholipid layers allowing them to be stable in the blood (Ferezou & Bach 1999). However, the excess phospholipids or emulsifiers can aggregate and form liposomes leading to the sequestering of enzymes such as lipoprotein lipase and causing reduced clearance of TGs (Ferezou & Bach 1999; Rochow et al. 2010). The reduced clearance of TGs was associated with high plasma TG level and accumulation of cholesterol and phospholipids in low-density lipoproteins (Haumont et al. 1989). Haumont *et al* (1989) suggested that lipid emulsions with lower phospholipid content might be preferable for preterm infants who have impaired removal of emulsions in the blood (Haumont et al. 1989).

Table 1. Summary table of five commercially available lipid emulsions in terms of their lipid sources, ratio of (n-6) to (n-3) fatty acids, and composition of fatty acids

Product	Intralipid	ClinOleic	SMOFlipid	Lipidem	Omegaven
Lipid source	100% Soybean oil	80% Olive oil 20% Soybean oil	30% Soybean oil 30% Coconut oil 25% Olive oil 15% Fish oil	50% Coconut oil 40% Soybean oil 10% Fish oil	100% Fish oil
(n-6)/ (n-3) ratio	7:1	9:1	2.5:1	7:1	1:8
Composition	Mainly n-6 fatty acids	Mainly n-9 fatty acids	(n-3), (n-6), (n-9) fatty acids and medium fatty acids	Medium chain fatty acids	Mainly n-3 fatty acids

Table 2. Summary table of main fatty acids in oil sources

	Soybean oil	Olive oil	Coconut oil	Fish oil
Main fatty acids	C16:0, C18:0, C18:1, C18:2, C18:3	C16:0, C18:1, C18:2	C8:0, C10:0	C20:5, C22:6

1.3.3 Lipoprotein Profiles

Lipoproteins are the functional unit of transporting water-insoluble lipids in the blood. There exist several classes of lipoproteins according to their densities. The lightest particles, chylomicrons are formed in the intestine and transport exogenous TGs. Very low density lipoproteins (VLDL) are synthesized in the liver and transport endogenous TGs. Low density lipoproteins (LDL) transport cholesterols in the blood and high density lipoproteins (HDL) scavenge cholesterols from cells and transport it back to the liver.

The level of LDL is used as a part of cardiac risk assessments for adults, which can predict an individual's risk of developing heart diseases. The LDL deposits excess cholesterol in blood vessel walls and contributes to hardening of the arteries and heart diseases. Additionally, an increase of VLDL in the blood can slow down of the conversion rate of VLDL to LDL as well as lead to the accumulation of intermediate particles. This can contribute to the development of atherosclerosis and coronary heart diseases (Sacks et al. 2000). Whether high level of LDL and VLDL during early life affects health outcomes in later life is unknown.

Plasma lipoproteins in newborn infants at birth consist mostly of HDL while VLDL and LDL are present at low concentrations. During the first postnatal week, VLDL, LDL, and HDL increased significantly compared to at birth and this increase persisted up to a month (Biervliet et al. 1986). Furthermore, another literature reported that LDL levels were higher in preterm infants than term infants whereas no significant difference of HDL levels was found in both populations (Ghaemi et al. 2014).

Literature has shown that high TG levels were correlated with high VLDL levels ($r=0.69$, $p<0.001$) (Masarei et al. 1971). This indicates that infants with high TG levels would demonstrate high VLDL compared to infants with normal plasma TG levels. In a case report, markedly high elevation of VLDL was observed as well as significant lower level of lipoprotein lipase (LPL) in a premature boy who developed hypertriglyceridemia (48.1 mmol/L) (Nilsson et al. 1996). The activity of the enzyme LPL is the rate-limiting enzyme for TG removal. Hence hypertriglyceridemia may be caused by a primary deficiency or a secondary decrease of the LPL enzyme (Shimada et al. 1995).

1.4 Fatty Acids

1.4.1 Structure, Nomenclature, and Function of Fatty Acids

Fatty acids are composed of carbon-carbon (C-C) chains with a carboxylic acid group (-COOH) at one end and a methyl group (-CH3) at the other. Fatty acids are named according to the number of carbons, degree of (un)saturations, and position of the first double bond. Medium chain fatty acids (MCFAs) are C6:0 – C12:0 without any double bonds. Long chain saturated and unsaturated fatty acids are C14:0 – C22:0 without and with double bonds, respectively. Long chain polyunsaturated fatty acids (LC-PUFAs) contain more than one double bond including essential fatty acids (linoleic acid and α -linolenic acid).

LC-PUFAs are derived from the essential fatty acids such as α-linolenic acid and linoleic acid. The α-linolenic acid is the parent of (n-3) fatty acids. It can synthesize the eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) through the biosynthetic pathways as demonstrated in Figure 1. However, the conversion rate from the α-linolenic

acid to EPA and DHA is very insufficient for preterm infants so dietary intake of EPA and DHA is recommended (Innis 2007; Koletzko 1999). An important role of (n-3) fatty acids is the anti-inflammatory effects. Another essential fatty acid is linoleic acid, which is the parent of (n-6) fatty acids and has pro-inflammatory effects when there is an excess of (n-6) fatty acids (Schmitz & Ecker 2008). Therefore, the distinct functions of two fatty acid families make the balance between dietary (n-6) and (n-3) fatty acids an important consideration for optimal growth and development.

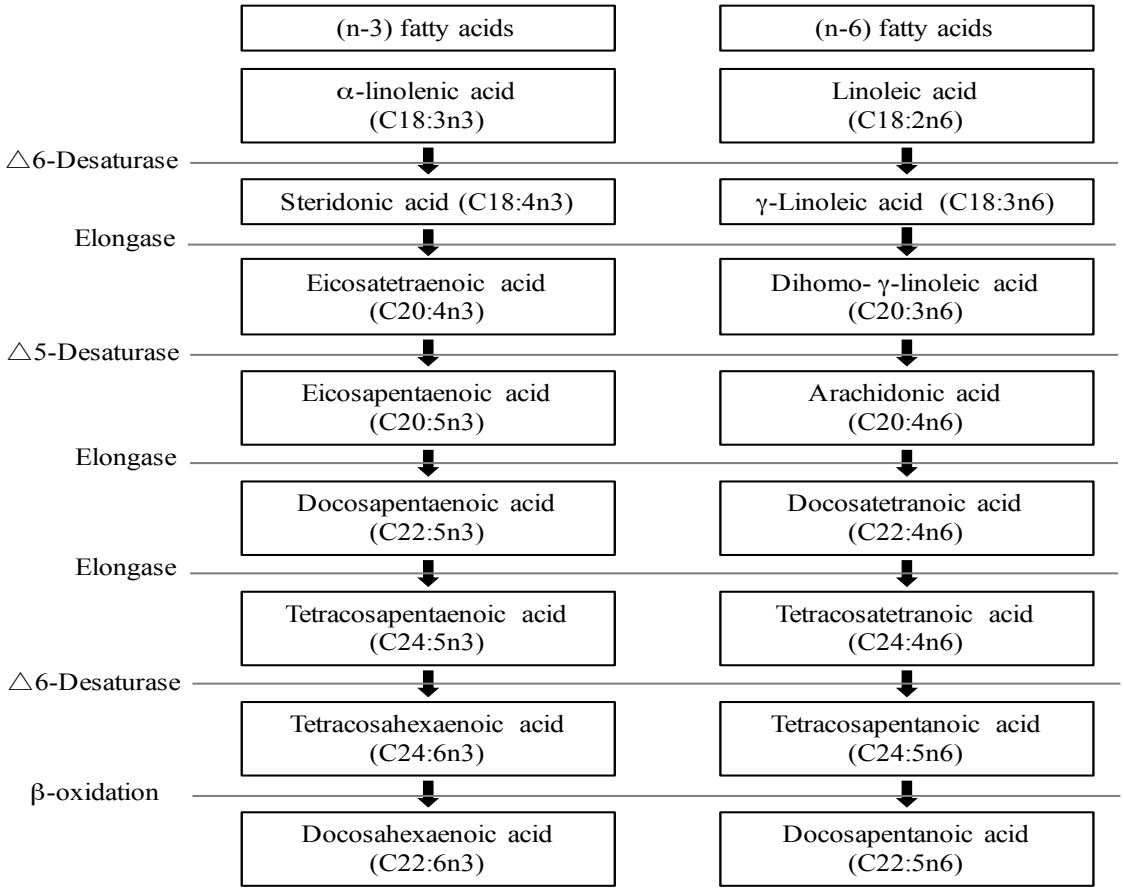

Figure 1. Metabolism of (n-6) and (n-3) fatty acids involving several enzymes of desaturation, elongation, and β-oxidation. This pathway synthesizes more long chain-polyunsaturated fatty acids: adapted from (Schmitz & Ecker 2008)

Fatty acids are an important source of energy but different classes of fatty acids have different functions. The MCFAs can passively diffuse from the gastrointestinal tract to the portal system without any transporter so these can be oxidized and metabolized easily (St-Onge & Jones 2002). Furthermore, the longer the chain length, the more concentrated the energy source but the more difficult the fatty acid is to metabolize. The LCFAs need carnitine to transport fatty acids to the mitochondria membrane for oxidation. However, preterm infants are deficient of carnitine compared to term infants resulting in a decrease in energy production from the LCFAs (Shenai & Borum 1984; Tokuriki et al. 2013). Moreover, as per their nutrition sources, breast milk contains carnitine (Warshaw et al. 1980) but parenteral nutrition is absent of it (Shenai & Borum 1984). When preterm infants received long-term parenteral nutrition, then carnitine supplementation might be valuable (Dahlstrom et al. 1990). However, there was no demonstrated clinical benefit to add carnitine in the parenteral nutrition when infants received short-term parenteral nutrition. It is because breast milk, which is composed of carnitine, is gradually increased as the clinical feeding regime (Van Aerde 2004). Furthermore, the LC-PUFAs have a structural function on cell membranes and central nervous system. They are essential to support growth as well as brain and retina development (Lauritzen et al. 2001; Martin 2014; Innis 2007).

1.4.2 Fatty Acids in Breast Milk

Fatty acids in breast milk have an even number of carbons and varying numbers of double bonds. Sometimes, fatty acids with odd number of carbons are found, which might be derived from maternal dietary intake. However, it does not seem to be of nutritional

importance to the infant (Martysiak-Zurowska 2008). Breast milk contains saturated and monounsaturated fatty acids as well as a full range of (n-3) and (n-6) PUFAs including DHA and AA (Makrides et al. 1995; Much et al. 2013; Hossain et al. 2016). DHA and AA are important fatty acids for brain and retina development. Iranpour *et al* (2013) found that the DHA and AA content in preterm breast milk was higher than in term breast milk but the difference was not significant (Iranpour et al. 2013). This indicates that optimal amounts of fatty acids from dietary sources are required for certain stages of preterm infants since they have very limited ability to synthesize these fatty acids from the primary precursors such as linoleic acid and α-linolenic acid.

Due to the lack of endogenous (n-3) and (n-6) fatty acids, infants must receive these fatty acids from breast milk and accumulate them in adipose tissue. Fatty acids in breast milk are derived from the maternal diet. The differences in maternal (n-3) and (n-6) fatty acids can result in differences in (n-3) and (n-6) fatty acids in human milk (Innis 2014; Koletzko et al. 1992). A high (n-3) fatty acid content has been found in the breast milk of mothers consuming a large proportion of dietary fat from fish and high (n-6) fatty acids in breast milk from ones that had little animal fat (Koletzko et al. 1992).

1.4.3 Fatty Acids in Lipid Emulsions

The commercially available lipid emulsions are formulated with various oil sources, implicating that each lipid emulsion is composed of different classes of fatty acids. The first one is composed of 100% soybean oil-based emulsion, which mainly contains (n-6) fatty acids including essential fatty acids. After its introduction into clinical practice, there were concerns about immune-compromising effects from excess of (n-6)

fatty acids and the increased risk of infections since the liver has a limited ability to metabolize plant sterols from soybean oil (Utermohlen & Tucker 1986; Schmitz & Ecker 2008; Weaver 2014). Administrating 100% soybean oil-based emulsion is associated with significant clinical complications such as hypertension, liver dysfunction, lipid embolism, and bronchopulmonary dysplasia (BPD) (Brans et al. 1986; Sosenko et al. 1993).

To solve these issues from 100% soybean oil-based emulsions, other lipid emulsions were investigated. The MCFAs, which are derived from coconut oil, were introduced into clinical practice in order to reduce the amount of (n-6) fatty acids in a lipid emulsion. Garancho-Montero *et al* (2002) demonstrated greater improvement in retinol binding protein and nitrogen balance in septic patients upon administrating (n-6) fatty acids and MCFAs lipid emulsion (Garnacho-Montero et al. 2002).

Another lipid emulsion was formulated with further reduction of (n-6) fatty acids content and completion with (n-9) fatty acid from olive oil. The studies have evaluated a good short- and long-term tolerability of (n-6) and (n-9) fatty acids and no signs of essential fatty acid deficiency in preterm infants (Munck et al. 1996; Goulet et al. 1999; Göbel et al. 2003; Webb et al. 2008; Deshpande et al. 2009).

The newest lipid emulsion was based on MCFAs and (n-3) fatty acids, derived from fish oil. MCFAs and (n-3) PUFAs containing lipid emulsion had great anti-inflammatory and immunosuppressive effects (Skouroliakou et al. 2010), less retinopathy of prematurity (Beken et al. 2014), decreased BPD, and more favorable lipoprotein profiles (Skouroliakou et al. 2012). It may also have important protective properties for the liver (Muhammed et al. 2012). In randomized, double blinded, and clinical trials in

preterm infants, MCFAs and (n-3) PUFAs containing lipid emulsion was safe and well tolerated (Skouroliakou et al. 2010; Goulet et al. 2010; Tomsits et al. 2010). Preterm infants administrated with MCFAs and (n-3) PUFAs containing lipid emulsion demonstrated a decrease in plasma bilirubin and increase in (n-3) PUFA without changing lipid peroxidation (Goulet et al. 2010).

Certain lipid emulsions may be more beneficial in certain situations: infants on fentanyl infusions (sedatives) cannot receive lipid emulsions containing fish oil due to incompatibilities between the drug and lipid emulsion. The fatty acid characteristics and compatibilities of lipid emulsions are very important factors to minimize complications without compromising nutrition enhancement for preterm infants.

1.5 Lipid metabolism

At birth, the fetus switches an energy supply from carbohydrates to lipids. Hence, efficient lipid metabolism is important for energy utilization and optimal growth.

1.5.1 Digestions of Lipids

The digestive physiology of preterm infants is a little different due to the smaller size of digestive organs as well as the quality and quantity of enzymes and bile, pH values, and gut permeability. Therefore, infant lipid digestion relies on various lipases such as gastric lipase (GL), pancreatic TG lipase (PTL), bile salt-dependent lipase (BSDL), and pancreatic lipase-related protein 2 (PLRP2)After fat ingestion, TGs are hydrolyzed by GL in the stomach. The GL preferentially hydrolyzes fatty acids on the sn-3 position of TGs, indicating functional specificity for certain fatty acids. This resulted in free fatty acids and sn-1,2 diglycerides (Bernback et al. 1989). Moreover, gastric lipolysis

of milk fat is extensive up to 60% due to the optimum gastric pH of the GL in infants (Hamosh et al. 1981; Hamosh et al. 1989). The longer-chain fatty acids are further digested by PTL and BSDL in the duodenum. Digested products are emulsified by bile salts and free fatty acids which are released by the GL to induce binding between the colipase-lipase complex and fat globules (Manson & Weaver 1997). The colipase-dependent lipase hydrolyzes fatty acids on the sn-1,3 position of TGs producing sn-2 monoglycerides and free fatty acids. However, preterm infants have much lower concentrations of the colipase-dependent lipase since their pancreas is not fully developed after birth in addition to having limited bile salt (Hernell & Bläckberg 1994). Furthermore, breast milk contains bile salt-stimulated lipase (BSSL), which is a similar enzyme of pancreatic BSDL (Abrahamse et al. 2012). Preterm infants fed breast milk can hydrolyze TGs using BSSL. The BSSL or BSDL hydrolyzes TGs at all three positions resulting in the formation of free fatty acids and glycerol (Andersson et al. 2011).

