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Effects of Dose and Parenteral Lipid Composition on Liver Function in Neonatal Piglets on Total Parenteral Nutrition

by

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ABSTRACT

Both parenteral lipid dose and fatty acid (FA) composition have been proposed as risk factors for neonatal intestinal failure associated liver disease (IFALD). This research compared conventional lipid (Intralipid®, n-6FA), dosed both high (10 g/kg/d) and low (5 g/kg/d), to fish oil (Omegaven®, n-3FA), dosed low (5 g/kg/d), in neonatal piglets. Piglets were given iso-nitrogenous TPN for 14 days and compared to sow fed controls. Outcome measures included bile flow, total body and brain weight. Bile flow was increased with fish-oil treatment and lowered with high dose Intralipid® ($p < 0.05$) while not different between low dose Intralipid® and controls. All TPN groups weighed less than controls ($p < 0.05$). Both low dose treatments were associated with reduced brain weight compared to the other groups ($p < 0.05$). These findings suggest that while low dose lipid treatments reduce the risk of developing IFALD, growth in neonates may be compromised.

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List of Abbreviations

ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
AST	Aspartate Aminotransferase
CCK	Cholecystokinin
CRP	C-Reactive Protein
GGT	Gamma-glutamyl transferase
IFALD	Intestinal Failure Associated Liver Disease
IL-6	Interleukin-6
PN	Parenteral Nutrition
TNF-α	Tumor Necrosis Factor- Alpha
TPN	Total Parenteral Nutrition

Fatty Acid Abbreviations

AA	Arachidonic Acid
ALA	Alpha-Linolenic Acid
DHA	Docosahexaenoic Acid
EPA	Eicosapentaenoic Acid
LA	Linoleic Acid
n-3 LC PUFA	n-3 Long Chain Polyunsaturated Fatty Acid
n-6 LC PUFA	n-6 Long Chain Polyunsaturated Fatty Acid

Chapter 1 INTRODUCTION

1. 1 Introduction

Intestinal failure can be defined as an impaired ability to absorb nutrients, restricting both growth and development (Goulet et al. 2004). Infants with intestinal failure often require parenteral nutrition (PN) to ensure normal growth and development during a period where enteral nutrition is unable to be absorbed and/or tolerated (Balistreri, Heubi, and Suchy. 1983; Geyer. 1960; Goulet and Ruemmele. 2006). The challenge is balancing optimal nutrition while managing co-morbidities that relate directly to the provision of PN, in particular life threatening liver disease, now known as intestinal failure associated liver disease (IFALD).

IFALD includes a spectrum of liver diseases from steatosis, to cholestasis and fibrosis, as well as end stage liver disease. The causation is recognized to be multifactorial including sepsis, prematurity, nutritional deficiencies, and a lack of bile flow as a result of limited enteral stimulation (Carter and Karpen. 2007; Diamond et al. 2011; Goulet and Ruemmele. 2006; Peyret et al. 2011).

Recently, parenteral lipids have been implicated in having a role in the development of IFALD (Diamond et al., 2011; O. a. F. R. Goulet, 2006; Hyde et al., 2008.; Mary R, 1987; Peyret et al., 2011; Roesner M, 1987). However, the components or factors of lipid administration that contributes to IFALD development have yet to be elucidated (Venick and Calkins. 2011; Calder and Deckelbaum. 1999; Carter and Karpen. 2007). Regardless, strategies that manipulate parenteral lipid dose or fatty acid composition have emerged aimed at preventing and reducing the severity of IFALD in young children.

Clinical evidence has shown that a modification in fatty acid profile of PN can influence IFALD (Chan, McCowen, and Bistrian. 1998; Diamond et al. 2012; Dupont and Carpentier. 1999; Koletzko and Goulet. 2010; Seida et al. 2013b). Fish oil-based lipids that are predominant in n-3 fatty acids (n-3), in contrast to the current soy based therapy that are predominant in n-6 fatty acids (n-6), have been suggested to reduce the severity of IFALD (Koletzko and Goulet. 2010). Yet, to the author's knowledge, a study examining a fish oil-based lipid treatment at the start of parenteral therapy instead of after the use of soy-based therapy has yet to be performed. Another clinical strategy to reduce the severity of IFALD in neonates is to restrict the dosage of soy based parenteral lipids (Cober et al. 2012a). Cober and team have examined the effects of significant n-6 PN emulsion reduction and the effect on IFALD. They demonstrated that there was an improvement in the clinical symptoms of IFALD; however, this was at the cost of inducing biochemical essential fatty acid deficiency (EFAD) in the infants receiving lipid restriction.

However, to the author's knowledge, a direct comparison of both the alternative lipid sources and PN dose restriction has not been performed. Therefore, the purpose of this study is to examine both dose reduction strategies and modified fatty acid profiles in the development of IFALD in a neonatal model.

1.2. Parenteral Nutrition Support

Total Parenteral Nutrition (TPN) was designed as a way to combat severe malnutrition that was encountered when patients were unable to absorb required nutrients directly from the gastrointestinal tract (Dudrick. 2005; Dudrick et al. 1968). Early parenteral solutions were comprised of glucose and saline solution (Vinnars and

Wilmore. 2003). Additions of proteins, vitamins, and minerals to parenteral solutions supplied most required nutrients, however in order to prevent essential fatty acid deficiency and provide energy, lipids became an important part of parenteral solutions.

Premature neonates are particularly susceptible to nutritional deficits, being born with lower fat and lean mass and impaired ability to absorb nutrients. In Canada, approximately 24,500 births (7% of all live births per year) are preterm (Beck, et al. 2010), with an overall mortality rate of 50 deaths per 1000 births (Sampalis and Williams. 2003). Most preterm infants require PN support for at least the first few weeks of postnatal life. This is principally due to the combination of increased nutrient requirements for rapid growth and development, immature gastrointestinal function and limited tolerance for oral feeding (Goulet and Ruemmele. 2006; Nehra, Fallon, and Puder. 2011). Furthermore, short bowel syndrome (SBS) is a common in preterm infants and so this population has also emerged as one of the most susceptible to the complications of PN therapy, as will be discussed.

1.2.1 History of Parenteral Nutrition

Prior to the advent of PN, patients with complex medical conditions, where enteral feeding was not an option, would die as a result of malnutrition, irrespective of available treatments for their illness (Dudrick. 2005; Shamsuddin. 2003). As a result, it became essential to create a form of nutrition that would be able to be given parenterally. Such a therapy would also benefit the care of the extremely premature infants, who developmentally were not yet able to tolerate optimal amounts of enteral nutrition, thus limiting their survival.

Sir Christopher Wren authored the first recorded report of administering an intravenous 'nutrient' solution in 1665, reportedly giving a mixture of ale, opium and beer to animals (Knochel. 1985; Vinnars and Wilmore. 2003). According to Geyer (Geyer. 1960) and Vinnars and Wilmore (2003), initial parenteral solutions were composed of dextrose and saline solutions, as early as 1896. Early 10% glucose solutions were delivered at a very high rate. Complications - later labeled as 'glucose fever' - suspended the administration of these solutions. Glucose fever was so named because a key feature was a fever secondary to an extremely high blood glucose level (over 600 mg/dL) during administration of glucose infusions. Other symptoms include a dry mouth, sleepiness, and blindness if the high glucose levels persisted.

In 1915, using an infusion pump to control the rate at which the glucose solution was given, it was found that a continuous dose of glucose could be tolerated without 'glucose fever' (Vinnars and Wilmore. 2003). As a result, it was identified that both extreme infusion rates and high glucose concentrations factored into the development of glucose fever. A high concentration of glucose administered at a low rate would not be as adverse as the same glucose concentration given at higher rates over shorter periods (Vinnars and Wilmore. 2003).

After the establishment of safe glucose administration, the focus turned to meeting the essential nitrogen requirements of these patients. At the advent of parenteral solutions, whole protein was given, necessitating breakdown of provided proteins by the patient (Vinnars and Wilmore. 2003; Shamsuddin. 2003). In the late 1930's a huge advance in parenteral solutions occurred when hydrolysated proteins were given parentally. Hydrolysation of proteins increased amino acid bioavailability by placing

whole proteins in a strong acid prior to placing them in parenteral solutions (Knochel. 1985; Vinnars and Wilmore. 2003). Following hydrolysation, the next breakthrough by G.H. Whipple in 1934 was demonstration that an adult dog could meet all its protein requirements parenterally while being fed a protein free enteral diet (Whipple. 1942). This work earned him the Nobel Prize for Physiology and Medicine. Puppies were later shown to grow when fed an intravenous protein infusion (Allen, Stemmer, and Head. 1956). This revelation was significant for neonatal medicine, as the study showed that the same level of protein delivered enterally or parenterally could achieve equal growth rates.

Following the advent of glucose and amino acid solution parenteral solutions, it was recognized that a source of lipid was required, both for energy and to meet the essential fatty acid requirement. It was found that deficiency symptoms such as skin rash and alopecia could be reversed with the addition of linoleic acid (LA, 18:2n6) to the diet (Shamsuddin. 2003). LA, it was later found is an essential precursor for arachidonic acid (Shamsuddin. 2003; MARKLEY. 1947; Sardesai. 1992).

Development of safe parenteral lipid solutions was a complex procedure with hundreds of parenteral lipid sources being rejected (Shamsuddin. 2003). Between the 1920's and the 1960's, primarily in Japan and the United States, hundreds of available lipid sources were tested, including castor oil, olive oil and cottonseed oil (Shamsuddin. 2003). In 1964, the United States banned the use of parenteral lipids as a result of the adverse effects in humans including jaundice, severe liver damage and bleeding (Shamsuddin. 2003; Vinnars and Wilmore. 2003). These effects were especially prominent with the cotton seed-based lipids (Shamsuddin. 2003).

This ban was lifted in 1975 as a result of the successful development and utilization of Intralipid® (Fresenius Kabi, 1961) in Europe (Shamsuddin. 2003; Vinnars and Wilmore. 2003). This soybean based lipid source continues to be the main parenteral lipid source in North America and one of four approved lipid sources for PN solutions in Canada. Other approved sources include: ClinOleic, Travamulsion, and Lyposyn II. The third generation lipid, Omegaven®, is available on a compassionate basis, subject to Health Canada approval. In 2013, Health Canada licensed SMOF lipid for adult patients and it is available off-label for pediatrics.

Finally, it is important to mention the discovery of pyrogenic substances by Florence Siebert. Pyrogens are a metabolic product of microorganisms that result in contamination as a result of improper sterilization (Swarbrick. 2007). Identification of pyrogens allowed for the creation of non-pyrogenic solutions, and has significantly improved patient care, decreasing morbidity and mortality previously associated with PN (Vinnars and Wilmore. 2003).

1.2.2. Modern Parenteral Nutrition

1.2.2.1 Protein

It has been shown using amino acid oxidation studies that neonates are able to convert excess nitrogen delivered in utero to energy (van Goudoever and Vlaardingerbroek. 2013; van den Akker et al. 2009; Chien et al. 1993; Thureen et al. 2003). However, it is now known that the limitation of even a single essential amino acid below age specific requirements can restrict protein synthesis and thus growth (Brunton, Ball, and Pencharz. 2000). This presents a particular problem for neonatal PN, given

amino acid requirements for this population continues to be poorly defined (van Goudoever and Vlaardingerbroek. 2013).

The sulfur amino acids cysteine and methionine present complications in current parenteral formulations. Cysteine (CYS) is easily oxidized in solution to cystine, which is insoluble. Therefore, its addition to commercially available parenteral amino acid solutions is problematic. However, cysteine is a crucial building block in the production of glutathione, a major antioxidant (Miloudi et al. 2012; Zlotkin and Anderson. 1982). Additionally, the more soluble sulfur amino acid methionine (MET) has been added in increased amounts in parenteral amino acid solutions to provide for the reduction or absence of CYS. Yet, MET is inefficiently converted to CYS in neonates (Zlotkin and Anderson. 1982).

Furthermore it appears MET is potentially hepatotoxic. Moss and colleagues (1999), compared enterally fed rabbits with additional IV MET, to rabbits that fed TPN, containing MET, and to chow fed rabbits. At the end of the experiment, rabbits fed IV MET all showed signs of hepatobiliary dysfunction as well as an altered liver histology, worse in the TPN fed group (Moss et al. 1999). This suggests that MET may be one specific amino acid that contributes to TPN liver damage. Furthermore work of Duce and colleagues (Duce et al. 1988) suggests that compromised liver function itself contributes to accumulation of MET through impaired enzyme function in the hepatic degradation of MET via transulfuration.

1.2.2.2 Carbohydrates

While technically not an essential nutrient, carbohydrates continue to be an important part of TPN as a readily available energy source and the preferred fuel for

central nervous system metabolism. Carbohydrates traditionally provide half the total parenteral energy supplied, with a calorie density of ~ 4 kcal/g. However, the use of carbohydrates as the sole non-protein energy source is less than optimal. In this regard a balance of carbohydrates and lipid energy is important. Using a rat model, Buzby and co-authors (Buzby et al. 1981) showed that manipulation of the carbohydrate to fat ratio in PN altered the degree of hepatic steatosis. In these rats, the introduction of a "balanced" non-protein energy regime of 75% carbohydrate to 25% fat was shown to avoid steatosis, while energy delivered solely as carbohydrates or lipid promotes hepatic steatosis (Buzby et al. 1981).

1.2.2.3 Lipids

The addition of lipids to parenteral solutions provides essential fatty acids and a dense source of calories, being ~ 9 kcal/g. A relatively small dose of parenteral lipids (~0.5 g/kg/day) can be provided to prevent the development of essential fatty acid deficiency (Gura, Parsons, and Bechard. 2005).

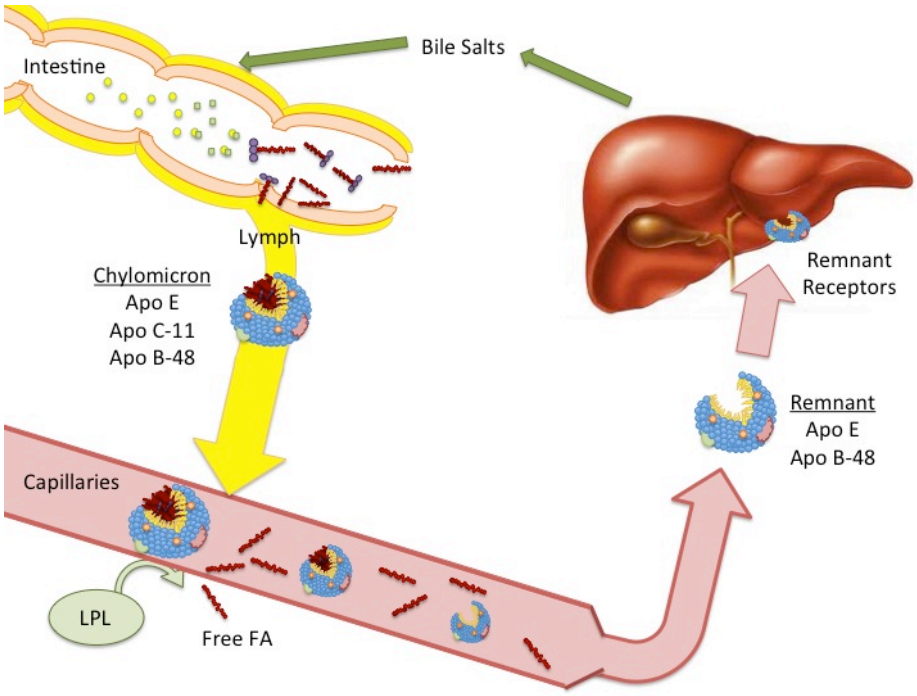
Parenteral lipid is composed of triglycerides, free fatty acids, glycerol, and phospholipids (Fresenius Kabi®). Parenteral lipid enters the blood stream as lipid droplets <5μm in size; similar to chylomicrons (Gura and Puder. 2010; Gottschlich. 1992), and are so classified as chylomicron-like particles (Gottschlich. 1992). However, parenteral lipid bypasses the major physiological processes normally involved with dietary lipid digestion.

Normal enteral lipid digestion begins with the emulsification of fat globules using bile salts (see **Figure 1.1**). These are then further broken down using pancreatic lipase into free fatty acids and monoglycerides. These are then packaged into micelles –

allowing transport of monoglycerides and free fatty acids across the unstirred layer and then they diffuse to the epithelial layer of the intestines. Intracellular addition of Apo proteins (ApoB-48, ApoE and Apo C-11) converts the micelle to chylomicrons, which are then transported to the lymphatic system. From here, the chylomicron is transported to the capillaries, where Lipoprotein Lipase (LPL) works to remove the triglycerides and free fatty acids from the chylomicron. Once the chylomicron is depleted of the triglycerides and free fatty acids, the remnant particle is transported to the liver to be converted into very low-density lipoprotein (VLDL).

In contrast, the primary site of digestion of parenteral lipid bypasses the gastro-intestinal tract directly to the circulation and liver. As mentioned above, lipids from parenteral solutions are labeled chylomicron-like particles, however, unlike intestinal derived chylomicrons, these particles just undergo lipolysis prior to uptake into the hepatocytes (Hultin et al. 1995). The suggested pathway for absorption is that chylomicron like particles bind transiently to the vascular endothelium where lipoprotein lipase removes some of the triglycerides. This process occurs several times before remnant receptors in the liver accept the triglyceride deficient lipid droplet. Once accepted, the liver endocytoses the lipid droplet and degrades it (Hultin et al. 1995; Mahley and Hussain. 1991; Viralo and Llovera. 1988).

Figure 1.1: Enteral lipid absorption



Evidence of parenterally supplied lipid utilization is supported by the rise in the plasma free fatty acids after administration of parenteral lipids (Bezard et al. 1994; Fomon and Heird. 1986; Green D.E. 1963). Using indirect calorimetry, Nordenstrom and colleagues (Nordenstroem et al. 1982) characterized the utilization of fat emulsions and found they were used more effectively than glucose as a source of calories in patients with nutritional depletion (Nordenstroem et al. 1982).

1.3 Fatty Acids

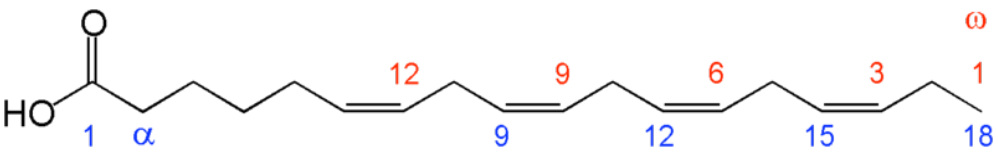
Fatty acids play an important role in many different cellular functions including being metabolized to CO_2 and H_2O for energy (Fomon and Heird. 1986), being incorporated into cell membranes and adipose tissue (Fomon and Heird. 1986; Kalish, Fallon, and Puder. 2012), and being metabolized into other fatty acids or other compounds including prostaglandins (Kalish, Fallon, and Puder. 2012; Fomon and Heird. 1986). Fatty acids also comprise a major part of the brain (Fomon and Heird. 1986). They are a source of fuel via mitochondrial beta-oxidation, and are stored as the bodies

main fuel reserve in adipose tissue. In addition, they mediate cell signaling and gene transcription, through incorporation into membrane phospholipids that directly modifies membrane structure and fluidity (Fomon and Heird. 1986). Finally, they function as precursors for biologically active compounds, such as eicosanoids, active in cardiovascular, immune and other important functions (Kalish, Fallon, and Puder. 2012).

Fatty acids consist of a carboxylic acid with a long chain and are classified according to the chain length and/or saturation. Saturation refers to the presence of double bonds within the fatty acid tail. A monounsaturated fatty acid contains a single double bond, while a polyunsaturated fatty acid contains multiple double bonds (Bezard et al. 1994; Campbell and Reece. 2002). Saturation becomes very important in cell membranes, as an increase in saturation decreases membrane fluidity (Campbell and Reece. 2002; Kalish, Fallon, and Puder. 2012; Ramirez, Amate, and Gi.). Short chain fatty acids are between 2-4 carbons long, medium chain fatty acids are between 6-10 carbons long, long chain fatty acids from 12-26 carbons in length (Kalish, Fallon, and Puder. 2012) and very long chain from 28 and greater carbons in length.

For fatty acids, the basic structure of chain length can vary greatly. Thus, fatty acid nomenclature starts at the tail end of the fatty acid: designated as the omega (n) position. Fatty acids are then defined by the first occurrence of a double bond, delineated by an –x, x representing the start of the double bond. See **Figure 1.2** for details.

