PARENTERAL BETAINE AS A STRATEGY TO PREVENT FATTY LIVER AND IMPROVE DOCOSAHEXAENOIC ACID AND ARACHIDONIC ACID DISTRIBUTION IN PARENTERALLY FED NEONATAL PIGLETS

By

Gagandeep Kaur

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Department of Biochemistry

Faculty of Science

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Abstract

Parenterally fed infants carry the risk of developing fatty liver. Hepatic fat is removed by very low density lipoprotein (VLDL) secretion, which requires sufficient phosphatidylcholine (PC) synthesis. PC synthesis depends on choline (CDP-choline pathway) or methionine (PEMT pathway). The PEMT pathway is more important in infants because its PC is rich in DHA and AA, which are used for brain development. Methionine supplementation may enhance PC synthesis but can cause hyperhomocysteinemia. However, betaine could enhance PC synthesis by sparing choline as well as by converting homocysteine to methionine. The objective of this thesis was to assess the effects of parenteral betaine, methionine or its combination on fatty liver and brain DHA/AA. The hepatic lipid parameters were unaltered, but betaine supplementation improved DHA, AA and saturated fatty acids incorporation in brain phospholipids, suggesting that betaine can be used as a novel parenteral nutrition ingredient to improve brain DHA and AA status.

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^{3}H	Tritium
5-MTHF	5-methyltetrahydrofolate
AA	Arachidonic acid
ACSL	Long-chain fatty acid CoA synthase
ADP	Adenosine diphosphate
ALA	Alpha-linolenic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BHMT	Betaine-homocysteine methyltransferase
CCK	Cholecystokinin
CDP	Cytidine diphosphate
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DMG	Dimethyl glycine
DPA	Docosapentaenoic acid
Ex	Excess
EFA	Essential fatty acid
EFAD	Essential fatty acid deficiency
EPA	Eicosapentaenoic acid
FAD	Fatty acid desaturase

FAO	Food and Agriculture Organization
FABP	Fatty acid binding protein
FAT/CD 36	Fatty acid translocase/cluster of differentiation 36
FATP	Fatty acid transport protein
FDA	Food and Drug Administration
GAA	Guanidinoacetate
GC	Gas chromatography
GIT	Gastrointestinal tract
IV	Intravenous
LA	Linoleic acid
LCPUFA	Long chain polyunsaturated fatty acid
LDLr	Low density lipoprotein receptor
Lyso-PC	Lysophosphatidylcholine
Met	Methionine
Mfsd2a	Major facilitator superfamily domain containing protein 2
MAT	Methionine adenosyl transferase
MCT	Medium chain triglyceride
MUFA	Monounsaturated fatty acid
n-3	Omega-3
n-6	Omega-6
NEC	Necrotizing enterocolitis
PA	Palmitic acid
PC	Phosphatidylcholine

PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine N-methyltransferase
PI	Phosphatidylinositol
PL	Phospholipid
PN	Parenteral nutrition
PNALD	Parenteral nutrition associated liver disease
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
SA	Stearic acid
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SFA	Saturated fatty acid
TAG	Triacylglycerol
THF	Tetrahydrofolate
TLC	Thin layer chromatography
TPN	Total parenteral nutrition
UNU	United Nations University
VLDL	Very low density lipoprotein
VLDLr	Very low density lipoprotein receptor
WHO	World Health Organization

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1. Introduction

1.1 Total Parenteral Nutrition

Total parenteral nutrition (TPN) is a lifesaving therapy for patients with intestinal failure, in whom sufficient absorption of nutrients via the enteral route is intolerable (Fell et al., 2015). TPN is the intravenous infusion of nutrients to meet complete nutritional demands. The major goal of TPN is the maintenance of body hydration, electrolyte balance and promotion of growth and neurodevelopment without posing any risks (Calkins et al., 2014). It is a beneficial therapy especially for premature infants who cannot tolerate optimal amounts of enteral nutrition, thus limiting their survival. In Canada, 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, 2003). In the initial few weeks of postnatal life, preterm infants often require TPN support to meet their nutritional demands for rapid growth and development (Goulet & Ruemmele, 2006).

Early parenteral nutrition (PN) was a mixture of glucose and saline solutions (Geyer, 1960; Vinnars & Wilmore, 2003). To meet the nitrogen requirement, whole protein was added to PN formulations (reviewed by Shamsuddin, 2003; reviewed by Vinnars & Wilmore, 2003). In the late 1930's, the next breakthrough was the use of parenterally infused hydrolyzed proteins (Knochel, 1985; reviewed by Vinnars & Wilmore, 2003). In the 1960s, PN formulations to meet the nutritional demands for intravenous administration of carbohydrates, amino acids, electrolytes, and minerals were designed (reviewed by Fell et al., 2015). However, it was later found that long-term PN led to essential fatty acid deficiency, which was characterized by impaired growth, delayed development, skin rash

and renal and pulmonary abnormalities (Fell et al., 2015). Thus, to meet the requirement of energy as well as essential fatty acids, lipids were eventually added to the PN formulations.

1.1.1 Composition of Total Parenteral Nutrition

1.1.1.1 Proteins

Amino acid mixtures for PN solutions that are commercially available provide all essential amino acids, the total of which varies in amounts ranging between 38-57% of total amino acids (Yarandi et al., 2011). Among the non-essential amino acids, alanine, arginine, proline, glycine, serine, and tyrosine are commonly included, while others differ from manufacturer to manufacturer (Yarandi et al., 2011). Recent studies have shown that restriction of any amino acid lower than age-specific recommendations can alter protein synthesis (Brunton et al., 2000). Cysteine, a sulfur-containing amino acid, is conditionally essential in preterm infants since the liver cannot efficiently convert methionine to cysteine (Riedijk et al., 2007). However, because of its instability in solution, it easily gets oxidized to cystine which is insoluble (Yarandi et al., 2011). To correct for this, increased amounts of the more stable sulfur amino acid, methionine are added to PN formulations. However, some studies have suggested that methionine can be potentially hepatotoxic (Moss et al., 1999), so caution is warranted.

1.1.1.2 Carbohydrates

Carbohydrates form an important part of the PN, having the major role of providing energy (Bolder et al., 2009). When appropriately mixed with amino acids and lipids, carbohydrates can improve nitrogen balance (Bolder et al., 2009). Glucose solutions should be used as the standard carbohydrate in PN formulations accounting for nearly 60% of non-protein energy.

1.1.1.3 Lipids

Lipids are an important source of energy-dense non-protein calories as well as essential fatty acids (linoleic acid and alpha-linolenic acid) (Raman et al., 2017). They also help to maintain the integrity of the cell membrane, are secondary messengers in cell signaling, and serve as precursors to inflammatory mediators like eicosanoids and prostanoids. Recommended fat doses for parenterally fed infants vary from 2.5-3 g/kg/day (Fell et al., 2015). PN devoid of lipids can lead to essential fatty acid deficiency (EFAD), especially in preterm infants who have limited fat stores (Mayhew & Gonzalez, 2003) and an increased requirement of EFA. Incorporation of omega (n)-3 and n-6 polyunsaturated fatty acids (PUFA) in an appropriate ratio helps avoid EFAD. Currently, Intralipid, with a (n-6) to (n-3) ratio of 7:1, is the most common lipid emulsion in use in TPN. However, newer lipid emulsions with different fatty acid compositions like Omegaven, SMOFlipid, and ClinOleic are also commercially available and have been approved for use in Europe (Burrin et al., 2014). Because TPN lipid emulsions use soy lecithin as an emulsifier, lipid solutions are rich in PC.

1.1.2 Complications of Total Parenteral Nutrition

Though life-sustaining, TPN comes with its own set of complications. Some of these are short-lived and allow recovery of the patient, while some are highly risky and associated with high rates morbidity and mortality (Fell et al., 2015). Some causes of fatalities include disruption of the gut mucosal architecture (Seres et al., 2013), compromised gut function, excess inflammation, and sepsis. Preterm infants are more susceptible to the deleterious effects of long-term PN than adults, mainly because of their immature gastrointestinal tract (Goulet & Ruemmele, 2006). Also, catheter-related sepsis,

venous thrombosis and liver abnormalities frequently occur in neonatal populations receiving long-term PN. One of the major complications associated with PN is parenteral nutrition-associated liver disease (PNALD). It remains one of the most severe complications leading to early mortality in neonates.

1.2 Parenteral Nutrition-Associated Liver Disease

PNALD is an established complication with high incidences of mortality and morbidity in the pediatric population (Calkins et al., 2014). The prevalence varies with age group, with a high rate of 40—60% of the cases in infants and neonates (Kelly, 2006). In adults on long-term PN, however, the rate is between 15-40% (Kelly, 2006); the reason for this variation between the two populations is unknown (Bharadwaj et al., 2015) A broad clinical spectrum of hepatobiliary abnormalities are associated with PNALD: steatosis, cholestasis, fibrosis, cirrhosis and liver injury (Drongowski & Coran, 1989; Kelly, 2006) with hyperbilirubinemia emerging first.

1.2.1 Pathology of PNALD

The actual pathogenesis of PNALD is still obscure (Wang et al., 2006; Xu & Li, 2012). Liver dysfunction associated with PNALD commonly includes steatosis, cholestasis and fibrosis or cirrhosis, as previously mentioned. Steatosis is the accumulation of fatty acids in the form of triacylglycerol in the hepatocytes (Vilgrain et al., 2013). Cholestasis may be defined in two different ways. Pathologically, it is the impairment of bile flow or bile obstruction caused either intra- or extrahepatically (Feldman & Sokol, 2013). Clinically, it is defined as elevated serum conjugated bilirubin (greater than 34 μmol/L) (Alkharfy et al., 2014). Cholestasis can further progress to fibrosis and then to cirrhosis

over the course of time (Nandivada et al., 2015). Both cholestasis and steatosis can be reversed with the termination of PN (Bharadwaj et al., 2015) . Additionally, children are more susceptible to the development of fibrosis whereas adults are more vulnerable to develop steatosis of the liver (Naini & Lassman, 2012; Zambrano et al., 2004).

1.2.2 Etiology of PNALD

The development of PNALD is multifactorial, having both patient-related as well as PN-related factors. Patient-related causes include prematurity, gastrointestinal abnormalities, and infections (Calkins et al., 2014). The non-patient-related or PN solution-related risk factors include the duration of PN and its composition.

Prematurity is a crucial risk factor for PNALD. Additionally, small size and low birth weight make neonates more vulnerable to the development of PNALD and put them at a greater risk when compared to normal birth weight infants (Javid et al., 2011; Zambrano et al., 2004). A number of studies have shown that small for gestational age infants, on a shorter PN exposure, are more likely to develop PN-associated cholestasis (Lee et al., 2013; Robinson & Ehrenkranz, 2008). Preterm infants are more prone to PNALD mainly because of their immature hepatobiliary system (Duro et al., 2008). Intestinal surgery is another cited risk factor for PNALD in infants (Javid et al., 2011). In infants, intestinal failure is mostly due to necrotizing enterocolitis (NEC) (Duro et al., 2011). However, depending on the severity of NEC, resection of the small bowel may be necessary which amplifies the dependence on PN (Gude, 2013). Therefore, the direct link between surgery, NEC, and PNALD may seem overestimated as infants with surgical complications are more likely to require long-term PN support.