1.5.2 Absorption of Lipids

Unsaturated fatty acids are more easily absorbed than saturated fatty acids due to better solubilization by bile salts, greater gastric lipolysis, and preferentially binding to intracellular fatty acid-binding protein (Ling & Weaver 1997; Hamosh et al. 1989). The coefficient of fat absorption (CFA) is modulated by the chain length and degree of unsaturation of fatty acids: CFA decreases with increasing chain-length of fatty acids but increases with a high number of double bonds of fatty acids (Martin 2014). The oleic acid (C18:1) and linoleic acid (C18:2n6) can be about 90% absorbed whereas saturated fatty

acids such as palmitic acid (C16:0) and steric acid (C18:0) are absorbed less (Chappell et al. 1986).

Moreover, absorption of saturated fatty acids depends on the position of the fatty acid (i.e. sn-1,2,3) in TGs (Mattson & Volpenhein 1962). The most predominating saturated fatty acid is C16:0 in breast milk and most of them are present at the sn-2 position of TGs, which is rather well water soluble and is readily absorbed (Sauerwald et al. 2001). However, fatty acids originated from vegetable oils, especially for lipid emulsions, are largely presented at the sn-1,3 positions of TGs, which can be cleaved easily by GL and less well absorbed (Straarup et al. 2006). Thus, as much as 30% of dietary lipids depending on various degrees of malabsorption and type of feeds may be excreted in the stool of preterm infants (Hernell & Bläckberg 1994).

The medium chain TGs containing MCFAs are highly water soluble and readily absorbed without the presence of large quantity of PTL and BSSL (Hamosh et al. 1989). The potential benefit of utilizing MCFAs rather than LCFAs is a rapid process for energy absorption in the sense that portal venous transport of the albumin-bound MCFAs to the liver and carnitine-independent transport into the mitochondria are included. However, energy content of MCFAs is about 16% lower than that of LCFAs. The LCFAs contain high energy but need carnitine transport into the mitochondria and subsequent oxidation. Thus, it is interesting to note that degrees of lipid absorption can be explained by the amount and composition of lipids given along with large inter-individual differences in the capacity to utilize dietary lipids.

1.6 Metabolic Disposition of Lipid Emulsions

1.6.1 High Triglycerides in Blood

With intravenous lipid administration, high plasma TGs have been observed in preterm infants. The clinical studies have shown that preterm infants who received intravenous lipid emulsion about 1.0-3.0 g/kg/d had plasma TG levels of 0.9-2.4 mmol/L (Andrew et al. 1976; Cooke & Burckhart 1983; Cooke et al. 1987; Haumont et al. 1989). However, preterm infants who were fed breast milk up to 7g/kg/d showed their plasma TG levels of 0.6-1.6 mmol/L (Greer et al. 1987; Tseng et al. 1990; Decsi et al. 1993; Rochow et al. 2010). Moreover, a study reported that preterm infants receiving 1.4 g/kg/d of parenteral lipid emulsion showed plasma TG levels of greater than 3.0 mmol/L (Adamkin & Gelke 1984). The fat intake from breast milk was more than double the amount than that of lipid emulsions. However, the plasma TG levels were higher in preterm infants who received lipid emulsions. An intake of 5-7 g/kg/d of fat would be desirable to meet nutrition needs for preterm infants, but this is often unachievable due to high TGs present in blood. Moreover, preterm infants grow 2.5 times faster than term infants so they are in need of concentrated energy in order to achieve proper growth and development at this critical stage in life. Low fat intake from lipid emulsion administration might cause cumulative caloric deficit and growth restriction.

High TG levels in blood are an indicator of fat intolerance. To minimize undesirable consequences, the TG cut-off value is generally set to be 1.7 mmol/L for preterm infants (Barness et al. 1981). An excess of 1.7 mmol/L (i.e. 150 mg/dL) of TG levels in blood results in saturation of LPL activity and decreased clearance of the emulsion (Andrew et

al. 1976). Initially, this value was derived from the reference TG value for adults since high TGs are often an indicator of increased cardiovascular risk later in adult life according to the American Heart Association guidelines (Smith et al. 2006).

The intolerance of lipid emulsion suggests that currently available lipid emulsions might not be an optimal source of nutrition for preterm infants (Haumont et al. 1989; Rochow et al. 2010). It has been speculated that this might be because of the fatty acid composition within TGs in lipid emulsions which can influence metabolism. Furthermore, High TG levels in plasma appear to be acute since TG levels return to normal when lipid administration is halted or infusion rate is reduced. Therefore, this may not present a serious short-term problem but long-term effects of high TG levels during neonatal life are unknown.

1.6.2 Possible Adverse Effects of Lipid Emulsion Administration

One of the possible adverse effects on administration of lipid emulsions especially in premature infants is chronic lung disease. A study performed by Sosenko *et al* (1993) suggested that early administration of lipid emulsions to premature infants (<800g) at birth increases mortality rate and the risk of pulmonary hemorrhage (Sosenko et al. 1993). Another study also found an increased risk of chronic lung disease (Hammerman & Aramburo 1988), whereas other studies showed no increase in the risk of respiratory impairment (Alwaidh et al. 1996).

With adequate metabolism, infused lipid emulsions can result in an increase in free fatty acids which compete with free bilirubin for albumin binding sites (Barness et al. 1981). A high free fatty acid to albumin ratio may be associated with an increased risk of

hyperbilirubinemia. However, this has not yet been demonstrated to have a significant effect on hyperbilirubinemia in preterm infants (Barness et al. 1981). Moreover, liver dysfunction was associated with lipid emulsion infusion in newborn infants as well as lipid emulsion related cholestasis (Toce & Keenan 1995; Colomb et al. 2000).

Although some possible adverse effects of lipid emulsion infusion are still debated, the benefits of intravenous lipid administration in premature infants outweigh the potential risks, suggesting that TG levels should be monitored regularly and infusion rate should be adjusted if it is necessary.

1.7 Project Rationale

From the preliminary data from the **F**atty **A**cid Profiles in **N**utrition **S**ources for preterm infants (**FANS**) study, relative profiles of fatty acids in commercially available lipid emulsions do not match with relative profiles of fatty acids in breast milk. The unnatural profiles of fatty acids in commercially available lipid emulsions might create a situation where an excess amount of one fatty acid prevents proper hydrolysis of other fatty acids from TGs and/or fatty acid uptake. In addition, relative quantification can bias results because changes in the amount of one fatty acid influence the relative amount of the other fatty acids. This makes it difficult to truly understand fatty acid patterns and fluxes in the condition of hypertriglyceridemia. This can also result in the misinterpretation of biochemical diagnoses including nutritional and metabolic disorders (Hon et al. 2012; Lagerstedt et al. 2001; Abdelmagid et al. 2015). Therefore, absolute quantification of fatty acids in lipid emulsions and breast milk rather than showing the relative percentage in fatty acid profiles allows for comprehension of the optimal amount

for each fatty acid in lipid emulsions. For preterm infants, comparing the absolute amount of fatty acids in nutrition sources (i.e. breast milk, lipid emulsions, human milk fortifier) to the absolute amount of fatty acids in their plasma may elucidate a mechanism, which contributes to lipid metabolism.

Furthermore, artificial chylomicrons in lipid emulsions might be the one possible contributor to adverse effects of lipid emulsion used for preterm infants. The excess of artificial chylomicrons in lipid emulsions might lead to the reduced TG clearance in blood. This investigation would lead to the identification of related issues and would help to improve in currently available lipid emulsions. It has great potential to increase the efficiency in TG delivery to preterm infants without the associated risks of hypertriglyceridemia or liver damage.

1.7.1 Study Objective 1 and Hypothesis

The first objective was to optimize and validate the gas chromatography-mass spectrometry method for absolute quantification. I hypothesized that the absolute quantification of fatty acids is a more accurate measurement than relative quantification of fatty acids.

1.7.2 Study Objective 2 and Hypothesis

The second objective was to quantify the absolute amount of fatty acids in breast milk and commercially available lipid emulsions. I hypothesized that fatty acid profiles in commercially available lipid emulsions differ from fatty acid profiles in breast milk.

1.7.3 Study Objective 3 and Hypothesis

The third objective was to determine how plasma fatty acid profiles of infants received lipid emulsions differs from infants who were fed breast milk. I hypothesized that plasma fatty acid profiles are associated with recent dietary fatty acid intake.

1.7.4 Study Objective 4 and Hypothesis

The fourth objective was to evaluate whether certain fatty acids or lipoproteins were consistently elevated in conditions of hypertriglyceridemia in preterm infants. I hypothesized that certain fatty acids or lipoproteins are consistently elevated in hypertriglyceridemic plasma samples from preterm infants.

CHAPTER 2. Study Design and Methods

2.1 Study Design

2.1.1 FANS study

This thesis project was based on the Fatty Acid Profiles in Nutrition Sources for preterm infants (FANS) study. The FANS study is a multi-center, observational, and prospective study conducted at McMaster Children's Hospital in the Department of Pediatrics, Division of Neonatology (Hamilton, Canada). Other collaborators are the University of Greifswald (Greifswald, Germany), Beth Israel Deaconess Medical Center (Boston, USA), and Coastal Carolina Neonatology (Wilmington, USA). This study was approved by the Hamilton Integrated Research Ethics Board (Project 12-220). All newborn infants in Neonatal Intensive Care Unit (NICU) were eligible for this study except infants with sepsis development at the time of recruitment.

2.1.2 Clinical Procedures

The study coordinator recruited participants in the NICU. Once consent was obtained from their parents, a standing order with sample collection information was placed in the bedside binder and signed by a nurse practitioner or physician. About 200 μL of blood was collected for each infant on clinical routine blood collection days (Mondays) since this request for extra blood did not pose any additional risk to the infants. The team of physicians and nurses decided whether it would be favourable to draw extra blood based on the infant's condition. About 2 mL of breast milk, one day prior to the blood collection, was collected to represent recent dietary fatty acid intake for infants. The NICUs in McMaster University and University of Greifswald use the

ClinOleic and SMOFlipid. Lipids emulsions were not procured regularly since fatty acid content in commercial lipid emulsions are constant. After blood samples were collected in the NICU, plasma was immediately isolated from the blood in the laboratory: blood was centrifuged at 3000 rpm at 5 °C for 15 minutes. Fat content of breast milk samples was analyzed using a validated milk analyzer (Unity Scientific, Brookfield, USA). Collected plasma and breast milk samples were stored in -80 °C freezer in the laboratory. Nutritional (total daily volume of enteral and/or parenteral feeds), demographic (sex, gestational age, days of life, date of birth), anthropometric (birth weight, weight at time of sample collection), and clinical information (TG levels) were recorded on the day of blood collection and one day prior to the blood collection for infants from the charts or Sovera (Health information management system). The summary of clinical procedures is presented in Figure 2.

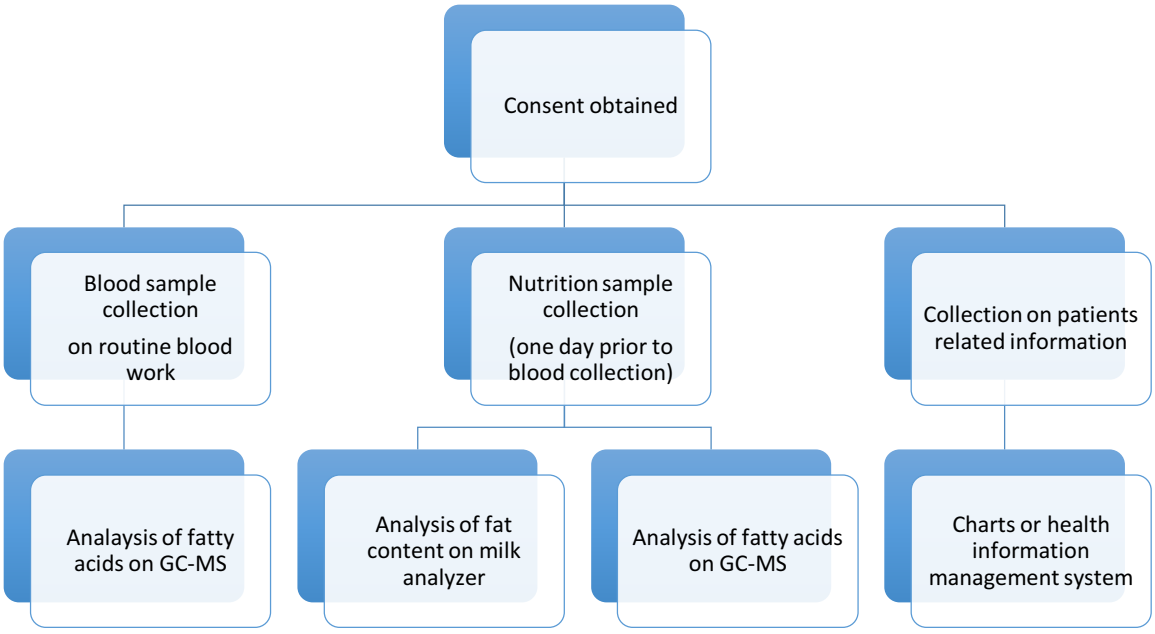

Figure 2. Schematic summary of clinical procedures for the FANS study

2.1.3 Collection of Hypertriglyceridemic Plasma Samples

In order to investigate fatty acid profiles in hypertriglyceridemic plasma, leftover hypertriglyceridemic samples were collected from Hamilton Regional Laboratory Medicine Program without consenting. It was because hypertriglyceridemia patients could not be identified proactively prior to the fat analysis in blood as well as leftover samples would normally be discarded. Collected plasma samples were stored in -80°C freezer in the laboratory. Patient related information on nutrition, demography, and anthropometry was collected.

2.1.4 Nutritional Grouping for Plasma Samples

The recent nutrition (one day prior to blood collection) can affect patterns and amounts of fatty acids in plasma. Comparing fatty acid composition in plasma from infants fed 100% breast milk (BM) or 100% lipid emulsions (ClinOleic and SMOFlipid) would be ideal without compromising nutrition as one of confounders. However, due to the nature of clinical feeding regimes, parenteral nutrition is introduced after birth and infusion rate of parenteral nutrition is gradually decreased with respect to a stepwise increase feeding volume of BM. When infants can tolerate full BM, then BM is fortified with human milk fortifier to meet their caloric intake. Therefore, when infants fed mixed nutrition, it was difficult to differentiate which fatty acids and how much of these came from which nutrition since some of fatty acid composition from the nutrition sources overlap.