Figure 1.2: Free fatty acid numbering



(Kalish et al. 2012)

Thus an n-3 fatty acid as demonstrated in **Figure 1.2** has the first double bond occurring at the third carbon from the tail end of the fatty acid. To further identify a fatty acid, the numbers following the n and double bond classification describe the total number of carbon bonds and double bonds within the tail of the fatty acid. Thus the classification of n-3 18:4, (stearidonic acid, as pictured in **Figure 1.2**) describes a fatty acid tail that is 18 carbons long, with four double bonds, the first occurring three carbons from the tail (Gura and Puder. 2010)

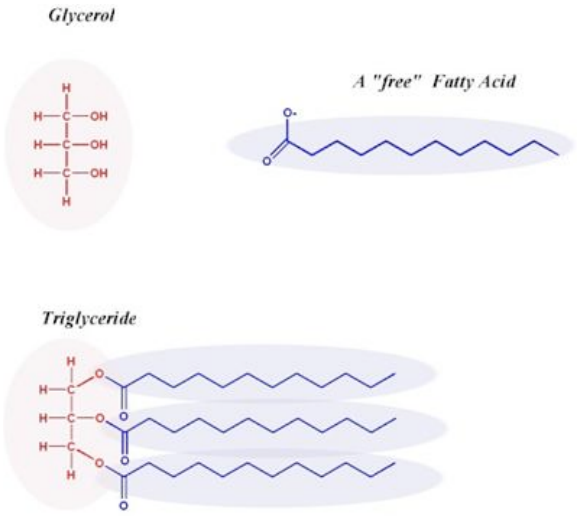
Because of their chemical structure, free fatty acids are extremely energy dense, contributing 9 kcal/g of energy (Kalish, Fallon, and Puder. 2012). In comparison to proteins and carbohydrates at an energy density of 4 kcal/g, the ability of free fatty acids to store energy becomes essential for maintaining energy balance (Kalish, Fallon, and Puder. 2012). This ability to store energy is enhanced by the hydrophobic properties of a free fatty acid. Because free fatty acids are hydrophobic, they are able to be stored in an anhydrous environment, like adipose tissue.

In comparison, if the human body were to use glycogen as its main energy source, 1g of glycogen binds to approximately 2g of water. This greatly reduces the energetics (1.33kcal/g glycogen) as well as drastically increasing the weight to be energetically equivalent to fatty acids. Thus the human body would require 31kg of glycogen to match the energetically equivalence of 5 kg of fatty acids stored as lipids.

Storage of fatty acids requires conversion into lipid involving a dehydration reaction using both free fatty acids and a glycerol backbone (Campbell and Reece. 2002). **Figure 1.3** shows the different components of a triglyceride, which is a lipid most commonly used in the storage of energy in fat deposits. The glycerol background is

composed of an alcohol with three additional carbons each bearing a hydroxyl group.

Figure 1.3: Components of a triglyceride



(Lee et al. 2006)

The C-H bonds in the hydrocarbon tail are responsible for the hydrophobicity expressed by lipids (Campbell and Reece. 2002; Kalish, Fallon, and Puder. 2012). In a triacylglycerol lipid, all three hydroxyl groups are bound to a free fatty acid. These fatty acids are not required to be the same, thus a mixture of saturated and unsaturated fatty acids of differing length can be used in one triacylglycerol (Campbell and Reece. 2002). A phospholipid is comprised of the same components, however, instead of being bound to three free fatty acids, one of the hydroxyl groups is bound to a phosphate group (negative in charge). This negative charge enables hydrophilic properties exerted by phospholipid 'head'. Thus, the hydrophobic and hydrophilic properties of a phospholipid allow for the formation of the phospholipid bilayer found in cell membranes (Campbell and Reece. 2002).

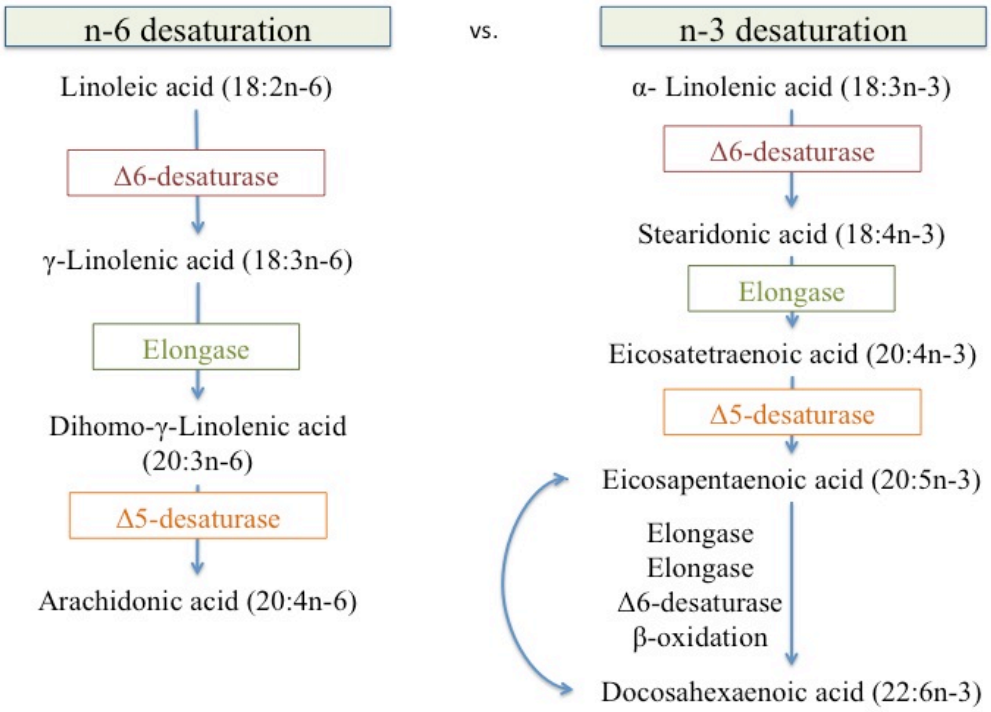
1.3.1 Essential Fatty Acids

The dietary essential nature of the fatty acids Alpha-Linolenic acid (n-3 18:3) (ALA), and LA depends upon the human body's inability to insert double bonds past the

ninth n-carbon position (Sardesai. 1992). As a result, these essential fatty acids need to be provided within PN and failure to provide them over a period of time can result in essential fatty acid deficiency (EFAD). Preterm neonates are particularly susceptible to an essential fatty acid deficit, as a result of their limited fat stores (Mayhew and Gonzalez. 2003) and increased requirement for EFA. They can display symptoms of EFAD including alopecia, skin rash, soft brittle nails, excessive thirst, and frequent urination (Herrera and Amusquivar. 2000; Manson et al. 1999; Hay. 1991) within 72 hours of PN delivery with no lipids added (Mayhew and Gonzalez. 2003; Evans and Thureen. 2001).

Initial research by George and Mildred Burr in 1929 listed three dietary essential fatty acids: LA, ALA and arachidonic acid (AA, n-6 20:4)(Green D.E. 1963). Since the original publication, it has been recognized that only LA and ALA are truly dietary essential, while AA has been shown to be conditionally essential. For neonates in particular, this requirement in the diet is the result of a limitation of available enzymes and decreased enzyme activity resulting in an insufficient conversion of LA to AA (**Figure 1.4**) (Bezard et al., 1994; Green D.E., 1963; Sardesai, 1992). This is also true of the developmentally conditionally essential products of ALA desaturation, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Bezard et al. 1994).

Figure 1.4: Desaturation of essential fatty acids



(Lee et al. 2006)

Due to the lengthy desaturation process as seen in **Figure 1.4** from Lee et al. (2006), elongation and desaturation of ALA and LA is an energy costly process, with relatively low efficiency in both adults and infants (Innis. 1993). Valenzuela and colleagues (Valenzuela, Sanhueza, and Nieto. 2006) indicate that less than 5% of ALA is converted to DHA. This has serious implications for an infant, when the DHA requirements are highest as a result of higher rates of postnatal growth and brain development (Crawford. 2000; Lapillonne, Eleni dit Trolli, and Kermorvant-Duchemin. 2010; Carlson. 2001). In series of studies done by Brenna and colleagues (2009, 2012) it was found that only supplementation of pre-formed DHA increases plasma levels of DHA in infants, but not the addition of ALA. Even when ALA levels were increased ten times the level of DHA, retinal concentration of DHA did not match levels of those being

fed pre-formed DHA (Brenna et al. 2009; Brenna. 2012). As a result of the high requirement and the potential usage of only pre-formed DHA, it is important to consider what the true DHA requirement for premature infants might be and should DHA be considered essential in infant diets including pediatric PN to maintain normal brain and retinal development (Green D.E. 1963).

1.3.2 Parenteral Fatty Acids

While there are four different parenteral lipid sources that are currently available and approved in Canada, we will be specifically examining two of these parenteral lipids that vary significantly according to fatty acid composition: Intralipid® and Omegaven®. Intralipid® is the most commonly used PN emulsion, composed primarily of soybean oil. This plant-based lipid therapy is high in n-6 fatty acids and low in n-3 fatty acids, with the latter being provided in the form of ALA. The resulting formulation has an n-6 to n-3 fatty acid ratio of 7:1. The alternative emulsion, Omegaven® has been used in Canada since 2007 on a compassionate basis for treatment of IFALD. This is a fish oil based lipid emulsion, which predominantly contains n-3 fatty acids - including a large amount of pre-formed EPA and DHA. As a result, Omegaven® has an n-6 to n-3 fatty acid ratio of 1:8. As seen in **Tables 2.1** and **2.2**, these two lipid sources have markedly differing fatty acid composition. They also differ in Vitamin E content. Vitamin E is a powerful anti-oxidant that is used in Omegaven® to prevent the n-3 LCPUFA's, like DHA that are readily oxidized, producing reactive oxygen species (Traber and Atkinson. 2007).

1.4 Complications of PN support

Many significant complications can arise from PN use. By removing enteral stimulation, use of PN affects gut mass and function (Goulet et al. 2004; Duro, Kamin,

and Duggan. 2008; Goulet and Reummele. 2006). When accompanied by even a small proportion of enteral nutrition, gastrointestinal (GI) atrophy is slowed (Grand, Sutphen, and Montgomery. 1979). Compromised gut mass and function with TPN is thought to contribute to small bowel bacterial overgrowth, translocation and an increased risk of sepsis (Atinmo et al. 1976; Balistreri, Heubi, and Suchy. 1983; Diamantia et al. 2007).

Complications of PN use are also affected by age and stage of development. The premature infant is at greatest risk of the long-term complications from PN, including catheter-related sepsis, venous thrombosis, liver disease and retardation of bone growth. Unfortunately, intestinal failure is also more common in premature infants than in adults, necessitating TPN (Goulet and Reummele. 2006). This is because, along with immaturity of the gastrointestinal tract, premature infants are at increased risk of gastrointestinal conditions like necrotizing enterocolitis, antenatal intestinal atresia and gastroschisis (Goulet et al. 2004; Duro, Kamin, and Duggan. 2008). These can cause SBS, the most common cause of severe intestinal failure in neonates (Diamond, Pencharz, and Wales. 2009; Duro, Kamin, and Duggan. 2008). These conditions reduce the length of the small intestine, either by the required surgical resection or through congenital losses, and so increase the need for PN support for survival and normal growth (Spencer et al. 2005). SBS results in the need for prolonged PN, secondary to intestinal failure. The length of PN support required is determined by the length and function of the remaining gastrointestinal tissue (Nehra, Fallon, and Puder. 2011).

One of the most common and significant complications of intestinal failure, and with SBS in particular, is the liver disease. PN disrupts the entero-hepatic circulation of bile acids (Halpern and Dvorak. 2008; Holschneider et al. 1974) compromises gut barrier

function and so increases the potential for sepsis (Duro, Kamin, and Duggan. 2008; Spencer et al. 2005). Inadequate enteral nutrition further reduces bile flow through enteral stimulated hormonal mechanisms. Therefore, long-term use of PN is associated with liver disease, particularly cholestasis in neonates (Duro, Kamin, and Duggan. 2008; Nehra, Fallon, and Puder. 2011; Spencer et al. 2005). This liver disease remains the most severe complication of neonatal intestinal failure, leading to early mortality or a need for intestinal transplantation (Goulet and Ruemmele. 2006).

1.5 Intestinal Failure Associated Liver Disease in Neonates

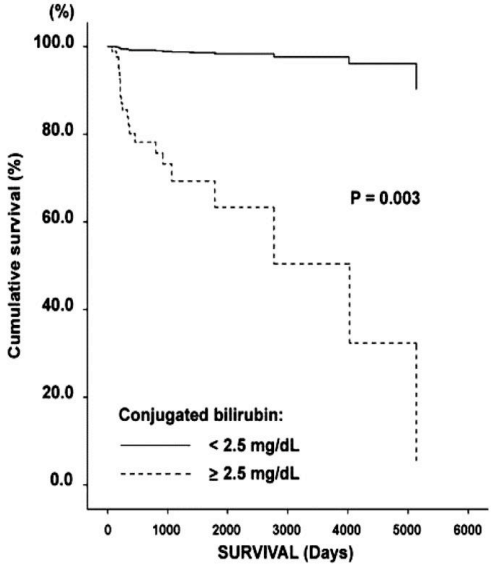
Intestinal failure associated liver disease (IFALD) can be defined as an elevation in the liver function test values (conjugated bilirubin, Alanine Transaminase (ALT), Aspartate Aminotransferase (AST), and Gamma-glutamyltransferase (GGT)) in patients receiving PN and contains a wide clinical spectrum of hepatobiliary dysfuction including: steatosis, cholestasis, cirrhosis and liver failure (Kelly. 2006). In adult cases, IFALD also carries a specific amount of time that these tests need to be elevated (approximately 6 weeks), however, in neonates, the progression of disease is often very rapid, and a repeated elevation of liver function tests (on two occasions) is enough to diagnose IFALD (Goulet and Reummele. 2006).

IFALD is a multi-factorial disease where there are many contributing factors that lead to its development. In infants, factors can include: a shortened bowel due to SBS, recurrent sepsis, prematurity, and/or the duration of PN (Goulet et al. 2004; Kelly. 2006; Goulet and Reummele. 2006). It is estimated to occur 40-60% of children with SBS, and in up to 85% in neonates with SBS (Diamond et al. 2011). Of these, ~25% proceed to

end stage liver disease, which carries a mortality rate of ~30% while infants wait for appropriately sized organs for transplantation (Chungfat et al. 2007).

IFALD is known to be the most significant cause of mortality in intestinal failure patients - particularly in the neonatal population (Carter and Karpen. 2007; Nehra, Fallon, and Puder. 2011). We see in **Figure 1.5** by (Spencer et al. 2005) the cumulative survival rate significantly dropped for infants with SBS with a conjugated bilirubin >2.5 mg/dL (42.75 μmol/L) over time, while those infants without a raised bilirubin level maintained a higher level of survivability over the same period.

Figure 1.5: Cumulative survival rate (%) in children over time with IFALD using conjugated bilirubin



(Spencer et al. 2005)

1.5.1 Pathology

The development of IFALD is multifactorial, inclusive of factors that can be related directly to the PN solution and other factors, notably the immaturity of the hepatobiliary system in the developing neonate. As mentioned above, liver pathology associated with IFALD includes steatosis, cholestasis and fibrosis or cirrhosis. Steatosis

is the accumulation of fat deposits within the liver indicating impairment in the synthesis or removal of triglycerides. Cholestasis is the primary form of IFALD seen in the neonatal population and is an impairment of bile flow either by an obstruction in the extrahepatic biliary tree, or a reduction in the intrahepatic ability to form and transport bile salts. Fibrosis is akin to scarring given severe liver damage with hepatocellular necrosis. The accumulation of connective tissue disrupts the structure and function of tissues affected, creating the histopathological entity of nodular cirrhosis.

Cholestasis has several definitions; in a pathological sense it refers to a systemic accumulation of biliary constituents resulting from impaired bile excretion, liver to bowel. In the clinical context, it is usually considered to exist when conjugated bilirubin is elevated between 2-3mg/dL (34.2 – 51.3 μmol/L) (Duro, Kamin, and Duggan. 2008; Nehra, Fallon, and Puder. 2011; Spencer et al. 2005). At the current time, discontinuation of PN is the only proven method to resolve IFALD (Nehra, Fallon, and Puder. 2011; Goulet et al. 2004).

1.5.2 Aetiological Factors Independent of PN

The fact that IFALD is most common in the preterm population with intestinal failure suggests that immaturity of the developing hepatobiliary system is likely to play a prominent role (Grand, Sutphen, and Montgomery. 1979; Duro, Kamin, and Duggan. 2008). Neonates have a small bile acid pool at birth (Heubi, Balistreri, and Suchy. 1982; Watkins et al. 1975), and this is exacerbated in the pre-term neonate, where the gastro-intestinal tract is the one of the last organ systems to mature (Duro, Kamin, and Duggan. 2008; Goulet et al. 2004).

SBS is often associated with significant loss of ileum in neonates, especially in conditions such as NEC and ileal atresia. As most of the bile salt reabsorption occurs in the ileum (Holschneider et al. 1974), neonatal patients with SBS deplete an already deficient bile acid pool (Heubi, Balistreri, and Suchy. 1982; Holschneider et al. 1974; Watkins et al. 1975; Halpern and Dvorak. 2008).

Intestinal failure is associated with decreased tolerance of enteral nutrition (Cowles et al. 2010). Enteral nutrition works to improve the enterohepatic circulation, and increase bile flow through hormonal mechanisms, such as cholecystokinin (CCK), which is released from duodenal mucosal cells when stimulated by the presence of fat in the GI tract which then stimulates the release of bile from the gall bladder as well as digestive enzymes including pancreatic lipase. A further result of minimal enteral stimulation, intestinal atrophy, increases the risk of bacterial translocation, as was discussed. Both bacterial sepsis and the production of endotoxins associated with bacterial sepsis have been associated with cholestasis (Atinmo et al. 1976; Van Camp, Tomaselli, and Coran. 1994). Sepsis has been commonly associated with the development of IFALD (Sungurtekin et al. 2011). As the number of septic events increases, there is a concurrent increase in the bilirubin levels (Spencer et al. 2005).

1.5.3 Aetilological Factors Related to PN

The specific role of amino acids as a cause of cholestasis is debated (Adamkin. 2003). Early studies showed inadequate growth in neonates as a result of inadequate amino acid concentrations – with amino acid formulations reflecting adult requirements instead of neonatal (Heird. 1998). This resulted in both growth and metabolic issues for neonates on long-term parenteral nutrition.

Initially, a dose dependent relationship between cholestatic liver disease and amino acid delivery was explored by Vilesis and colleagues (1980). Using a prospective design, infants were examined for development of parenteral nutrition associated cholestatic jaundice on varied dosages of amino acids, keeping all other parenteral elements equal between groups. Cholestatic jaundice was defined as a direct bilirubin above 2 mg/dL (34.2 μmol/L) and was assessed weekly. Study results indicated that those infants receiving the high level of amino acid delivery, showed a significantly higher bilirubin level than those who had received the low level of amino acids. However, the authors concede that there is a possibility for an amino acid imbalance with regards to infant requirements particularly considering the total sulfur amino acids, which could possibly influence the development of cholestasis (Vileisis, Inwood, and Hunt. 1980).

To further understand the relationship between parenteral amino acids and cholestasis, Hata and colleagues (Hata et al. 1994) altered the composition of TPN solutions given to neonatal rabbits for 7 days. Parenteral solutions were formulated with one group having high dextrose concentrations, and another high levels of amino acids. At the end of the study, the high dextrose TPN solution displayed both biochemical and histological signs of cholestasis, while those given high levels of amino acids showed normalized biochemical and histological liver profiles. As a result, the authors concluded that non-protein calorie overload induced cholestasis, rather than high levels of protein administration (Hata et al. 1994).

Tuchweber and colleagues (Tuchweber et al. 1990) utilized guinea pigs, and found that intrahepatic cholestasis caused by an amino acid and dextrose solution was

reversed by the addition of taurine, which was not added to pediatric formulations at the time (Helms et al. 1987; Adamkin, Sims, and Radmacher. 1985). However, the subsequent addition of taurine to standard amino acid solutions has not prevented IFALD. Therefore, the role of amino acids in neonatal PN formulations as a cause of IFALD needs further elucidation.

The relationship between glucose delivery and IFALD seems to pertain mainly to steatosis, rather than cholestasis (Sax et al. 1986; Serfatya and Lemoinea. 2008). In diabetic patients it has been observed that use of insulin to regulate the blood glucose level in patients receiving PN resulted in a decrease in the incidence of cholestasis and biliary sludge (Mesotten et al. 2009). This is suggested to be a result of bile acid-independent fractions of bile flow being decreased by hyperglycemia (Garcia-Marin, Villanueva, and Esteller. 1988). However, outside of diabetes, steatosis has been more often linked to glucose administration, particularly in adult home PN patients (Sax et al. 1986; Serfatya and Lemoinea. 2008).