Lack of enteral feeding is thought to be another important driver of PNALD (Calkins et al., 2014). Food intake not only stimulates bile acid secretion but also important gastrointestinal hormones like cholecystokinin (CCK). Deficiency of CCK may impede gall bladder contractility and disrupt the enterohepatic circulation (Guglielmi et al., 2008). Moreover, lack of enteral feeding leads to gastrointestinal tract (GIT) atrophy, which further increases the potential for sepsis (Duro et al., 2008). Therefore, trophic enteral feeding may be seen as a beneficial strategy in parenterally fed infants to increase the enteral feeding tolerance and reduce the adverse effects associated with PN (Ben, 2008).

Trace elements such as copper and manganese are considered to be hepatotoxic (Jin et al., 2017). Both copper and manganese are excreted in the bile (>80%). A study by Blaszyk et al. (2005), showed that patients with significant cholestasis had higher amounts of hepatic copper when compared to those with no or minimal cholestasis. Similarly, accumulation of manganese has been seen in PN patients with cholestasis as well as in patients with normal hepatic function (Jin et al., 2017). Thus, there is a possibility that both these trace elements can contribute to cholestasis. Also, studies exist to show that infants with PNALD have high aluminum levels, although the mechanism for this accumulation is not clearly understood (Alemmari et al., 2012). However, some studies suggest that aluminum may alter bile flow and cause hepatic damage by pro-oxidant effects (Alemmari et al., 2012). Most recently, in a study by Hall and colleagues (2016), it was found that aluminum contamination in the PN of infants was thrice the recommended maximum approved by FDA (Hall et al., 2016). However, further investigation with respect to the concentration of these trace elements in tissues is required, mainly because of the heterogeneity in the PN patient population.

Recent research has shown that the longer the exposure to PN, the higher is the risk of developing PNALD. Based on multivariate analysis, two studies concluded that the incidence of cholestasis associated with PN was significantly related to the duration of PN (Beale et al., 1979; Pereira et al., 1981). In another study by Christensen et al. (2007), it was shown that infants on PN for 14-28 days had a lower incidence of cholestasis whereas those who underwent PN treatment for >100 days had the highest rate of 86%. Indeed, the occurrence of PNALD is nearly 50% in infants on PN for a minimum of sixty days (Burrin et al., 2014).

1.3 PNALD and lipid emulsions

Intravenous lipid emulsions came into existence in the early 1960s with soybean oil being the first commercially available lipid emulsion (Hojsak et al., 2016). Intralipid was the first FDA-approved lipid emulsion clinically recommended for PN (Fell et al., 2015). However, a number of studies have linked soybean oil with PNALD in infants (Bindl et al., 2000; Iyer et al., 1998; reviewed by Rangel et al., 2012). The drawbacks of soybean oil-based lipid emulsion revolve around the high content of n-6 PUFA and phytosterols. The n-6 PUFA in these lipid emulsions target the immune system, activating NF-kB pathway and enhancing cytokine generation and subsequently resulting in cholestasis (Clayton et al., 1993; Dichtl et al., 2002; Park et al., 2001; Van Aerde et al., 1999). Intralipid has also been associated with lipid peroxidation (Bharadwaj et al., 2015). Consequently, new-generation lipid emulsions composed of fish oil alone (Omegaven), as well as SMOFlipid, a blend with soybean oil, olive oil, fish oil, and medium chain triglycerides (MCTs), have gained popularity and shown positive outcomes in infants with PNALD (Burrin et al., 2014; Premkumar et al., 2014).

SMOFlipid is one such new generation intravenous fish oil based lipid emulsion with a high content of n-3 PUFA, especially EPA and DHA from fish oil (15%), along with soybean oil (30%), MCTs (30%), and olive oil (25%). The n-3 PUFA have anti-inflammatory potential and are important structural components of membrane phospholipids that are crucial for infant growth and development. Contrary to the conventional soybean oil lipid emulsion, SMOFlipid has a higher amount of the antioxidant α-tocopherol that prevents lipid peroxidation of the fatty acids (Deshpande et al., 2014; Ng et al., 2017). Recent studies have reported the beneficial effects of SMOFlipid in cholestatic infants (Rajagopal et al., 2016; Tomsits et al., 2010). SMOFlipid has also been associated with the reversal and prevention of liver abnormalities (Tomsits et al., 2010). Having a balanced blend of fatty acids, SMOFlipid has a great potential for improving the health outcomes of infants on PN.

1.4 Methionine

Methionine is an essential amino acid critical to the growth and development of neonates. Besides its major role in protein synthesis, it caters to the methyl demands of over 200 transmethylation reactions through S-adenosyl methionine (Petrossian & Clarke, 2011). The methionine cycle involves the transfer of the terminal methyl group of methionine (transmethylation) forming various methylated products (Figure 1.1) (Robinson & Bertolo, 2016). First, there is an irreversible conversion of methionine to S-adenosyl methionine (SAM) through methionine adenosyl transferase (MAT). SAM is a universal methyl donor, donating methyl groups for synthesis of many methylated metabolites including phosphatidylcholine (via phosphatidylethanolamine

methyltransferase) and creatine through methylation of guanidinoacetate, the two most abundant methylated products. S-adenosyl homocysteine (SAH), formed as a result of methyl group removal from SAM is converted to homocysteine by losing an adenosine molecule in a reaction catalyzed by SAH hydrolase. Homocysteine can be remethylated back to methionine through methionine synthase, or alternatively through the enzyme betaine homocysteine methyltransferase (BHMT) in the liver; 5-methyltetrahydrofolate and betaine (obtained by the oxidation of choline) act as the respective methyl donors in these remethylation pathways. The effects of betaine on increasing methionine availability have been studied in a few animal models, although its quantitative contribution to whole body methionine requirement is still unclear. Besides transmethylation and protein synthesis, methionine also acts as a precursor for the amino acid cysteine via transsulfuration of homocysteine. Both methionine and cysteine are sulfur-containing amino acids and the amount of dietary cysteine is known to influence the dietary requirement of methionine (Shoveller et al., 2003a; Shoveller et al., 2003b). For example, addition of cysteine to the diet will spare the methionine requirement since methionine is no longer needed for that pathway. In the same way, we hypothesize that addition of methylated products (i.e., creatine) might spare methionine from being used for its synthesis (Robinson & Bertolo, 2016). Moreover, addition of methyl donors could also change the dietary methionine requirement by increasing remethylation of homocysteine and making methionine more available. These concepts warrant further investigation into the methionine requirement in neonates.

1.4.1 Transmethylation reactions

Methionine demands for transmethylation are significantly higher in the initial weeks of early life (Robinson & Bertolo, 2016). The first step of transmethylation is the adenosylation of methionine to form SAM, which utilizes a significant proportion of this indispensable amino acid (~50%) in 2-week-old piglet (McBreairty et al., 2016). The major transmethylation products synthesized by SAM are PC, creatine, methylated DNA and proteins (Robinson et al., 2016). A substantial proportion of the labile methyl groups (~70%) is utilized in creatine synthesis (Brosnan et al., 2009), although the partitioning among transmethylation products is quite dynamic. A recent study from our laboratory reported that piglets fed with guanidinoacetate, the precursor for creatine, profoundly altered the methionine partitioning among transmethylation products (McBreairty et al., 2015). Also, because PE requires three SAM molecules to form PC, a change in PC synthesis can profoundly change the amount of methionine diverted to this pathway. Methionine deficiency or excess can also influence its partitioning across the transmethylation pathways. However, further research is needed to quantify the dependency of the methylation products on methionine availability.

1.4.2 Remethylation

As previously discussed, SAM is converted to SAH, which on hydrolysis forms homocysteine. Homocysteine can undergo two fates - remethylation to methionine or irreversible transsulfuration to form cysteine. Remethylation to methionine occurs either by accepting a methyl group from betaine via BHMT in liver or kidney or from 5-methyltetrahydrofolate via methionine synthase in a variety of tissues. A recent study confirmed that both betaine and folate have equal potential to form methionine for protein

synthesis (McBreairty et al., 2016). Moreover, we estimated that in a neonatal piglet, remethylation via these methyl donors contributes to nearly 20% of the whole body methionine flux (Robinson et al., 2016).

In addition to removal of homocysteine via transsulfuration (and synthesis of cysteine), remethylation is also an important route of homocysteine disposal. Elevated plasma homocysteine has been closely related to the development of cardiovascular diseases (Kumar et al., 2017) and more recently to impaired cognition (de Jager, 2014). Methyl donors, namely betaine, choline, and folate, have proved to be beneficial in alleviating hyperhomocysteinemia, although their supplementation has not guaranteed the elimination of the disease (Jadavji, 2014; Robinson & Bertolo, 2016).

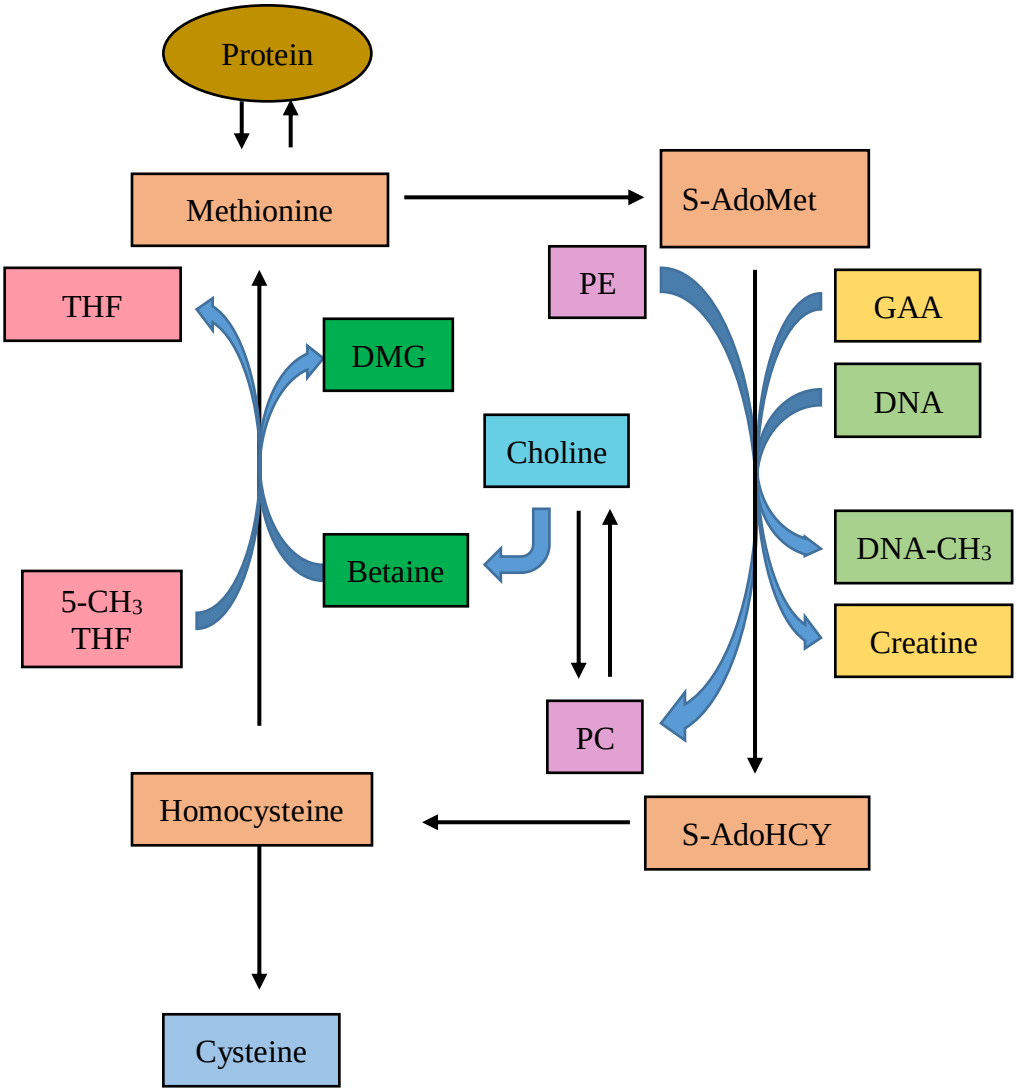

Figure 1.1: A schematic representation of the methionine cycle highlighting relevant metabolites.