Breast milk is a gold standard nutrition for newborn infants. Comparison of fatty acid profiles in plasma from infants fed 100% BM or >70% BM (i.e. greater than 70% of

nutrition coming from BM and the rest coming from other nutritional sources such as lipid emulsion, human milk fortifier, formula) demonstrated no statistically significant difference to each other. This indicated that the cut-off value for main nutrition (BM, ClinOleic, and SMOFlipid) can be assigned to 70%. This led an increase of sample size for each nutritional category. The calculation for the nutritional distributions (as a percentage) was performed after converting each nutrition intake to a unit of g/kg/d. For example, if an infant with a weight of 1300g (at the time of blood collection) was fed 2 mL of breast milk and 4.8 mL of SMOFlipid during 24 hours (one day before blood collection). Then, intake of breast milk was 0.06 g/kg/d (i.e. 2 mL/100 mL* 4.0 g/1.3kg =0.06 g/kg/d, note: fat content in breast milk was 4.0 g/dL) and intake of SMOFlipid was 0.74 g/kg/d (i.e. 4.8 mL/5/1.3kg = 0.74g/kg/d, note: volume of SMOFlipid is divided by 5 since SMOF lipid contains 20g of fat per 100 mL). The percentage of SMOFlipid is 0.74/ (0.06+0.74)*100% = 93%. The nutrition distribution for this particular plasma was 93% SMOFlipid and 7% BM, meaning that fatty acids in plasma came mostly from the SMOFlipid. This plasma belonged to a nutritional category of >70% SMOFlipid. Seventy percent is an arbitrary number to set as nutritional cut-off value to increase sample size in each nutritional category without any statistical significant difference in fatty acid composition. However, whether seventy percent as a cut-off value is clinically relevant is unknown.

2.2 Method 1: Gas Chromatography – Mass Spectrometry (GC-MS)

The plasma and nutritional samples were analyzed using gas chromatograph (Agilent 7890, Agilent Technologies; Santa Clara, California, USA) fitted with an

autosampler (Agilent 7693) and a single quad mass detector (Agilent 5975 MSD). Chromatographic separations were performed on a Supelco fused silica capillary column, 100m x 0.25mm x 0.2 μm film thickness (Supelco 1L111 GC column, Sigma-Aldrich; Bellefonte, Pennsylvania, USA). The oven temperature was 130 °C for 10 minutes, then gradually ramped up 2 °C/min to 240 °C held for 2 minutes. Helium was used as a carrier gas at 1.2 mL/min. Auxiliary column temperature was maintained at 250 °C. Three pre- and post-washes of the syringe with hexane were conducted before and after injections. The injection volume was 0.1 μL with a splitless mode. Total run time for a sample was 61 minutes.

2.2.1 Extraction and Esterification of Fatty Acids from Plasma

To extract fatty acids in plasma, 5 μL potassium oxalate solution (3.5 % potassium oxalate monohydrate in water), 50 μL pure ethanol, 50 μL pure tert-butyl methyl ether (TBME), 100 μL petroleum ether and 10 μL internal stock solution (Stock solution of internal standard containing C19 ethyl ester was prepared in hexane: 4.07 mg/mL) were added to 100 μL plasma. The mixture was vortexed for 2 minutes, then centrifuged for 5 minutes at 14000 rpm (Centrifuge 5424, Eppenndorf; Hamburg, Germany). The organic layer containing the fatty acids was extracted. For a second extraction, 50 μL TBME and 100 μL petroleum ether were added to the aqueous layer. The combined organic layer was dried under nitrogen gas. To transesterify the fatty acids into fatty acids methyl esters (FAMEs), the dried organic layer was reconstituted with 500 μL methanolic sodium hydroxide (0.125 g sodium hydroxide/50 mL methanol) and vortexed for 2 minutes. The mixture was heated to 60 °C (Reacti- Therm III heating

module; Rockford, Illinois, USA) for 15 minutes for the derivatization of fatty acids to FAMEs. After completion of the heating phase, 100 μL hexane, 100 μL water, and 100 μL butylated hydroxytoluene (BHT) solution (1 mg BHT/10 mL hexane) were added to the reaction mixture and then vortexed for 2 minutes. The FAMEs from the organic phase was extracted. A second extraction was performed adding 100 μL BHT solution into the reaction mixture to maximize the FAMEs extraction. Extracted sample was injected into the GC-MS device.

2.2.2 Extraction and Esterification of Fatty Acids from Nutritional Sources

To draw representative sample (100 μL) from the pool of breast milk and lipid emulsions, collected nutritional samples were homogenized for fifteen seconds using an ultrasonic vibrator (VCX 130; Sonics and materials Inc.; Newtown, Connecticut, USA). Breast milk and lipid emulsion samples (100 μL each) were diluted by a factor of ten with water since breast milk contains ten times the concentration of fat found in plasma. The same as above described extraction and transesterification method was used for breast milk and lipid emulsion samples with one modification of 25 minutes derivatization time instead of 15 minutes to ensure a complete chemical reaction.

2.2.3 Data Acquisition and Quantification

The FAMEs were identified by retention times and mass fragmentation patterns using GCMSD Data Analysis software (Enhanced ChemStation E. 02.02.1431, Agilent Technologies Inc.; Santa Clara, California, USA) (Table 9, Appendix A). The amounts of FAMEs in the samples were expressed as relative and absolute amounts. The relative quantification was calculated as:

$$Fatty\ acid\ percentage\ (\%) = \frac{Peak\ area\ of\ individual\ fatty\ acid}{Sum\ of\ peak\ areas\ of\ all\ fatty\ acids} \times 100\ \%$$

For the absolute quantification (unit: mg/dL), the concentrations of fatty acids were calculated relative to the concentration of the internal standard (C19:0 fatty acid):

$$Fatty\ acid\ concentraction\ \left(\frac{mg}{dL}\right) = \frac{peak\ area\ of\ individual\ fatty\ acid}{peak\ area\ of\ internal\ standard} \times$$

$$concentration\ of\ the\ internal\ standard\ \left(i.e. 36.98\frac{mg}{dL}\right)$$

All FAMEs were referenced using the same internal standard.

2.2.4 Validation Experiments

As an internal validation, intra-day repeatability was determined by analyzing 10 replicates of one plasma and one breast milk sample, whereas inter-day reproducibility was measured from the same plasma and breast milk sample run over 10 non-consecutive days. The average coefficient of variation (CV) for fatty acids was calculated. Recovery rates were calculated by adding known concentrations of fatty acid compounds of C16:0, C18:0, C18:1, and C18:2 (Nu-Check-Prep; Elysian, Minnesota, USA) to plasma and breast milk samples. Absolute recovery was indicated by a ratio of the observed value to the corresponding expected value. The limit of detection (LOD) and limit of quantification (LOQ) were set to be the lowest concentration with a signal-to-noise ratio of 3:1 for LOD and 10:1 for LOQ. The method was validated using two commercially

available human plasma standards and these samples were used as a quality control (sample #1: standard reference material 1950, National institute of standards and technology (NIST); Gaithersburg, Maryland, USA & sample #2: Human Plasma Quality Control, Bio Rad; Irvine, California, USA).

As an external validation, plasma samples (n=10) were sent to the Lipid Analytical Laboratory at University of Guelph (Guelph, Ontario, Canada) to validate the absolute quantification method by comparing the results. The Lipid Analytical Laboratory used the Folch method to extract total lipids, and the gas chromatography – flame ionization detector to quantify fatty acids with a lab proprietary method. We recalibrated our method according to the reference laboratory using a correction factor. The method was revalidated with the NIST standard.

2.3 Method 2: 1D Gel Electrophoresis

One-dimension gel electrophoresis separates lipoproteins (HDL, LDL, VLDL, Chylomicrons) based on their size and charge. To investigate which lipoprotein is elevated in hypertriglyceridemic plasma compared to normotriglyceridemic plasma, plasma samples were analyzed on 1D gel electrophoresis (Hydragel Lipo + Lp (a) K20, Sebia, France). Plasma sample (10 μL) was loaded onto an applicator for 2 minutes and the applicator contacted on a gel for 7.5 minutes. Lipoproteins on the gel were separated by SEBIA K20 chamber for 90 minutes (settings: voltage- 60V, current-22mA, and power-60W) and dried the gel for 45 minutes. The gel was stained with Sudan Black for 15 minutes and was destained and dried. The laboratory scanner (MFC-7840W, Brother Industries, Japan) was used to scan the gel. The Image J (version 1.48; Wayne Rasband,

National institutes of health, USA) was used for relative quantification of each lipoprotein. Commercially available human plasma standard (Human Plasma Standard, Bio Rad; Irvine, California, USA) was used as a quality control sample. For relative quantification of lipoproteins, each sample was normalized to the quality control sample within a gel. To compare levels of lipoproteins between gels, the quality control samples between the gels were normalized to the quality control sample in the one designated gel.

2.3.1 Validation Experiment on 1D Gel

The instruction protocol for 1D gel electrophoresis recommended using fresh serum samples. It is because fibrinogen from plasma might affect the interpretation of results. Moreover, VLDL might be degraded and might migrate together with LDL due to storage effect. However, the collected samples were frozen plasma samples. Thus, the experiments were conducted to compare lipoprotein levels between fresh serum (n=10) and frozen plasma samples (n=10).

2.4 Fat Content Measurement in Breast Milk using a Milk Analyzer

The fat content in BM samples can be assumed to be 4.0 g/dL. However, the fat in BM between mothers of infants and between feeds is highly variable (Weber 2001), indicating that more fatty acids would be observed with higher fat level. The Fat content was measured using a near-infrared milk analyzer (SpectraStar; Unity Scientific, Brookfield, Connecticut, USA). Prior to measurements, breast milk samples were homogenized to have representative sample preventing from any fat aggregation using a sonicator for 30 seconds (VCX 130: Chemical Instruments AB, Sollentuna, Sweden). The

fat values were adjusted according to the internal validation protocol of the milk analyzer (Fusch 2010).

2.5 Statistical Analysis

Statistical analysis was performed using SPSS (version 21.0; SPSS Inc., Chicago, IL, USA), Microsoft Excel 2010 (Microsoft Co.; Redmond, Washington, USA), and PPCOR program for a partial correlation (R version 3.2.2; Free Software Foundation Inc., Boston, MA, USA). Descriptive statistics were reported the mean, standard deviation, median, minimum and maximum for all data. An independent two-tailed t-test was performed in order to compare means on fatty acid profiles and lipoprotein profiles between normo-and hypertriglyceridemic plasma samples. One-way analysis of variance (ANOVA) was performed to determine whether there were any significant fatty acid differences in plasma from infants fed different nutrition (BM vs ClinOleic vs SMOFlipids). If there was a significant difference, a post hoc multiple comparison using Tukey's HSD test was performed. For the matched pair analysis of dietary fatty acid intake and plasma fatty acid concentrations, a partial correlation was applied.

Results were displayed as bar graphs comparing fatty acid profiles expressed as absolute amount (mg/dL) or percentage (%) across the groups were studied. Fatty acids in the bar graphs were presented as mean and standard deviation. Statistical significance was assigned to *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

2.5.1 Sample Size

The sample size calculation in this study was based on the data from the previous FANS study, which analyzed relative amounts of fatty acids in plasma from infants fed

breast milk and Intralipid. Among fatty acids found in plasma and nutritional sources, the C18:0 was chosen to calculate sample size since it was one of the predominant fatty acids. The sample size calculation was performed using the application provided by the website (http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html). The confidence interval was defined to be 95% (alpha error) and power to be 80%. Further, a normal distribution was assumed for the sample size calculation. The third objective of the study was to determine how plasma fatty acid profiles of infants received lipid emulsions differed from infants fed breast milk. From the previous FANS data, the mean of C18:0 in plasma samples from infants fed breast milk was 24.4 ± 14.1 (%) (n=31) and the mean of C18:0 in plasma samples from infants administrated Intralipid emulsion was 17.9 ± 14.0 (%) (n=15). The average standard deviation was 14.1. The sample size calculated for the third objective was 75 participants for each group. The fourth objective of the study was to evaluate whether certain fatty acids or lipoproteins were consistently elevated in the condition of hypertriglyceridemia in preterm infants. The previous data showed that the mean C18:0 in normotriglyceridemic plasma was 19.7 ± 13.8 (%) (n=113) and the mean of C18:0 in hypertriglyceridemic plasma was 13.77 ± 5.99 (%) (n=22). The average standard deviation was 12.9. The sample size for the fourth objective was 75 participants for each group.

CHAPTER 3. Results

3.1 Study Sample Demographics

A total of 136 infants were included in the study with a sex distribution of 79 males and 56 females. The average birth weight was 1579 g with a range from 510 g to 4090 g and average gestational age was 30.7 weeks with a range from 23.3 weeks to 41.0 weeks. In total 294 plasma and 112 breast milk samples were collected from 136 infants. Further, of this sample, 206 had corresponding plasma TG level: normal TGs (< 1.50 mmol/L; n=116) and high TGs (≥ 1.50 mmol/L; n=88). The remainder of samples did not have triglyceride values within ± 7 days of blood collection. Moreover, of total 294 plasma samples, 214 had corresponding nutrition of >70% BM (n=118), SMOFlipid (n=41), and ClinOleic (n=55) one day prior to blood collection. Others were excluded from the sub-analysis since nutrition was mixed without having one primary nutrition as well as contained various of other nutrition products. Detailed participant characteristics for samples in total and subgroups are summarized in Table 3.

Each sample was treated as an independent sample in spite of the fact that occasionally more than one sample came from one participant. It was because the study was the pilot and exploratory to investigate fatty acid profiles in nutrition and resulting plasma.

Table 3. Participant characteristics at birth and at study

Sample size	All (n=294)	TG level		Nutrition		
		Normal TG (n=116)	Hyper TG (n=88)	>70% Breast milk (n=118)	>70% SMOFlipid (n=41)	>70% ClinOleic (n=55)
Infants (n)	Preterm:113, Term:23	Preterm:32, Term:7	Preterm:56, Term:11	Preterm:28, Term:2	Preterm:18, Term:3	Preterm:29, Term:13
Sex (n)	M: 79, F:56	M:19, F:20	M:41, F:25	M:17, F:12	M:10, F:10	M:22, F:20
Birth weight (g)	1345 (510, 4090)	1610 (590,3360)	885 (510,4090)	915 (516,3860)	1120 (590,3860)	1615 (610,4090)
GA (weeks)	30.1 (23.3, 41.0)	31.4 (24.0,40.4)	27.3 (23.3,41.0)	28.4 (23.3,41.0)	28.6 (23.9,41.0)	33.9 (23.6,41.0)
DOL at study (day)	16 (2, 153)	23 (3,153)	14 (3,137)	28 (5,137)	16 (3,137)	8 (2,139)
Weight at study (g)	1830 (540, 5150)	2060 (600,4400)	1080 (540,5150)	1716 (630,5150)	2519 (580,5150)	1920 (590,4460)
Fat intake (g/kg/d)	4.4 ± 2.5	4.7 ± 2.9	3.5 ± 2.3	6.0 ± 2.0	2.7 ± 1.7	1.8 ± 0.9
TG level (mmol/L)	1.7 ± 0.9	0.9 ± 0.7	2.1 ± 0.7	1.8 ± 0.8	1.2 ± 0.9	1.8 ± 0.8

Data are shown as median (minimum, maximum) and mean ± SD. GA: gestational age, DOL: days of life, TG: triglycerides, M: male, F: female

3.2 Results on GC-MS

3.2.1 Validation on GC-MS

For the method validation on absolute quantification of plasma, the coefficients of variation (CVs) of intra-day (n=10) and inter-day (n=10) ranged from 8% to 13% and from 4% to 20%, respectively. Recovery rates of fatty acids on plasma ranged from 87% to 102%. For breast milk, the CV for the intra-day (n=10) and inter-day (n=10) ranged from 7% to 15% and from 2% to 27%, respectively. Recovery rates of fatty acids on breast milk ranged from 88 % to 116%. The lowest limit of detection and quantification were 42 μg/dL and 0.14 mg/dL respectively. For the accuracy and precision of the developed GC-MS method, fatty acid measurements from our laboratory were plotted against fatty acid measurements from the reference laboratory. The mean regression equation indicated $y=1.01x + 0.01$ with $r^2=0.97$ using relative quantification and $y=1x$ with $r^2=0.90$ using absolute quantification, where x was the reference value and y was measured value (Figure 3). Sixteen fatty acids were identified and quantified by GC-MS analysis and they are common fatty acids in nutrition, which have a role in energy intake. Identification of fatty acids by the retention time and molecular weight is presented in Table 9 (Appendix A).