Minerals such as copper - which have been suggested to be hepatotoxic when added to PN – tend to have their excretion linked to biliary function and therefore accumulation with cholestasis does not infer causation (Blaszyk et al. 2005; Spee et al. 2006). This is also the case with manganese, with studies identifying high manganese levels in infants showing cholestatic liver disease not proving hepatotoxicity (Reynolds, Kiely, and Meadows. 1994). A randomized control trial by Fok and associates (Fok et al. 2001), found no difference in incidence of cholestasis between infants that received parenteral manganese supplementation and those that did not. However, the degree of

hyperbilirubinaemia was more profound in those infants that received PN with manganese. Therefore the role of manganese in IFALD remains controversial.

1.6 Intestinal Failure Associated Liver disease the Role of Parenteral Lipid Composition and Dose in IFALD

1.6.1 The Composition of Soy-based Lipid and Risk of IFALD

Soy-based lipids, having been available longer than other emulsions for PN, have been studied in detail. As a result, a link between the duration of soy-based lipids and IFALD has been studied extensively. However, it is not immediately apparent which aspect of the soy-based lipid influences IFALD development to a greater degree. Aspects including the high n-6 fatty acid content and resulting eicosanoids, high levels of phytosterols and a lack of vitamin E have all been implicated in the development of IFALD.

Inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and their eicosanoids nitric oxide, prostaglandin E_2 and leukotriene have been examined for their role in a prolonged inflammatory response due to parenteral nutrition delivery. Eicosanoids - immune system modulators derived from the oxidation of 20 carbon polyunsaturated fatty acids, primarily AA, are produced when the polyunsaturated fatty acids are released from the phospholipid membrane of inflammatory cells (Calder, 2012). The predominant theory is eicosanoids derived from AA are generally pro-inflammatory (Martins, 2007), while those derived from EPA and DHA are generally less so. However, in a systematic review of available literature, Johnson and Fritsche (2012) conclude that there is no indication that simply consuming or providing n-6 LC-PUFA results in a pro-inflammatory situation. Of the available 20-carbon PUFA, AA is

generally more readily available. However, when the concentration of n-3 LC PUFA in the diet is increased, the n-3 LC PUFA's are preferentially selected for use over the n-6 LC PUFA's due to Δ-6 desaturase activity. This is suggested to result in a decreased production of AA derived eicosanoids resulting (Calder. 2006). In particular, AA-derived eicosanoids including prostaglandins, and leukotrienes, and reactive oxygen species, have all been implicated as being related to the very high n-6 parenteral lipid administration (Calder. 2006).

There are a number of different types of eicosanoids: prostaglandins, prostacyclins, thromboxanes, and leukotrienes have been studied the most (Calder, 2012). Altering the fatty acid from n-6 to n-3 has been found to produce eicosanoids with differing structure than that of those derived from n-6 fatty acids, however in a study by Rees et al. (2006) it was found that the dose of EPA required to alter the eicosanoid production was relatively high - at intakes between 1.35 g/day and 2.7 g/day. In a study by Bagga and colleagues (2003), they showed that the expression of prostaglandin COX-2 and IL-6 secretion was reduced in cell cultures exposed to EPA and DHA in an inflammatory situation.

'Non-classic eicosanoids' including resolvins, known as anti-inflammatory mediators, are synthesized in two different manners. Through supplementation with DHA and the addition of Aspirin, the 17R resolvin series was identified using mediator informatics. Utilizing the mediator informatics approach, the discovery of the 17S resolvin pathway; which can be produced exogenously, not requiring the supplementation of Aspirin or DHA (Ariel and Serhan, 2007). Both the 17R and 17S series belong to the D resolvin series. The D resolvin series named for the precursor DHA; has been

indicated in limiting the polymorphonuclear leukocyte presence in brain, skin, and peritoneum tissues (Ariel and Serhan, 2007). The E resolvin series named for the precursor EPA; are also found to reduce the inflammatory reaction (Calder, 2012). Because n-3 LC-PUFAs are an integral part of the formation of resolvins, supplementation (with or without Asprin) encourages the production of resolvins in an inflammatory situation (Ariel and Serhan, 2007). Included in the non-classical eicosanoids are lipoxins. Derived from AA, lipoxins have been described as having anti-inflammatory properties. It appears that the primary function of lipoxins is to inhibit inflammation. This includes inhibiting neutrophil chemotaxis, and blocking TNF-α secretion (Stables and Gilroy, 2011).

Production of inflammatory cytokines including TNF-α, IL-1β, and IL-6, is stimulated by AA derived eicosanoids (Calder, 1997). By altering the eicosanoid substrate to an n-3 fatty acid (EPA derived eicosanoids in particular) has the potential to alter the cytokines that are produced. Human studies have shown that increases in EPA and DHA result in an ex vivo decrease in the production of TNF-α, IL-1β, and IL-6 (Calder 1997, Khalfoun et al. 1997).

Another suggested causative influence for IFLAD is the presence of phytosterols in plant-based lipid emulsions. Phytosterols are a plant-based sterol, similar to cholesterol. Unlike cholesterol, phytosterols are unable to be processed and incorporated into bile acids – which can result in an accumulation over time in the liver (lyer, Spitz, and Clayton. 1998; Clayton, Whitfield, and Iyer. 1998). They can be viewed as a contaminant of soy-based lipids as, of yet, there is no method to separate phytosterols from such plant based oils.

Using a mouse model, El Kasmi and colleagues examined the role of phytosterols in liver injury, cholestasis, and liver macrophage activation (El Kasmi et al. 2013) using several different experiments contrasting fish oil, soybean oil, saline solutions and chow fed control mice. Their experiments showed the presence of phytosterols (administering soybean oil) worsened cholestasis and macrophage activation. Those treatments that did not receive phytosterols (those groups given fish oil, or saline) showed similar outcomes to the chow fed controls. Using a neonatal piglet model, Clayton and colleagues (Clayton, Whitfield, and Iyer. 1998) administered phytosterols parenterally, and independent of other nutrient components of PN and concluded that phytosterols reduced measured bile flow. They postulated this was the result of their incorporation into the cell membrane transport proteins responsible for bile secretion.

Parenteral lipids have also been indicated in increasing the oxidative stress experienced by those on parenteral lipid therapies. Oxidative stress has been linked to lipid peroxidation products using animal studies. Peroxidation products including malondialdehyde occur at higher levels in cholestatic livers (Weinberger, Watorek, and Strauss R. 2002) with these products being associated with leukocyte infiltration and decreased antioxidant activity (Krahenbuhl et al. 1995; Weinberger, Watorek, and Strauss R. 2002).

1.6.2 The Composition of Fish Oil: Use and Treatment of IFALD

In contrast to soy-based parenteral lipid, pure fish oil is predominant in n-3 fatty acids, has a high vitamin E content and does not contain phytosterols. The use of fish oil in parenteral lipid therapies rose to prominence in North America with the publication of "Use of a fish oil-based lipid emulsion to treat essential fatty acid deficiency in a soy

allergic patient receiving PN" (Gura, Parsons, and Bechard. 2005). This case study describes the reversal of EFAD, defined by both a normalization of the triene to tetraene ratio as well as a disappearance of the characteristic rash. This reversal of EFAD in a soy allergic patient led the authors to hypothesize that fish oil could be used successfully used to treat IFALD while providing adequate n-3 essential fatty acids. They assumed that the elimination of soy parenteral lipid and replacement with fish oil would reverse IFALD, because of the long chain n-3 content.

This hypothesis was tested first in case studies (Gura et al. 2006). Two neonates experiencing IFALD while fed soy-based PN therapy, were changed to fish oil-based therapy at 1 g/kg/d, with the remaining non-protein caloric deficit being provided as carbohydrates. Serum bilirubin was used to define cholestasis and C-reactive protein (CRP) levels were measured to monitor systemic inflammation. After 8 weeks of only fish oil therapy, both cases had resolution in cholestasis and inflammation, implied by elevated bilirubin and CRP.

In both cases a significant dose reduction accompanied the change in parenteral lipid compositions, changing from a soy-based parenteral lipid dose of 3 g/kg/d to fish oil-based lipid dose of 1 g/kg/d. Therefore it is unclear if the reversal of IFALD was due to the alternative lipid composition or overall reduction in total lipid dose. Another shortcoming in this study was the confounding effect of enteral nutrition given in one infant, having 100% of nutrition requirements met by PN as well as an additional 50% given enterally. An additional limitation of the study was the use of only a single biochemical marker for liver injury and inflammation. These two studies did prompt larger case series to be conducted in both Canada and the United States.

The next study done by Puder and associates (Puder et al. 2009), was the largest case-control study reported to date on this topic. Historical controls were identified and compared to infants on fish oil-based treatment in the modern era. Using infants with SBS, Puder found that the time to cholestasis reversal (defined by serum bilirubin level declining to <2 mg/dL (34.2 μmol/L)) was drastically reduced in those infants given parenteral fish-oil. However, it is important to note that the dosages of total lipid were again drastically different. The fish-oil therapy, Omegaven®, was given at doses of 1 g/kg/d, while the historical controls, on Intralipid®, had dosages ranging from 1-4 g/kg/d. So while, the time to reversal of IFALD may have been different, the confounding issue of lipid load still could not allow us to draw definite conclusions as to the reason for IFALD reversal.

Subsequently a Canadian study reported the use of both Intralipid® and Omegaven® in combination, with 1 g/kg/d dosages for each, as well as sole Omegaven® therapy (Diamond et al. 2009). The combination approach was done in an effort to balance the fatty acid profile, supplying both n-6 and n-3 LC PUFA's, as well as to maintain conventional total lipid dosing. Once again, using infants with SBS, it was shown that using a balanced fatty acid profile 9 out of 12 infants were removed from the intestinal transplant list with a complete reversal of hyperbilirubinemia. However, this study again did not have a contemporary control group.

Subsequently, there has been a plethora of reports of the use of fish oil in the treatment of IFALD, both as monotherapy as well as in conjunction with other lipid therapies. A brief summary of key publications is shown in **Table 1.1**

Table 1.1 Comparison of literature detailing Omegaven® use and outcomes

Author	Rollins	St-Jules	Groleau	Lee	Klein	Hall
Lipid	IL/O	O	1:1 & O	SMOF & O	IL/O	O
Dosage	2 g/kg/d	1 g/kg/d	2 &1 g/kg/d	-	3 than 1 g/kg/d	0.2 g/kg/d
# treated	25	10	15&5	7	10	60
Type	Case Study	Retrospective	Case Study	Retrospective	Prospective	RCT
Control	None	None	None	None	None	Contemporary
Outcomes	10 resolved IFALD with enteral intake	Resolution of IFALD	Quicker IFALD resolution on O	Bilirubin and CRP rose with sepsis	n-3 circulation increased	No significant difference between controls and O
	6 died/ required transplants	Normal growth		O reduced septic markers	n-6 circulation decreased	Organ dysfunction decreased with O
	6 required sole O therapy					

Literature compared based on their use of Omegaven as a parenteral lipid therapy. Study lipid doses, type of trial, number of patients studied, and control group is displayed for each trial. Abbreviations: O: Omegaven®, IL: Intralipid®, 1:1: 1 g/kg/d Intralipid® : 1 g/kg/d Omegaven®, SMOF: SMOFlipid®, IFALD: Intestinal Failure Associated Liver Disease, RCT: Randomized Controlled Trial, CRP: C-Reactive Protein, n-3: omega-3 fatty acid, n-6: omega-6 fatty acid Authors as noted: (Groleau, Thibault, and Marchland. 2014; Hall et al. 2014; Klein et al. 2013; Lee et al. 2013; Rollins et al. 2010; St-Jules, Waatters, and Iwamoto. 2014).

1.6.3 Lipid Dose Minimization and Treatment of IFALD

Lipid minimization in North America has become a significant trend with both the contemporary soy-based lipids as well as the novel fish oil-based therapies. Having done a review of the relationship between lipid dose and IFALD in 2010, Cober and colleagues suggested that a restriction of soy-based lipids could be used to treat IFALD (defined as a total bilirubin >2.5 mg/dL or 42.5 μmol/L) (2012). Comparing to matched historical control - where each infant pair was matched based on weight, diagnosis, and gestational age - Cober and colleagues evaluated 31 infants treated with dose restriction of soy-lipid to 1 g/kg/d on two days a week to the controls who received 3 g/kg/d continuously.

After 9 weeks of study, it was found that lipid minimization did reduce the bilirubin levels in lipid-restricted infants as compared to the historical controls. IFALD was defined as a bilirubin of >2.5 mg/dL (42.75 μmol/L). Analysis compared the slope of the average bilirubin levels over a week for each matched pair, finding a significant difference in slopes between historical controls - which displayed a slight increase (estimated 0.008 mg/dL/d), and the decreasing bilirubin levels of the lipid restricted group. However, it is important to note that when septic events were accounted for, it was found that the lipid-restricted group had a significant sustained increase in bilirubin levels that was not seen with similar conditions in the historical controls. As a result of these slopes being calculated based on average weekly bilirubin levels this elevation in bilirubin levels may not completely reflect the whole bilirubin story.

Of the infants studied, 42% of lipid restricted infants resolving IFALD, compared to the controls at 10% and growth parameters were found to be not different between groups. Testing for EFAD occurred only once per month and did not occur after a patient

was deemed to have resolved IFALD based on their bilirubin levels. As a result, only 13 of the 31 infants on lipid restriction had more than one test for EFAD and of these, eight went on to develop biochemical signs of EFAD (using a strict definition of a triene:tetraene ratio of > 0.05). EFAD diagnoses were treated by adding an additional day of 1 g/kg/d delivery of lipids. Subsequent EFAD diagnoses resulted in an increase in lipid dose to 2 g/kg/d.

Finally, it is important to note that while not statistically significant, this study also identified increased mortality in the lipid-restricted group. This was deemed insignificant as the historical controls were determined to be not as "severely ill" as patients in the lipid-restricted group. The lipid-restricted fatalities appeared to have other co-morbid etiologies implicated, however, even Cober and colleagues could not rule out nutritional involvement in the poor outcomes. As the authors have stated, the lipid-restricted group and historical controls were matched as closely as possible including co-morbidities. As a result, while not statistically significant, the fact that these conditions resulted in fatalities in one group and not the other bears further scrutiny.

More recently, a study by Nehra and colleagues (Nehra et al. 2013) examined daily lipid minimization of soy-based therapies, to 1 g/kg/d, in comparison to neonates who received 2-3 g/kg/d lipids. This was again a retrospective review study examining hospital records from 2007-2011. Cholestasis was the primary outcome measure and was defined as a total bilirubin >2 mg/dL. In this study, a reduction in the amount of soy lipid given daily did not show a positive influence on the cholestatic outcome with both groups showing a similar incidence of cholestasis (51% vs. 43.8%, 1 vs. 2-3 g/kg/d). The amount of time on parenteral nutrition, incidence of sepsis and amount of enteral

nutrition were not significantly different between groups. While the 2-3 g/kg/d group did display a higher number of co-morbidities at baseline, the retrospective study found that there were no other significant differences at the end of treatment for both groups, suggesting that lipid minimization did not affect cholestatic outcomes.

It is important to note that most of the fish oil-based studies have also been utilizing a lipid minimization technique. As was previously stated, this makes it difficult to establish if it is the dose reduction of the lipid or the composition change that facilitates improvement in IFALD. As a result, we are currently unable to ascertain which is the most effect IFALD treatment.

1.7 Assessment of IFALD in Neonates

1.7.1 Minimally Invasive Liver Chemistry Tests

In evaluation of IFALD the most important liver chemistry tests are those that indicate biliary injury or cholestasis. The hepatic enzymes or that indicate biliary injury include GGT and Alkaline Phosphatase (ALP).

The enzymes more likely to reflect worsening hepatocellular damage are ALT and AST. Strictly speaking, however, these tests do not measure liver function per se. Serum bilirubin is a measure of liver function, because it measures the livers role in conjugation and clearance of biliverdin, the breakdown product of heme. Additional liver function tests that can be abnormal given severe hepatic dysfunction with IFALD include the international normalized ratio (INR), a measure of the livers synthetic role in coagulation; and albumin, a measure of liver protein synthesis.

1.7.2 More Invasive Tests

1.7.2.1 Liver Biopsy and Histology

Liver biopsies are used to assess the degree of liver damage that is present, often identifying more severe and end stage conditions, including fibrosis and cirrhosis. However, in a neonate, liver biopsies are not often performed. This test is invasive with a 1% risk of severe bleeding and death as a complication of the percutaneous needle biopsy procedure when performed blindly. Open biopsies can only be performed, given other concurrent open abdominal procedures, and have a complication rate of <1% (Hays et al. 1967). Furthermore, it is not clear that histology accurately predicts outcome for IFALD, perhaps given that the pathology can be restricted to specific areas of tissue and one biopsy sample may poorly represent the entire organ (Hays et al. 1967).

1.7.2.2 Direct Bile Flow Measurement

To date to the author's knowledge, the direct measurement of bile flow has only been performed in animal models. While this may be viewed as the 'gold standard' of measurement of cholestatic liver disease, the invasive laparotomy and bile duct cannulation make this procedure not possible in clinical practice and patient research. The earliest studies of bile flow and PN were done in piglets and established that bile flow directly correlates to biochemical markers of IFALD (Shulman and Fiorotto. 1987). Unlike the mouse or rat model, where bile flow is continuous and cannulation of the bile duct is extremely difficult, the piglet model displays a GI tract that is more representative of a neonatal biliary system.

1.8 Piglet Model

1.8.1 Strengths

The neonatal piglet has been used as a model for many different human research studies including but not limited to: metabolic disease studies (Litten-Brown, Corson, and

Clarke. 2010; Miller and Ullrey. 1987), xenotransplantation (Litten-Brown, Corson, and Clarke. 2010), immunology (Miller and Ullrey. 1987), toxicology (Miller and Ullrey. 1987), and gastroenterology and nutrition (Litten-Brown, Corson, and Clarke. 2010; Miller and Ullrey. 1987; Ball et al. 1996). The availability of genetically homogenous populations and the similarities in anatomy, physiology and growth patterns to humans makes the pig a preferential model for human nutrition studies (Ball, House, Wykes, & Pencharz, 1996; Litten-Brown et al., 2010; Miller & Ullrey, 1987).

In neonatal medicine in particular, it is important to consider how research will affect the vulnerable subject. Invasive study endpoints, such as tissue biopsies or large volumes of blood collection, are unethical or impractical for a neonatal patient (Ball et al. 1996). Neonates that require intensive care especially face many challenges including infections, surgical complications, and significant metabolic demands that impact research findings (Ball et al. 1996). This creates a population for which it is difficult to standardize research interventions (Ball et al. 1996). One of the biggest challenges of studying IFALD in neonates with intestinal failure is the heterogeneity among this population (Morgan III,W., J.Yardley, G.Luk, P.Niemiec, and D.Dudgeon. 1987; Litten-Brown, Corson, and Clarke. 2010; Ball et al. 1996). These are most often preterm neonates requiring significant medical intervention, having a variety of underlying illnesses in addition to intestinal failure, and often recovering from several different comorbid complications at any given time (Hasselmann and Reimund. 2004). Therefore, a valid animal model is often very useful for neonatal research, particularly if selected to be physiologically and developmentally comparable to a preterm human and if allows duplication of interventions that commonly occur with the intensive care of neonates. A

useful model would also allow for controlled interventions, complex technical procedures as performed in neonates and the collection and analysis of multiple blood and tissue samples (Morgan III,W., J.Yardley, G.Luk, P.Niemiec, and D.Dudgeon. 1987; Litten-Brown, Corson, and Clarke. 2010; Ball et al. 1996).

One of the greatest strengths of a piglet model for IFALD in the premature neonate is that piglets are born relatively altricial, with the growth and development of some tissues and organs occurring postnatally (Book and Bustad. 1974b). This includes development of the gastro-intestinal tract, which closely mimics the final stages of growth of a GI tract in a premature neonate (Book and Bustad. 1974b). Particularly when examining IFALD and its development in premature neonates, utilization of the piglet model allows researchers to closely study the outcomes in a model with similar development.