5-CH_3 THF, 5-methyltetrahydrofolate; THF, tetrahydrofolate; S-AdoMet, S-adenosyl methionine; S-AdoHCY, S-adenosyl homocysteine; GAA, guanidinoacetate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DMG, dimethylglycine

1.5 Betaine

Betaine is a modified amino acid (N,N,N trimethyl glycine), produced in vivo by the irreversible oxidation of choline via choline dehydrogenase in a two-step process (Ejaz et al., 2016; Robinson & Bertolo, 2016; Wang et al., 2013). First, there is a conversion of choline to betaine aldehyde in a reaction catalyzed by choline dehydrogenase. This is followed by conversion of betaine aldehyde to form betaine (Craig, 2004). Besides de novo synthesis in the mitochondria of liver and kidneys, betaine occurs naturally in spinach, beets, whole grains and shrimps (Zhao et al., 2018).

Physiologically, the major role of betaine is as an osmolyte, primarily within the cells where it protects the cells from osmotic stress (Craig, 2004; Zhao et al., 2018). Moreover, betaine is an essential methyl donor in the remethylation of homocysteine to form methionine. This reaction is catalyzed by betaine-homocysteine methyltransferase, occurring primarily in the liver and kidneys (Zhao et al., 2018). Recently, betaine supplementation has proved to be effective against many diseases (Craig, 2004; Robinson & Bertolo, 2016). Betaine supplementation is a promising agent in the attenuation of alcoholic and non-alcoholic fatty liver disease (Deminice et al., 2015) and betaine consumption has been correlated with the prevention of hepatic steatosis (Craig, 2004; Wang et al., 2014a). Earlier reports on betaine ingestion have shown that betaine consumption decreased triacylglycerol accumulation in animals fed a high fat diet (Kawakami et al., 2012; Kwon et al., 2009; Wang et al., 2010). The lipotropic effects of betaine are attributed to its methyl donor capacity (Craig, 2004). Recent studies have reported that betaine consumption in animal models of fatty liver disease led to increased availability of SAM (Deminice et al., 2015). Moreover, piglets born to sows exposed to

betaine in the diet have increased serum methionine as well as hepatic betaine and SAM levels (Cai et al., 2014). Similarly, betaine supplementation in human subjects have led to increased methionine concentrations (Cai et al., 2014).

SAM availability regulates the transmethylation reactions in the methionine cycle, particularly the formation of PC from PE, which utilizes SAM as a methyl donor in three sequential reactions. Additionally, the study by Deminice et al. (2015) observed elevated hepatic PC concentrations, as well as PC/PE ratio, in rats fed a high fat diet supplemented with betaine, although PEMT gene expression remained unchanged. Enhanced PC is associated with increased transport of hepatic fat via very low density lipoprotein (VLDL) secretion (Kharbanda et al., 2009), thus alleviating fatty liver. Because PNALD is associated with hepatic fat accumulation, we hypothesized that betaine supplementation might also have a potential role in hepatoprotection during PN feeding.

In addition to its role in hepatic lipid metabolism, betaine also affects muscle lipid metabolism. Effects of betaine supplementation on cholesterol metabolism in muscle are varied and are influenced by animal and dietary factors (Li et al., 2017). Betaine is known to upregulate the expression of genes involved in lipogenesis and lipid transport in the muscle of pigs. For example, betaine supplementation has been shown to promote the uptake of free fatty acids in muscle by upregulating the expression of certain transporters, namely, fatty acid translocase/cluster of differentiation 36 (FAT/CD36), fatty acid transporter 1 (FAT1) and fatty acid binding protein 3 (FABP3) (Li et al., 2017). Furthermore, betaine supplementation also increases fatty acid oxidation in muscle by altering peroxisome proliferator-activated receptor (PPAR) alpha and its target gene carnitine palmitoyltransferase 1 (CPT1) through activation of AMP-activated protein

kinase (AMPK). Similar observations with betaine exposure have been reported in rat liver which show that betaine plays an important role in mitochondrial β-oxidation (Li et al., 2017). Dietary betaine is also reported to significantly affect unsaturated and saturated fatty acids in pigs. Betaine supplementation increased the amount of C16:0 (Yang et al., 2009) and C18:0 fatty acids, but decreased the amount of C18:2 (Hur et al., 2007) in the muscle of pigs; these changes improve the meat quality for commercial purposes. However, the impact of betaine supplementation on whole body fatty acid metabolism is still unclear.

Betaine has been beneficial to patients with homocystinuria (Jadavji, 2014). A few studies have reported that betaine supplementation can alleviate plasma homocysteine levels (Engelbrecht et al., 1997; Schwahn et al., 2004; Tawari et al., 2002), although results are variable (Holme et al., 1989; Strauss et al., 2007). Maternal betaine supplementation is known to reduce brain homocysteine levels in the Mthfr $^{-/-}$ offspring (Schwahn et al., 2004). Moreover, betaine concentrations, as well as BHMT levels in the brain, are normally low and thus metabolism of homocysteine by betaine is not efficient (Jadavji, 2014). Also, betaine is associated with improved brain structure and myelination in homocystinuric individuals which is a result of lowering plasma homocysteine and elevating plasma methionine along with SAM levels in cerebrospinal fluid (Engelbrecht et al., 1997). Additionally, a randomized controlled trial in Dutch individuals, positively associated plasma betaine levels with improved memory, sensimotor speed and executive function (Eussen et al., 2007) while the reverse was true for homocysteine. This effect of betaine on cognition was thought to be a result of increased choline availability. The role of choline in neurological development is well established and discussed below. However, no study,

to our knowledge, has studied the effect of betaine supplementation on fatty acid composition in the brain. There is some evidence suggesting that betaine supplementation in high fat fed dams upregulates the expression of fatty acid binding protein 7 (FABP7) and fatty acid binding protein 5 (FABP5) in the fetal brains (Joselit et al., 2017). FABP7 and FABP5 have both been associated with the transport of DHA, while FABP 5 is also a known carrier for saturated fatty acids (Chouinard-Watkins et al., 2018; Joselit et al., 2017). Saturated fatty acids are important for myelin synthesis during early development, while DHA is critical to both visual and cognitive development (Calder, 2016; Edmond et al., 1998). These data suggest that betaine supplementation might affect the fatty acid profile in the brain, either by affecting fatty acid transport, or by sparing choline and by enhancing methionine availability, thereby facilitating biosynthesis of phosphatidylcholine. However, no study has been conducted investigating the effects of parenteral betaine supplementation in brain fatty acid metabolism.

Despite the beneficial effects of betaine in human metabolic diseases, the dietary betaine requirement is still unclear. An estimated daily betaine requirement of 131 mg is based on a Western diet (Ross et al., 2014). For infants, the betaine content of breast milk depends on maternal dietary intake while infant formulas contain betaine well within the range of breast milk content. Betaine intakes in neonates are variable and thus the specific requirement is unknown. In spite of high variability, infant intakes of betaine are well below levels used in supplements (3-6 g betaine/d) (Robinson & Bertolo, 2016). However, with the increasing evidence of the positive effects of betaine in early life as well as adulthood, it is important to understand how variable intakes of this nutrient affect the metabolites of one-carbon metabolism.

1.6 Overview of Phosphatidylcholine

Originally called lecithin, phosphatidylcholine (PC) was first identified as a constituent of egg yolk in 1847 (Cole et al., 2012). Structurally, PC consists of two fatty acids esterified to a glycerol backbone. The third hydroxyl group is linked to a choline molecule via a phosphodiester linkage (Cole et al., 2012). Typically, liver PC has a saturated fatty acyl chain at the sn-1 position and a PUFA at the sn-2 position (Yamashita et al., 1997). PC is the most abundant phospholipid of all classes of lipoproteins in mammals (Noga & Vance, 2003; Watkins et al., 2003). Physiologically, it is required for the secretion of bile and VLDL (Yao & Vance, 1988; 1989) and also acts as a lung surfactant (Pérez-Gil, 2008).

1.6.1 PC biosynthesis

PC synthesis occurs in the liver by two different pathways (Figure 1.2). The primary pathway is the CDP choline, or Kennedy, pathway, accounting for up to 70% of the hepatic PC synthesis and the secondary pathway is the PEMT pathway, accounting for the remaining 30-40% of the total PC in hepatocytes (Pynn et al., 2011; Watkins et al., 2003). Synthesis of PC via the CDP choline pathway depends on dietary choline supply, unlike the PEMT pathway which only requires the substrates, PE and SAM (Vance et al., 1997).

Choline is indispensable for PC synthesis as well as for its multitude of functions in conditions including but not limited to fatty liver, cognitive health, and atherosclerosis (Li & Vance, 2008). Inhibition of enzymes in the CDP-choline pathway has been associated with certain abnormalities in rodent models (Vance et al., 2007). Apart from exogenous intake of choline, the PEMT catalyzed synthesis of PC is the endogenous source of choline,

which makes transmethylation for de novo synthesis important for the supply of not only PC, but also of choline for the body.

In the PEMT pathway of PC synthesis, PC is formed from PE by three sequential methylation reactions in which S-adenosyl methionine is the methyl donor. The PEMT enzyme is predominantly present in the liver where it significantly contributes to the PC pool. The PEMT pathway thus becomes a critical source of PC synthesis when choline in the diet is limiting. The vital role of the PEMT pathway has been demonstrated through PEMT knockout mice studies. PEMT knockout mice with sufficient dietary choline sustained normal liver function and PC/PE levels. However, when the diet was limiting in choline the knockout mice were presented with liver failure within 3 days along with PC levels being reduced to half the original (Vance et al., 2007). This demonstrates that the PEMT pathway is essential to rescue choline in case of choline restriction. Similarly, PEMT elimination has been associated with hepatosteatosis and reduced hepatic PC content, in the presence of recommended choline ingestion (Robinson & Bertolo, 2016). These findings demonstrate that both of these pathways work in harmonization to ensure adequate PC levels for normal hepatic function (Robinson & Bertolo, 2016).

Choline deficiency is observed in pregnant and lactating women, but otherwise rarely manifests in humans since dietary choline intakes are often adequate (Li & Vance, 2008). However, a study showed that patients with non-alcoholic steatohepatitis and brain impairment exhibited signs characteristic of choline deficiency, despite normal levels of choline consumption (Abdelmalek et al., 2001; Agut & Ortiz, 1991). As previously discussed, the oxidation product of choline, betaine, has proved to be a promising agent in the treatment of non-alcoholic steatohepatitis via generation of PC molecules, probably

through the PEMT pathway (Abdelmalek et al., 2001; Li & Vance, 2008), but also possibly by sparing choline for the CDP choline pathway. Yet, it is still unknown if the methyl group supply, either provided directly by methionine or through remethylation by betaine, is adequate to meet the demands of the PEMT pathway.