A. Relative quantification B. Absolute quantification

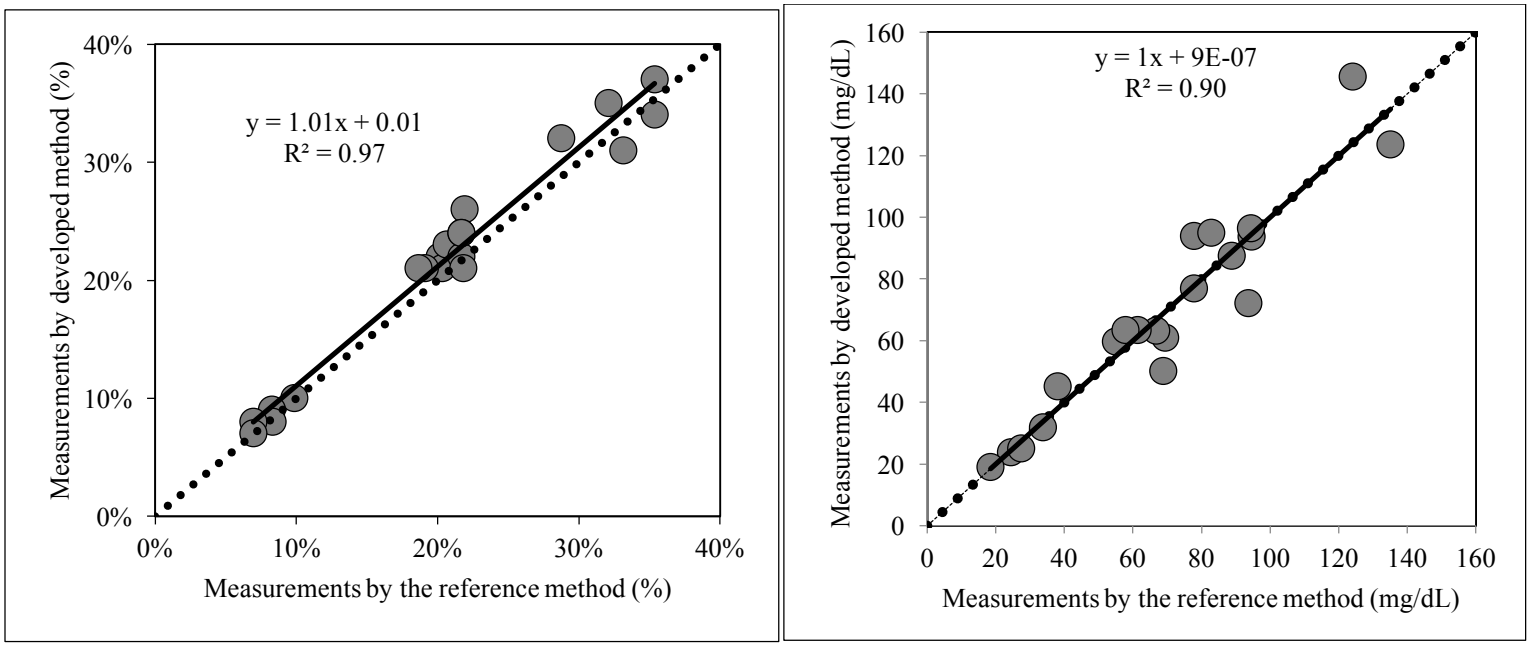

Figure 3. Method validation on relative (A) and absolute (B) quantification of fatty acids; comparison of fatty acid measurements by the reference method (x-axis) and developed method (y-axis).

3.2.2 Fatty Acid Profiles in Breast Milk and Human Milk Fortifier

Breast milk samples (n=112) collected from 34 local mothers were composed of MCFAs, MUFAs, and a full range of PUFAs in Figure 4. Essential fatty acids (C18:2n6 and C18:3n3), small quantity of DHA (C22:6n3), and AA (C20:4n6) were presented in breast milk. The fatty acid profiles in breast milk with relative and absolute amounts are presented in Table 4. In breast milk, the (n-6) fatty acids were 317.3 mg/dL (51.9, 1038.6) [median (min, max)] and the (n-3) fatty acids were 47.3 mg/dL (0.0, 134.0). The average ratio of (n-6) to (n-3) fatty acids on breast milk from local mothers was 6.8:1.

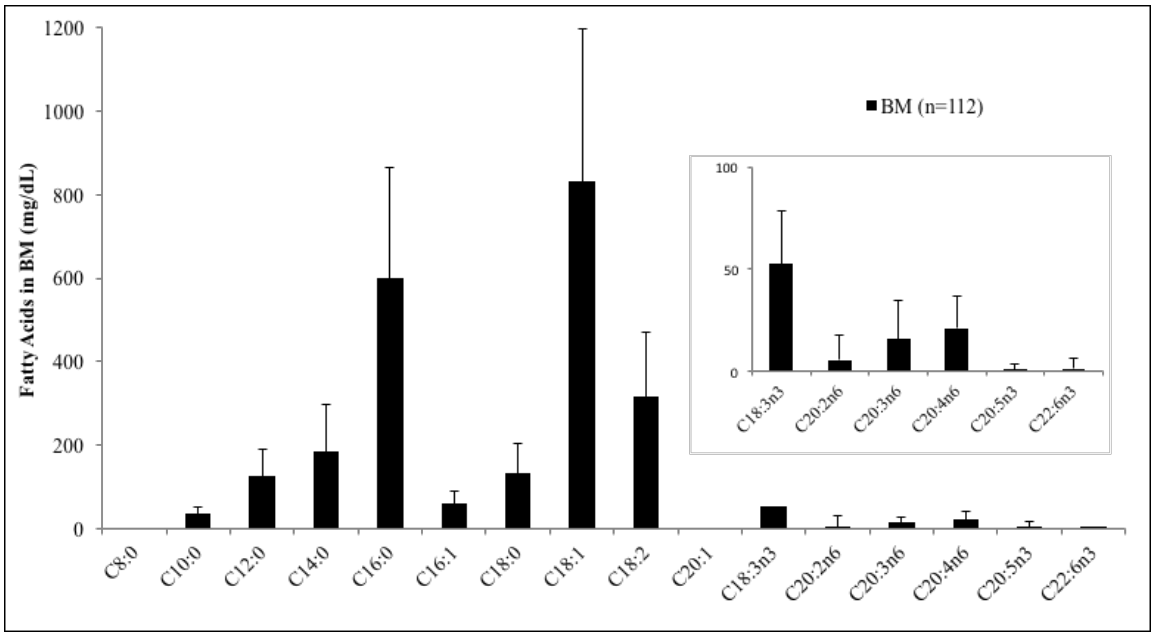

Figure 4. Fatty acid profiles in breast milk (n=112) collected from 34 mothers. Data is presented as mean ± SD. Amounts of long chain polyunsaturated fatty acids (C18:3n3-C22:6n3) are very small: this region is zoomed in the same figure at a free place.

Human milk fortifier (n=10) consisted of MCFAs (C8:0 and C10), MUFA (C18:1), some PUFAs including essential fatty acids in Figure 5. However, no LC-PUFAs were observed in the human milk fortifier. The human milk fortifier compensates with other fatty acids (MCFAs), which are either present very little or are not present in breast milk.

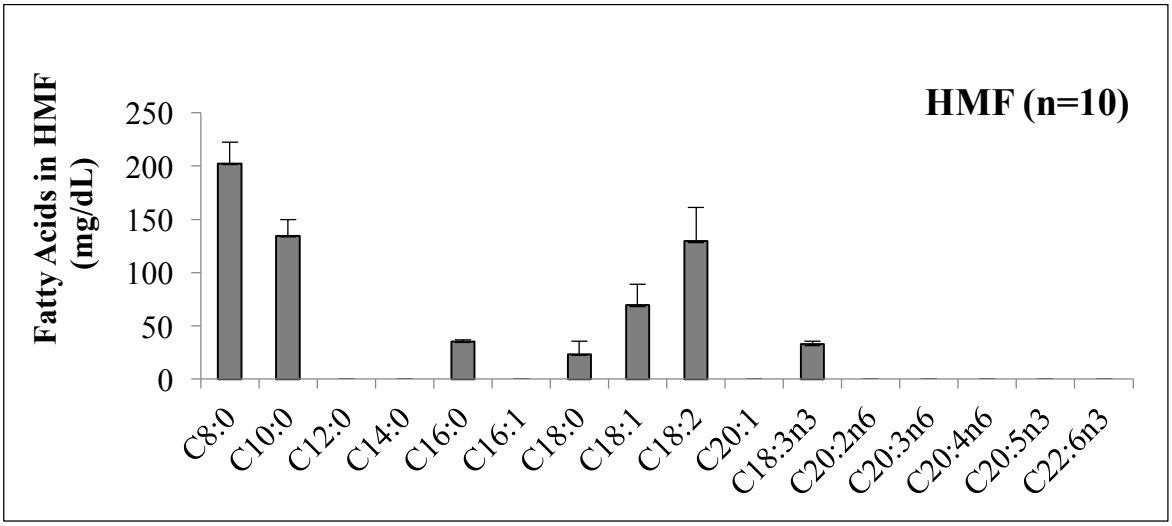

Figure 5. Fatty acid profiles in human milk fortifier (HMF; n=10), which breast milk is fortified with in the McMaster NICU. HMF is Enfamil® Human Milk Fortifier Powder (Mead Johnson Nutrition, Glenview, USA). Data is presented as mean ± SD.

3.2.3 Fatty Acid Profiles in Lipid Emulsions

The fatty acid composition in commercially available lipid emulsions (SMOFlipid, ClinOleic, Lipidem, Intralipid, and Omegaven) is shown in Figure 6. The fatty acid profiles of the lipid emulsions did not resemble each other. The SMOFlipid and Lipidem contained the highest proportion of MCFAs (C8:0 and C10:0) but the ClinOleic, Lipidem, and Omegaven did not contain MCFAs (C8:0, C10:0, C12:0). The ClinOleic consisted of primarily MUFA (C18:1n9) but no DHA and AA. The Intralipid consisted of primarily PUFA (C18:2n6) but little to no DHA and AA. The fatty acid profiles in lipid emulsions with relative and absolute amounts are presented in Table 4. The ratio of (n-6) to (n-3) fatty acids was calculated for each lipid emulsion product as follows: SMOFlipid (2.7:1), ClinOleic (9.7:1), Lipidem (2.7:1), Intralipid (7.8:1), and Omegaven (0.1:1).

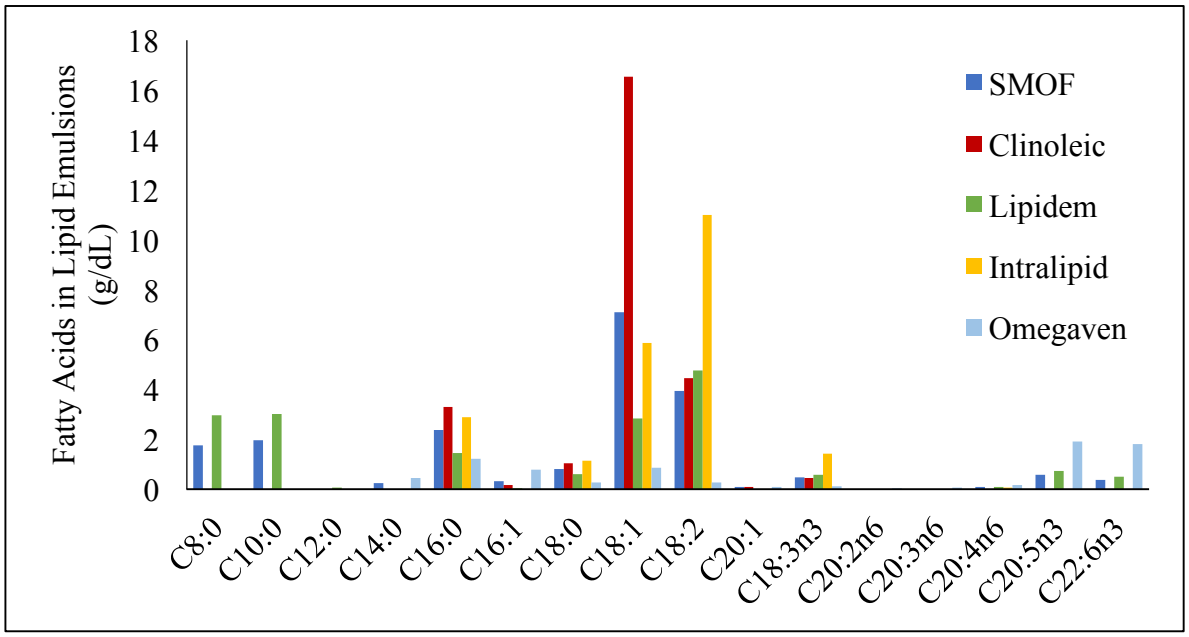

Figure 6. Fatty acid profiles in commercially available lipid emulsions. Data is presented as a mean value.

3.2.4 Comparison between Breast Milk and Lipid Emulsion Products

Fatty acid profiles in commercially available lipid emulsions did not match with fatty acid profiles in breast milk, which is a gold standard for newborn infants in Figure 7. All lipid emulsions and breast milk contained essential fatty acids. However, lipid emulsions contained no or varying amounts of MUFAs and LC-PUFAs depending on lipid emulsions. Comparison table for fatty acid composition in nutritional sources with relative and absolute amounts is presented in Table 4.