Relevant to nutritional studies, piglets and neonates are found to have similar metabolic growth in life stages over the life cycle (Miller and Ullrey. 1987). As such, taking into account the metabolic rate expressed by piglets and by neonates (of which piglets is approximately five times higher (Ball et al. 1996)), life stages such as birth, weaning and maturity, show similar metabolic patterns of development and as such allow for easier comparisons. In contrast, direct comparisons between neonates and their murine counterparts is limited as their metabolic rate and pattern of development is different. In addition, murine mammals are born extremely altricial, making neonatal studies with invasive procedures in vivo most often impossible.

As a medium size animal model the piglet allows for complex surgical interventions and collection of larger sized tissue samples (Book and Bustad. 1974a;

Litten-Brown, Corson, and Clarke. 2010). Due to similar sizes and physiology medical technologies can be advanced in piglet studies and then translated to human babies (Miller and Ullrey. 1987; Litten-Brown, Corson, and Clarke. 2010).

At birth piglets have little to no fat stores and a very low initial ability to synthesize lipids, thus, the intake of colostrum is essential to maintain a positive energy balance (Farnworth and Kramer. 1987) as well as providing the necessary immuno-globulins necessary for immune function. In normal circumstances, piglets are able to suckle approximately every hour and thus replace their energy stores (Miller and Ullrey. 1987). The term human neonate in comparison has a larger store of fat to utilize after birth (Manson and Weaver. 1997). Preterm infants, however, are found to be more like the piglet in that they were not able to accumulate the same fat store during late gestation, and thus require a larger degree of nutritional support (Manson & Weaver, 1997).

The neonatal piglet was therefore suggested as an acceptable model for nutritional studies relevant to the human neonate because of its many similarities including, gastrointestinal development, both in rate and pattern of development, and body composition (Ball et al. 1996; Book and Bustad. 1974a; Litten-Brown, Corson, and Clarke. 2010; Miller and Ullrey. 1987). In addition, there is a high level of comparability between histological samples of both the liver and gastrointestinal tract between piglets and neonates (Zabielski, Godlewski, and Guilloteau. 2008). Zabielski (2008) looked at how factors that control GI development compare between piglets and humans finding a great similarity and comparability between piglet and human histological gastrointestinal samples. When accounting for metabolic age, both growth and physiology of the small

intestine epithelial cells bears striking resemblance between both species (Zabielski, Godlewski, and Guilloteau. 2008).

The neonatal piglet has also been used to study amino acid requirements of neonates given TPN feeding (Wykes, Ball, and Pencharz. 1993; Bertolo et al. 1998; Miller and Ullrey. 1987). This usage has allowed for evaluation of the amino acid levels and, in part, for the development of a pediatric parenteral formulation (Wykes, Ball, and Pencharz. 1993). The piglet has also been used to study SBS and IFALD (Heemskerk et al. 1999; Miller and Ullrey. 1987; Turner et al. 2011).

The neonatal piglet was first established as a model for biomedical research in 1987, with a wide variety of applicable areas of research including immunology, metabolic syndromes, and gastrointestinal research (Miller and Ullrey. 1987). In order for these studies to have been successful it is relevant that both human neonates and piglets have similarities in gastrointestinal set up and function for lipid absorption and digestion. Both possess a functional gall bladder, used to store and allocate bile (Zabielski, Godlewski, and Guilloteau. 2008); as well as sharing similar pathways for absorption of macro and micronutrients (Litten-Brown, Corson, and Clarke. 2010; Miller and Ullrey. 1987).

Furthermore, important to the design of this study, dietary lipids have key roles for growth and development of the human neonate and piglet. The neonatal brain undergoes a growth spurt at comparable times in both the piglet and neonate. This growth spurt is accompanied by lipid accumulation. Research by Amusquivar et al. (2008) and Hyde et al. (2005) has found that the fatty acids fed postnatally to piglets, influence the fatty acid profile in the brain, as well as other tissues. Research conducted

by Innis (Innis. 2007) examined the fatty acid profiles in both piglets and neonatal babies and found that the composition relative dosages were similarly distributed. This allows for a greater degree of comparison between these two species, as well as allows for treatment effects to be extrapolated.

Another benefit of using a piglet model to describe and observe the effects of different lipid sources is the ability to investigate the fatty acid composition of end organ tissues, normally unavailable in neonatal studies, unless postmortem. This allows us to examine the effects of alteration of the route and composition of nutrition delivery on end organs including histological profiling, evaluating fatty acid profiles of specific organs and compare differing treatments.

1.8.2 Limitations

One of the characteristics of the piglet model, which is both a strength and a weakness, is the difference in growth patterns. The piglet has been known to have a 1000% increase in body weight (1,200g to 12,000g) in a six-week period. The human neonate, born weighing on average 3400g, experiences a significantly slower growth rate, seeing an increase on average of only 1200g in the same time period (Miller and Ullrey. 1987). Because of higher growth rate, piglets have higher nutritional requirements on a per kg basis than human neonates and, as such, are at higher risk for growth failure and its consequences when intake is restricted or below requirements (Book and Bustad. 1974a). This creates an advantage for translating nutritional findings to human neonates, as the stringent dietary requirements in the piglet will allow for a faster determination of dietary complications.

Fat-free body tissue composition (ash, protein and water) between the pig and human at different stages of life is found to be similar (Miller and Ullrey, 1987). As well, the portions of total life to reach chemical maturity are almost identical at 4.4% and 4.6% for man and pigs respectively. Unlike fat free body tissue composition, fat composition between the human neonate and the piglet do differ. As discussed above, at birth, the piglet has minimal fat reserves; approximately 1-3% of the body composition is lipids (Farnworth and Kramer. 1987), while the term neonate has approximately 16% fat to use following birth (Book and Bustad. 1974a; Farnworth and Kramer. 1987; Miller and Ullrey. 1987).

Brain development in mammals is known to have similar basic stages, with the differentiating factor being the maturity of brain development at birth (Dobbing and Sands. 1979). As a model for human brain growth, the piglet has been shown to undergo similar periods of development prior to birth and postnatally (Dobbing and Sands. 1979; Purvis, Clandinin, and Hacker. 1982). When examining the fatty acid content of both piglet and neonate brains, it was found that during the last trimester of development in particular, both piglet and neonatal brains displayed similar fatty acid profiles including the n-3 LC PUFA accretion (Purvis, Clandinin, and Hacker. 1982). It is also important to note the brain synthesis of DHA for both piglets and neonates is very low, rather plasma DHA (which if not fed, results from bio-synthesis in the liver) is utilized for membrane production (Davis-Bruno and Tassinari. 2011). Thus, a decrease in brain DHA often results from a limited DHA production due to competition for desaturation enzymes (**Figure 1.4**)(Davis-Bruno and Tassinari. 2011).

Both the piglet and the neonate derive important immunological function from the ingestion of colostrum. For 24 to 48 hours after birth, the intestinal lumen is able to absorb immune macromolecules from colostrum via enterocytes (Kelly and Coutts. 2000; Stokes et al. 2004). After that time period, the gut begins to function as an immunological barrier. Although piglets and neonates derive many gamma globulins from colostrum, there is a significant difference between piglet and neonate immunology. While the human is able to gain gamma globulins in utero, in the third trimester, the piglet relies on colostrum alone to provide gamma globulins. This creates an immune dependency in the piglet that differs from human neonates (Book and Bustad. 1974a; Miller and Ullrey. 1987). It also allows significant contrasts in potentially immune mediated problems, between colostrum deprived and colostrum fed piglets, example in necrotizing enterocolitis (Ball et al. 1996). These studies then have relevance to preterm humans.

1.8.3 Validity of a piglet model for Investigation of IFALD

The ability to use the piglet as a model for IFALD allows us to control many of the confounding variables that have been discussed regarding the use of parenteral lipids to treat IFALD in clinical studies. Use of piglets provides many advantages to traditional clinical randomized controlled trials. Firstly, piglets are raised in a controlled environment with genetic records that allow us to control for heterogeneity. This allows us to eliminate much of the heterogeneity effects that human clinical trials are subject to (Cober et al. 2012a), providing a more consistent result. Pigs also have a large litter size and frequent litters, making sampling from the same genetic population feasible and cost effective.

In particular, using piglets as a model allows for use of invasive procedures that are unable to be performed in a neonatal population. Of these, the ability to restrict or eliminate enteral advancement in the piglet provides an ethical alternative, which is not available in a neonatal population. One important difference, that is both an advantage and a potential limitation of our model, is the rapid growth of the piglet. Such rapid growth requires significantly increased calories and means that the doses used in these piglets 5g/kg/d or 10g/kg/d may seem extreme to clinicians used to giving human neonates dose varying from 1-4g/kg/d of lipids.

Use of piglets as an animal model with representative gastrointestinal physiology (Gu and Li. 2003; Heemskerk et al. 1999), allows for a more thorough investigation of IFALD that can be done in either cell culture studies or murine models. Similarly, at our study endpoint, we are able to assess invasive biological indicators including bile flow and sample tissues not normally available in neonatal models. Bile flow in particular, requires an invasive laparotomy, cannulization of the biliary tree above pancreatic involvement, and lengthy anesthetic procedures making this measurement infeasible in neonatal populations, forcing clinicians to rely on bio-indicators. The piglet model also continues to strengthen the investigation of IFALD by enabling sampling of various tissues, many of which are inaccessible in the neonate. In particular, brain tissue collected from piglets allows us to understand the role fatty acid delivery plays in brain fatty acid accretion post parturition.

Finally, with proper aseptic line care and prophylactic antibiotic use, occurrence of sepsis in the piglet is low. However it is important to note that it is impossible to completely eliminate septic events in any population with a central venous catheter.

While not ideal, the occurrence of sepsis in piglets can be looked at as representative of clinical conditions and allows us to investigate the co-morbidities associated with septic events in neonates in a controlled setting.

1.9 Knowledge Gaps and Controversies

Considering the literature reviewed above, a number of controversies remain. Firstly, the definition of IFALD and the measures used to assess both its development and resolution continues to be debated (Cober et al. 2012a; Diamond et al. 2009; Filler et al. 1969; Fukatsu. 2012; Gura et al. 2008). Amongst the literature reviewed, the definitions of IFALD vary in the use of total versus conjugated bilirubin - at which level is defined as IFALD, as well as which other liver chemistry provide the most accurate picture of IFALD. A quick examination of the literature will show IFALD definitions ranging from >2 mg/dL (34.2 μmol/L) of total bilirubin (Gura et al. 2006; Puder et al. 2009) up to a severe IFALD definition of >5.9 mg/dL (100 μmol/L) conjugated bilirubin (Diamond et al. 2009). This distinction between total and conjugated bilirubin also factors into the definition as total bilirubin represents both conjugated and unconjugated concentrations in plasma, which represent potential defects in hepatic biliary uptake or conjugation. Conjugated bilirubin however, tends to represent parenchymal liver disease and/or biliary obstruction (Limdi and Hyde. 2003; Knight. 2005; Martin. 1992). A lack of a standardized definition will impact how we evaluate the efficacy of fish-oil therapies.

Secondly, and of critical importance, is the lack of randomized controlled studies in this field to date. There is a significant need for randomized controlled trials evaluating both dosage and lipid therapies in a contemporary era (Seida et al. 2013a). Use of retrospective controls, given higher doses of lipid, means that the improvement noted

with fish-oil and lipid restriction may rather reflect an association with improved care and use of lower lipid doses over all. That this consideration is important is supported by studies that show improved outcomes from IFALD with contemporary multidisciplinary intestinal failure rehabilitation alone (Diamond et al. 2007; Sant'Anna et al. 2012).

Other controversies relate to the variable treatment approaches. Such variable approaches mean that systematic reviews of the current literature, even those that have examined cohort studies alone, are also likely to be flawed (Seida et al. 2013a). Future studies need to standardize the dosage of lipids given, the biochemical definitions of IFALD, the duration of treatment, the maximum compensatory glucose dose allowed and the need to standardize enteral advancement in all treatment cohorts.

The varied composition of the lipids studied, independent of fatty acid composition is another confounding issue. A lower Vitamin E intake due to the content in Intralipid® has been implicated in significant hepatic injury. Particularly, formation of reactive oxygen species from AA metabolites interacts with hepatic tissue causing injury (Weinberger, Watorek, and Strauss R. 2002). As a result, differing concentrations of Vitamin E within parenteral lipids can potentially result in differing degrees of hepatic injury, often being more severe in treatments with little to no Vitamin E.

The converse is true when phytosterol concentration increases. Phytosterols have been implicated in cholestasis development, influenced by dosage and length of lipid treatment (Clayton, Whitfield, and Iyer. 1998; El Kasmi et al. 2013; Kurvinen et al. 2011). Unfortunately, plant-based lipid sources contain phytosterols in conjunction with other lipid soluble sterols, making their removal difficult if not impractical. In contrast, fish-oil based lipid sources do not contain phytosterols, and as a result are not linked to a

dramatic rise in bilirubin and IFALD (Fallon, Le, and Puder. 2010; Koletzko and Goulet. 2010; Fuchs et al. 2011; Venick and Calkins. 2011), often being used to resolve IFALD (Gura et al. 2006; Groleau, Thibault, and Marchland. 2014; Koletzko and Goulet. 2010). These diverse concentrations of phytosterols in conventional soy-based therapy versus dose restricted soy-based therapy versus fish-oil based therapy do not allow a researcher to pinpoint the mitigating factor in IFALD development.

Finally, a significant knowledge gap that present in current literature is the lack of long-term studies that have assessed the effects of early parenteral nutrition therapies on neurocognitive outcomes. As the advent of these novel therapies is considered fairly recent in scientific literature; with fish oil-based therapy first being used in North America in 2005 (Gura, Parsons, and Bechard. 2005) and dose restriction appearing in 2012 (Cober et al. 2012b), there has been insufficient time to do long-term follow up studies. Even when we consider the conventional soy-based therapy, only a few long-term studies have been undertaken, the most prominent being a 25-year study by Quirós-Tejeira (Quirós-Tejeira et al. 2004).

As a result, while these therapies may produce desirable short-term results like the reversal of IFALD, we currently have no data on patients' health and development into childhood (>10 years). This is of specific concern when considering the alteration and/or the restriction of fatty acids that are essential for cognitive development at the point where essential fatty acid demands are highest. Particularly for DHA and AA, where their combined dry weight of brain tissue is approximately 35%, limiting their supply could significantly affect future health.

To fill one of these knowledge gaps, we propose to study parenteral lipid dose and fatty acid composition in the development of IFALD using a neonatal piglet model. We propose to use a neonatal piglet model of total parental nutrition (no enteral nutrition), varying only in the parenteral lipid solution used. We will compare the two lipids that are currently being used to treat babies with IFALD so as to have relevant translational information, given that similar controlled trials have not been conducted in human babies to date. Our primary endpoints will be to examine intra and extra-hepatic cholestasis by evaluating bile flow for each piglet, as well as examining the nutritional status of our piglets by measuring growth, and fatty acid concentrations in the brain.

Therefore, we will assess both standard and restricted dosages of the conventional soy-based lipid in relation to the restricted dosage of the fish-oil lipid. Given the rapid growth of piglet the actual doses of lipid provided will not be the same as those given to human infants, but based on our knowledge of piglet growth and nutritional requirements, will be set at five times that of the doses given babies. Therefore, we will define standard or high dose as 10g/kg/d (compare to 2g/kg/d in babies) and low or restricted dose as 5g/kg/d (compare to 1g/kg/d babies).

We will also compare the TPN treated groups to sow fed control piglet, which would be expected to have normal liver chemistry and function for age and would also represent the gold standard for nutritional parameters and development. We chose sow fed controls for this trial instead of enteral control that were raised in the lab to establish the maximum potential that our piglets could achieve, striving to compare our outcomes with the nutritional gold standard. Ideally, the goal for neonatal parenteral nutrition

would be an inability to differentiate between those children on parenteral nutrition and those receiving breast milk.

1.10 Hypothesis and Objectives

1.10.1 Hypothesis

1.10.1.1 Parenteral lipids at a dosage of 5 g/kg/d will reduce the severity of cholestasis in a neonatal piglet receiving total parenteral nutrition as compared to conventional dosing of 10 g/kg/d.

1.10.1.2 Parenteral lipids at restricted doses will not allow for optimal growth in neonatal piglets.

1.10.1.3 Brain weight in lipid-restricted therapies will be significantly less than those receiving conventional dosages of 10 g/kg/d or sows milk.

1.10.1.4 Additionally, fatty acid profiles will change with parenteral lipid delivery. Specifically, piglets receiving higher n-3 LC PUFA will display higher n-3 LC PUFA percentage in brain tissue.

1.10.2 Objective

The overall objective is to utilize a neonatal piglet model to compare and contrast soy-based parenteral lipid at standard and restricted doses to fish-oil based lipid at a restricted dose in the development of IFALD in parenterally fed, neonatal piglets, receiving no enteral nutrition. The second objective is to compare key nutritional outcomes in these parenterally fed piglets, given parenteral lipid at different doses and varying fatty acid composition, to sow reared control piglets –

Specific objectives are as follows:

1.10.2.1 To assess the development of IFALD in parenterally fed piglets using bile flow as a measure of both intra and extrahepatic cholestasis. This measurement will be compared and contrasted with current clinical bio-indicators of liver function.

1.10.2.2 To assess nutritional status utilizing the gross measure of body weight, and biochemical parameters including albumin.

1.10.2.3 Brain development will be assessed based on total brain weight and fatty acid profile. Each treatment will be evaluated against the other parenteral therapies as well as the gold standard of sow fed piglet brain parameters.

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Chapter 2: EFFECTS OF DOSE AND PARENTERAL LIPID COMPOSITION ON LIVER FUNCTION IN NEONATAL PIGLETS ON TOTAL PARENTERAL NUTRITION

2.1 Introduction

Intestinal failure associated liver disease (IFALD) is a multi-factorial disease that continues to challenge neonates who require parenteral nutrition (PN) (Diamond et al. 2012; Carter and Karpen. 2007; Nehra, Fallon, and Puder. 2011). PN is life saving nutrition support comprised of essential nutrients including amino acids, glucose and fatty acids. PN has markedly altered survival for preterm neonates with intestinal failure, given that they are at greater risk of nutrient deficiencies. These deficiencies result from increased growth demands and limited nutrient stores. Preterm infants are also known to be at greater risk of developing IFALD (Kurvinen et al. 2011; Peyret et al. 2011). Parenteral lipid in PN is an important source of calories for the growth of these infants, but is now being recognized as having a major role in the development of IFALD (Diamond et al. 2012; Dupont and Carpentier. 1999; Hyde et al. 2008.; Nehra, Fallon, and Puder. 2011).

The current standard parenteral lipid in North America is soy-based Intralipid® (Fresenius Kabi, Bad Homburg), which is high in the n-6 long chain poly-unsaturated fatty acids (n-6 PUFA), particularly linoleic acid (LA). Soy-based lipid is also restricted in n-3 long chain polyunsaturated fatty acids (n-3 PUFA), with some alpha-linolenic acid (ALA), but no eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) (Deshpande and Simmer. 2011; Wanten and Calder. 2007) creating an overall n-6 LC PUFA to n-3 LC PUFA ratio of 7:1. This overabundance of n-6 LC PUFAs may result in an imbalance in the fatty acids delivered compared to what may be optimal for immune function and cognitive development (Rushton and Ankney. 1996; Wainwright. 1992). Alternative

parenteral lipid therapies have been developed containing fish oil to provide n-3 LC PUFA. Omegaven® (Fresenius Kabi, Bad Homburg) skews the n-6 LC PUFA to n-3 LC PUFA ratio in the opposite direction, to 1:8 (Wanten and Calder. 2007; Deshpande and Simmer. 2011). Potentially, neither Intralipid® nor Omegaven® provide adequate balance of polyunsaturated fatty acids for growth and development, given the extreme amounts of n-6 LC PUFA in the former and of n-3 LC PUFA in the latter. Particularly when compared to breast milk, which can vary in the n-6 LC PUFA to n-3 LC PUFA ratio from 2:1 to 4:1, depending on each individual mother (Innis, 2008).

Long term PN utilizing soy-based lipid at doses above 2.5 g/kg/d has been found to significantly increase the odds of developing end stage IFALD (Diamond et al. 2011). Potential mechanisms for IFALD development may include but are not limited to: 1) exacerbation of inflammation; including inflammation secondary to sepsis from bacterial translocation (Van Camp, Tomaselli, and Coran. 1994); 2) central venous catheter infections; 3) exacerbation of oxidative stress (Weinberger, Watorek, and Strauss R. 2002); and 4) potentially hepatotoxic phytosterol contaminants of the plant based lipid (lyer, Spitz, and Clayton. 1998; Clayton, Whitfield, and Iyer. 1998). In contrast, current research suggests the use of fish oil-based lipid can reduce the incidence of IFALD (Diamond et al. 2009; Gura, Parsons, and Bechard. 2005; Koletzko and Goulet. 2010; Park, Nespor, and Kerner Jr. 2011; Venick and Calkins. 2011; Xu et al. 2012). This may be a result of a reduction in the pro-inflammatory state, or a reduction in oxidative stress as Omegaven® contains additional vitamin E, or through the absence of phytosterols.