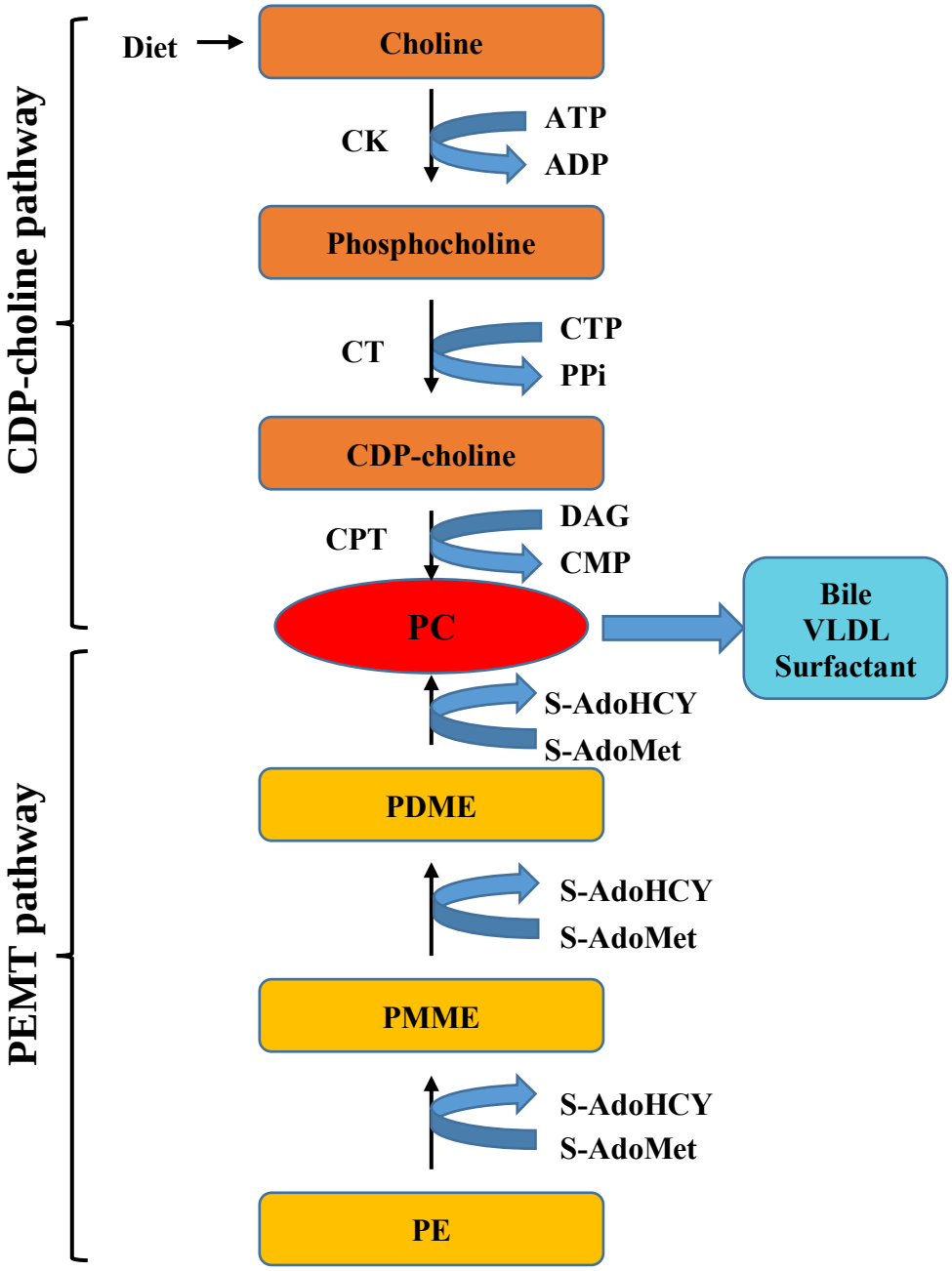

Figure 1.2: Biosynthetic pathways of phosphatidylcholine synthesis (Adapted from: Cole et al., 2012).

CK, choline kinase; CT, CTP:phosphocholine cytidylyltransferase; CTP, cytidine triphosphate; CPT, CDP-choline: 1,2-diacylglycerol:cholinephosphotransferase: ATP,

adenosine triphosphate; ADP, adenosine diphosphate; DAG, diacylglycerol; CMP, cytidine monophosphate; PDME, phosphatidyldimethylethanolamine; PMME, phosphatidylmonomethylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT phosphatidylethanolamine N-methyltransferase; S-AdoMet, S-adenosyl methionine; S-AdoHCY, S-adenosyl homocysteine

1.6.2 PC and VLDL secretion

Lipids are hydrophobic molecules which cannot circulate freely in the plasma to reach the target tissues. This limitation is overcome by carrier molecules called lipoproteins which have a hydrophobic core of TG and cholesteryl esters surrounded by a monolayer of PL and cholesterol. In addition, specific apolipoproteins are attached to lipoproteins which bind to specific cell receptors and play an important role in the targeted transport of lipids. Depending on their density, these particles are classified as chylomicrons, VLDL, intermediate density lipoproteins, low density lipoproteins and high density lipoproteins.

Phosphatidylcholine is the most abundant PL involved in VLDL assembly and secretion, constituting 60-80% of the phospholipid on its surface (van der Veen et al., 2017). Thus, it is not surprising that PC synthesis and VLDL secretion share a strong connection. Both pathways of de novo PC synthesis are equally and independently important in VLDL secretion. Experiments in rodents on a choline-deficient diet provide evidence of impaired VLDL secretion due to insufficient PC synthesis (Rinella & Green, 2004; Rizki et al., 2006; Z. Yao & Vance, 1990). Best and Huntsman were the first to demonstrate that choline intake lowered hepatic TAG accumulation (Best & Huntsman, 1932). Further studies showed that 3 days on a choline-deficient diet reduced hepatic PC concentration in addition to TAG accumulation, when compared to choline-supplemented

rats (Yao & Vance, 1988; 1990). Studies involving incubation of rat hepatocytes exhibited restricted PC synthesis through either pathway on VLDL particles when the medium was deficient in methionine and choline. When the medium lacked substrates for both pathways, PC, apolipoproteins, and TAG in the secreted VLDL were lower than when either of the substrates was present in the medium (Yao & Vance, 1988). However, if the cell culture medium included only methionine and no choline, then VLDL secretion was not reduced (Kulinski et al., 2004). Indeed, choline's uniqueness lies in the fact that it cannot be substituted for other methylamines in the absence of methionine for PC synthesis (Yao & Vance, 1989).

The role of either pathway in VLDL homeostasis was further clarified with experiments in knockout models. TAG and apolipoprotein secretions were inhibited in the hepatocytes of PEMT knockout mice, due to abnormal VLDL secretion (Noga & Vance, 2003; Noga et al., 2002). Similarly, mice lacking CTα, the rate-limiting enzyme in the ubiquitous CDP-choline pathway, presented with lower VLDL secretion along with reduced plasma TAG and PC than the wild-type mice (Jacobs et al., 2004; Jacobs et al., 2008). Indeed, PEMT activity was upregulated without counteracting for the loss of CTα activity. These findings show that PC biosynthesis through either pathway regulates VLDL secretion.

1.7 Overview of Polyunsaturated fatty acids

N-3 and n-6 fatty acids are essential long chain PUFA having a number of beneficial effects. The precursor of the n-3 family, alpha linolenic acid (ALA), is found in soy, canola and flax seeds, whereas linoleic acid (LA), the parent compound of the n-6 family, is

commonly found in vegetable oils like safflower and corn oil (Saini & Keum, 2018). However, the rates of conversion of these precursors to their metabolites, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for n-3, and arachidonic acid (AA) for n-6 family, is not very efficient and thus, these fatty acids rely on external sources to meet the body's requirements (Saini & Keum, 2018). Fish oil is a good source of PUFA, especially DHA and EPA. The most commonly occurring n-3 fatty acids, EPA and DHA, are precursors to active lipid mediators like resolvins and protectins which have been known to impart anti-inflammatory property to these fatty acids (Masterton et al., 2010). On the contrary, AA produces pro-inflammatory eicosanoids. Thus, a balanced proportion of n-3 and n-6 fatty acids in the diet is required to maintain homeostasis of inflammatory pathways (Swanson et al., 2012). The human brain is enriched with PUFA, particularly DHA and AA that are essential to normal brain development and function (Liu et al., 2015).

1.8 Brain fatty acid composition

The human brain is enriched with lipids, the majority of which are phospholipids and cholesterol (Hamilton & Brunaldi, 2007; Liu et al., 2015). The brain lipid composition is very stable with a distinctive fatty acid composition (Dyall, 2015; Hamilton & Brunaldi, 2007). High concentrations of DHA, AA, and SFA, particularly palmitate, are predominantly found in the brain (Dyall, 2015). SFAs, predominantly palmitic acid and stearic acid, constitute nearly 45% of the total brain fatty acids (Gimenez da Silva-Santi et al., 2018). PUFAs account for approximately 35% of the total brain fatty acids, with DHA and AA comprising 50% and 40% respectively, of the brain PUFA (Guest et al., 2013; Liu et al., 2015). EPA, another important (n-3) PUFA often associated with brain disorders,

undergoes rapid β-oxidation, resulting in low levels of 1% or even less of the total PUFA in the brain (Chen et al., 2013; Liu et al., 2015; Guest et al., 2013; Yalagala et al., 2018). The MUFAs make up roughly 25% of the total brain fatty acids, with oleic acid as the most abundant fatty acid (Gimenez da Silva-Santi et al., 2018). The distribution of fatty acids among phospholipids differs within the brain (Dyall, 2015; Liu et al., 2015). DHA is predominantly esterified to phosphatidylethanolamine whereas EPA, as well as AA, are mostly found in phosphatidylinositol (Dyall, 2015; O'Brien et al., 1964; Svennerholm, 1968). DHA and AA are the most important LCPUFA for brain growth and function and their levels in the brain depend on dietary intake as well as endogenous synthesis from precursor fatty acids.

1.8.1 Long chain polyunsaturated fatty acids (LCPUFA) in the brain

In the early phase of the post-natal period, the human brain undergoes a growth spurt, a stage of rapid DHA and AA accretion, vital to brain development (Smink, Gerrits, Gloaguen et al., 2012). DHA and AA have been positively associated with cognitive health, neuron development, and cell signaling (Garg et al., 2017; Innis, 2008; Lauritzen et al., 2016). Both of these fatty acids are involved in long term brain potentiation, a process important for memory build-up (Garg et al., 2017; Sanchez-Meija et al., 2008). In the brain, phosphoglycerides, DHA and AA mediate signal transduction (Garg et al., 2017). Additionally, AA plays a critical role in neuronal firing, maintaining the plasticity of hippocampal neuron membrane, shielding the brain from oxidative stress, and forming new proteins in the brain (Hadley et al., 2016). Overall, these two fatty acids are indispensable for brain growth and development (Garg et al., 2017; Hadley et al., 2016). DHA and AA depend on their nutritionally essential precursors, ALA and LA, respectively, for their

synthesis (Innis, 2008). However, the conversion efficiency of these precursors to form LCPUFA via a series of elongation and desaturation steps is quite low, ranging between 0.2-21% (Liu et al., 2015). Thus, preformed DHA and AA must be supplied exogenously to achieve adequate levels for optimal brain function.