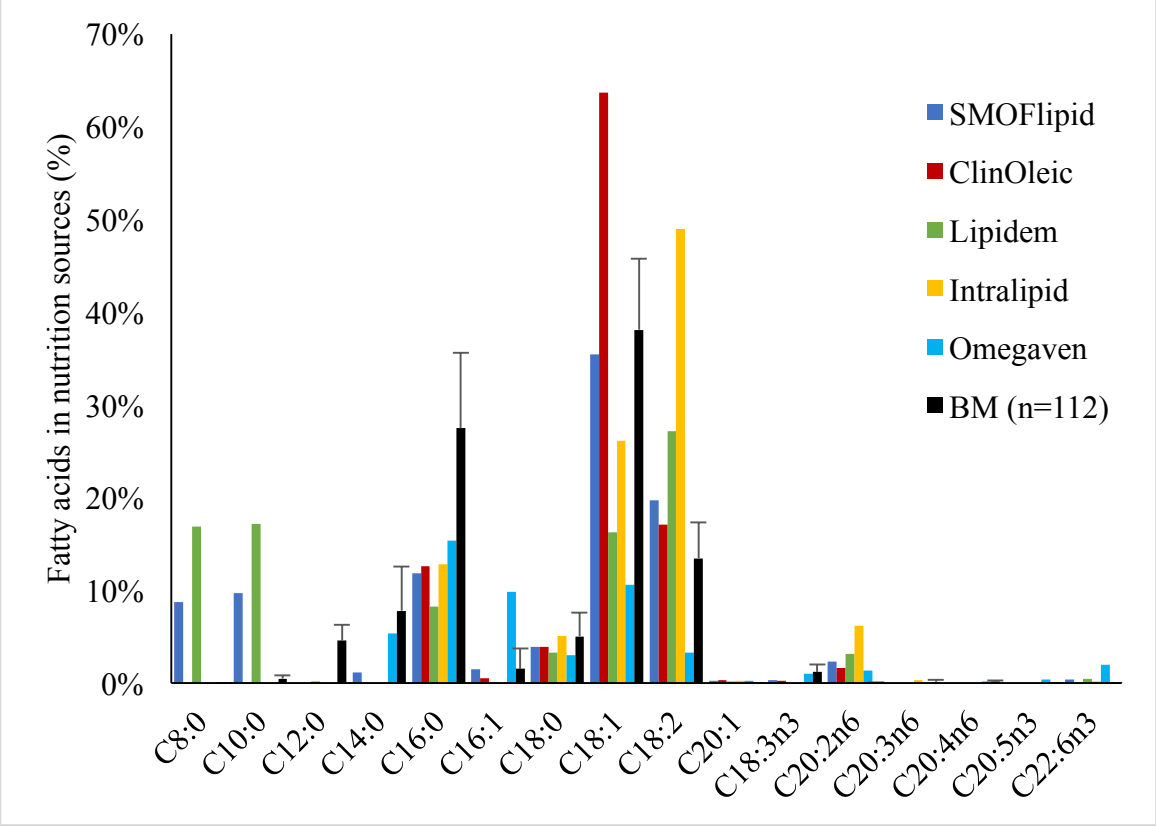

Figure 7. Comparison of fatty acid profiles on five lipid emulsions and breast milk using relative quantification. Note: relative quantification is utilized for this comparison due to deviant fat content between nutritional sources. Fat content in lipid emulsions is 20 g/dL and average fat content in breast milk is 4.0 g/dL.

Table 4. Fatty acid composition in commercially available lipid emulsions and average breast milk samples (BM; n=112) from 34 local mothers using the relative (A) and absolute quantification (B).

A. Relative quantification (unit:%)

	SMOFlipid	ClinOleic	Lipidem	Intralipid	Omegaven	BM (n=112)
C8:0	8.7	0.0	16.9	0.0	0.0	0.0 ± 0.0
C10:0	9.7	0.0	17.2	0.0	0.0	0.5 ± 0.3
C12:0	0.0	0.0	0.2	0.0	0.0	4.6 ± 1.7
C14:0	1.2	0.0	0.0	0.0	5.4	7.8 ± 4.8
C16:0	11.8	12.6	8.3	12.8	15.4	27.5 ± 8.2
C16:1	1.5	0.6	0.1	0.0	9.9	1.5 ± 2.2
C18:0	3.9	3.9	3.3	5.1	3.0	5.0 ± 2.5
C18:1	35.5	63.6	16.3	26.1	10.6	38.1 ± 7.7
C18:2	19.7	17.1	27.2	49.0	3.3	13.4 ± 3.9
C20:1	0.3	0.2	0.0	0.0	1.0	0.0 ± 0.0
C18:3n3	0.3	0.2	0.0	0.0	1.0	1.2 ± 0.8
C20:2n6	2.3	1.7	3.1	6.2	1.3	0.03 ± 0.01
C20:3n6	0.0	0.0	0.0	0.3	0.0	0.1 ± 0.2
C20:4n6	0.0	0.0	0.0	0.0	0.2	0.2 ± 0.1
C20:5n3	0.0	0.0	0.0	0.0	0.4	0.0 ± 0.0
C22:6n3	0.4	0.0	0.5	0.2	2.0	0.01 ± 0.03

B. Absolute quantification (unit: mg/dL)

	SMOFlipid	ClinOleic	Lipidem	Intralipid	Omegaven	BM (n=112)
C8:0	1765.5	0.0	2969.4	0.0	0.0	0.0 ± 0.0
C10:0	1970.7	0.0	3012.4	0.0	0.0	36.4 ± 15.5
C12:0	0.0	0.0	64.3	0.0	0.0	126.0 ± 63.9
C14:0	258.5	0.0	0.0	0.0	447.6	185.4 ± 112.0
C16:0	2284.2	3294.7	1465.4	2903.1	1225.9	598.7 ± 267.3
C16:1	331.4	172.5	50.9	0.0	797.2	58.9 ± 31.5
C18:0	806.4	1035.3	604.4	1159.0	263.8	133.5 ± 70.2
C18:1	7102.9	16543.8	2854.4	5873.8	856.7	830.7 ± 365.9
C18:2	3957.2	4464.9	4754.6	10991.4	296.4	316.2 ± 154.0
C20:1	95.2	88.8	0.0	0.0	105.7	0.0 ± 0.0
C18:3n3	491.0	459.2	571.6	1417.9	131.1	52.4 ± 26.4
C20:2n6	0.0	0.0	0.0	0.0	42.9	5.2 ± 12.3
C20:3n6	0.0	0.0	0.0	0.0	57.6	15.4 ± 18.9
C20:4n6	103.4	0.0	106.4	67.1	180.0	21.0 ± 16.0
C20:5n3	588.5	0.0	730.3	0.0	1929.3	0.3 ± 2.9
C22:6n3	382.6	0.0	496.4	0.0	1815.5	0.9 ± 5.4
Total	20237	26059	17680	22412	8139	2409.9 ± 948.2

Date is presented as mean for lipid emulsions and mean ± SD for breast milk

3.2.5 Nutrition Sources Affects Fatty Acid Profiles in Plasma

Neonate plasma samples (n=294) contained MCFAs, MUFAs, and PUFAs from C8:0 to C22:6n3. The C18:1, C16:0, C18:2, and C18:0 in order were quantitatively dominant fatty acids in plasma. Neonate plasma samples were grouped by their nutrition; 100% breast milk, 100% SMOFlipid, and 100% ClinOleic. The 100% SMOFlipid (n=23) and 100% ClinOleic (n=36) groups were compared to the 100% breast milk group (n=39) in Figure 8. The C8:0 ($P<0.001$), C20:5n3 ($p<0.001$), and C22:6n3 ($p<0.001$) in plasma were significantly higher in 100% SMOFlipid group compared to 100% breast milk group. The C12:0 ($P<0.05$), C18:2 ($p<0.001$), and C20:4n6 ($p<0.001$) in plasma were significantly lower but the C16:1 ($p<0.001$) and C18:1 ($p<0.001$) were significantly higher in the 100% ClinOleic compared to the100% breast milk groups.

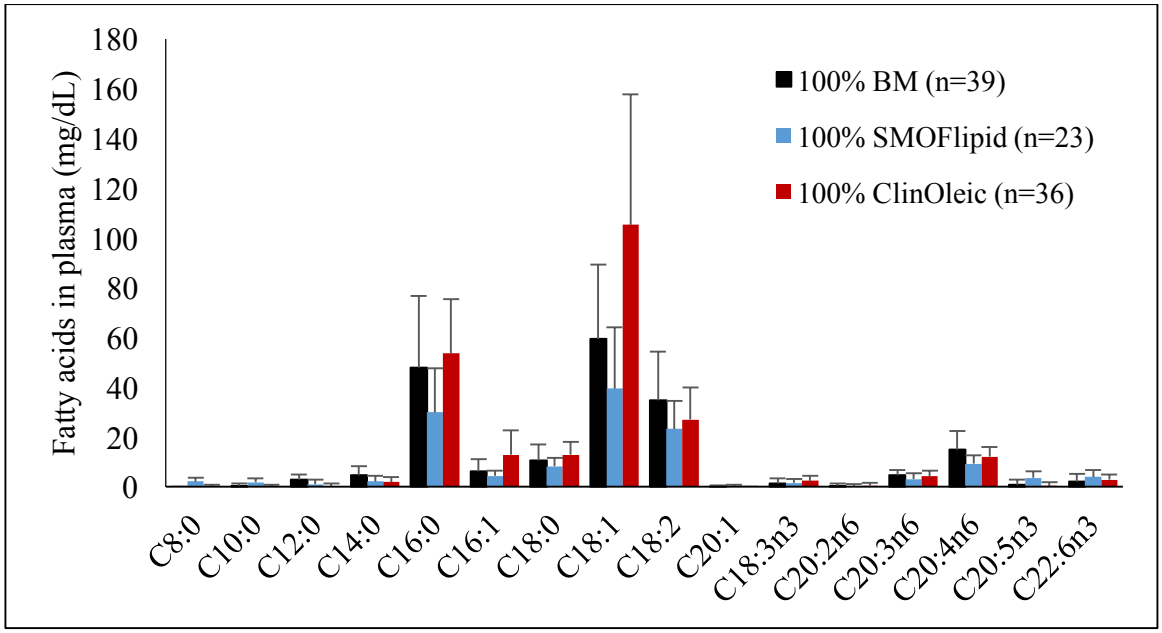

Figure 8. Fatty acid composition in plasma grouped by the nutrition sources of 100% breast milk, SMOFlipid, and ClinOleic. Data is presented as mean ± SD.

Since fatty acid composition in plasma from infants fed 100% breast milk and >70% breast milk with other nutrition were not statistically different, the ratio between mixed nutrition was set to be 70% to increase sample size in each category. The >70% SMOFlipid (n=41) and >70% ClinOleic (n=55) groups were compared with >70% BM group (n=118) in Figure 9. Very similar results seen in 100% nutrition groups were found also in >70% nutrition groups with addition of a few more fatty acids. The C8:0 ($P<0.001$), C10:0 ($P<0.001$), C20:5n3 ($p<0.001$), and C22:6n3 ($p<0.001$) in plasma were higher in >70% SMOFlipid group than those in the >70% BM group since the SMOFlipid contains high amount of these fatty acids than BM. However, the C12:0 ($P<0.001$) was lower in >70% SMOFlipid group due to no presence of C12:0 in the SMOFlipid. The C12:0 ($P<0.001$), C14:0 ($P<0.001$), C18:2 ($p<0.001$), C18:3n6 ($p<0.05$), C20:3n6 ($p<0.05$), C20:4n6 ($p<0.05$), and C22:6n3 ($p<0.05$) were significantly lower but the C16:1 ($p<0.001$), C18:0 ($p<0.001$), and C18:1 ($p<0.001$) were significantly higher in the >70% ClinOleic compared to the >70% BM groups. The C18:1 ($p<0.001$) was distinctly high in the >70% ClinOleic group since the ClinOleic contains relatively high proportion of C18:1 fatty acid. The fatty acid profiles in plasma grouped by >70% nutrition sources with relative and absolute amounts are presented in Table 5.

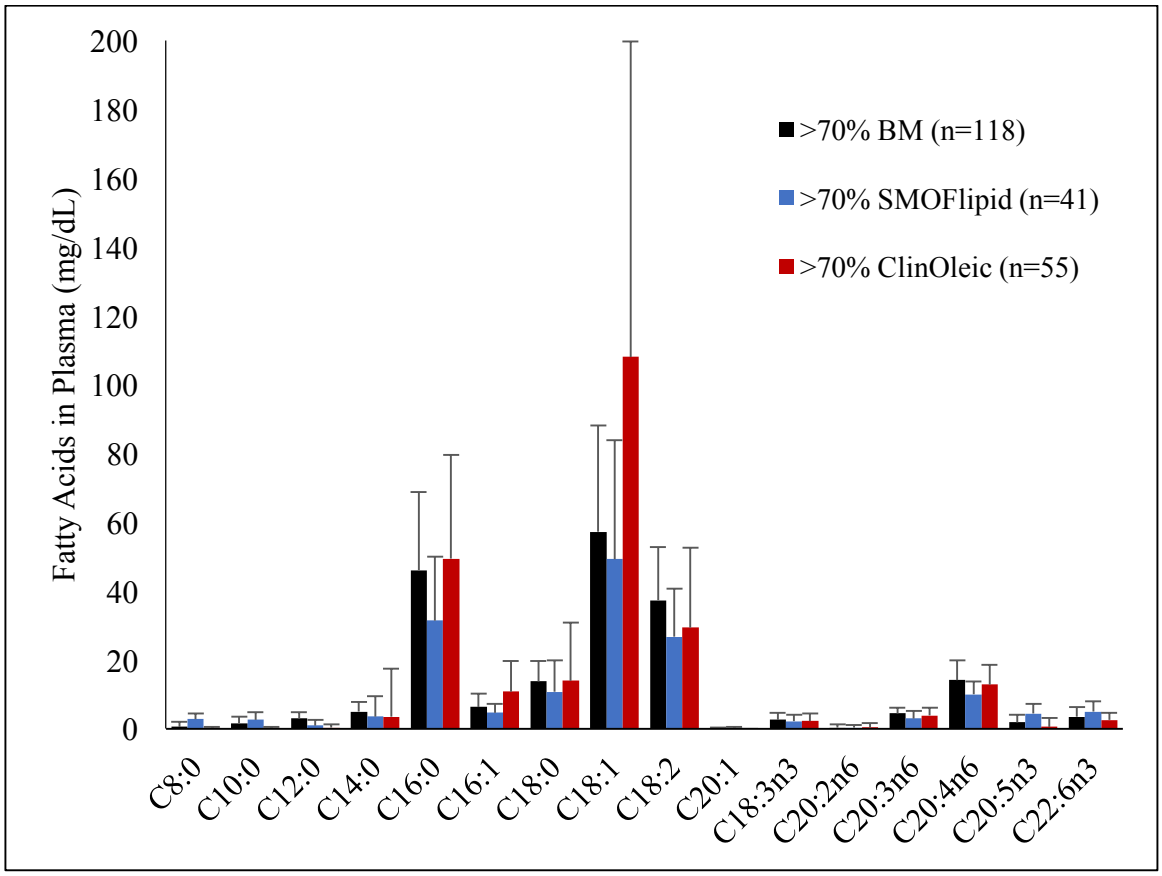

Figure 9. Fatty acid composition in plasma categorized by nutrition sources of >70% breast milk, SMOFlipid, and ClinOleic. Data is presented as mean ± SD.