The total dosage of lipid delivered is important and complicates our current understanding of the role of parenteral fish oil in treating IFALD. Compared to the

historical use of Intralipid® at doses of 2 to 4 g/kg/d, the dose of Omegaven® used to treat IFALD has usually been restricted to 1 g/kg/d (Filler et al. 1969; Hasselmann and Reimund. 2004; Park, Nespor, and Kerner Jr. 2011). Recently, a lower dose of Liposyn® (Hospira, Saint-Laurent, Quebec) was studied for the prevention of IFALD, with findings similar to those reported for Omegaven® (Cober et al. 2012a). The Lyposyn® approach; however, utilizes an even more restrictive lipid dose, raising concerns about growth and adequate essential fatty acid intake in these infants. As a result, the optimal composition and dose of parenteral lipid for neonates with intestinal failure remains controversial (Cober et al. 2012a; Cohen, Horton, and Hobbs. 2011; Deshpande and Simmer. 2011; Diamond et al. 2011). This is of particular importance when one considers the limited length of follow up in these studies and the potential for altering growth and long-term neurocognitive outcomes (Cober et al. 2012a; Diamond et al. 2011; Kurvinen et al. 2011).

Additionally, factors including enteral advancement and septic events work to further confound the data. Enteral feeding in particular is advanced on a per patient basis according to what each individual can tolerate. While general enteral advancement is often reported (Gura et al. 2006; Puder et al. 2009; Venick and Calkins. 2011), the degree with which enteral nutrition is advanced is often under-reported, thus confounding the resolution of IFALD. Septic events also work to confound the literature, with a general number of septic events being reported, while severity and duration are not.

The aim of this study was to assess liver function and growth, including total body weight, brain weight, and fatty acid content of the brain in neonatal piglets given total parenteral nutrition with equivalent doses of Intralipid® and Omegaven® (5g/kg/d), representing lipid dose restriction. This was compared to the conventional dose of

parenteral lipid shown to be required for adequate growth of PN fed piglets in our laboratory (10g/kg/d of Intralipid®)(Wykes, Ball, and Pencharz. 1993). Sow fed piglets were also included, representing the gold standard for postnatal growth and development of pre-weaned piglets. Our hypothesis was that lipid dose restriction would improve liver cholestasis, regardless of the fatty acid composition, but would not support adequate growth of neonatal piglets, including brain growth.

2.2 Materials and Methods

2.2.1 Animals and Surgical Procedures

All procedures were approved by the Faculty of Agriculture, Life and Environmental Sciences Animal Policy and Welfare Committee, University of Alberta, and were conducted in a bio-secure swine research facility, according to the guidelines of the Canadian Council of Animal Care.

Male newborn Landrace-Large White cross piglets (2-5 days old) were obtained from the University of Alberta Swine Research and Technology Center. These piglets underwent general anesthesia and insertion of a jugular venous catheter. Jugular catheter insertion allowed for continuous delivery of iso-nitrogenous PN. After catheter insertion, analgesia (Buprenorphine, (Shering Plough, Kirkland, Quebec) at 0.05 mg/kg) was provided twice daily for 48 hours post surgery. There was no enteral nutrition delivered.

Parenteral nutrition delivery was based on our validated model that demonstrated normal growth and body composition of PN fed piglets (Wykes, Ball, and Pencharz. 1993). All piglets were fed PN providing equivalent amino acids, dextrose, vitamins and minerals. The solutions were prepared in our laboratory under sterile conditions. Only lipid delivery varied across treatment groups. In the low dose groups total lipid dose was

set at 50% of the usual calories from lipid used in our established model. Four treatment groups were studied: Omegaven5 (n=8) received PN with 5 g/kg/d Omegaven®; Intralipid5 (n=6) received PN with 5 g/kg/d Intralipid®; Intralipid10 (n=9) received PN with lipid dose at 10 g/kg/d Intralipid®; finally Sow Fed Control (n=8) were sow fed control piglets. The delivery of individual fatty acids, according to group, dose and lipid composition is shown in **Tables 2.1-2**.

Lipid was added to the amino acid based PN solution just prior to infusion to create an all-in-one admixture. PN delivery commenced immediately after jugular catheter insertion. Nutritional targets for intakes included 16 g/kg/d amino acids, 29 g/kg/d glucose and 13.5 mL/kg/h total fluid intake. Total energy varied from 0.9MJ/kg/d in the low dose groups (Omegaven5 and Intralipid5) to 1.1MJ/kg/d in the standard dose group (Intralipid10).

All PN fed piglets were housed similarly in individual cages, including a swivel system to allow for freedom of movement, and were weighed daily. Cages were housed in a temperature-controlled room at 25^0C with a 12-hour light/dark cycle. Broad-spectrum antibiotics (Ampicillin (Sandoz, Boucherville, Quebec) at 20 mg/kg, Trimethoprim-sulfadoxine (Merck Animal Health, Kirkland, Quebec) at 16 mg/kg were administered from days 0-4 and days 8-12 to aid in the prevention of line sepsis. Sow fed piglets were raised under standard farm conditions until terminal laparotomy at the equivalent age of trial piglets.

2.2.2 Liver Chemistry and Function

Serum and plasma samples were collected at baseline (day 0) and at the end of trial (day 14) for measurement of liver chemistry including: bile acids, total bilirubin,

alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), alanine-transaminase (ALT), and aspartate aminotransferase (AST). As a primary outcome measure of liver function, bile flow was determined at the terminal surgical procedure as previously described by Van Aerde and colleagues (Van Aerde et al. 1999b). Standard methods were used for all treatment groups and control piglets and the temperature of piglets maintained between 37-38^{0}C, with no greater variation than +/- 0.5, through use of a heating blanket. The gallbladder was emptied and the cystic duct ligated to prevent back flow of bile. The common bile duct was then cannulated with a 7Fr polyurethane catheter cut 8 cm long and secured with a 3-0 silk suture. Bile flow was allowed to normalize for 5 minutes prior to sample collection. Bile flow was measured into pre-weighed micro-centrifuge tubes for a period of 10 minutes. Between sample collections a 5-minute rest period was permitted to ensure normalized bile flow. Bile flow was deemed representative once sample collections were less than 10% different between 3 individual collections, or 6 total sample collections had taken place. At the completion of bile flow determination, humane euthanasia was conducted and liver, and brain tissues, excised, weighed, flash frozen in liquid nitrogen and stored in an -80°C freezer to await further analysis.

2.2.3 Histology

Liver tissues removed were immediately fixed in 10% neutral buffered formalin and fixed for a minimum period of 24 hours. Following fixation, tissues were trimmed with a scalpel to a thickness of 2–3 mm and placed in tissue cassettes, and processed into paraffin. Paraffin blocks were sectioned on a microtome to a thickness of 5 microns, placed on a microscope slide and stained with hematoxylin and eosin stain, following

standardized techniques. Slides were examined by an experienced pediatric hepatopathologist (C.M. Sergi), who was blinded to grouping. Severity of liver disease was graded using a previously reported modified scale (Turner et al. 2011; Hua et al. 2013). The modification provides scoring for nine parameters: vacuolar degeneration, spotty necrosis, cholestasis, apoptosis, Kupffer cell hyperplasia, sinusoidal dilatation, portal oedema, tissue iron granules and polymorphonuclear leukocyte infiltrates. Each specimen was graded as 0/normal, 1/mild-moderate and 2/severe, with a maximum possible score of 18 points.

2.2.4 Oil Red O Staining

As a quantitative assessment of hepatic lipid content, frozen liver sections were fixed in formalin, cut and mounted prior to oil red O (ORO) staining. Prepared slides were stained using Oil Red O Solution (Sigma-Aldrich, St. Louis, MO, United States) and then dehydrated using propylene glycol in preparation for counterstaining with Mayer's Hematoxylin (Sigma-Aldrich, St. Louis, MO, United States). Following counterstaining, slides were rinsed with warm, distilled water and mounted with Geltol (Chem-Tel, Tampa, Florida, United States). Slides were then assessed using a published index for bronchiolar lipid-laden macrophages, modified to be applied directly to the staining of hepatocytes in both peri-portal and peri-central regions (Reid-Nicholson et al. 2010). This score ranges on a scale from 0-4, where 0 represents normal unstained hepatocytes and 4 represents hepatocytes with 100% ORO lipid stain present.

2.2.5 Bio-Markers of Inflammation

As a marker of systemic inflammatory status, serum C-Reactive Protein concentrations were assessed using a CRP ELISA test kit, detection range reported as

$1.82 \times 10^{-13} – 2.32 \times 10^{-13}$ µg (Genway, San Diego, California, United States). The concentration of specific serum cytokines, TNF-α and IL-6 levels were measured using porcine ELISA test kits, according to manufacturer instructions (R&D Systems, Minneapolis, Minnesota, United States). Detection range for TNF- α was 23.4 - 1,500 pg/mL, and IL-6 was reported as 18.8 - 1,200 pg/mL. The average of the duplicate data with a coefficient of variance less than 10% was used for statistical analysis.

2.2.6 Total Lipids in the Brain

A modified Folch extraction (Folch, 1957) was utilized to extract brain lipids and the fatty acid composition determined as described in Pratt et al. (Pratt et al. 2002) and Field et al. (Field, Van Aerde, and Clandinin. 2010). Briefly, 100 mg of brain tissue was ground for 1 minute in 500µL of PBS solution, and then pelleted by microcentrifuge. The supernatant was transferred to a clean glass tube for lipid extraction (0.025% $CaCl_2$ and 2:1 Chloroform:Methanol at a ratio of 4:1) overnight. The bottom layer of supernatant was transferred to a glass vial and dried under nitrogen. Samples were methylated by using hexane and BF3/methanol and by heating for 1 hour at 110°C to prepare the fatty acid methyl esters for gas chromatography. Water was added and left 2 hours to allow separation of layers. The bottom layer was collected, dried down with nitrogen, methylated with BF_3, e-suspended in 300 µL and injected into the gas chromatograph (Varian 3800; Varian Instruments, Mississauga, Ontario, Canada) under the conditions described by Pratt et al. (2002).

2.2.7 Data Analysis and Statistics

Following tests for normality, data was expressed as mean and standard deviation or medians and range, if not normally distributed. Comparisons between groups were

analyzed by ANOVA when normally distributed or by Kruskal-Wallis one-way analysis of variance, when not normally distributed, all using SPSS (version 21; SPSS Inc, and IBM Company, Chicago IL). Post hoc differences between two individual groups were analyzed using Tukey or Mann-Whitney tests as applicable to data distribution. Comparisons were made using Chi-squared analysis when applicable. An alpha value of $p \leq 0.05$ was considered significant.

2.3 Results

2.3.1 Piglet Performance

There were no significant difference between all groups for both age and body weight at baseline (**Table 2.3**). The percentage of intended PN delivery among treatment groups was not different ($p = 0.47$). Prophylactic antibiotics (Ampicillin, trimethoprim, and sulfadoxine) were given at days 0-4 and days 8-12 to prevent sepsis. Suspected sepsis was defined as fever, vomiting, and lethargic behavior. At the onset of suspected sepsis, blood cultures were drawn and the piglet was treated with additional antibiotics for routine prophylaxis (adding enrofloxacin (Bayer, Kansas, United States) at 2.5 mg/kg, clindamycin (Sandoz, Boucherville, Quebec) at 0.01 mg/kg, and gentamycin (Sandoz, Boucherville, Quebec) at 5 mg/kg individually if no response) for the remaining length of the study. Sepsis was confirmed by a positive culture in one piglet in the Intralipid5 group (Staphylococcus epidermidis) and 3 piglets from the Intralipid10 group (Staphylococcus epidermidis, Enterococcus faecalis, and Klebsiella pneumoniae). The proportion of piglets with a positive culture differed significantly among groups, and was highest in the Intralipid10 group (p-value ≤ 0.05). Data was used from all piglets, regardless of sepsis status.

Final total body weight (**Table 2.3**) showed significant differences with Omegaven5, Intralipid5 and Intralipid10 being significantly lower ($p \leq 0.05$) than the sow fed controls. Low dose therapies; Omegaven5 and Intralipid5, were not shown to be different from Intralipid10. Nor were the low dose therapies significantly different from each other.

Liver weights at termination (**Table 2.3**) showed significant differences with Omegaven5, Intralipid5, and Intralipid10 being significantly higher ($p \leq 0.05$) than the sow fed control piglets. Compared to Intralipid10, the low dose therapies, Omegaven5 and Intralipid5 were not significantly different ($p \geq 0.05$). Omegaven5 and Intralipid5 did not differ from each other ($p \geq 0.05$). Small bowel weight however, showed significantly lower ($p \leq 0.05$) weights in those receiving parenteral nutrition (Omegaven5, Intralipid5 and Intralipid10). Omegaven5 was found to be significantly higher ($p \leq 0.05$) than those receiving Intralipid10. Intralipid5, however, was not different from either Omegaven5, or Intralipid10.

Brain weights (**Table 2.3**) at termination revealed significantly lower ($p \leq 0.05$) weights for both Omegaven5 and Intralipid5 in relation to the sow fed controls. Intralipid10 piglets, however, did not show a significant difference between sow fed controls or the low dose therapies. Omegaven5 and Intralipid5 were not found to differ from each other.

2.3.2 Liver Function and Chemistry

Average bile flow, expressed as μg/g total liver weight (**Figure 2.1**), displayed no significant difference between the low dose therapies (Omegaven5 and Intralipid5) and the sow fed controls. Intralipid10 was also found to not differ from the sow fed piglets.

When compared to Intralipid10, Omegaven5 piglets showed a significantly higher ($p \leq 0.05$) bile flow, while Intralipid5 piglets showed bile flow not different from Intralipid10. The low dose therapies, Omegaven5 did not show a significantly different bile flow from Intralipid5.

All liver chemistry results are shown in **Table 2.4**. Total bilirubin was inversely correlated to bile flow ($r = -0.45$, $p = 0.009$). Serum total bilirubin concentration was not different between Omegaven5, Intralipid5 and sow fed piglets, while Intralipid10 was found to have a significantly higher ($p \leq 0.05$) serum bilirubin than the other treatments. Omegaven5 and Intralipid5 were not different from each other. Serum bile acids did not differ between the low dose therapies (Omegaven5, and Intralipid5) and sow fed piglets. Intralipid10 piglets, however, showed a significantly higher ($p \leq 0.05$) bile acid concentration than both the sow fed and Omegaven5 piglets. Bile acids for Intralipid5 were found to be not different from either Omegaven5 or Intralipid10. There was a significant ($p \leq 0.01$), inverse, correlation $r = -0.48$ between bile flow and bile acids. ALP and ALT concentrations did not significantly differ among groups ($p = 0.65$ and $p = 0.43$ respectively). GGT was significantly lower in sow fed controls compared to Omegaven5 and Intralipid10 ($p=0.05$).

2.3.3 Histology

Histological score was significantly ($p \leq 0.05$) higher in the Omegaven5 group in relation to the Sow Fed Control piglets (**Figure 2.2a**) while Intralipid5 and Intralipid10 were found to be not different from either Omegaven5 or Sow Fed Control histology. However, using a score of 0-1 as normal, based on our prior experience; (Hua et al. 2013), it was found that the proportion of piglets within the treatment groups that had an

abnormal liver score (≥ 2) was 100% for Omegaven5, 100% for Intralipid5 and 92% for Intralipid10, compared to 0% for sow controls.

The ORO results also showed significant differences between groups (**Figure 2.2b**). The ORO score for Omegaven5 was significantly lower than both Intralipid5 (0.63 vs. 2.38; $p \leq 0.01$) and Intralipid10 (0.63 vs. 1.78; $p \leq 0.05$); and did not differ from sow fed control piglets (all scored 0). Representative liver pathology with ORO staining between groups is shown in **Figure 2.3a-b.**

2.3.4 Bio-markers of Inflammation

C-Reactive Protein concentration (CRP) and the concentration of the individual pro-inflammatory cytokines Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) are shown in **Table 2.5**. There were no significant differences in CRP from baseline to termination for all groups. CRP concentration in all treatment groups was not statistically different from sow fed control ($p = 0.45$). There were no significant changes in IL-6 or TNF-α levels from baseline to trial completion for any group and no significant differences among groups in IL-6 and TNF-α at termination (**Table 2.5**).

2.3.5 Brain Fatty Acid Analysis

Brain fatty acids were expressed as a percent of total fatty acids (**Table 2.6**). The relative percent of total saturated fatty acids stearic and oleic acid did not differ amongst groups ($p = 0.35$ and 0.33, respectively). The n-6 fatty acids, linoleic acid (LA) and arachidonic acid (AA) were found to differ among groups. LA percentages were found to be significantly lower for Omegaven5 in relation to sow fed controls; Intralipid5 however, did not differ from the sow fed piglets. Intralipid10 was found to be significantly higher than the other treatments. AA levels were also significantly lower in

Omegaven5 in relation to sow fed piglets. Intralipid5 and Intralipid10, however, were not significantly different from the sow fed control or Omegaven5. The brain n-3 fatty acid concentrations were also affected by diet (**Table 2.6**). The relative proportion of EPA was higher in the sow fed control piglets compared to both Omegaven5 and Intralipid10 ($p \leq 0.05$). In the Omegaven5 group, the relative proportion of docosapentaenoic acid (DPA) and DHA were higher compared to the other groups ($p \leq 0.05$ for both DPA and DHA).

2.4 Discussion

To our knowledge, this is the first neonatal comparison between n-6 LC PUFA (Intralipid®) and n-3 LC PUFA (Omegaven®) predominant parenteral lipids given at a low dose and their effect on measures hepatic and brain function/structure. It is also important, in the absence to date of a randomized controlled trial in human neonates, to use a representative neonatal animal model (Turner et al. 2011; Hyde et al. 2008; Wykes, Ball, and Pencharz. 1993).

At this time, the different strategies that manipulate parenteral lipid dose or fatty acid composition have generally compared outcome to historical or conventional lipid dosing. Most of these 'new' strategies report dramatically improved outcomes from neonates with IFALD (Gura et al. 2008; Cober et al. 2012a; Fuchs et al. 2011). These approaches; however, have not been compared in randomized clinical trials with appropriate controls.

Many ethical and practical barriers exist to conducting clinical trials in the neonatal population. The use of neonatal piglets as a model for the preterm human has enabled us to directly compare currently used clinical strategies to treat IFALD while measuring important but invasive outcomes such as bile flow and fatty acid profiles in the

developing brain (Turner et al. 2011; Wykes, Ball, and Pencharz. 1993; Van Aerde et al. 1999a; Amusquivar et al. 2008; Book and Bustad. 1974a; Hyde et al. 2005; Miller and Ullrey. 1987). As previously stated, current clinical strategies vary widely in delivery of both n-6 and n-3 LC PUFA, often providing extremely different amounts and proportions of LC PUFAs. The long-term impact on growth and cognitive development in human infants treated in this manner is currently unknown (Quirós-Tejeira et al. 2004). In this regard, the neonatal piglet is a useful model for a nutritional intervention with such outcomes of interest, because of their rapid growth - approximately five times that of human infants (Book and Bustad. 1974b; Miller and Ullrey. 1987; Litten-Brown, Corson, and Clarke. 2010; Hyde et al. 2008).

Our findings demonstrate that restriction of parenteral lipid negatively influences both body and brain weight and the composition of the TPN influences the LC PUFA content of the brain. This has potential to impact cognitive development in neonates (Rushton and Ankney. 1996). In our study, all parenterally fed piglets had a reduced final total body weight in relation to piglets that received sows milk. This occurred even when a higher dose of lipid PN (increased energy) was compared within the PN comparisons (low dose vs. high dose lipid treated groups). Consistent with malnutrition, all parenterally fed piglets had lower concentrations of albumin compared to same aged sow fed piglets (**Table 2.4**). This suggests protein losses either from gut mucosal atrophy (Goulet et al. 2004) or a potential amino acid restriction in the PN provided limiting the growth of parenterally fed piglets. In our earlier study, we were able to support the growth of piglets comparable to sow-fed piglets, using an identical parenteral amino acid solution (Wykes, Ball, and Pencharz. 1993). However, the present study was conducted

over two weeks, while the initial Wykes study duration was one week and the same amino acid solution may not have been able to meet the rapid increase in growth that occurs in the piglet (Wykes, Ball, and Pencharz. 1993). This argument that has been applied to current parenteral amino acid solutions for human neonates (Brunton, Ball, and Pencharz. 2000). Furthermore, the added impact of developing liver disease (as indicated by the finding of abnormal liver chemistry and mild histological pathological abnormalities) in the parenterally fed piglets could have impacted hepatic synthesis of albumin. Additionally, when examining the brain weights, they were also found to be lower in those receiving PN than that of our sow fed control piglets. This may; therefore, implicate either total energy restriction or a limitation of specific fatty acids having a negative impact on brain growth. However, at this time we are unable to ascertain if it is an energy restriction or fatty acid restriction that impacted brain growth. We do note that while not statistically significant, those piglets that received more energy and fatty acids (Intralipid10) displayed a trend towards greater brain growth than those that were restricted (Omegaven5 and Intralipid5) and the fatty acid composition of the major LCPUFA in the Intralipid® group were not different from the sow fed, suggesting that energy restriction was the major contributor to the decrease in brain growth.