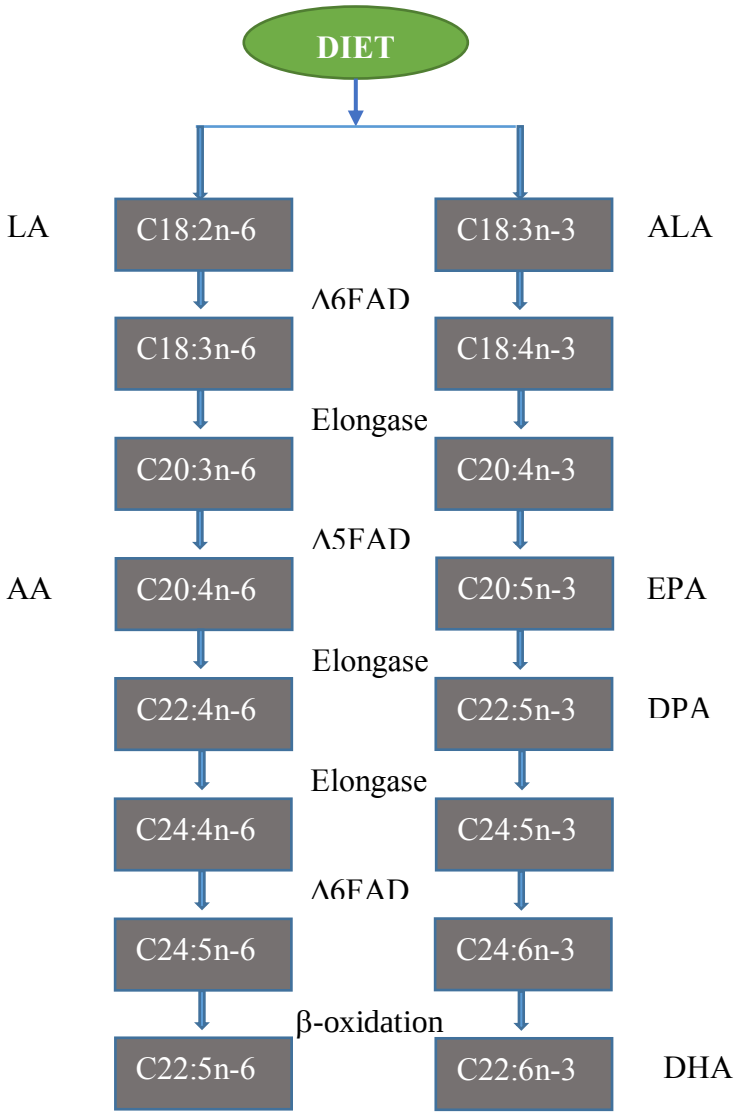

Figure 1.3: Synthesis of LCPUFA from essential fatty acids (Adapted from: Ibarguren et al., 2014)

LA, linoleic acid; ALA, alpha-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid, Δ6FAD, delta-6 fatty acid desaturase; Δ5FAD, delta-5 desaturase

Apart from endogenous production from ALA and LA, DHA and AA levels in neonates depend on the maternal intake of these fatty acids prepartum, as well as during lactation (Kabaran & Besler, 2015). However, because of variable intakes of DHA and AA in breastfeeding women across the globe, the mean intake of these LCPUFA is well below the recommended level (Huffman et al., 2011). As discussed previously, the accretion of DHA and AA in the brain is significantly increasing until 2 years postnatally (Hadley et al., 2016; Martinez, 1992), and the infant is vulnerable to essential fatty acid deficiencies during the growth spurt (Nyaradi et al., 2013). It is vital to ensure that the demands of DHA and AA for brain structural and functional development are adequately met by the diet.

In the brain, DHA and AA supply depend mainly on the plasma uptake as the endogenous synthesis within the brain is limited by the low and static levels of enzymes required for synthesizing these LCPUFA (Bazinet & Layé, 2014). However, the mechanism of delivery of LCPUFA to the brain is still unclear. There are two forms in which these fatty acids can be delivered to the brain: first, in their unesterified or free form bound to albumin, and second, esterified within a lysophospholipid associated with albumin or a lipoprotein. Thus, lipoproteins, lysophospholipids (particularly, lyso-PC), and free fatty acids are the major contributors to fatty acids entering the brain from the plasma pool. However, DHA in particular exists predominantly bound to PC and contributes immensely to the lipoprotein candidate pool in the plasma (Lacombe et al., 2018). The mechanism of entry into the brain and across the blood brain barrier (BBB) can be either through simple passive diffusion or with the aid of active transporters.

The BBB expresses lipoprotein receptors on the surface of the endothelium. DHA and other PUFAs in their esterified form bound to the circulating lipoproteins can interact

with the lipoprotein receptors followed by endocytosis within the endothelial cell membrane. However, evidence in receptor knock-out mice suggests that these lipoprotein receptors (LDLr and VLDLr) are not necessary for maintaining brain PUFA concentrations (Chouinard-Watkins et al., 2018). Nevertheless, their role in transporting the fatty acids to the brain is still important. The lipoprotein thus endocytosed is hydrolyzed by endothelial lipase to release the PUFA which can reach the neural membrane either passively by associating with FABP or actively by FATP (Lo Van et al., 2016). FABP-5 is expressed at highest concentration in the brain and its deficiency has been associated with the reduced influx of radiolabeled DHA in mice models (Lo Van et al., 2016). FABP-7 preferentially binds to DHA over other fatty acids and has been related to DHA transportation in fetal brains and neonatal brain development (Joselit et al., 2017; Lacombe et al., 2018).

Alternatively, lipoprotein lipase can act on the lipoprotein esterified with PUFA to produce the free fatty acid or lyso-PC (Bazinet & Layé, 2014; Lacombe et al., 2018). The lyso-PC PUFA is carried by a unique transporter Mfsd2a (major facilitator superfamily domain-containing protein-2) which is expressed in the endothelium (Nguyen et al., 2014). The absence of this transporter is known to drastically reduce the brain DHA levels and has been identified as a critical transporter of lyso-PC DHA (Nguyen et al., 2014).

Albumin carrying the PUFA either in its non-esterified form or esterified to lyso-PC can undergo facilitated transport involving FAT/CD 36 (fatty acid translocase/cluster of differentiation 36) or passive diffusion across the BBB into the endothelial cell. In comparison to lyso-PC DHA, the unesterified DHA uptake from the plasma into the brain is ten times greater, thereby suggesting that plasma pool of unesterified DHA is the major candidate contributing to brain DHA accretion (Chen et al., 2015).

Once in the neuronal cells, the PUFAs, especially DHA and AA, interact with long-chain fatty acid CoA synthase (ACSL) to form CoA thioesters and are esterified to phospholipids. Some PUFAs readily undergo β-oxidation, particularly EPA, whose concentration in the brain is nearly 250-300 times lower than DHA (Chen et al., 2011). Recent evidence has demonstrated that EPA is maintained at its low concentrations in the brain via extensive metabolism including rapid de-esterification from brain phospholipids, decreased uptake from plasma unesterified pool, and increased conversion to (n-3) DPA (Chen et al., 2013).

1.8.2 SFA and monounsaturated fatty acids (MUFA) in the brain

Saturated and monounsaturated fatty acids are vital structural components of the human brain. De novo synthesis of SFA occurs independently in the brain to give palmitic acid (C16:0) as the final product. Further elongation of C16:0 forms stearic acid (C18:0), which on desaturation is converted to MUFAs. The neuronal membrane phospholipids are dense with palmitic and stearic acid, accounting for 19% and 17%, respectively, of the total SFA in the brain (Edmond et al., 1998). Both SFA and MUFA have been linked to cognitive health in humans (Dumas et al., 2016). In early life, there is a rapid accretion of these fatty acids in the brain promoting myelination (Edmond et al., 1998). The MUFAs, particularly C18:1, constitute 30-40% of the total fatty acid content of the myelin sheath and low MUFAs have been linked with increased incidence of depressive disorders and Alzheimer's disease (Fernandes et al., 2017). Conversely, dietary intake of excess SFA has recently been associated with neuroinflammation and behavioral disorders. However, studies of SFA and MUFA in early brain development in neonates are limited and further research targeting these fatty acids in infant brain development will be beneficial.

1.9 LCPUFA, PEMT induced PC and methyl nutrients

The liver is crucial for the production of LCPUFA supplied to the brain via their incorporation into VLDL before secretion. The phospholipids, which make up an important component of VLDL, are considered one of the major determinants for the transportation of PUFAs, especially DHA and AA, to the brain. Among the phospholipids in the brain, PC and PE are the predominant species, essential to neural membrane development. DHA and AA together constitute nearly 25% of total phospholipid fatty acids in the brain (Hadley et al., 2016), with >80% of DHA being incorporated into phospholipids. DHA incorporation into brain phospholipids is extensively targeted at PC while AA is unique to the PI class of phospholipids; both fatty acids occupy the sn-2 position of the glycerophospholipid.

PC, as discussed previously, can be formed by two biosynthetic pathways. It is well established in the literature that PEMT-derived PC is particularly enriched with DHA and AA and plasma DHA levels have been correlated to PEMT activity (da Costa et al., 2010; Pynn et al., 2011; West et al., 2013). Studies with PEMT knock-out mice have demonstrated reduced PC-DHA in the brain during development (da Costa et al., 2010). Within the neurons, where the PEMT expression is also low, this pathway is not known to significantly contribute to PC associated with LCPUFA. Thus, any alterations in the LCPUFA-PC in the brain by PEMT is characteristic of its hepatic activity (da Costa et al., 2010). Likewise, a majority of DHA-PC produced in the liver is via the PEMT pathway, which eventually gets secreted into the circulation through VLDLs for distribution to tissues.

Recent studies have demonstrated the effect of modified concentrations of choline on PEMT activity (West et al., 2013). Choline generates PC via the CDP-choline pathway and is the precursor for the endogenous synthesis of betaine. Both choline and DHA consumption have been related to improved neurodevelopment in infants (Andrew et al., 2017). There is some evidence relating higher choline intake with higher plasma PC-DHA in non-pregnant women (West et al., 2013). The same group of researchers further suggested that high choline consumption may augment the flux through the methylation pathway by enhancing methyl donor availability via its derivative betaine. Certainly, studies have identified the methyl groups in labeled choline in the PEMT derived PC (Chew et al., 2011; West et al., 2013; Yan et al., 2011), further strengthening the link between the methyl donors, PC-LCPUFAs and PEMT activity. Although no study has directly studied the influence of betaine on PC-DHA and PC-AA, a recent report by Joselit et al. (2017) revealed that betaine supplementation in maternal high fat fed dams increased the expression of FABP 7, a protein with high DHA binding affinity, in the fetal brain. Thus, the importance of betaine as well as methionine, in improving the mobilization of PC-LCPUFA (via PEMT pathway) needs further research.