Table 5. Fatty acid composition in plasma grouped by nutrition (>70% breast milk, SMOFlipid, and ClinOleic) calculated using relative (RQ) and absolute quantification (AQ). Relative and absolute quantification are expressed as % and mg/dL, respectively

	>70% BM (n=118)		**>70% SMOFlipid (n=41)**		**>70% ClinOleic (n=55)**	
	RQ	AQ	RQ	AQ	RQ	AQ
C8:0	0.1 ± 0.3	0.6 ± 1.5	0.5 ± 0.5	2.8 ± 1.7	0.0 ± 0.1	0.1 ± 0.5
C10:0	0.1 ± 0.3	1.6 ± 1.9	0.5 ± 0.6	2.7 ± 2.1	0.0 ± 0.1	0.1 ± 0.5
C12:0	0.5 ± 1.6	3.0 ± 1.9	0.1 ± 0.5	0.9 ± 1.7	0.0 ± 0.1	0.3 ± 1.0
C14:0	1.4 ± 1.2	5.0 ± 2.8	1.0 ± 3.8	3.5 ± 6.0	0.2 ± 0.5	3.5 ± 13.9
C16:0	25.6 ± 6.6	46.1 ± 22.7	23.8 ± 9.2	31.5 ± 18.5	23.3 ± 8.5	49.4 ± 30.3
C16:1	1.9 ± 1.4	6.4 ± 3.9	1.7 ± 1.6	4.7 ± 2.5	3.6 ± 3.3	10.8 ± 8.9
C18:0	7.2 ± 3.3	13.8 ± 5.9	6.4 ± 5.7	10.8 ± 9.1	4.3 ± 1.8	14.0 ± 16.9
C18:1	31.6 ± 7.6	57.2 ± 31.0	34.9 ± 12.3	49.3 ± 34.5	48.9 ± 9.3	108.1 ± 91.6
C18:2	21.6 ± 5.4	37.3 ± 15.4	19.7 ± 7.4	26.7 ± 14.1	12.5 ± 4.5	29.4 ± 23.3
C20:1	0.0 ± 0.0	0.0 ± 0.3	0.0 ± 0.0	0.1 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
C18:3n3	0.4 ±0.5	2.7 ± 1.9	0.2 ± 0.4	2.2 ± 1.9	0.1 ± 0.4	2.2 ± 2.1
C20:2n6	0.0 ± 0.1	0.3 ± 1.0	0.0 ± 0.0	0.3 ± 0.9	0.0 ± 0.2	0.4 ± 1.2
C20:3n6	1.1 ± 0.7	4.5 ± 1.6	0.7 ± 1.1	3.0 ± 2.1	0.7 ± 0.7	3.9 ± 2.2
C20:4n6	7.2 ± 2.6	14.2 ± 5.7	6.4 ± 2.4	10.0 ± 3.7	5.5 ± 3.2	12.9 ± 5.6
C20:5n3	0.3 ± 0.8	1.9 ± 2.2	1.9 ± 1.8	4.4 ± 2.7	0.2 ± 1.0	0.6 ± 2.6
C22:6n3	0.9 ± 1.0	3.5 ± 2.8	2.1 ± 1.5	4.9 ± 3.0	0.3 ± 0.6	2.6 ± 2.0

Data is presented as mean ± SD.

Correlations on fatty acids between plasma and feeds (fatty acid intake) are shown in Table 6. Significant positive correlations were found in C8:0 ($r= 0.31$, $p<0.001$), C10:0 ($r= 0.46$, $p<0.001$), C12:0 ($r= 0.48$, $p<0.001$), C14:0 ($r= 0.23$, $p<0.05$), C20:5n3 ($r= 0.55$, $p<0.001$), and C22:6n3 ($r= 0.34$, $p<0.001$) when all nutrition groups were combined except C16:1 ($r=-0.31$, $p< 0.001$) with negative correlation.

Table 6. Correlation on fatty acids between plasma and feeds for neonates

Fatty Acids		Nutritional Groups			
		All	>70% BM	>70% SMOF	>70% ClinOleic
	n	153	70	31	39
C8:0	r	0.31	-0.10	0.45	-
	p	0.00***	0.41	0.02*	-
C10:0	r	0.46	0.06	0.41	0.01
	p	0.00***	0.66	0.03*	0.97
C12:0	r	0.48	0.22	-0.33	-0.04
	p	0.00***	0.07	0.09	0.81
C14:0	r	0.23	0.03	0.57	-0.11
	p	0.01**	0.82	0.02*	0.54
C16:0	r	-0.14	-0.20	0.02	-0.42
	p	0.10	0.11	0.93	0.01*
C16:1	r	-0.31	-0.28	-0.33	-0.40
	p	0.00***	0.02*	0.08	0.02*
C18:0	r	0.03	-0.12	0.23	-0.19
	p	0.71	0.33	0.24	0.28
C18:1	r	-0.09	-0.28	0.33	-0.23
	p	0.30	0.02*	0.09	0.18
C18:2	r	0.07	-0.15	0.18	-0.02
	p	0.43	0.23	0.37	0.93
C18:3n3	r	0.06	-0.23	0.36	0.01
	p	0.44	0.06	0.06	0.96
C20:2n6	r	-0.06	-0.10	-	-
	p	0.48	0.43	-	-
C20:3n6	r	0.06	0.01	-0.03	-
	p	0.46	0.91	0.90	-
C20:4n6	r	0.05	-0.13	-0.04	0.07
	p	0.52	0.28	0.85	0.68
C20:5n3	r	0.55	0.15	0.53	-
	p	0.00***	0.21	0.00**	-
C22:6n3	r	0.34	0.27	0.17	-
	p	0.00***	0.03*	0.38	-

* P value <0.05, ** p-value <0.01, and *** p-value <0.001 were considered statistically significant.

3.2.6 Fatty acids Profiles in Normo- and Hypertriglyceridemic Plasma

Average triglyceride levels in normo- and hypertriglyceridemic plasma were 0.9 ± 0.7 mmol/L and 2.1 ± 0.7 mmol/L, respectively. Fatty acid profiles in normotriglyceridemic samples (n=116) were significantly different to fatty acid profiles in hypertriglyceridemic samples (n=88) using both relative and absolute quantification. The mean percentages of fatty acids were statistically different for C16:1 ($p<0.001$), C18:0 ($p<0.05$), C18:1 ($p<0.001$), C18:2 ($p<0.001$), C18:3n3 ($p<0.001$), C20:2n6 ($p<0.05$), C20:4n6 ($p<0.001$), and C22:6n3 ($p<0.05$) (Figure 10A). The mean absolute concentrations of fatty acids were significantly different for C14:0 ($p<0.05$), C16:0 ($p<0.001$), C16:1 ($p<0.001$), C18:0 ($p<0.001$), C18:1 ($p<0.001$), C18:2 ($p<0.001$), C18:3n3 ($p<0.001$), C20:2n6 ($p<0.001$), C20:3n6 ($p<0.001$), C20:4n6 ($p<0.001$), and C22:6n3 ($p<0.05$) (Figure 10B). Absolute amounts of all fatty acids except C20:5n3 were elevated in hypertriglyceridemic plasma. However, the relative quantification did not show the same trend of fatty acid elevations as the absolute quantification. In the relative quantification, C16:0, C18:0, C18:2, C18:3n3, C20:4n6, C20:5n3, and C22:6n3 fatty acids were lower in hypertriglyceridemic plasma compared to normotriglyceridemic plasma.

(A) Relative quantification (B) Absolute quantification

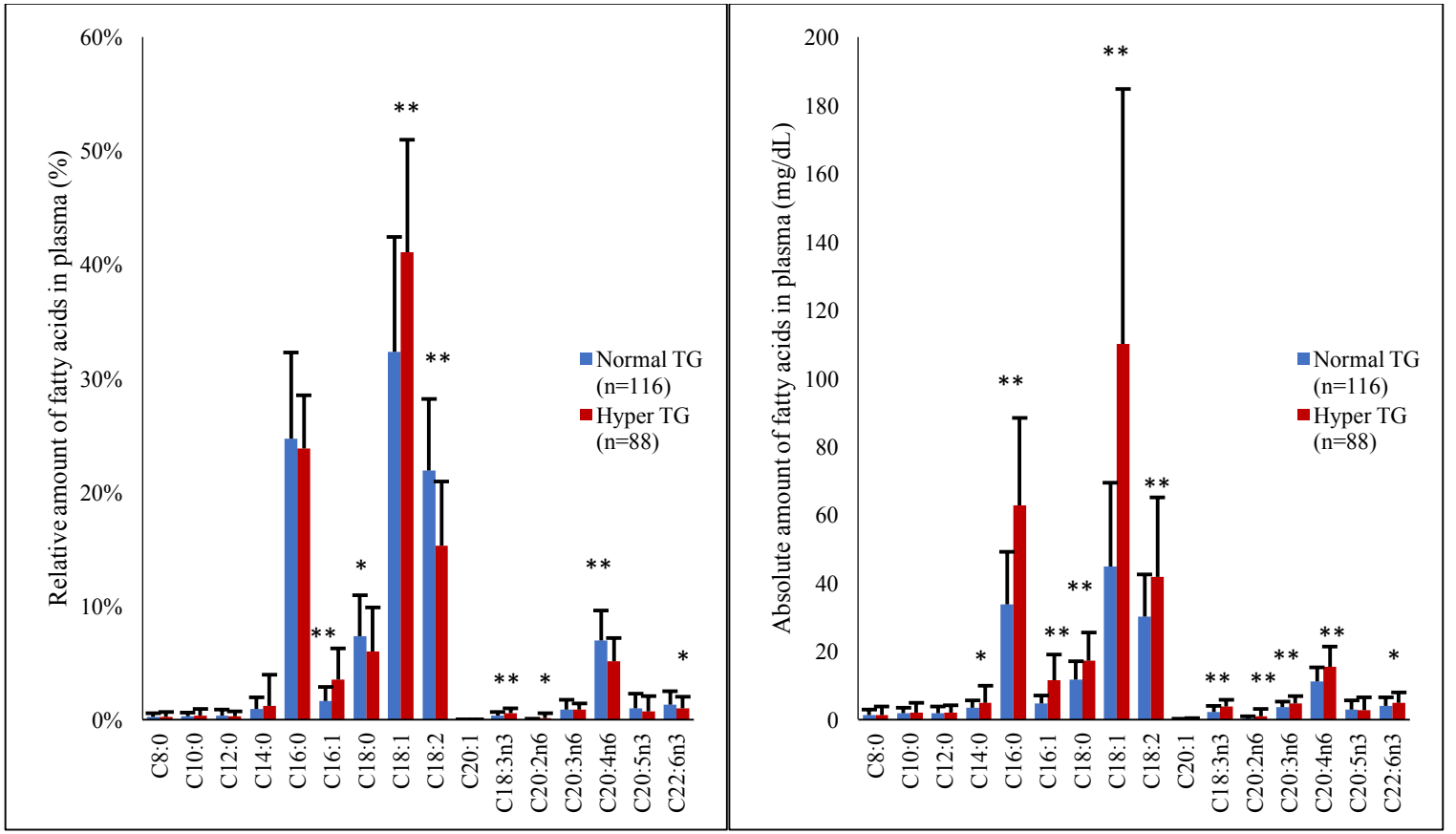

Figure 10. Distribution of fatty acids in normotriglyceridemic and hypertriglyceridemic plasma samples. Relative quantification (A) and absolute quantification (B) of fatty acids are presented using the same set of samples. Data presented as mean ± SD. * P- value <0.05 and ** p-value <0.001 were considered statistically significant.

Among the plasma samples with high TG levels (n=88), 76% of them were from infants who received partially or 100% lipid emulsions. Additionally, 17% plasma samples from infants fed full breast milk showed high TG levels. However, among the plasma samples with normal TG levels (n=116), 58% of them were from infants who received partially or 100% lipid emulsions. This indicates that lipid emulsion nutrition is one of factors that are associated with high triglyceride levels.

Fatty acid profiles in normotriglyceridemic and hypertriglyceridemic plasma were categorized by nutrition groups: >70% breast milk, >70% SMOFlipid, and >70% ClinOleic. Fatty acids in normotriglyceridemic plasma grouped by their nutrition demonstrated relatively similar profiles except distinctively high C18:1 in the >70% ClinOleic (Figure 11A). It can be explained by high concentration of C18:1 in the ClinOleic compared to other nutrition sources. The average ratio of fatty acids between normotriglyceridemic and hypertriglyceridemic plasma was 1.7. The ratio of certain fatty acids was distinguishably high between normo- and hypertriglyceridemic plasma for each nutrition group. The ratios of C20:2n6 in the breast milk group, C14:0 and C20:3n6 in the SMOFlipid group, C8:0 and C16:1 in the ClinOleic group were above 3. In hypertriglyceridemic plasma, the C18:1 in the >70% ClinOleic group was higher than one in other groups and C18:2 in the >70% breast milk group was higher than other groups (Figure 11B).

(A) Fatty acid profiles in normotriglyceridemic plasma (B) Fatty acid profiles in hypertriglyceridemic plasma

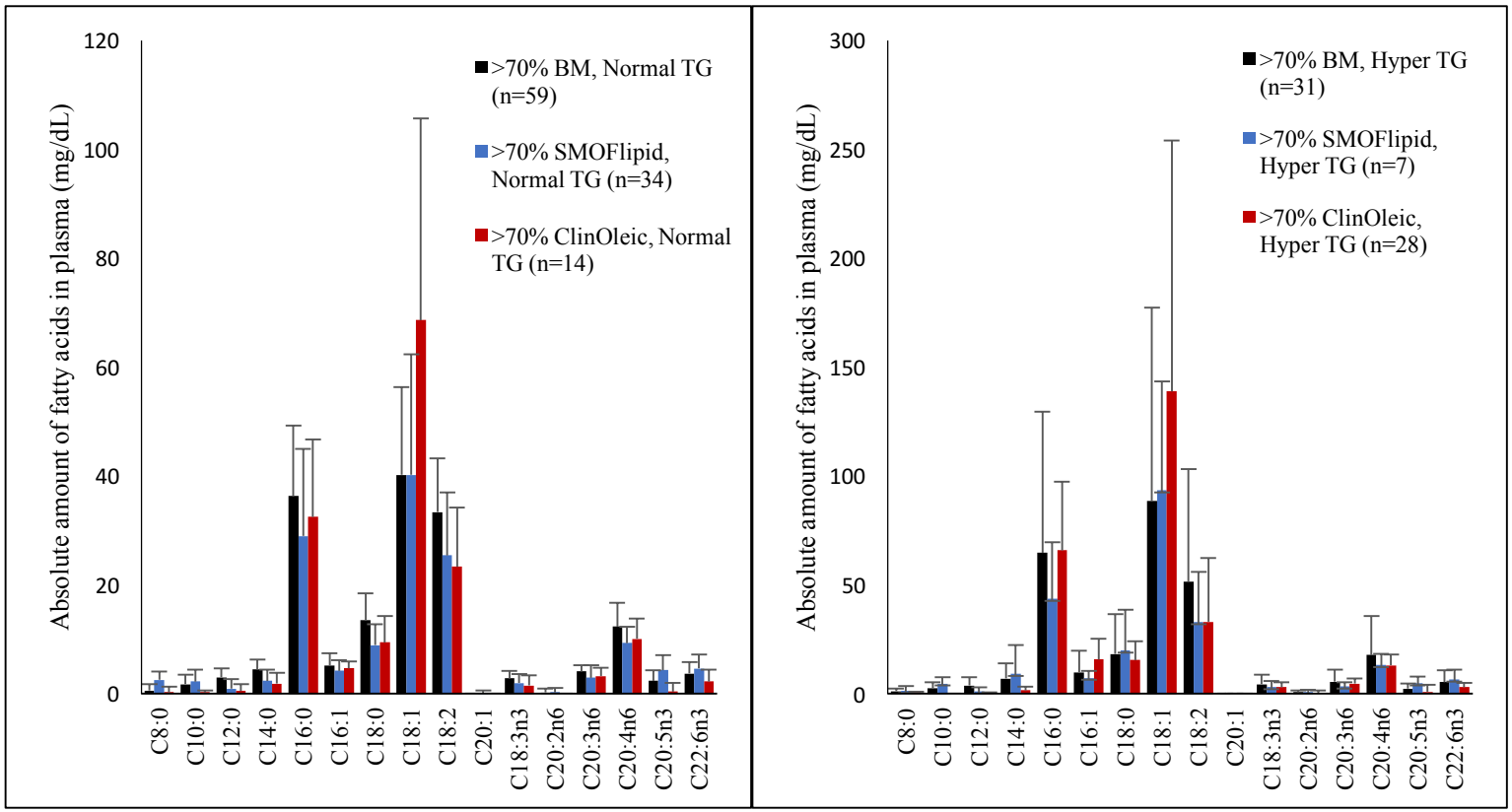

Figure 11. Fatty acid composition in plasma categorized by nutritional sources (>70% Breast milk, SMOFlipid, ClinOleic nutrition groups) and triglyceride levels: Fatty acids profiles in normotriglyceridemic (A) and hypertriglyceridemic (B) plasma. Data are presented as mean ± SD.