Examining specific fatty acid data, compared to the sow-fed and the intra-lipid fed piglets there was a lower AA content and higher DHA content in the brain of Omegaven5 piglets. This is consistent with the minimal dosage of n-6 fatty acids given in fish oil-based parenteral therapies. It is notable; however, that there is also a trend to low LA in the Intralipid5 treatment group and to low AA in both the Intralipid treatment groups (consistent with minimal AA delivery in these two groups and more limiting LA delivery

in the Intralipid5 group). These findings are interesting when we consider that DHA, traditionally thought to be of most importance for the neonatal brain, was higher in the Omegaven5 group than all other groups. Comparing our treatment groups to our sow fed piglets, we see there was no difference in AA levels for either Intralipid10 or Intralipid5 as compared to sow fed control. This suggests that while AA has minimal delivery in soy-based lipid therapies, the conversion of LA to AA is sufficient to maintain homeostatic levels, even in a malnourished piglet. Omegaven5 however, does show a significantly lower AA level as compared to our sow fed piglets, suggesting an inadequate supply of n-6 LC PUFAs. This is further supported by the LA levels in Omegaven5, which are significantly lower than all other groups.

Examining the n-3 LC PUFA concentrations in relation to our sow fed piglets, unsurprisingly, Omegaven5 displayed higher levels of DPA, and DHA. However, the EPA concentrations were lower than those of sow fed piglets and not different from both Intralipid® treatments, suggesting potential preference for EPA conversion to its corresponding substrates DPA and DHA. Interestingly, there was no difference in ALA levels across all groups.

DHA is known to be an important factor in the development and function of brain tissue (Innis. 2007). While the role of n-3 lipids on brain development has been a primary focus of study, research utilizing rodents has established that appropriate amounts of n-6 fatty acids are also necessary for proper brain growth and that a deficiency of AA results in a delay of postnatal rat pup development (Amusquivar, Ruperez et al. 2000). Human studies also show DHA and AA are the most abundant brain fatty acids (Helland et al. 2003). Both rodent and neonatal research is further supported by a post mortem study

examining human neonatal brain tissue (Martinez. 1992). Brain fatty acids from children who had died due to non-neurological causes were examined, finding that both n-3 and n-6 LC PUFA concentration increased for up to the first two years of life. The DHA/AA ratio in preterm infants on parenteral nutrition (both short and long-term) was 7:1, when compared to the DHA/AA ratio of term, enterally fed children.

Examining the DHA/AA ratio present in our treatment groups, (Omegaven5, 1.42; Intralipid5, 0.88; Intralipid10, 0.89; Sow Fed Control, 0.79) it is evident that the Omegaven5 group has a significant increase in available n-3 LC PUFA in relation to the n-6 LC PUFA. This is of particular importance when examining the amount of n-6 fatty acids present in the lipid infusions (**Table 2.2**). The primary source of n-6 LC PUFA in Intralipid® is LA, with AA delivery being very low. This is also a factor with Omegaven® where the total amount of n-6 LC PUFA delivered is extremely restricted – however still providing more LA than AA. Burdge and colleagues (2002) observed that both human and piglet neonates do not initially synthesize enough of the necessary hepatic enzymes to convert LA and ALA to the longer chain AA and DHA. With a significant portion of n-6 fatty acids being provided in the shorter LA form the effects of potential restriction of both DHA (in soy based therapies) and AA (in both soy and fish-oil based therapies) and the effect on neonatal brain function, becomes a significant unknown in parenteral lipid therapies.

In our study the Omegaven5 group did have high n-3 LC PUFA brain content, notably DHA was even higher than in sow controls. As we do not have a functional outcome measure, such as behavior, we cannot know if the increased DHA/AA ratio improved brain function in our Omegaven5 fed piglets, independent of the brain size

restriction. However, while our study did not measure cognitive function, there is good existing support for total brain weight relating to cognitive development. Rushton and Ankney assessed the relationship between brain weight and cognitive development utilizing magnetic resonance imaging of the brain (MRI) (Rushton and Ankney. 1996). A moderate correlation between estimated brain weight and the cognitive development, using a battery of standardized psychometric tests, across all ages and sexes was identified. Examining our low dose therapies, brain weight was actually markedly restricted (by ~20%) during this critical neonatal developmental stage. By using a treatment scenario in piglets that mimicked current dose restriction parenteral lipid therapies for human babies, we have demonstrated that the use of lipid restriction in rapidly growing neonatal animals can affect brain development in both weight and fatty acid profile. Therefore, functional studies need to be conducted in developing neonates given different parenteral lipid treatments, particularly with dose restriction.

In contrast to restrictions in total body weight, brain and intestinal weights in PN fed piglets; the liver weights for all parenteral therapies were significantly higher than those of sow fed piglets. Further examination of our results revealed no significant correlation between the final body weight and liver weights ($r = -0.068$ $p= 0.71$). This lack of correlation is supported by research done by Adeola and colleagues (1995) who found that organ weight is influenced by the method of diet delivery. Adeola and colleagues compared orally fed, parenterally fed and sow fed piglets for 8 days. Piglets were measured for body composition and in vivo protein synthesis. The results showed a strong correlation between the method of nutrient delivery and the organ weights; with orally fed piglets displaying larger gastro-intestinal tracts and those fed parenterally

displaying significantly heavier livers. Correspondingly, our treatment groups, which received equivalent parenteral nitrogen delivery, displayed significantly higher liver weights, with correspondingly low small intestine weights.

Bile flow is an important liver function and determination of bile flow is a direct marker of cholestastic liver disease (Green. 2003). Total dose of soy-based lipid, fatty acid composition of the lipid (Tuchweber et al. 1996), and phytosterols (Clayton, Whitfield, and Iyer. 1998; Kurvinen et al. 2011; lyer, Spitz, and Clayton. 1998) have all been shown to influence bile flow in animal models. In our study, we found that the lowest bile flow was in the Intralipid10 treatment group and the highest bile flow in the Omegaven5 group. In fact, bile flow was much higher than control values for some Omegaven5 treated piglets (see **Figure 2.1**). Therefore it appears that parenteral lipid therapy that contains more n-6 LC PUFAs and coincidentally includes phytosterols; decreases bile flow in a dose dependent manner. This was not observed in those piglets receiving Omegaven5 therapy.

Bile flow is known to be affected by inflammation (Bowers et al. 1987). In work performed mainly in rat populations, inflammatory markers like TNF-α influenced the bile salt transporter expression when stimulated by lipopolysaccharide (Green, Beier, and Gollan. 1996). These markers for the most part are in the range of sow fed controls. Perhaps they were not the best measurements as a trend to more sepsis was noted in the Intralipid® fed groups, but did not translate to higher CRP or cytokine expression. Thus we are unable to come to any specific conclusions about the effects of inflammation on bile flow in our study and in particular could not conclude that n-3 treatment reduces inflammation as the mechanism to improve bile flow.

Steatosis is one of the early pathological findings of IFALD, particularly in adults. We examined steatosis by measuring hepatic lipid retention in our piglets using Oil Red O staining. As seen in **Figures 2.1 and 2.3b**, there was little to no steatosis found in either Omegaven5 or sow fed control piglets. This has also been examined in Non-Alcoholic Fatty Liver Disease (NAFLD) models in children and it is suggested that n-3 fatty acids found in fish oils influence steatosis by lowering blood triglyceride levels and increasing insulin sensitivity (Nobili et al. 2013). Importantly, the groups containing soy-based lipids (Intralipid5 and Intralipid10), showed the greatest degree of steatosis and lowest bile flows of all treatments. This effect of steatosis on liver function is also seen in NAFLD, where association with non-alcoholic steatohepatitis shows disease progression from cholestasis to fibrosis and cirrhosis (Serfatya and Lemoinea. 2008). However, further examination in this area is required to elucidate if a relationship between steatosis and bile flow exists and to understand the impact on liver function in an IFALD model.

Impaired bile flow with soy-based lipids may also be explained by the lower vitamin E intake from Intralipid® as compared to Omegaven®. Reactive oxygen species interact with hepatocytes causing significant tissue injury (Weinberger, Watorek, and Strauss R. 2002). Increased oxidative stress has been shown in PN fed neonates receiving primarily soy-based lipids (Miloudi et al. 2012; Skouroliakou et al. 2010a; Versleijen et al. 2012). As well, cholestasis has been correlated to elevated serum phytosterols, which are absent in fish-oil based lipids. El Kasmi and colleagues found that phytosterols have a greater direct influence on bile flow and their removal increases bile flow (El Kasmi, Anderson et al. 2013). Overall while this study does demonstrate that Omegaven® improves bile flow, more than restriction of Intralipid® alone, it does not

clarify by what mechanism. Furthermore, the use of Omegaven® in our study, while a potent stimulator of bile flow, did not completely eliminate IFALD as evidenced by biochemistry and histology.

Currently, clinical use of indirect biomarkers of liver function - including bilirubin, bile acids, ALT, GGT, and ALP, remain the most common method of diagnosing and monitoring IFALD in human neonates. However, the optimal test has yet to be determined. The most significant biochemical indicator of impaired liver function for our study, that is commonly used clinically, was serum bilirubin (Diamond, Pencharz, and Wales. 2009; Diamond et al. 2009; Gura, Parsons, and Bechard. 2005; Gura et al. 2008; Cober et al. 2012a). It has been suggested that GGT is a good early marker for cholestatic liver disease, as GGT levels spike quickly after injury and normalize quickly after hepatic injury is no longer apparent (Bulle et al. 1990). While GGT did not correlate well with bile flow in our study, it is noteworthy that it was increased in all parenterally fed treatment groups and was at basal levels in sow fed controls only. Therefore, it may well be an earlier marker of IFALD than restricted bile flow, and may relate to a mechanism that is somewhat independent of extra-hepatic biliary obstruction.

In contrast, in human patients, there are practical difficulties in obtaining routine liver biopsies. Our animal model, however, permitted assessment of liver histology in all treatment groups. Utilizing our modified liver scoring system, there was no difference in mean histological score for treatment groups. However, we have previously shown that the usual score for sow fed control piglets was ≤ 1 (Hua et al. 2013). Therefore if we look at the proportion of piglets per group with a score of 2 or more, in relation to the established normal, each group showed some histological changes of IFALD. It should

be mentioned that within our model only the early stages of IFALD were identified, with stages including fibrosis and cirrhosis not evident in our histological data.

The advantage of the neonatal piglet as a research model is the similar rate and pattern of development of the gastrointestinal tract as the preterm human neonate (Litten-Brown, Corson, and Clarke. 2010; Book and Bustad. 1974b; Gu and Li. 2003). Certainly the piglet model has been shown to develop parenteral nutrition related liver pathology that is close to IFALD pathology as seen in neonates (Hua et al. 2013; Turner et al. 2011). The presence of a functional gall bladder is relevant to the human condition and bile duct cannulation was feasible, which would not be the case if using neonatal rodents.

In summary, in a neonatal piglet model we have been able to study the effects of lipid dose restriction (both with n-3 and n-6 predominant parenteral lipid therapy) and conventional lipid dosing on IFALD and neonatal growth and brain development (indicated by weight and fatty acid content). Low dose n-3 therapy was associated with a marked improvement in bile flow and improved biochemical markers of IFALD. To a lesser degree, low dose n-6 lipid was found to improve bile flow and biochemical markers of IFALD in comparison to conventional dosing. However both dose restriction regimens significantly altered the fatty acid profile in the brain of treated piglets. Long-term studies into the effects of an altered brain lipid profile should be conducted to better understand the impact of both decreasing brain n-6 LC PUFA content and increasing n-3 LC PUFA content that occurs with lipid dose restriction therapies. Finally, neither low dose therapy completely ameliorated all biochemical and histological evidence of IFALD and the progression to severe cholestasis with either approach over a long enough time period could not be excluded by our study.

Table 2.1: Parenteral lipid solution: fatty acid composition

	Intralipid®	Omegaven®
% by weig htof total fatty acid s		
Myristic Acid (14:0)	-	5.1
Palmitic Acid (16:0)	11	11.7
Palmitoleic Acid (16:1)	-	9.2
Stearic Acid (18:0)	4	4.4
Oleic Acid (18:1n-9)	24	15.1
Linoleic Acid (18:2n-6)	53	4.4
α-Linolenic Acid (18-3n-3)	8	1.8
Dihomo-γ-linolenic acid (20:3n-6)	-	0.6
Arachidonic Acid (20:4n-6)	0.1	2.1
Eicosapentaenoic Acid (20:5n-3)	-	19.2
Docosapentaenoic Acid (22:5n-3)	-	2.1
Docosahexaenoic Acid (22:6n-3)	-	12.1

Fatty acid content as reported by Wanten and Calder. (2007)

Table 2.2: Fatty acid delivery (g/kg/d) in parenterally fed piglets receiving either Omegaven5, Intralipid5 or Intralipid10

	Omegaven5	Intralipid5	Intralipid10
	g/kg/d		
Myristic Acid (14:0)	0.255	-	-
Palmitic Acid (16:0)	0.585	0.55	1.1
Palmitoleic Acid (16:1)	0.46	-	-
Stearic Acid (18:0)	0.22	0.2	0.4
Oleic Acid (18:1n-9)	0.755	1.2	2.4
Linoleic Acid (18:2n-6)	0.22	2.65	5.3
α-Linolenic Acid (18-3n-3)	0.09	0.4	0.8
Dihomo-γ-linolenic acid (20:3n-6)	0.03	-	-
Arachidonic Acid (20:4n-6)	0.105	0.005	0.01
Eicosapentaenoic Acid (20:5n-3)	0.96	-	-
Docosapentaenoic Acid (22:5n-3)	0.105	-	-
Docosahexaenoic Acid (22:6n-3)	0.605	-	-
Phytosterols (mg/kg/d)	0	17.4	34.8
Vitamin E (mg/kg/d)	14.8	1.9	3.8

Fatty acids as delivered in individual therapies Omegaven5, Intralipid5 and Intralipid10 shown in g/kg/d. Amounts were calculated using fatty acid values presented in Table 2.1

Table 2.3: Characteristics of parenterally fed piglets receiving either Omegaven5, Intralipid5 or Intralipid10 and sow fed piglets.

		Omegaven 5 (n=8)		Intralipid 5 (n=6)		Intralipid 10 (n=9)		Sow Fed Control (n=8)		
	Units	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	p-value
Age	days	4.13	0.64	4.17	0.75	3.89	1.05	-	-	NS
Weight	kg	2.29	0.26	2.18	0.16	2.15	0.24	2.20	0.00	NS
End Weight	kg	5.01 a	0.35	4.5 a	0.35	4.30 a	0.63	6.11 b	0.80	≤ 0.05
TPN Delivered	%	85.38	3.89	85.83	5.12	80.11	4.81	-	-	NS
Trial Duration	days	13.88	0.35	14.00	0.00	13.89	0.60	14.00	0.00	NS
Liver Weight	g	210.9 a	19.20	205.53 a	10.26	199.6 a	40.63	156.61 b	16.99	≤ 0.05
Small Bowel Weight	g	108.78 a	9.21	92.4 ab	7.68	84.33 b	10.60	182.45 c	19.82	≤ 0.05
Brain Weight	g	36.60 a	2.84	36.00 a	3.84	39.80 ab	5.17	45.90 b	3.44	≤ 0.05

Omegaven®5 (n=8), Intralipid®5 (n=6), Intralipid®10 (n=9), and Sow Fed Control (n=8). Data reported as mean, and standard deviation; comparisons made by ANOVA; α=0.05. Post-hoc comparisons made using Tukey HSD. Significant differences ($p \leq 0.05$) among treatments are labeled with different letters; non-significant different differences were represented with NS.

Table 2.4: Biochemical outcomes at termination for parenterally treated piglets compared to sow fed piglets.

		Omegaven 5 (n=8)		Intralipid 5 (n=6)		Intralipid 10 (n=9)		Sow Fed Control (n=8)		
	Units	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	p-value
Albumin	g/L	15.00 a	3.07	14.83 a	1.60	15.00 a	2.74	30.75 b	3.58	≤ 0.05
Bile Acids	umol/L	15.28 a	12.41	32.52 ab	18.47	38.9 b	21.35	15.26 a	6.39	≤ 0.05
Bilirubin	umol/L	6.86 a	4.85	9.67 a	2.94	21.11 b	10.68	10.13 a	4.39	≤ 0.05
ALP	IU/L	836.75	298.26	754.00	148.43	717.78	173.74	949.63	292.90	NS
GGT	IU/L	173.25 a	135.78	88.67 ab	74.54	174.67 a	86.56	25.13 b	10.84	≤ 0.05
ALT	IU/L	19.63	24.85	9.17	2.14	9.89	1.62	24.75	8.29	NS

Omegaven®5 (n=8), Intralipid®5 (n=6), Intralipid®10 (n=9), and Sow Fed Control (n=8). Abbreviations: ALP – Alkaline Phosphatase, GGT – Gamma-Glutamyl Transpeptidase, ALT – Alanine Transaminase. Data reported as mean, and standard deviation; comparisons made by ANOVA; α=0.05. Post-hoc comparisons made using Tukey HSD. Significant differences ($p \leq 0.05$) among treatments are labeled with different letters; non-significant different differences were represented with NS.

Table 2.5: Plasma markers of inflammation at termination for parenterally treated piglets compared to sow fed piglets

		Omegaven 5 (n=8)		Intralipid 5 (n=6)		Intralipid 10 (n=9)		Sow Fed Control (n=8)		
	Units	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	p-value
Term CRP	IU/L	16.23	20.91	7.69	2.27	16.39	5.93	12.48	3.25	NS
Term IL6	IU/L	18.91	11.47	14.16	10.88	22.96	20.88	16.01	9.22	NS
Term TNFa	IU/L	814.85	811.82	725.97	1092.04	1743.12	1293.68	1769.80	2697.24	NS

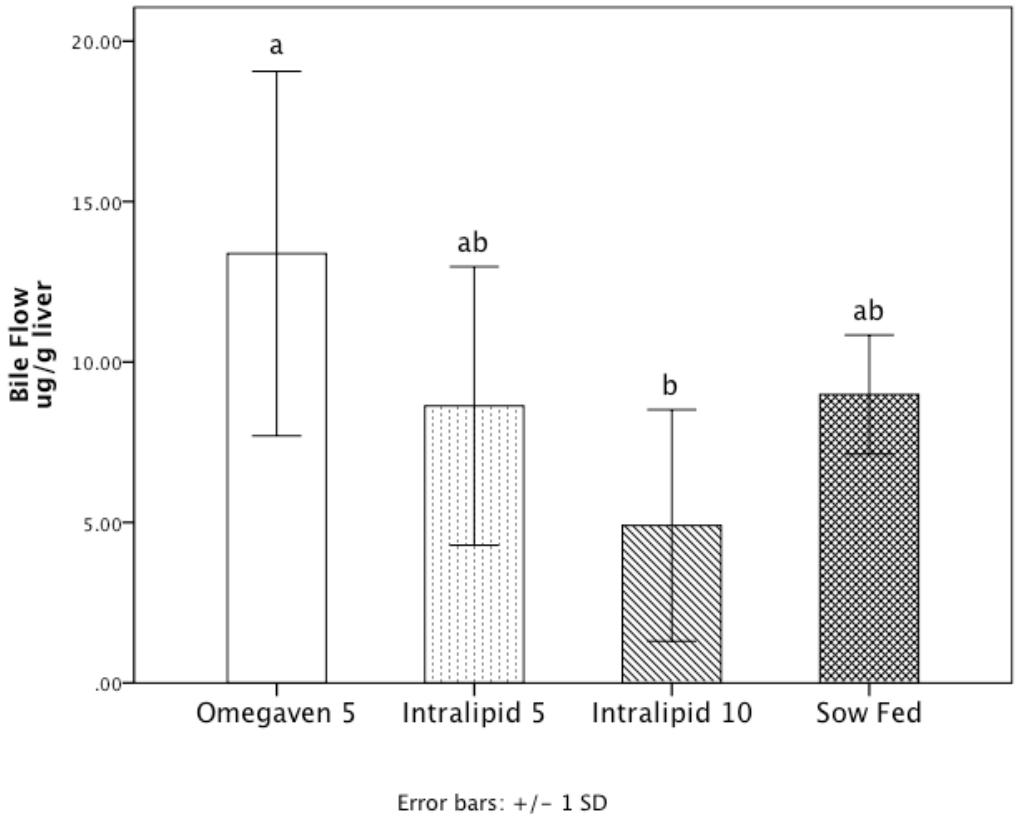
Omegaven®5 (n=8), Intralipid®5 (n=6), Intralipid®10 (n=9), and Sow Fed Control (n=8). Abbreviations CRP: C-Reactive Protein, IL6: Interleukin-6, TNF-α: Tumor Necrosis Factor – alpha. Data reported as mean, and standard deviation; comparisons made by ANOVA; α=0.05. Post-hoc comparisons made using Tukey HSD. Non-significant different differences were represented with NS.