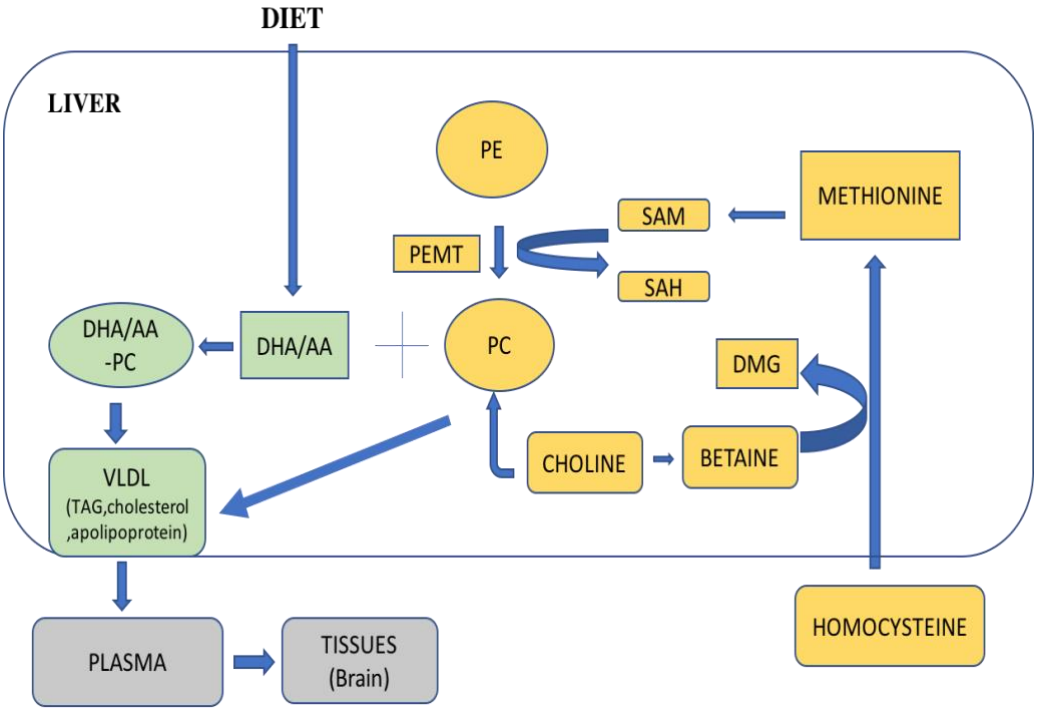

Figure 1.4: Schematic representation of DHA/AA incorporation into VLDL and its delivery to the brain via VLDL.

PC, phosphatidylcholine; PE, phosphatidylethanolamine; DMG, dimethylglycine; PEMT, phosphatidylethanolamine N-methyltransferase; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; VLDL, very low density lipoprotein; TAG, triacylglycerol

1.10 Piglet as an animal model

Rodents have long been used in laboratory research owing to their rapid reproductive capacity, similarities in gene and biochemical pathways, and easy environmental stability (Holemans at al., 2003). However, rodent gastrointestinal tracts differ from that of humans in terms of immunological development, anatomy and maturation stage. Unlike humans, rodents have less mature gastrointestinal tract at the time of birth and more rapid birth and weaning periods (Sangild, 2006). Moreover, rodents lack cholesterol ester transfer protein (CETP), which renders it incapable of exchanging cholesterol between lipoproteins. In contrast, the neonatal piglet is an ideal model for studying lipid and amino acid metabolism. The dietary requirements of piglets are remarkably similar to those for newborn infants (Baracos, 2004; Miller & Ullrey, 1987). The piglet closely mimics the infant's anatomy, physiology and metabolism and therefore closer in translation to human than rodent models (Baracos, 2004; Miller & Ullrey, 1987). The neonatal piglet has been used to study amino acid requirements in both oral feeding, as well as in parenteral feeding (Miller & Ullrey, 1987; Wykes et al., 1993). In particular, the methionine requirement has been established, both in enterally and parenterally fed neonatal piglets with excess and deficient levels of cysteine (Shoveller et al., 2003a; Shoveller et al., 2003b).

Dietary lipids have a key role in growth and development in human neonates as well as in piglets. The piglet brain shows a growth spurt at similar developmental stages as in a human neonate (Smink et al., 2012). Additionally, the piglet's brain anatomy and morphology are very similar to those in a neonate (Schmidt, 2014). Moreover, the

gastrointestinal anatomy and physiology, including lipid absorption and digestion, is very similar to that in humans (Innis, 1993). Recent studies have shown that fatty acid profile of the brain and other tissues are influenced by postnatal fatty acid feeding (Hyde et al., 2008; Hyde et al., 2005). Furthermore, the relative fatty acid profiles in neonates and piglets are similar (Innis, 2007). These similarities allow for a greater degree of comparison between the two species. Thus, the piglet is a clinically relevant model for studying lipid metabolism in parenterally fed neonates.

1.11 Rationale, Hypotheses and Objectives

Methionine is an essential amino acid important for protein synthesis as well as non-growth requirements in neonates. It is a primary source of methyl groups which are in high demand in the growing neonate. Recent evidence suggests that in suckling piglets, half of the dietary methionine is equally utilized for synthesis of creatine and of PC, and the remainder is used to meet demands for protein synthesis. PC synthesis via PEMT is quantitatively most sensitive to methyl supply. Methyl incorporation into PC was found to be restricted when methyl flux towards creatine synthesis was increased. Thus, the transmethylation pathways compete for limiting methyl groups, whose supply in infant nutrition needs to be optimized to meet the overall growth demands in the neonate.

However, supplementation of methionine to enhance methylation to PC has drawbacks. Dietary methionine proportionately increases plasma homocysteine during TPN which can be toxic. However, betaine supplementation reduces plasma homocysteine while at the same time it can alleviate fatty liver disease (Kharbanda, 2013). Betaine can donate a methyl group to enhance methionine availability as well as spare choline, both of

which are substrates for PC biosynthesis. PC synthesis is critical in TPN to prevent fatty liver via VLDL secretion.

TPN has often been associated with liver dysfunction. However, recent evidence suggests SMOFlipid may help prevent cholestasis. Subsequently, many Canadian pediatric clinics have adopted SMOFlipid as the lipid emulsion of first choice. Moreover, SMOFlipid is a good source of (n-3) PUFA, delivery of which to the brain is largely dependent on PEMT-derived PC. However, betaine is a novel TPN ingredient and it is still unknown if betaine supplementation can enhance hepatic PC synthesis by enhancing S-adenosyl methionine availability and sparing choline for PC synthesis during TPN feeding.

Hypotheses

We hypothesized that betaine supplementation will prevent fatty liver caused by TPN feeding by enhancing methylation of PE to PC and by sparing choline for hepatic PC synthesis in Yucatan miniature piglets, while also lowering homocysteine concentrations. Subsequently, the enhanced PC synthesis will increase delivery of DHA and AA to the brain via increased VLDL secretion.

Objectives

1. To assess the effects of parenteral betaine, methionine and its combination on hepatic and plasma lipid parameters to determine if betaine supplementation can prevent fatty liver caused by TPN feeding.
2. To assess the effects of parenteral betaine, methionine and its combination on hepatic total and phospholipid fatty acid composition.

3. To assess the effects of parenteral betaine, methionine and its combination on DHA and AA distribution in the brain.

2. Materials and Methods

2.1 Remarks

The animal work and isotope infusions for this study were performed by a previous M.Sc. student. Liver, brain, and plasma samples from that study were used for this thesis. Although conducted by another student, Sections 2.2 to 2.5 are included herein to describe the original protocols for the animals used in this thesis.

2.2 Animal Procedures

The Yucatan miniature piglet model was chosen as a model for our study. The Institutional Animal Care Committee at Memorial University of Newfoundland approved all animal procedures used. Additionally, procedures adhered to Canadian Council on Animal Care guidelines. A total of thirty-two Yucatan pigs, ranging from ages 8-12 days old, from the breeding colony at Memorial University of Newfoundland, St. John's, NL, were used (Figure 2.1). Based on previous transmethylation partitioning variance estimates, six pigs per group was determined to be sufficient to detect a 10% difference, calculated with a significance level of 0.05 and power of 0.80. In this study, 8 pigs were used in each of the 4 experimental groups and pigs within each group were balanced for gender and weight.

On the day of animal surgeries, piglets were transported from the breeding colony to the Biotechnology building at Memorial University of Newfoundland by Animal Care Services staff. Prior to surgery, pigs were weighed and anaesthetized with a 22 mg/kg dose of ketamine hydrochloride (Bimeda Canada, Cambridge, ON, Canada) and a 0.5 mg/kg dose of acepromazine (Vetoquinol, Quebec, Canada), injected intramuscularly. As well, to reduce airway secretions, pigs were given a subcutaneous injection of a 0.05

mg/kg dose of atropine sulfate (Rafter and Products, Canada). Lastly, the pigs received two intramuscular injections of 0.03 mg/kg dose of buprenorphine analgesic (Temgesic, Reckitt Benckiser Healthcare, UK); first prior to surgery and, the second 12 h following surgery. Following induction of anaesthesia, the piglets were cleaned with soap and Proviodine solution. During surgery, anesthesia was maintained with 1.5% isoflurane (Abbott Laboratories Inc., Canada) with oxygen (1.5 L/min), and respiratory rate, heart rate, oxygen saturation, and body temperature were monitored. Each surgery consisted of two venous catheters being implanted. The first was introduced through the left external jugular vein and advanced to the cranial vena cava and was used for diet infusion, as well as isotope infusion during the last two days of the study. The second was introduced into the femoral vein and advanced to the caudal vena cava and was used for blood sampling during the isotope infusion.

Following surgery, anti-bacterial veterinary ointment was applied to all incisions and an IV injection of 0.5 mL of anti-bacterial Borgal (Trimethoprim 40mg/mL and sulfadoxin 300 mg/mL; Intervet Canada Ltd., Canada) that was diluted to 10.0 mL with saline was given. Piglets were then immediately moved to the animal housing room, with 12 h light and dark cycles and a maintained temperature of 28 °C. The piglets were housed in individual cages that allowed for both visual and aural contact with other piglets. As well, a dual-port swivel and tether system (Lomir Biomedical, Montreal, Quebec, Canada) allowed continuous IV feeding while also permitting the piglets to move freely in their cages. The cages also contained toys for stimulation.

On the last day of the study, a piglet from the control group (pig# 33) was found dead and hence no tissues or blood were collected from that piglet. Plasma samples could

not be collected from pig# 6 of the control group and pig# 11 of the excess methionine group due to catheter-related issues, and were unavailable for analyses. Brain samples from pig# 1 and #6 of the control group could not be located and were not analyzed.

2.3 Diets and Diet Preparation

The control diet (Appendix I), containing a full profile of amino acids based on parenteral nutrition solutions for infants (Vaminolact; Fresnius Kabi, Germany), and slight modifications for piglets, was prepared in the laboratory using crystalline L-amino acids (Evonik Industries AG, Hanau-Wolfgang, Germany or Sigma, St. Louis, MO, USA). These amino acids were mixed together and slowly added to 60-70 °C pyrogen free water; nitrogen gas was added to prevent oxidation. Once dissolved, the nitrogen gas was removed. Next, D-glucose was added directly to the amino acid solution followed by other major minerals (Appendix II). Pyrogen-free water was then used to bring the solution to the desired volume of 10.6 L. This was followed by filtration of the diets using 0.22 μm filters (ACROPAK, Pall Corporation, Switzerland) into sterile intravenous bags (Baxter Corporation, Mississauga, ON, Canada) in a laminar flow hood. These bags were then refrigerated and stored away from light until needed. The other three experimental diets (Appendix III) were prepared similarly, with alanine being adjusted to ensure isonitrogenous diet solutions.

2.3.1 Diet Groups

Control diet (Control): This diet was designed to provide 100% of all amino acid requirements. This diet did not contain betaine, as betaine is normally not included in parenteral nutrition diets for infants.

Betaine diet (Betaine): The betaine diet consisted of the control diet plus betaine added as betaine hydrochloride at a rate of 235 mg/kg/day. The added betaine was the molar equivalent to 80% of the piglet's methionine requirement (Shoveller et al., 2003a). This diet was used to determine the effect of betaine on methionine availability via remethylation reactions, as well as its effect on choline utilization.