3.3 Results on 1D gel electrophoresis

3.3.1 Validation on 1D Gel Electrophoresis

The levels of lipoproteins in plasma and stored at -80 ˚C were not statistically different to those collected as fresh serum samples (p=0.47). It proves that collected plasma samples at -80 ˚C can be measured by 1D gel electrophoresis without compromising on quality of data.

3.3.2 Lipoproteins in Normo- and Hypertriglyceridemic Plasma

Adjusted relative lipoprotein levels in the normo- and hypertriglyceridemic plasma were summarized in Table 7. The adjusted relative HDL levels in hypertriglyceridemic plasma were significantly lower than normotriglyceridemic plasma ($p<0.001$). The adjusted relative (LDL+VLDL) levels were significantly high in hypertriglyceridemic plasma compared to normotriglyceridemic plasma ($p<0.001$). There was no statistical difference found in the adjusted relative chylomicrons between hypertriglyceridemic and normotriglyceridemic plasma (p=0.46). There was a negative linear trend observed between TG levels and ratios of HDL/(VLDL+LDL) in Figure 12.

Table 7. Adjusted relative values of HDL, LDL, VLDL and chylomicron in normo- and hypertriglyceridemic plasma

	% HDL	% (LDL + VLDL)	% chylomicrons	Average HDL/(LDL + VLDL)
Normal TG (n=18)	52.9 ± 18.1	37.9 ± 17.0	9.2 ± 4.9	1.9 ± 1.3
High TG (n=24)	21.4 ± 11.5	67.0 ± 17.3	11.6 ± 14.8	0.4 ± 0.3

Data is presented as mean ± SD.

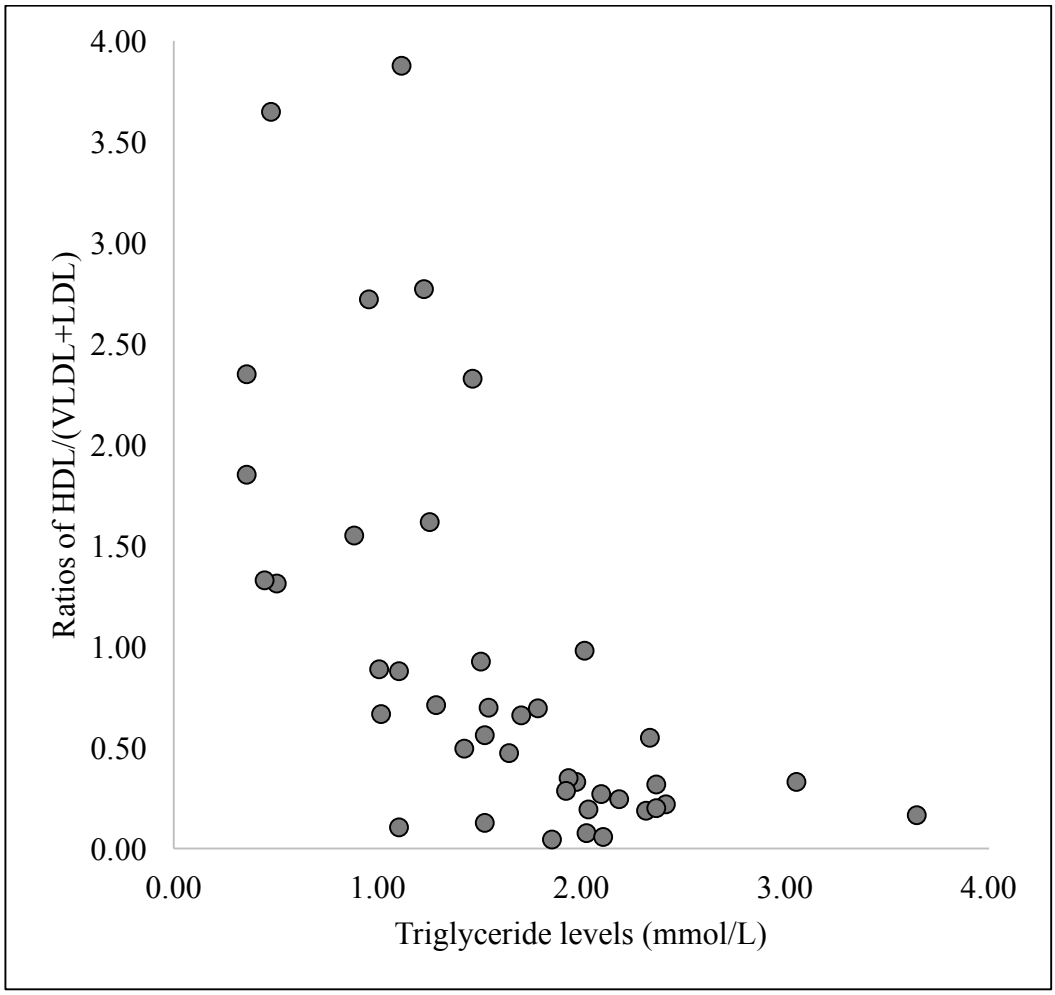

Figure 12. Comparison between triglyceride levels and ratios of HDL/(VLDL+LDL) in plasma from preterm infants (n=42). Lower triglyceride levels tend to have higher ratio of HDL/(VLDL+LDL)

The adjusted relative lipoprotein levels in normo- and hypertriglyceridemic plasma were categorized by the nutrition in Table 8. The average ratio of HDL/(LDL+VLDL) in plasma collected from infants fed breast milk was higher than those received lipid emulsions.

Table 8. Adjusted relative HDL, LDL, VLDL and chylomicron values in normo- and hypertriglyceridemic plasma categorized by nutrition (breast milk, SMOFlipid, ClinOleic)

	Nutrition	% HDL	% (LDL + VLDL)	% chylomicrons	Average HDL/(LDL + VLDL)
Normal TG	>70% BM (n=6)	71.2 ± 5.2	22.2 ± 4.7	6.7 ± 4.0	3.2 ± 1.1
	>70% SMOFlipid (n=6)	42.6 ± 19.6	48.2 ± 20.0	9.2 ± 5.4	0.9 ± 1.0
	>70% ClinOleic (n=6)	45.0 ± 10.0	43.3 ± 10.2	11.7 ± 4.5	1.0 ± 1.0
High TG	>70% BM (n=6)	31.7 ± 12.9	57.3 ± 13.3	11.0 ± 3.8	0.6 ± 1.0
	>70% SMOFlipid (n=5)	14.7 ± 11.5	55.8 ±26.4	29.5 ± 23.7	0.3 ± 0.4
	>70% ClinOleic (n=13)	19.3 ± 8.0	75.5 ± 9.3	5.0 ± 6.6	0.3 ± 0.9.

Data is presented as mean ± SD

CHAPTER 4. Discussion

4.1 Developed GC-MS method

The developed GC-MS method allows for precise, accurate, and reproducible analysis of fatty acids from total lipids in plasma. The intra- and inter-day variations were met by the AOAC guidelines for CVs of repeatability and reproducibility (AOAC 2013). Absolute plasma fatty acid concentrations in the NIST standard analyzed by this method were in agreement with fatty acid content in the NIST specification as well as validated with the external reference laboratory. Of importance, a base catalyst was selected over an acid catalyst due to the appropriate range of absolute amounts of fatty acids and the same fatty acid patterns as the NIST standard. Using the acid catalyst, concentrations of fatty acids were about 50% lower with inconsistent fatty acid patterns. It is unclear why dissimilar fatty acid concentrations were observed using either acid or base catalyst. Theoretically, the same outcomes are expected since the esterification should form esters from carboxylic acids and alcohols in the excess presence of concentrated acid or base acting as the catalyst. The next step should be the transesterification which is the process of exchanging the organic group of the ester with the organic group of an alcohol. However, generating fatty acid profiles in a single analytical run may be challenging due to the complicated biological matrix, such as the complex structure of isomers of conjugated double bonds (Rosenfeld 2002). Ideally, a combination of the two catalysts may be the most effective: a base catalyst is added for the esterification of lipid-bound fatty acids and as a second step, an acid catalyst would be used for free fatty acids (Badings & De Jong 1983). However, due to limited resources and time, a base catalyst

was used with the application of a correction factor, which was generated by the validation of fatty acids from the reference laboratory. For the quality assurance, the NIST and human plasma standard were analyzed prior to each run of samples. As per analytical trials to improve quality of analysis, a longer reaction time of 2 hours, internal standard of triglycerides-bound C17, Folch extraction method, triple extractions were tested but the results did not significantly differ than the results following the protocol in the method.

The developed method can measure both relative and absolute amounts of fatty acids. In contrast with this study, many nutrition studies reported only percentages of fatty acids. The problem of presenting findings in this manner is the difficulty to compare results between studies because the percentage of each fatty acid relies on number of fatty acids investigated. This indicates that the change in a single fatty acid relatively influences other fatty acids. The quantitative approach is more useful to compare results among other literature since concentration values are not dependent on the relative abundance of other fatty acids. Moreover, literature has shown that relative quantification does not represent a true reflection of fatty acids presented in biological fluids (Hon et al. 2012; Abdelmagid et al. 2015; Lagerstedt et al. 2001). Lagerstedt *et al* (2001) demonstrated a high false positive rate using relative percentages of fatty acids on the biochemical diagnosis of nutritional and metabolic disorders. They reported that 14 of 31 cases, which tested as abnormal by the relative quantification, conveyed normal concentrations by the absolute quantification (Lagerstedt et al. 2001). Thus, absolute

quantification of fatty acids is essential for enhancement of the understanding of fatty acid composition and metabolic conditions for newborn infants.

4.2 Fatty Acid Profiles in Nutrition Sources

The average relative percentage of fatty acids in breast milk found in this study is similar to the fatty acid composition reported in other literature along with high abundance of C16:0, C18:1, and C18:2 fatty acids in breast milk (Berenhauser et al. 2012; Much et al. 2013; Makrides et al. 1995). Average DHA and AA were slightly higher in preterm breast milk compared to term breast milk. However, the difference was not statistically significant, which is consistent with another study (Iranpour et al. 2013). This may be because preterm infants need more of DHA and AA during their development stage compared to term infants. A wide range of inter- and intra-individual variations of fatty acids were displayed in breast milk. The average ratios of (n-6)/(n-3) fatty acids from Canadian local mothers were 6.8:1 and 11.6:1 using the absolute and relative quantification, respectively. The ratios of (n-6)/(n-3) fatty acids using the relative quantification were found to be quite different between mothers in varying countries: 25:1, 12:1, 10:1, 6:1 for Greek, Italian, Spanish, and Swedish mothers (Antonakou et al. 2013). This might be affected by the maternal diet and consumption of fatty acid supplementation (Koletzko et al. 1992; Antonakou et al. 2013). Obtaining dietary records from mothers would be beneficial in order to investigate correlation on fatty acids between maternal diet and breast milk. However, this was not part of this study.

Preterm infants are incapable (or have very limited ability) of synthesizing LC-PUFAs by chain elongation and desaturation of essential fatty acids due to low enzyme

activities (Uauy et al. 2000). It is very important to provide adequate DHA and AA during early infancy since they have significant roles on retina and brain development (Martin 2014; Innis 2007). Breast milk DHA was higher when mothers have consumed DHA supplementation (Berseth et al. 2014). Maternal DHA intake has shown positive effects on visual and neurodevelopment in infants at 30 months of age with no adverse effects (Jensen et al. 1999). Since maternal diet intake is a determinant of fatty acid profiles in breast milk, having a well balanced-diet and omega 3 supplements if necessary is beneficial to support the infant's growth and development.

Human milk fortifiers that are routinely used to fortify breast milk are to support the needs of preterm infants and affect LC-PUFAs intake. Amounts of essential fatty acids in human milk fortifier powder analyzed for this study are consistent with levels reported in the nutrient specification in the manufacture website (Mead Johnson & Company). The current ESPGHAN guideline recommends 11- 27 mg DHA/100 kcal and 16 – 39 mg AA/100 kcal for preterm infants (Agostoni et al. 2010). However, this particular human milk fortifier powder which was in use for the study did not contain other important LC-PUFAs such as DHA and AA. Taking into consideration the fact that preterm breast milk collected for this study did not contain sufficient amount of DHA with an exception for AA, the level of DHA did not meet the requirement. A study demonstrated that levels of DHA and AA were enhanced using another human milk fortifier product containing 12 mg DHA and 20 mg AA for 100 mL breast milk (Berseth et al. 2014). Supplementing DHA and AA in human milk fortifier may be beneficial for preterm infants based on the current findings which have shown support for

improvements in visual and neurodevelopmental outcomes from LC-PUFAs supplementation (Skouroliakou et al. 2010; Jensen et al. 1999; Makrides et al. 1995).

Fatty acid profiles in the lipid emulsions do not resemble each other nor are they similar to the ones in breast milk. Among commercially available lipid emulsions, the fatty acid profiles in the SMOFlipid is the most comparable to the ones of breast milk since the SMOFlipid consists of MCFAs, MUFAs, and LC-PUFAs coming from mixed oil sources of soybean, coconut, olive, and fish. However, it contains high proportions of MCFAs especially C8:0 and C10:0, compared to breast milk. The MCFAs have been shown to be easily oxidized in the liver and quickly converted to ATP for cellular energy compared to long chain fatty acids (St-Onge & Jones 2002). They are unlikely to be stored as fat because the body is able to efficiently use it for energy. Moreover, literature has reported that the SMOFlipid is a safe and well tolerated lipid emulsion with decreased plasma bilirubin and increased (n-3) fatty acids (Tomsits et al. 2010; Goulet et al. 2010).

However, due to drug incompatibility with Fentanyl, the application of SMOFlipid in very sick infants, which would benefit from PUFAs supply in favourable of (n-6)/(n-3) ration is limited. In these cases, an alternative lipid emulsion is used in McMaster NICU called the ClinOleic, which does not contain fish oil. The ClinOleic is rich in the MUFA of oleic acid. Although the oleic acid (C18:1n9) is not considered to be essential dietary fatty acid since it can be endogenously synthesized by desaturation of C18:0 fatty acid, it is a major fatty acid in brain myeline lipid (Rioux & Innis 1992). However, whether excess C18:1n9 fatty acid may accumulate and cause any adverse effects is unknown. Although some studies have shown no adverse effects using the

ClinOleic for preterm infants (Munck et al. 1996; Deshpande et al. 2009; Göbel et al. 2003), long-term data on the effect of the ClinOleic is not currently available. Thus, long-term effects must be investigated since the ClinOleic does not contain DHA and AA.