Table 2.6: Selective fatty acid content (% of total identified fatty acids) of brain tissue in parenterally fed piglets compared to sow fed control.

		Omegaven 5 (n=6)		Intralipid 5 (n=6)		Intralipid 10 (n=9)		Sow Fed Control (n=4)		
	Units	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	p-value
Stearic Acid	%	25.17	1.96	24.95	2.05	25.12	1.94	23.20	0.78	NS
Oleic Acid	%	14.20	0.39	14.40	1.14	14.05	1.03	13.25	1.19	NS
Linoleic Acid	%	0.47 a	0.03	0.85 b	0.22	1.43 c	0.25	0.93 b	0.13	≤ 0.05
α-Linolenic Acid	%	0.43	0.05	0.52	0.08	0.48	0.15	0.51	0.12	NS
Dihomo-γ-linolenic Acid	%	0.50 a	0.14	0.63 ab	0.14	0.78 b	0.19	0.48 a	0.04	≤ 0.05
Arachidonic Acid	%	8.13 a	0.28	8.94 ab	0.86	8.88 ab	0.71	9.73 b	0.43	≤ 0.05
Eicosapentaenoic Acid	%	0.16 a	0.03	0.21 ab	0.02	0.20 a	0.04	0.31 b	0.11	≤ 0.05
Docosapentaenoic Acid	%	0.84 a	0.15	0.39 b	0.13	0.35 b	0.09	0.44 b	0.10	≤ 0.05
Docosahexaenoic Acid	%	10.08 a	1.46	7.94 b	0.42	7.92 b	1.30	7.71 b	0.37	≤ 0.05

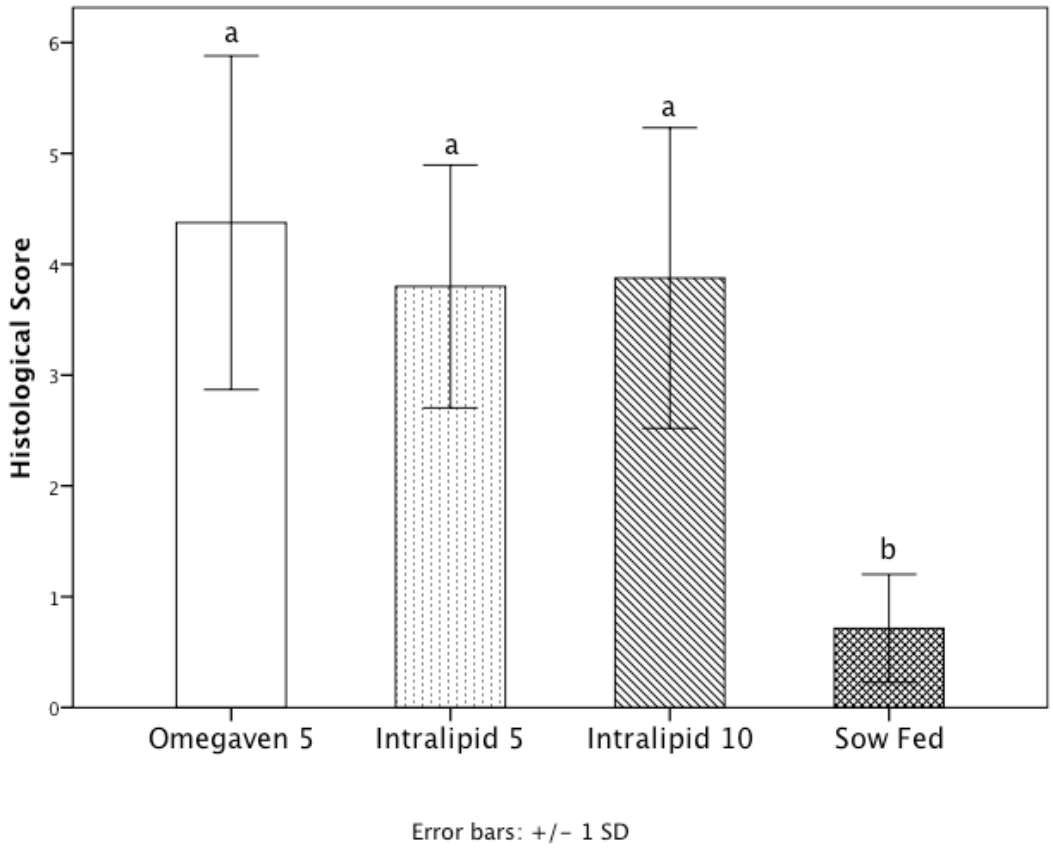
Omegaven®5 (n=8), Intralipid®5 (n=6), Intralipid®10 (n=9), and Sow Fed Control (n=8). Data reported as mean, and standard deviation; comparisons made by ANOVA; $\alpha=0.05$. Post-hoc comparisons made using Tukey HSD. Significant differences ($p \leq 0.05$) among treatments are labeled with different letters; non-significant different differences were represented with NS.

Figure 2.1: Bile Flow (µg/ g liver) in neonatal piglets receiving Omegaven5, Intralipid5, and Intralipid10 in relation to sow fed control piglets at day of termination



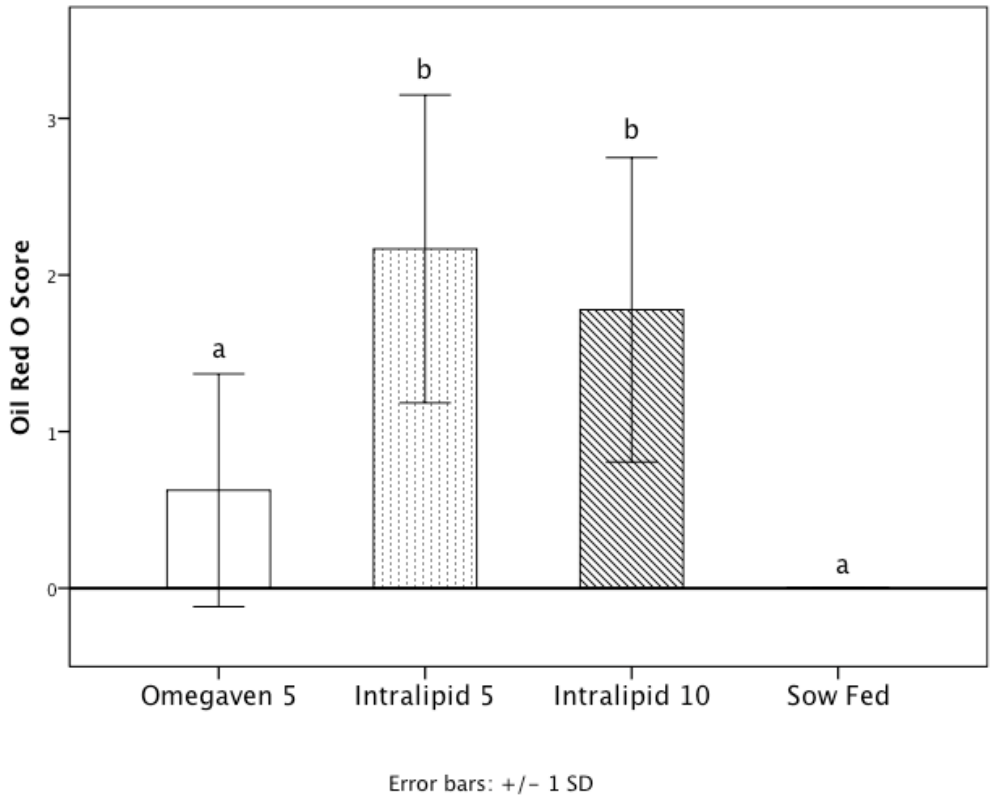
Omegaven5 (n=8), Intralipid5 (n=6), Intralipid10 (n=9), and Sow Fed Control (n=8). Mean and SEM is shown; comparisons made by ANOVA; α=0.05. Significant differences between treatments are labeled with different letters.

Figure 2.2a: Histological scoring for liver sections of Omegaven5, Intralipid5, and Intralipid10 in relation to sow fed control piglets at day of termination



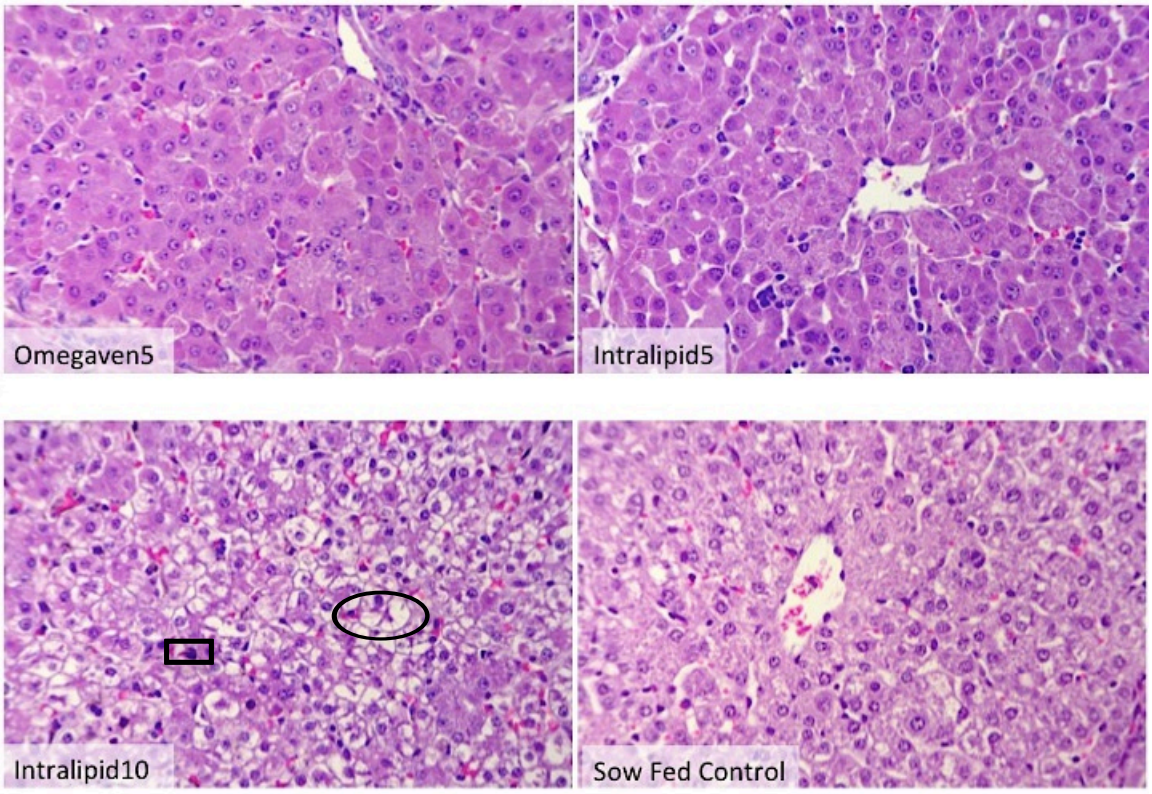
Omegaven5 (n=8), Intralipid5 (n=6), Intralipid10 (n=9), and Sow Fed Control (n=8). Mean and SEM is shown; comparisons made by ANOVA; α=0.05. Significant differences between treatments are labeled with different letters.

Figure 2.2b: Mean Oil Red O Score of parenterally fed Omegaven5, Intralipid5, and Intralipid10 piglets in relation to sow fed control piglets



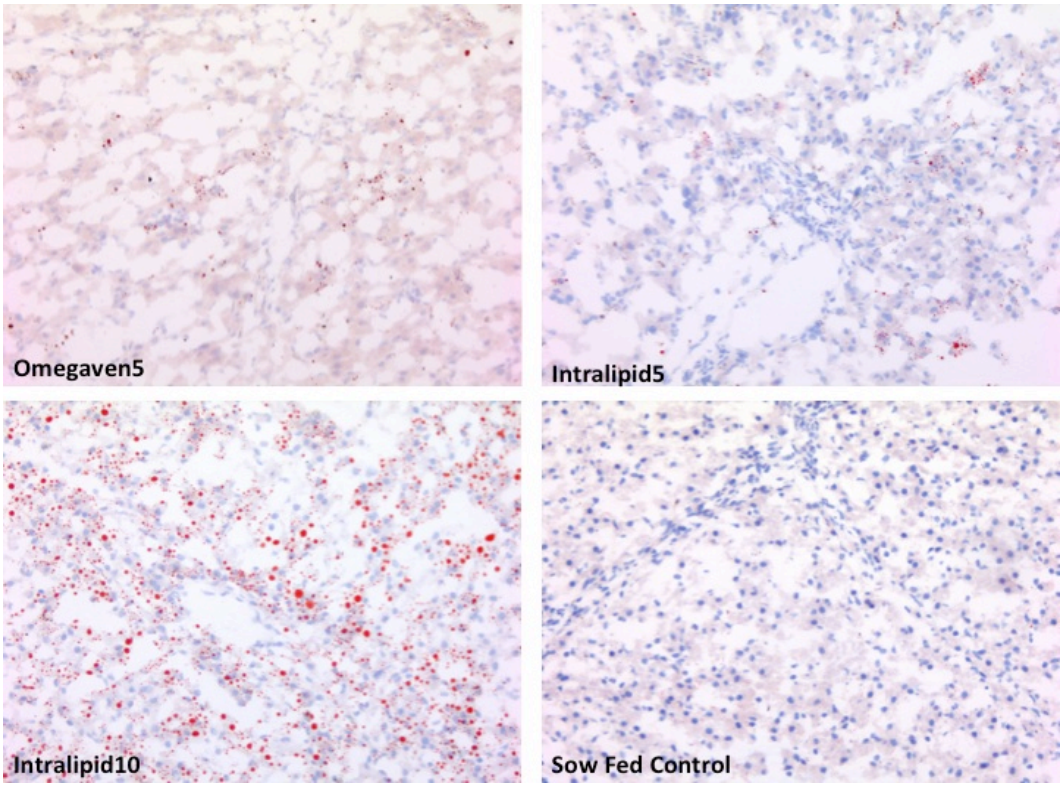
Omegaven5 (n=8), Intralipid5 (n=6), Intralipid10 (n=9), and Sow Fed Control (n=8). Mean and SEM is shown; comparisons made by ANOVA; α=0.05. Significant differences between treatments are labeled with different letters.

Figure 2.3a: Hepatocyte centered on the liver lobule for each parenteral group Omegaven5, Intralipid5, Intralipid10, and Sow Fed Control piglets



Slides represent the populations of Omegaven5 (n=8), Intralipid5 (n=6), Intralipid10 (n=9), and Sow Fed Control (n=8). Liver lobules are magnified at 200x. Slides include a central vein in a liver lobule. Dark blue stain represents foci of hemopoesis in sinusoids and kupffer cells, purple stain represents nucleus of individual hepatocytes, and clear areas represent vacuolization of cells. Light pink stain is an artifact of staining and should be ignored. Significant vacuolization can be seen in the Intralipid10 group as highlighted by the circle, the square, highlights an activated Kupffer cell.

Figure 2.3b: Oil Red O staining for lipid storage in hepatic tissue for parenterally fed piglet groups in comparison to sow fed control piglets.



Slides represent the populations of Omegaven5 (n=8), Intralipid5 (n=6), Intralipid10 (n=9), and Sow Fed Control (n=8). Oil Red O slides examined at 200x multiplication. Slides present a liver lobule with red stain identifying lipid storage with scoring as follows: 0: no lipid, 1: ¼ cytoplasmic opacification by lipid, 2: ¼ to ½ opacification, 3: ½ to ¾ opacification, and 4: 100% opacification of the slide. Blue stain represents the nucleus of hepatocytes. When looking at the slides, one can see a significant accumulation of fat deposits present in the Intralipid10 group, as represented by the red staining.

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Chapter 3: CONCLUSIONS

3.1 Summary

Going back to the original hypotheses,

1: Parenteral lipids at a dosage of 5 g/kg/d will reduce the severity of cholestasis in a neonatal piglet receiving total parenteral nutrition as compared to conventional dosing of 10 g/kg/d.

2: Parenteral lipids at restricted doses will not allow for optimal growth in neonatal piglets.

3: Brain weight in lipid-restricted therapies will be significantly less than those receiving conventional dosages of 10 g/kg/d or sows milk.

4. Additionally, fatty acid profiles will reflect parenteral lipid delivery with piglets receiving higher n-3 LC PUFA displaying higher n-3 LC PUFA percentage in brain tissue.

Overall the findings are consistent with these hypotheses, however a number of caveats exist and will be reviewed. In summary, however, we did find:

1. Parenteral lipid restriction at 5 g/kg/d did reduce the severity of a majority of physical and biochemical parameters of IFALD and cholestasis in relation to the 10 g/kg/d dosage, however not all parameters normalized.

2. Growth was restricted in all parenteral therapies in relation to the sow fed controls.

3. Brain weights reflected dosages, with dose restricted brain weights being less than those of sow fed piglets. However, conventional dosing was not different from either the restricted dosages or the sow fed control piglets.

4. Brain fatty acid profiles did reflect parenteral lipid delivery, with those receiving more n-3 LC PUFA's having higher levels in brain tissue than those receiving less.

It should be first stated that this research was important and novel. At this time, to our knowledge, this study is the only neonatal comparison of dose-restricted therapies and novel lipid sources, as used in current clinical practice, to contemporary controls. Clinical practice and enthusiasm over the use of Omegaven®, to treat IFALD, has been based largely on case study experience, rather than clinical trials. Factors including the degree of prematurity, the length of time the infant is on parenteral therapy, the use of enteral nutrition and the actual lipid doses have all been variable in the cohort studies to date (Groleau, Thibault, and Marchland. 2014; Hall et al. 2014; Klein et al. 2013; Lee et al. 2006; Rollins et al. 2010; St-Jules, Waatters, and Iwamoto. 2014). However, we have been able to effectively standardize these variables in this research study.

Arguably the biggest criticism of the literature to date is the lack of appropriate controls. Therefore, it is important that we used contemporaneous control animals, at the same age and developmental stage, and given the gold standard nutrition, sow's milk. The control represents optimal nutritional development for neonatal piglets. However, it must be acknowledged that these healthy controls were not reared under the same conditions, remaining in the barn with their sow and littermates. This may account for some differences in outcomes, independent of the different nutritional exposures. However, a contemporary disease control was also studied. Again, this is an advance over the published literature, which uses historical disease controls, sometimes 'matched' to the treatment cohort. Use of these controls, may exaggerate the effectiveness of novel treatments unintentionally. Firstly, the practice in neonatal units has changed recently, so that most preterm neonates and especially those at risk of IFALD, are rarely exposed to the doses and durations of parenteral lipid that were historically given. Secondly,

improving diagnostics, advances in line care to avoid sepsis and the advent of multidisciplinary teams have all improved outcomes for infants with intestinal failure, regardless of novel treatments (Sant'Anna et al. 2012; Diamond et al. 2007).

Certainly on the basis of our study there is now evidence that dose restriction is an effective treatment to prevent IFALD. In our model, consistent with the reports in human babies, dose restriction (fish or soy oil) improved liver disease – primarily bile flow - in comparison to more conventional dosing of soy based parenteral lipid. Interestingly however, utilizing other factors of to evaluate liver function, low dose therapies did not normalize all liver chemistries, nor provide normal liver histology. This lack of normalization, even with low dosages, reinforces the multifactorial nature of IFALD, and while dose restriction may aid in reducing the perpetrating factors, it does not completely eliminate the biochemical and physiological markers of IFALD.