Excess methionine (Ex Met): The methionine diet consisted of the control diet plus methionine added at a rate of 0.5 g/kg/day. The added methionine was equivalent to ~200% of the methionine requirement in piglets (Shoveller et al., 2003a). This diet was used to compare enhanced methionine availability by diet vs that by betaine-enhanced remethylation.

Betaine and excess methionine (Bet+Ex Met): This diet consisted of the control diet with betaine and excess methionine combined. Betaine and methionine were added at the same rate as described previously. This diet was used to determine if higher homocysteine induced by excess methionine can be offset by betaine supplementation.

2.3.2 Trace Elements

Pyrogen-free water was used to dissolve the trace elements (Appendix III), which were then filtered through a 0.22 μm syringe filter (Millipore Ireland Ltd., Ireland) into a sterile intravenous bag. The mixture was then refrigerated and stored away from light until needed.

2.3.3 Vitamin Mixture

The vitamin mixture was based on Multi-12/K1 pediatric multivitamin commercial solution doses. Pyrogen free water was used to dissolve all the vitamins (Appendix IV) with polysorbate being used to first dissolve the fat-soluble vitamins. Next, the solution was filtered using 0.22 um syringe filter into a sterile intravenous bag followed by refrigeration.

Prior to administering the diet, 3 mL of the Multi-12/K1 vitamin mixture, 1 mL of iron dextran (Ventoquinol Canada Inc., Canada) (providing 2 mg of iron per kg of body weight), 3 mL of the trace element mixture, 2 mL of choline (providing 0.15 mg/kg/h), and 145 mL of 20% SMOFlipid (Fresenius Kabi, Uppsala, Sweden) (Appendix V) were added to 750 mL of diet.

The diet provided approximately 15 g of amino acids/kg/d and 1.1 MJ of metabolizable energy/kg/d. Lipids provided 50% of non-protein energy. Vitamins were given at more than 100% of requirements and trace elements were given at 100-200% of requirements.

On the day of surgery, infusion pumps (Baxter Healthcare Corporation, Deerfield, USA) were used to continuously administer the experimental diets intravenously into the jugular catheter at 50% of the maximal rate (12 mL/kg/h). The morning after surgery, the rate of infusion was increased to 75% of the maximal rate and then further increased to 100% in the evening. The maximum rate of 100% was maintained for the remainder of the study. During this time, body weight of the piglets was recorded each morning and infusion rates were adjusted accordingly.

2.4 Isotope Infusion

On day 7, piglets received an infusion of stable isotopes of methionine and choline. During this infusion period, to account for the dietary contribution of isotopes infused, new diets (Appendix VI) were given that were identical to the experimental diets, except that they contained less methionine and the 2 mL choline was not added. Piglets received a prime dose of 19.97 μmol/kg of choline chloride (Trimethyl-D9) and L-methionine (Methyl-^{13}C) (Cambridge Isotope Laboratories Inc., MA, USA) via the jugular catheter. This was followed by a constant infusion of 9.97 μmol/kg/h for 8 h, which was again through the jugular catheter. Prior to administration of the prime, baseline blood samples were collected, and then half hourly throughout the infusion. Immediately after, the blood samples were transferred to heparinized vacutainers and centrifuged at 1300 x g (VWR Clinical 200, Hermle Labortechnik, Wehingen, Germany) for five minutes to separate the plasma, which was then stored at -80 ºC.

On day 8, piglets received a radioisotope infusion. First, a prime dose of 30 μCi/kg of L-[methyl-^{3}H] methionine (30-80 Ci/mmol; American Radiolabeled Chemicals) via the jugular catheter. This was immediately followed by a continuous infusion of 30 μCi/kg of L-[methyl-^{3}H] methionine for 6 h. Prior to the prime dose, a baseline blood sample was collected and then samples were taken every half hour throughout the study. All blood samples were centrifuged and frozen as on day 7.

2.5 Necropsy

Following the 6 h infusion, piglets were anaesthetized with 1.5% isoflurane gas and oxygen (1.5 L/min) via mask. Using a syringe, 20 mL of blood was taken directly from the heart. Next, tissue samples from the liver and the brain were collected and frozen with liquid nitrogen. Time of removal and freezing was recorded for all samples.

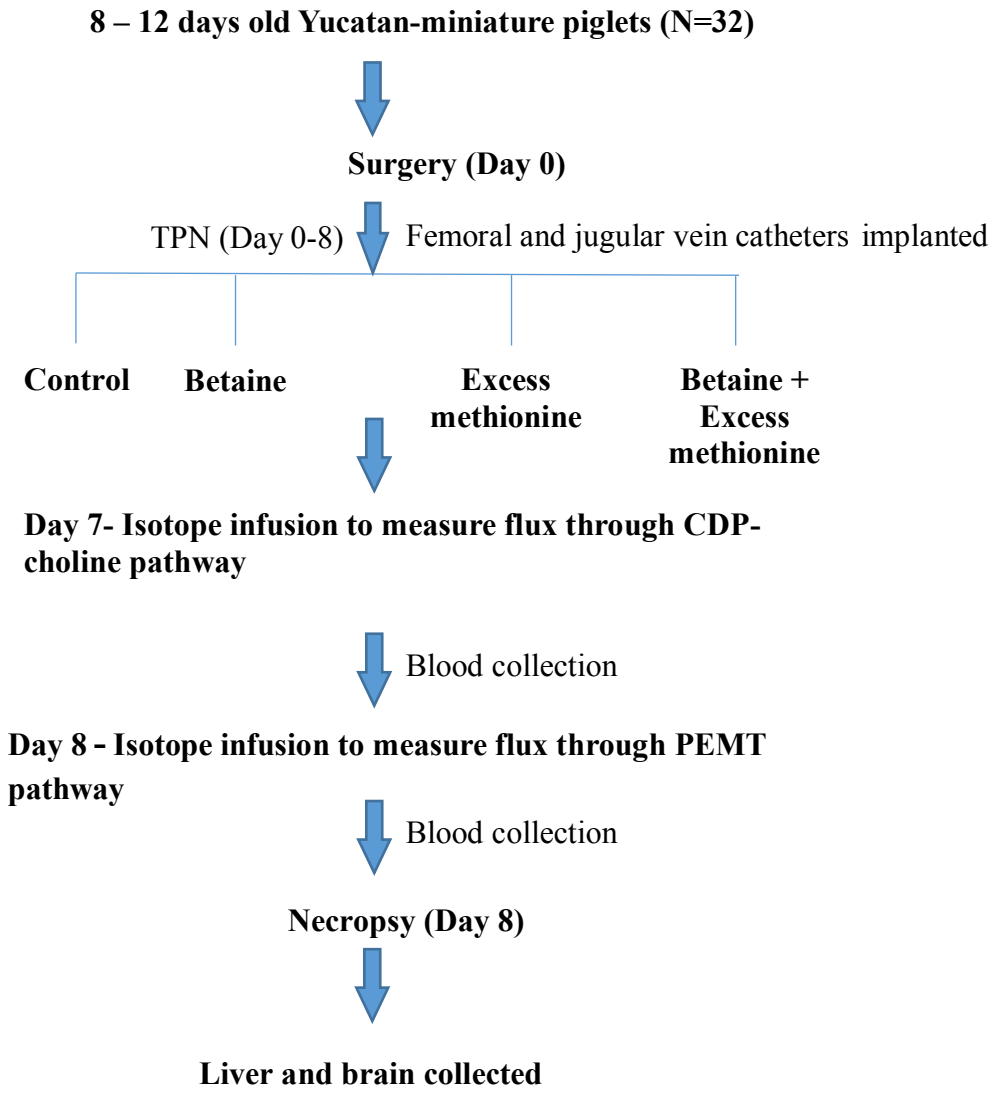

Figure 2.1: A schematic representation of the experimental design

2.6 Plasma Biochemical Analyses

The total plasma cholesterol and TAG concentrations were determined using enzymatic colorimetric assay kit for total cholesterol #234-60 and for triacylglycerol #236-60 (Sekisui Diagnostics PEI Inc. Charlottetown). Plasma samples collected on day 7 at 2.5 h after the start of the stable isotope infusion was used for analysis. One plasma sample per pig was used.

The enzymatic method used in the total cholesterol assay was a modification of that described by Allain and Roeschlau (Allain et al., 1974; Roeschlau et al., 1974). The assay is based on the principle that the cholesterol esters are hydrolyzed to free cholesterol (FC) by cholesterol esterase (CE). The FC is then oxidized to cholest-4-ene-3-one by cholesterol oxidase (CO), with the simultaneous production of hydrogen peroxide. The produced hydrogen peroxide then couples with 4-aminoantipyrine and p-hydroxybenzoate, in the presence of peroxidase, yielding a chromogen with maximum absorbance at 505 nm. The intensity of the color produced is directly proportional to the concentration of total cholesterol in the sample.

The triacylglycerol assay was a modification of the method described by Fossati and Prencipe (Fossati & Prencipe, 1982). Plasma triacylglycerols were hydrolyzed to glycerol and free fatty acids by lipase; glycerol was phosphorylated to glycerol-1-phosphate in the presence of ATP and glycerol kinase. Glycerol-1-phosphate was then oxidized by glycerol phosphate oxidase to yield hydrogen peroxide, which brings about oxidative coupling of p-chlorophenol and 4-aminoantipyrine producing a red colored quinoneimine dye complex with a maximum absorbance at 520 nm. The intensity of the color produced is directly proportional to the concentration of triacylglycerol in the sample.

2.7 Lipid Analyses

2.7.1 Hepatic Lipid Analyses

Total lipids were extracted from liver samples according to the method of Folch (Folch, 1957), and resuspended in isopropanol. Liver total cholesterol and TAG concentrations of total extracted lipids were determined using kit methods described above.

Fatty acid analysis of total extracted lipids was determined using gas chromatography (GC) after generating fatty acid methyl esters by transmethylation reaction. The samples were heated with 2 mL of transmethylation reagent (6% concentrated sulphuric acid and 94% methanol + few crystals of hydroquinone added as an anti-oxidant) for 2 h at 65°C in order to generate fatty acid methyl esters (Arvidson & Olivecrona, 2018). Methyl esters were extracted using hexane and water, then placed at -20 °C overnight to freeze any water in the tube; the hexane layer was then transferred to a new tube. Subsequently, the samples were dried under nitrogen gas and resuspended in carbon disulphide prior to GC analysis (Keough & Davis, 1979). Samples were run for 60 minutes on an Omega-wax X 320 (30 m x 0.32 mm) column from Supelco (Sigma-Aldrich, Canada) using a flame ionization detector. The GC parameters were set as: oven temperature, 200 C; injector temperature, 240 °C; and detector temperature 260 °C. The GC was ignited and allowed to run until a stable baseline was obtained. PUFA standards -2 and -3 (Sigma-Aldrich, Canada) were used as standards for identification of fatty acid peaks by retention time.

To analyze hepatic phospholipid fatty acid composition, phospholipids were separated from hepatic total lipids using thin layer chromatography (Keenan et al., 1982). Total extracted lipids were dissolved in chloroform and spotted on 20 x 20 cm flexible

plates coated with silica gel (Cat #: 4420222, Whatman Ltd. UK). Phospholipids were separated using the solvent system: hexane:ethyl ether:acetic acid (70:30:2 v/v) (Keenan et al., 1982). Phospholipid spots at the point of origin were scraped, and chloroform:methanol (2:1) was added followed by the addition of the internal standard, C17:0 (Sigma Aldrich, Canada). The above solution was vortexed well and the supernatant was collected into transmethylation vials, which were then analyzed for fatty acid composition of the total phospholipids, as described above.