The Intralipid is the oldest lipid emulsions used in the United States and much of North America. The primary concern of the Intralipid containing a large excess of C18:2n6 and no DHA and AA is related to the clinical complication of liver dysfunction, BPD, and infections (Sosenko et al. 1993; Utermohlen & Tucker 1986; Schmitz & Ecker 2008; Brans et al. 1986). The Intralipid is still utilized for early delivery of lipids to preterm infants regardless of these issues in North America since there is no other lipid emulsions approved by the US Food and Drug Administration. They have legally no other choices to provide suboptimal lipid emulsions. On the other hand, other lipid emulsions approved for use in Europe.

The ratios of (n-6)/(n-3) fatty acids for lipid emulsions investigated for this study were similar to the values reported by the manufacturers. The lowest ratios of (n-6)/(n-3) fatty acids were the SMOFlipid and Lipidem whereas the ClinOleic had the highest ratio. This can be indicative of the presence of fish oil, which contains (n-3) fatty acids in the formulation. However, high ratios of (n-6)/(n-3) fatty acids are associated with pathogenesis of many diseases including cardiovascular diseases, inflammatory diseases, and cancer. On the contrary, low ratios of (n-6)/(n-3) fatty acids exert suppressive effects. In the adult population, the ratio of 4:1 was related to a 70% decrease in total mortality of the secondary prevention of cardiovascular disease and the ratio of 2.5:1 reduced rectal

cell proliferation in patients with colon cancer (Simopoulos 2002). It is likely that a lower ratio of (n-6)/(n-3) fatty acids is more desirable in reducing the risk of chronic diseases.

There is one only paper which demonstrates the short-term exposure of Intralipid (2-3g/kg/d) during neonate life and it is associated with negative consequences of cardiovascular function at the age range of 23-28 years compared to age- and sex-matched healthy volunteers who did not receive lipid emulsion during neonate life (Lewandowski et al. 2011). There is still a need to investigate whether various lipid emulsions containing different ratios of (n-6)/(n-3) fatty acids during the neonatal period are associated with any consequences in later life. Furthermore, a multitude of literature has compared safety and tolerability of lipid emulsion products but they have not investigated on whether energy requirements from lipid emulsions are met or lead to optimal growth for preterm infants.

4.3 Fatty Acid Profiles in Plasma

The C16:0, C18:1, and C18:2 are high abundance fatty acids found in plasma from infants fed breast milk, SMOFlipid, and ClinOleic, which is in agreement with current literature (Hossain et al. 2016; Webb et al. 2008; Goulet et al. 2010). These fatty acids are also in abundance in young healthy Canadian adults (Abdelmagid et al. 2015). Fatty acid concentrations in infant's plasma are found to be relatively lower than those in young healthy adults but similar in fatty acid patterns, possibly due to amount of fat intake (Abdelmagid et al. 2015).

Fatty acids can be measured in various blood fractions and tissues (i.e. plasma, erythrocytes, and adipose tissue). The fatty acid composition in plasma lipids has been

observed as a reliable index reflecting recent dietary fatty acid intake (past hours to a few days) as well as fatty acid composition in erythrocytes are reflected by nutrition consumed several days ago (Arab & Akbar 2002). On the contrary, the fatty acid composition in adipose tissue has been shown to be a valid index for the habitual dietary fatty acid composition (Arab & Akbar 2002). Since infants receive lipid emulsions for a short period, investigating fatty acid composition in plasma was more suitable for this study.

The results of this study show that the fatty acid composition in dietary intake reflects, to some extent, fatty acid composition in plasma. Due to the high concentrations of C8:0, C10:0 (MCFAs), C14:0 (LCFA), and C20:5n3 (LC-PUFA) in feeds from infants receiving the SMOFlipid compared with other nutrition groups, these fatty acid differences were confirmed in plasma, where the concentrations of the aforementioned fatty acids were significantly higher in this group than among other groups. In addition, significant positive correlations were found particularly for these fatty acids. However, there were significant negative correlations found on C16:1 and C18:1 (breast milk feeds) and C16:0 (ClinOleic feeds) between dietary intake and the resulting plasma. This may be explained by different fatty acid utilization to support the hypothesis that there is a regulatory fatty acid balance using enzymes. For example, substantially elevated MUFA C18:1n9 in the nutrition was found to not accumulate in the plasma, which indicates that it can readily be metabolized.

The ratios of (n-6)/(n-3) fatty acids were slightly higher in plasma than actual nutrition for all nutrition groups. The ClinOleic group compared with other groups

resulted in a higher ratio of (n-6)/(n-3) observed in actual feeds as well as their plasma. This may be due to low concentrations of (n-3) fatty acids in the ClinOleic nutrition and lower endogenous biosynthesis of (n-3) LC-PUFAs from infants who have a low level of enzyme activity. Furthermore, factors such as level of LDL, level of infants' prematurity, and varying levels of fatty acid utilization can also explain the discrepancy in fatty acid concentration between nutrition and plasma. However, these factors were not within the scope of this study.

4.4 Fatty Acid Profiles in Hypertriglyceridemic Plasma

Hypertriglyceridemia has been reported to be associated with intravenous lipid administration in preterm infants. As we analyzed, using relative percentages of fatty acids would not allow accurate elucidation of whether specific or all fatty acids were elevated in hypertriglyceridemic plasma. However, absolute quantification revealed that all fatty acids were elevated in hypertriglyceridemic plasma except C20:5n3 (EPA) fatty acid which showed very similar concentration in both hypertriglyceridemic and normotriglyceridemic plasma. The optimal level of EPA is important since high levels of EPA significantly depressed the (n-6) fatty acid pathway, resulting lower AA in plasma and lower z-score findings of body weight, length, and head circumference (Carlson et al. 1992). It is likely that total fatty acid accumulations may play a role in elevated plasma TG levels. Moreover, normotriglyceridemic plasma categorized nutrition groups demonstrated comparably very similar fatty acid profiles except for very high C18:1 expressed in the ClinOleic group. Hypertriglyceridemic plasma categorized nutrition groups displayed similar fatty acid patterns of those seen in normotriglyceridemic plasma.

However, all fatty acids were on average 1.7 times more concentrated in hypertriglyceridemic plasma. This may be intuitive since the TG levels in the hyper TG group were two times higher than TG levels in the normal TG group. However, particular fatty acids in each plasma group were three times more concentrated in hypertriglyceridemic plasma compared to normotriglyceridemic plasma. This may be indicative of dissimilar fatty acid metabolism based on enzyme activity. Furthermore, despite the fact that the majority of infants who showed high TG received lipid emulsions, interestingly, some infants fed breast milk also showed high TG although breast milk is considered to be the gold standard nutrition. This suggests that lipid emulsions of nutrition are one associated factor of developing high TG. Further, other factors such as level of enzyme activities including LPL and carnitine and prematurity of infants may contribute to high TG in the blood.

The American Academy of Pediatrics suggested TG levels of 150 mg/dL (i.e. 1.7 mmol/L) as an acceptable normal upper limit for infants receiving intravenous lipids (Barness et al. 1981). According to this guideline, the NICU at McMaster Children's Hospital uses a conservative plasma triglyceride level of 1.5 mmol/L as a cut-off value to minimize the risk of undesirable consequences. However, this reference value was not derived from reference data on infants but from the data on adults. There is growing research on the safe reference range of TG levels for preterm infants. Adamkin & Geike (1984) reported that continuous infusion of lipid emulsion over a 24 hour period resulted in plasma TG levels no greater than 250 mg/dL (i.e. 2.8 mmol/L) in preterm infants without any undesirable consequences (Adamkin & Gelke 1984). Moreover, Fenton *et al*

(1997) suggested that the acceptable upper limit of TGs for preterm infants with low birth weight (<1500g) on lipid emulsion could be 2.5 mmol/L since infants fed breast milk showed a TG range of 2.5 mmol/L (Fenton et al. 1997). Another literature supported that the TG reference interval was higher during the first year of life for healthy infants (0.56-2.28 mmol/L for male; 0.73-2.64 mmol/L for female) compared to during the first to fifth years of life (0.67-1.65 mmol/L for male; 0.75-1.86 mmol/L for female) (Yip et al. 2006). Moreover, when preterm infants received 3g/kg/d of the SMOFlipids, plasma TG levels did not exceed the upper TG value of 2.8 mmol/L as some other literature suggested (Skouroliakou et al. 2015). Various NICUs seem to have their own reference TG range for preterm infants. It is important to note that safe cut-off TG levels in blood for adults are values during a fasting state. However, obtaining fasting lipid profiles may not be practical for preterm infants since they need continuous dietary intake to grow. The non-fasting TG ranges may be more feasible for this special population. In addition, the lack of established reference ranges for TG and fatty acids has resulted in the poor interpretability of lipid emulsion products. Importantly, it is unclear what level of elevated TGs is considered to be harmful for preterm infants (Valentine & Puthoff 2007). Further research is necessary to define a safe reference range of TG levels for newborn infants. It is also not known yet whether high TG levels during the neonatal period contribute to long-term effects on health, increased risk of cardiovascular diseases, diabetes, or obesity.

4.5 Lipoprotein Profiles in Hypertriglyceridemic Plasma

A significant increase in plasma (LDL and VLDL) levels and decrease in HDL levels were found in hypertriglyceridemic plasma, in agreement with previous literature (Masarei et al. 1971; Nilsson et al. 1996; Morillas et al. 1992). This finding may be interpreted as a result of low LPL activity in plasma, which is responsible for low metabolism of VLDL (Shimada et al. 1995; Morillas et al. 1992). The higher levels of VLDL and lower levels of HDL found in preterm infants compared to term infants (Morillas et al. 1992). This might be because preterm infants have a limited ability to clear intravenous lipids and smaller mass of adipose tissue (Morillas et al. 1992). One study reported that an increased intake of energy seems to enhance VLDL synthesis (Gibbons 1990). Since infants switch an energy supply from carbohydrates (main energy source during prenatal period) to lipids after birth, preterm infants begin to receive lipid emulsions and this increase intake of energy. This may suggest that an increase VLDL in addition to low LPL activity impairs the TG hydrolysis of lipoproteins.

4.6 Strengths and Weakness of the Study

As a whole, there were a number of remarkable strengths in this study. First, the GC-MS method, which is able to quantify absolute amounts of fatty acids, was developed involving validations using the NIST standard and external validation at the reference laboratory. The calculation of absolute amounts of fatty acids rather than percentages provides a more precise and accurate measurement. This subsequently allowed for proper statistical analysis between different nutritional groups and between actual feeds and resulting plasma. Secondly, an appropriate number of samples were collected for each

group analyzed by GC-MS which is in accordance to the estimated sample size. The standard deviation of analyzed samples for this study (4%) was much lower than the standard deviation (14%) used for the sample size calculation, indicating that the results are valid. Moreover, upon a literature review, this is the first study to investigate fatty acid profiles in hypertriglyceridemic plasma in preterm infants in relation to the nutrition of breast milk and several lipid emulsions.

One limitation of this study is that although some samples came from the same infants, each sample was treated as an independent sample as this was a pilot and exploratory study. Further, no correction was made for multiple comparisons except partial correlations for the same reason. Although adjusting confounding factors such as gender, gestational age, and weight would affect the fatty acid profiles, these were dismissed because total intake of fat and infusion rate of lipid emulsions are dependent on gestational age and weight.

The sample size for lipoprotein analysis was relatively low. Leftover samples from GC-MS analysis were utilized for this analysis and not many samples were left for this purpose. Additionally, due to lack of quality control sample with known absolute lipoprotein values, only relative percentages of lipoproteins were determined indicating that increase or decrease in one lipoprotein would cause a shift relative amount of all other lipoproteins. Although high variations in quality control samples were adjusted between gels for the lipoprotein analysis, high variation in the 1d gel electrophoresis method may have compromised the results.

CHAPTER 5. Conclusions

This study demonstrates that the developed GC-MS method can be utilized to analyze relative and absolute amounts of fatty acids in both nutritional sources and plasma for preterm infants. Absolute rather than relative amounts of fatty acids would help to establish optimal levels of fatty acids in nutrition and plasma. Fatty acid profiles in commercially available lipid emulsions do not resemble each other nor are they similar to fatty acid profiles in breast milk. Additionally, the fatty acid composition of nutritional sources somewhat affects fatty acid profiles in plasma. However, fatty acid chain length, the number of double bonds, and the positional distribution of fatty acids in dietary TG can also influence fatty acid absorption. It indicates that lipid emulsions with different fatty acid profiles may not be biologically equivalent due to the structural characteristics of fatty acids, which lends useful insight into future designs for new nutritional sources for preterm infants. Moreover, accumulation of all fatty acids (to varying degrees) as well as relatively high lipoproteins of (LDL+VLDL) resulted in hypertriglyceridemic plasma. Although due to individually varying rate of lipid clearance, infants' prematurity, and enzyme activity, the results, which particular fatty acids with high ratios between hyper- and normotriglyceridemic plasma may not be readily metabolized, cannot be gleaned from the study. Overall, optimal fatty acid composition in lipid emulsion is important especially for preterm infants who have distinctive metabolic needs to maintain adequate growth without high plasma TG levels.

Future studies in this topic and additional analysis of levels of LPL and carnitine are needed to further explore fatty acid metabolism and help to establish international

reference data on fatty acid profiles of preterm infants according to nutritional sources. In addition to fatty acid composition, the adverse effects of lipid emulsion could be a result of their physical structure, which contain excess of artificial chylomicrons. Artificial chylomicrons may not mimic biological transport in the blood, closely enough to be effective in delivering fatty acids, leading them to floating in the blood. Furthermore, it is also important to determine whether acute high TGs during neonatal life have long-term effects on health considering the DOHaD hypothesis.

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Appendix A

Table 9. Identification of fatty acids by the retention time and molecular weight using gas chromatography – mass spectrometry

Fatty acid	Common name	FAME name	Retention time ± 0.5 (min)	Molecular weight (g/mol)
C8:0	Caprylic acid	Octanoic acid, methyl ester	15.2	158
C10:0	Capric acid	Decanoic acid, methyl ester	16.6	186
C12:0	Lauric acid	Dodecanoic acid, methyl ester	18.7	214
C14:0	Myristic acid	tetradecanoic acid, methyl ester	21.5	242
C16:0	Palmitic acid	Hexadecanoic acid, methyl ester	25.6	270
C16:1n7	Palmitoleic acid	9-hexadecenoic acid, methyl ester	27.3	268
C18:0	Stearic acid	Octadecanoic acid, methyl ester	29.3	298
C18:1n9	Oleic acid	9-Octadecenoic acid, methyl ester	31.5	296
C18:2n6	Linoleic acid	9,12-Octadecadienoic acid, methyl ester	34.1	294
C20:1	Gondoic acid	13-Eicosenoic acid, methyl ester	36.6	324
C18:3n3	Alpha-linolenic acid	9,12,15-octadecatrienoic acid, methyl ester	37.3	292
C20:2n6	Eicosadienoic acid	11,14-eicosadienoic acid, methyl ester	38.9	322
C20:3n6	Eicosatrienoic acid	8,11,14-eicosatrienoic acid, methyl ester	40.8	320
C20:4n6	Arachidonic acid (AA)	5,8,11,14-tetraenoic acid, methyl ester	42.1	318
C20:5n3	Eicosapentaenoic acid (EPA)	5,8,11,14,17-eicosapentaenoic acid, methyl ester	45.3	316
C22:6n3	Docosahexaenoic acid (DHA)	4,7,10,13,16,19-docosahexaenoic acid, methyl ester	51	342