While liver function, bile flow, and chemistry, bilirubin, can be improved by both dose restriction studies, concerns raised about nutritional complications exist and were able to be explored further in this study. The clinical studies provide data that lipid restriction may not provide optimal nutritional support, in particular increasing the risk of EFAD (see section 1.3.1). One problem with the introduction of any new medical therapy in a vulnerable population, like neonates, is the time it would take to understand the impact on the developing brain. Fortunately, in our piglet model we directly examined the impact of lipid restriction and altered fatty acid profiles on brain size and fatty acid composition. Such information is vital when introducing treatments that have the potential for long-term adverse effects. Use of a neonatal animal model allows for a greater depth of understanding, with less ethical concerns. Using both soy oil

(Intralipid®) and fish oil (Omegaven®) the growth of the piglets was restricted. Whether this was the result of specific limitations in fatty acids or overall calorie / energy deficit, we are unable to ascertain from our results. Examining the brain weights, it is evident that parenteral lipid therapy, no matter the dosage or lipid, restricted brain weight in comparison to the sow fed control group.

Further examination of fatty acid composition of brains in each treatment group showed that lipid treatments alter the brain fatty acid composition. Most notably, the fish oil, which is high in n-3 long chain polyunsaturated fatty acids, displayed a significantly higher level of the end-products DPA and DHA, while the more saturated products ALA was not different from the sow fed piglets. This efficient conversion of ALA, suggests that while there is still competition for enzymes (**Figure 1.4**), desaturation in an n-3 rich environment is not only possible, but is reflected in important tissues. In comparison, examination of the key n-6 long chain polyunsaturated fatty acids showed no difference among treatment groups for the more saturated LA, however as we examine the essential AA levels, we find that those given the soy-based lipid did not show a difference in AA levels from sow fed piglets. There is a significantly lower amount of AA present in brain tissues of those given fish oil-based lipid. This significant finding suggests that there is the potential for AA to become very limiting in neonates who are fed exclusively Omegaven®, as the requirements for brain growth may outpace the availability of LA and AA in Omegaven®.

These may not be the only nutritional considerations that must be further evaluated for neonates treated with novel parenteral lipid therapies and dosing strategies. Current clinical strategies may be inadvertently providing an undersupply of non-protein

energy by swapping out calorie dense lipid for dextrose. Particularly in a susceptible population, such as infants with short bowel syndrome or IFALD, requirements have been shown to not match those of a healthy term infants (Adamkin, Sims, and Radmacher. 1985; Adamkin. 2003; Heird. 1998; Imura and Okada. 1998) on growth, cognition, and IFALD.

3.2 Limitations of this Study

There were several limitations to this study, the aforementioned caveats. Some of the limitations encountered originate from the use of a piglet model and some from the experimental design. One of the greatest weaknesses in our study is that we were unable to examine the lipids while controlling for calorie delivery. Initially, we attempted to produce iso-caloric as well as iso-nitrogenous treatment groups by adding glucose to our parenteral solution to make up for the energy deficit. This in fact mimics some acknowledged clinical practice with infants (Ling et al. 2011). Unfortunately, due to the piglet's extremely high metabolic rate such a significant amount of glucose was required that we saw significant complications, particularly severe renal dysfunction from diabetic nephropathy. Therefore, it was not practical or ethical to continue with this study design. Interestingly, to the author's knowledge, a thorough examination of the effects of excess non-protein energy supplied in the form of glucose in neonates given lipid minimized parenteral strategies has not been performed.

A solution to this experimental design problem would have been to study one additional treatment group, given Omegaven® at an equivalent 10 g/kg/d dose, as the disease control (Intralipid10). Use of doses >1g/kg/d in young children may increase the risks of complications from Omegaven®, those already implicated including burr-cell

anemia (Mallah et al. 2010) and risk of bleeding from platelet dysfunction; all related to the extremely high n-3 fatty acid content (Dicken et al.). The study was specifically designed to evaluate current clinical practices. Even though treatment groups were not iso-caloric, we achieved our intention with the study design. As previously mentioned, most clinicians are utilizing 1g/kg/d dosages of Omegaven to treat IFALD as was originally published (Gura et al. 2006; Puder et al. 2009; Cober et al. 2012a). In our piglet model, we considered conventional dosing to be 10g/kg/d, based on our first studies of TPN feeding piglets (Wykes). Therefore, we considered dose restriction to be 5g/kg/d dosage. We are extrapolating to a 'standard dose' of 2g/kg/d in an infant, a somewhat conservative doubling of the 1g/kg/d dose used in parenteral lipid restriction. Therefore, while our study design is flawed from an 'experimental' point of view, we believe it mimics the clinical strategies as was intended and so provides translational information on the impact of dose restriction for growing neonates. Realistically this includes such relevant information as the impact of lipid calorie restriction on growth.

Another limitation of this study was the lack of analysis of body composition, which would have allowed us to further understand the nutritional impact of piglets given the different parenteral lipid therapies. Our primary outcomes related to nutrition were measurements of total body weight and albumin levels (at baseline and termination). These measurements may be altered by several factors. Of particular importance, weight can be artificially elevated by oedema. Any degree of water retention would skew both of our nutritional status measures. Given that we observed abnormal albumin levels in our treatment groups there was potential for fluid retention in all piglets. This indicates that while there was a significant difference in body weights between the parenterally fed

piglets and those that received sow's milk, this difference may actually be much more significant than it appears. Furthermore, given we did not have an accurate measurement of water composition; we cannot exclude actual differences between treatment groups existed in relation to lipid treatment in oedema or in fat and lean mass. Even more confounding, while we know albumin levels were reduced with parenteral treatment, we cannot be sure that this was the result of protein deficiency, liver functional impairment of excess parenteral water delivery. Having measured body composition would have enabled us to better understand these issues.

Another shortfall of our study design is that our piglets represent a TPN model with gut atrophy, but not a short bowel model, the most usual diagnosis associated with IFALD. They had a complete gastrointestinal tract (GI tract) and this is important when considering the absorption of bile acids is mainly performed by the ileum. With our piglets containing a functional GI tract, we have to concede that they likely have greater potential for normal bile acid re-uptake and circulation that is not present in a short bowel model. This potential re-uptake and hepatobiliary circulation could influence the degree of hepatic dysfunction. It remains to be seen if the findings in our TPN model of successful IFALD prevention by lipid restriction, measured as normal bile flow over two weeks, could be observed given gut resection. However, one advantage of our model was that piglets were not provided any enteral nutrition. Therefore, of the role of CCK in the maintenance of the bile acid pool was kept to a minimum and the confounder present in the literature of variable advancement of EN was also removed.

Another limitation is the short study duration; piglets underwent study for 14 days of continuous total parenteral nutrition (TPN) delivery. If we take into account their

metabolic rate, and the time it takes for piglets and neonates to double their birth weight (according to Payne and Wheeler (Payne and Wheelerd. 1968), piglet ~10 days and neonates ~100 days), to assess equivalent developmental states, using our piglets we can only assess up to approximately 6 months of age in a neonate. This creates a major issue when examining our data in a clinical setting as most infants in both Intralipid® and Omegaven® studies are on PN for a significantly longer period of time. It is also important to note that those infants who receive Omegaven® are working on a 'recovery' instead of a 'prevention' model. Often times, infants initially receive soy-based PN, with careful monitoring for symptoms of IFALD like a bilirubin level greater than 2 mg/dL (34μmol/mL). This initial treatment can last a significant amount of time prior to diagnosis, and any reversal work done on a compassionate care basis, either by dose restriction or utilization of Omegaven®, often lasts much longer than our 10 week extrapolation extends (Nehra, Fallon, and Puder. 2011)

Our model was also unable to replicate this clinical practice of switching lipid sources after development of IFALD, as the significant growth of our piglets restricted the duration of the trial to 14 days. We did attempt to extend the 14 days study to 21 days, but found that the piglets outgrew their jugular catheters and required an additional surgery to insert another catheter. This additional surgery would increase the stress that piglets on trial were subjected to, which could potentially influence hepatic dysfunction independent of lipid treatment. Therefore, as a result of the short trial duration, it can be suggested that we saw only a mild version of liver dysfunction in our piglet model and we would be unable to examine the potential to reverse IFALD in our model.

Finally, while we focused on measuring liver function, brain fatty acids and inflammatory cytokines, but other important measures that were not preformed. Notably these include measurement of essential fatty acid deficiency and consideration of oxidative stress experienced by these piglets. Essential fatty acid deficiency (EFAD), measured through plasma fatty acids, was unable to be monitored on a regular basis during our study. This becomes a limitation within our study, as we cannot confirm that our piglets received adequate amounts of fatty acids over the duration of this study. While we did monitor for outward signs of EFAD, preliminary biochemical EFAD may have been a factor in our piglet's limited growth. In particular, measurement of fatty acids that were severely restricted, AA for example, could neither be confirmed nor denied as sufficient in its supply for any of the parenteral therapy groups.

This also extends to measurement of oxidative stress in our model. Knowing that n-3 long chain poly-unsaturated fatty acids (n-3 LC PUFA's) are easily oxidized, particularly in solution, our study limits our understanding of the potential oxidative stress and/or oxidative protection that is provided by the lipid solutions. Confounding the degree of oxidative stress encountered is the differing levels of the anti-oxidant Vitamin E. As previously mentioned, Vitamin E is added to Omegaven® to prevent the oxidation of n-3 LC PUFA's, this is not the case for soy-based lipids, which contain a minimum amount of Vitamin E (**Table 2.2**). As a result of differing levels, and a lack of measurement of oxidative stress, we are unable to elucidate why there is a suggested hepato-protective role of Omegaven® (Gura et al. 2006), if it relates to the presence of n-3 LC PUFA's or if it is influenced by the amount of Vitamin E present in solution. A better study design might have considered measuring essential fatty acid deficiency as

well as measured the oxidative stress experienced by the piglets. Oxidative stress in particular can be measured by a direct assessment of the amount of reactive oxygen species present, or by measuring anti-oxidants such as catalase or super oxide dismutase. Additionally, equalizing the vitamin E content of diets would eliminate the confounding variable that is currently present in our study as a result of varying amounts of vitamin E within our formulations.

Also found to differ in our parenteral solutions is the phytosterol levels, with the conventional dosing strategy receiving approximately double the amount of phytosterols than our low dose Intralipid® group. As phytosterols accumulate in the liver and have been implicated as a cause of hepatic dysfunction, the differing levels present in the diets could possibly account for the differences in hepatic function. As phytosterol levels were not standardized, we are unable to elucidate in any way their role in IFALD. Because the treatment group with the greatest degree of hepatic dysfunction was also the group that received the highest lipid dose, as well as the highest phytosterol concentration with minimal vitamin E delivery, we cannot effectively pinpoint which element has the greatest effect on hepatic function.

Timing of measurements came into play with assessment of sepsis in our piglets. As with a neonate, our piglets were susceptible to development of sepsis and were watched carefully for any signs or symptoms of potential sepsis. However, these indicators were all very subjective. Blood cultures were taken at the onset of suspected sepsis and piglets were treated with additional antibiotics (to reduce the severity of septic events) for the remainder of the trial. Cultures reported initial results at 24 hours; however, septic events and the bacteria responsible for the sepsis were not confirmed for

7 days, often after the trial had completed. This created a two-fold effect in our study. First, it is possible that antibiotic treatments could benefit or worsen the liver disease and so confound our findings. Secondly, both the delay in proving sepsis and the possibility to successfully treat piglets caused us to continue to utilize potentially septic animals for this study. This is perhaps less of a problem than it first appears. Sepsis is common for PN fed neonates and may of itself be an important factor in IFALD development, where it is almost universally observed. It may simply not be practical to study IFALD prevention without potential for sepsis. Certainly any clinical trials in infants with IFALD that attempted to remove all patients who experienced septic events would probably be infeasible, certainly they would greatly increase the recruitment period, hence expense, and shorten the potential intervention duration of the study, all restricting clinically relevant data. This is also the case in our piglet work. Having kept a record of those suspected of sepsis and those found to be correspondingly septic, examination of liver function tests, nutritional indicators and the cytokine data did not show a difference between septic animals and those identified as healthy.

An additional limitation we identified is that with the use of pre-formulated lipid solutions, many different fatty acids are given simultaneously. This limits our ability to identify fatty acids that positively or negatively influence hepatic function on their own. A stronger model would examine the effectiveness of each individual fatty, and their effect on hepatic function. This could also include examination of different sources of fatty acids, from plant-based sources to marine sources.

Finally, investigators were not blinded when preparing and administering parenteral solutions. This was simply an issue of practicalities, yet lack of blinding is a significant flaw for any clinical trial that can alter findings often in subtle ways.

3.3 Future Directions:

Because there continues to be many factors in the development of IFALD, there are also many different directions this research could go in. Firstly, as mentioned we were unable to study the 10 g/kg/d Omegaven® dosage in our piglet – equivalent to 2 g/kg/d in neonates. While most studies examining the use of Omegaven® utilize only 1 g/kg/d (Puder et al. 2009) or a combination of Omegaven® and Intralipid® (Diamond et al. 2009) at 1 g/kg/d each, the reported use of a dose that is equal to the convention lipid delivery, has only occurred in one study done by Richmond and colleagues to the author's knowledge. As a result, a thorough examination of Omegaven® at a higher dosage is recommended. Doing so would allow us to study potential complications and potential advantages, including for improved growth. A natural extension of this study would be to evaluate an equivalent dosage to that of current clinical practice utilizing fish oil-based lipid instead of soy-based lipid. This would help us evaluate the effect of an iso-caloric fish oil-based parenteral solution, additionally allowing us to evaluate Omegaven® and its potential role in an IFALD model.

Also mentioned in the limitations section, our study lacked several key factors that potentially reduce clinical applicability. Particularly, the lack of body composition, the use of a piglet with a complete GI tract, and the lack of enteral feeding could be rectified in an additional experiment. As our lab has already developed a short bowel piglet model with controlled enteral feedings (Heemskerk et al. 1999; Hua et al. 2013;

Turner et al. 2011), utilization of this model could allow us to work towards a greater understanding of the whole body effects that an n-3 lipid source has on infants. Particularly, our model has been designed to evaluate piglets with and without a functional ileum, allowing us to understand the effects of n-3's on hepatobiliary circulation and gastrointestinal involvement. At the end of this study, evaluation of body composition would provide key information to the nutritional status of these piglets. This study could also include the aforementioned clinical equivalency of 10 g/kg/d Omegaven®.

Additionally, it would be of value to further examine the role of sepsis and bacterial translocation in piglets on TPN, and in those provided some degree of enteral stimulation, in the aetiology of IFALD. Translocation studies have previously utilized the neonate (Atinmo et al. 1976; Van Camp, Tomaselli, and Coran. 1994), however this research is limited. It was noted that those infants who had undergone anastomosis procedures were more susceptible to translocation than those with an intact gastrointestinal tract. As bacterial translocation is suggested to be involved in the aetiology of IFALD development, understanding the degree of potential translocation and its effects on septic events, bile formation and hepatic dysfunction would provide clinicians with potential strategies to reduce the severity of IFALD in neonatal patients.

Finally this future study could be used to evaluate the effect of specific fatty acid enteral supplementation, not only examining the source (fish oil vs. algae, vs. plant based sources), but examining individual fatty acids, both n-3: ALA, EPA and DHA respectively, and n-6: LA and AA respectively, and their effect on hepatic function. Current research has focused on the lipid formulations available for clinical use (**Table**

2.1), however examining the influence of specific fatty acids delivered enterally, could allow for selective enteral treatment which could aid in IFALD reversal or prevention.

The second area that bears further study in IFALD research is the examination of altered lipid sources and their cognitive effects. As discussed previously, n-3's are preferentially taken up in the last part of gestation, particularly DHA. Cognitive effects of infants receiving lipid solutions that are not sufficient in DHA, or those that over supply DHA at the expense of AA should be examined in greater detail. Using the piglet model, a readily accessible indicator of central nervous system (CNS) development could be the retina. Assessment of retinal development and function could be used as a proxy for assessment of cognitive development, which would prove more challenging in piglets. This measurement, being repeatable, allows researchers to assess the CNS development over time. It is also possible to modify cognitive testing that occurs in the mouse and rat models to assess how the piglets respond based on several different lipid profiles. A more advanced cognitive test such as the rotor test could be utilized. This test involves placing the animal on an axel large enough for them to stand on, which is then rotated at an increasing speed. Cognitive function is measured by timing how long it takes and at which speed the subject falls off the axel. This test could be utilized in piglets very early in the study period and could be a repeated measure, without acclimatization to the test (which would skew the results).

Another potential trial that could be conducted is a reversal study. Within this study, IFALD is induced in our piglets. However, IFALD is not the endpoint. Once we have reached our established IFALD parameters (elevated bilirubin and liver function tests), the goal of this study would be, utilizing current clinical treatments, to attempt to

reduce the severity or even reverse IFALD. As a result, we would be able to incorporate and study many second and third generation parenteral lipids that are available on a compassionate care basis in Canada, as well as diverse lipid dosing strategies. The study endpoint would be at or below established liver function test results, and/ or a set period of time after the reversal protocol was introduced, whichever comes first. One limitation that must be considered for this proposed study is the growth rate of piglets and the rate at which IFALD is developed. As we have seen in our study, a 14-day trial can be extrapolated to approximately 10 weeks in a neonate, however this may not be enough time to induce IFALD and recover hepatic function using an alternative lipid. As such, it may be necessary to examine other models including mini-pigs who do not display the growth rates that are found in our piglets.

One final trial that could be performed is the examination of both Vitamin E and phytosterols and their influence in the development of IFALD. To reduce the amount of confounders present in this study (as it is difficult to eliminate both vitamin e and phytosterols from a parenteral lipid), examination of Vitamin E and Phytosterols simultaneously in a Latin square design (**Figure 3.1**) would allow you to test the influence varying dosages have on hepatic function. Suggestion of a Latin square design stems from the observation in literature that most studies examine either vitamin E dosage (Skouroliakou et al. 2010b; Koletzko and Goulet. 2010) or phytosterol content (Clayton, Whitfield, and Iyer. 1998; El Kasmi et al. 2013; Kurvinen et al. 2011). However, to the author's knowledge, a simultaneous examination of both these properties and their effects has yet to be performed. And since only one parenteral lipid solution

provides only Vitamin E, (Omegaven®), examination of simultaneous effects would strengthen clinical applicability.

Experimental design uses treatment 1 of Vitamin E with levels ranging from 0 to 40 mg/kg/d, which reflects the maximum dosage that is found in literature to date, and treatment 2 of phytosterols, ranging from 0 to 200 mg/kg/d also reflecting current literature dosages. The strength of this design is that you not only test each vitamin E and phytosterols independently, but you also test them simultaneously, at varying dosages, up to the maximum of both (experienced by group D in this model).

Figure 3.1: Latin Square Experimental Design

Phytosterol dosage (mg/kg/d) \ Vitamin E dosage (mg/kg/d)	0	10	20	30	40
0	A 0/0	B 10/0	C 20/0	D 30/0	E 40/0
50	B 0/50	C 10/50	D 20/50	E 30/50	A 40/50
100	C 0/100	D 10/100	E 20/100	A 30/100	B 40/100
150	D 0/150	E 10/150	A 20/150	B 30/150	C 40/150
200	E 0/200	A 10/200	B 20/200	C 30/200	D 40/200

Different treatments, Vitamin E and Phytosterols are laid out in a Latin square design with different dosages and groups of assessment (A-E). Dosage combinations are noted for each experimental treatment.

In this trial in particular, it is important to measure oxidative stress, cytokine production and liver function tests over the entire study to evaluate hepatic effects. In order to effectively test just Vitamin E and phytosterol content, this study would have to contain a base lipid solution comprised of individual fatty acids thus minimizing the contribution of both vitamin E and phytosterols in the base solution.

In summary, this study showed that dose restriction does reduce some of the clinical signs of IFALD; alteration of the fatty acid profile does influence brain fatty acid profiles; however, neither dose restriction, nor altered fatty acid profiles, completely normalized all aspects of the liver disease and growth may be compromised. Continued knowledge gaps include the contribution of Vitamin E and or phytosterol content, and the long-term effects of alteration of parenteral lipid on growth and cognitive development. Accordingly, more research in this field is essential, particularly, moving toward RCT's in pediatric populations and mechanistic research with attention paid to many of the confounders present in today's literature. To this end, continued research in this field is not only important to our understanding of IFALD but also essential when working towards a parenteral lipid that provides optimal growth and nutritive profiles.

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