2.7.2 Brain Lipid Analyses

Total lipids were extracted from brain using the method of Folch (Folch, 1957). Phospholipids were separated as described above. The fatty acid composition of total lipids and total phospholipids was quantified by GC as described above.

2.8 Statistical Analyses

The effect of diet on various biochemical parameters was analyzed using one-way analysis of variance (ANOVA) and Student Newman Keul's post hoc analysis to test significant differences among groups (Graph Pad Prism 5.0). Values were expressed as mean ± standard deviation. Differences were considered to be statistically different if the associated P value was < 0.05. Fatty acid compositions were expressed as relative weight percentage of the total extracted fatty acids; because data were skewed, data were arcsine transformed to normalize the data distribution, before subjecting to statistical analysis.

3. Results

3.1 Effect of Diet on Piglet Growth Rate

The effect of the dietary treatments on the piglet growth rate is shown in figure 3.1. There was no significant difference in growth rate among the treatment groups (Figure 3.1).

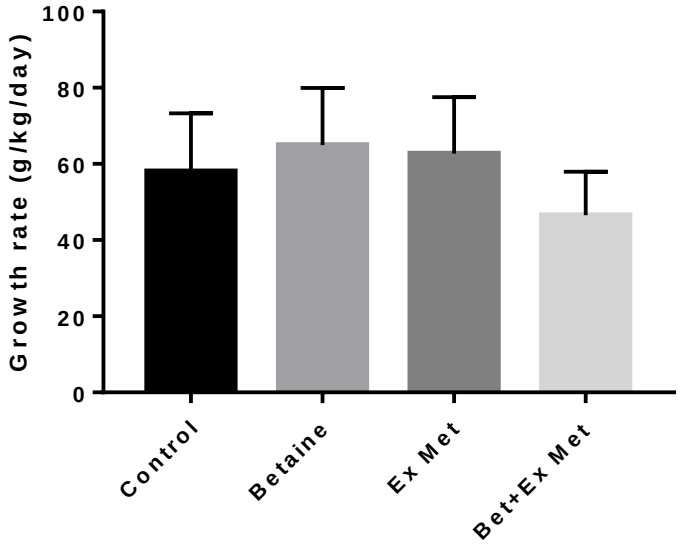

Figure 3.1: Effect of diet on piglet growth rate: Bars represent mean ± standard deviation (n = 7 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met). One-way ANOVA was used to test for an effect of the treatments.

Ex Met, Excess Methionine; Bet, Betaine

3.2 Effect of Diet on Hepatic Total Fat

The effect of diet on hepatic total fat is shown in figure 3.2. There was no difference in hepatic total fat among the treatment groups.

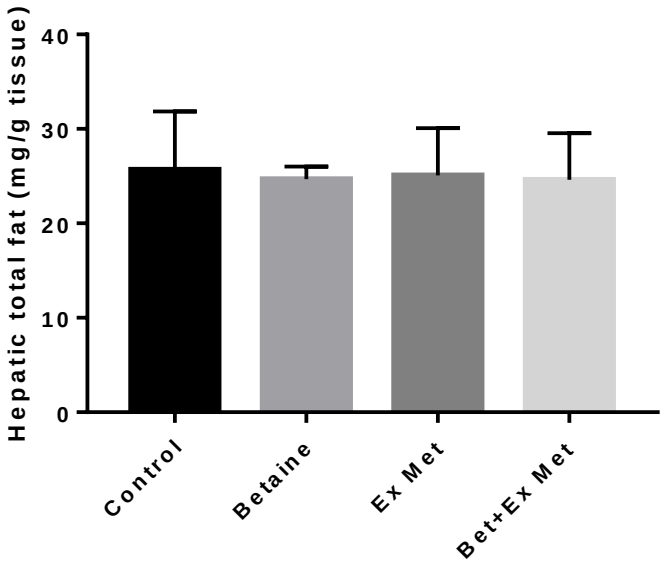

Figure 3.2: Effect of diet on hepatic total fat: Bars represent mean ± standard deviation. For piglet growth rate (n = 7 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met). One-way ANOVA was used to test for an effect of the treatments.

Ex Met, Excess Methionine; Bet, Betaine

3.3 Effect of Diet on Plasma Lipids

The plasma total cholesterol and TAG concentrations in the piglets receiving the control, betaine, excess methionine and betaine+excess methionine diets are shown in figures 3.3 and 3.4, respectively. No differences were observed in the plasma total cholesterol and TAG levels across treatment groups.

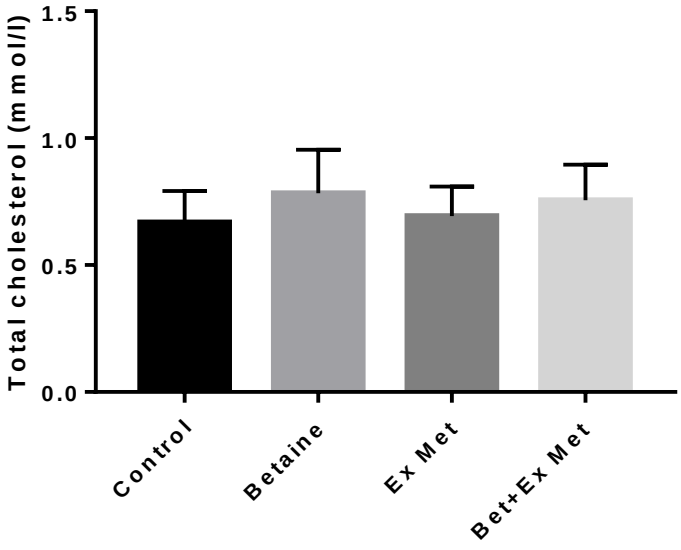

Figure 3.3: Effect of diet on plasma total cholesterol: Bars represent mean ± standard deviations. For plasma total cholesterol (n = 7 for Control, n = 8 Betaine, Ex Met and Bet+Ex Met). One-way ANOVA was used to test for an effect of the treatments.

Ex Met, Excess Methionine; Bet, Betaine

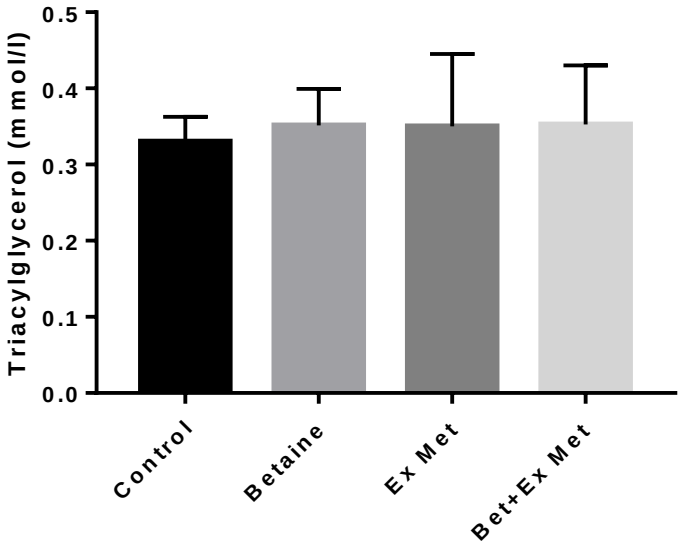

Figure 3.4: Effect of diet on plasma triacylglycerol: Bars represent mean + standard deviation. n = 7 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA was used to test for an effect of the treatments.

Ex Met, Excess Methionine; Bet, Betaine

3.4 Effect of Diet on Hepatic Lipids

The hepatic total cholesterol and TAG concentrations in the piglets receiving the control, betaine, excess methionine and betaine+excess methionine diets are shown in figures 3.5 and 3.6, respectively. No differences were observed in the hepatic total cholesterol and TAG levels among treatment groups.

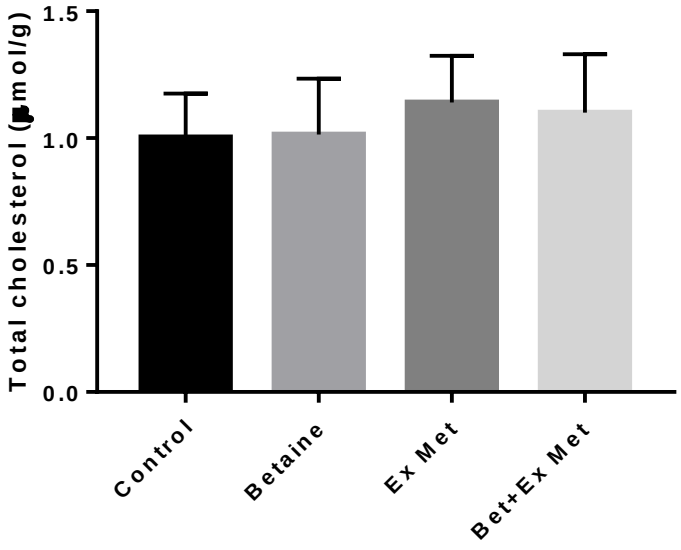

Figure 3.5: Effect of diet on hepatic total cholesterol: Bars represent mean ± standard deviation. n = 7 for Control, n = 8 for Betaine, Ex met and Bet+Ex met. One-way ANOVA was used to test for an effect of the treatments.

Ex Met, Excess Methionine; Bet, Betaine

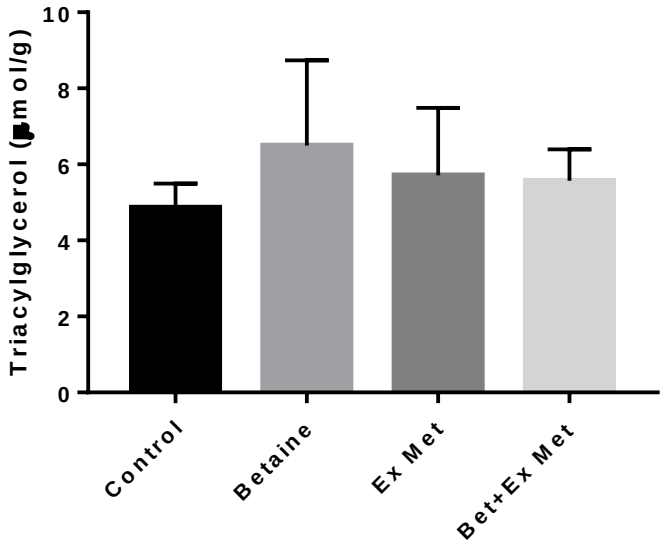

Figure 3.6: **Effect of diet on hepatic triacylglycerol:** Bars represent mean ± standard deviation. n = 7 for Control, n = 8 for Betaine, Ex Met and Bet+Ex met. One-way ANOVA was used to test for an effect of the treatments.

Ex Met, Excess Methionine; Bet, Betaine

3.5 Effect of Diet on Hepatic Total Fatty Acids

The fatty acid composition of total lipids in the liver of piglets receiving the control, betaine, excess methionine and betaine+excess methionine diets are shown in table 3.1. No differences were observed among the treatment groups in individual or total SFA, MUFA and PUFA.

Table 3.1: Effect of diet on hepatic total fatty acids

		Control	Betaine	Ex met	Bet+Ex met
	Fatty acid		**(% w/w)**		
SFA	C14:0	1.99±0.20	2.05±0.12	2.07±0.19	2.16±0.18
	C16:0	13.17±0.28	13.22±0.45	13.27±0.57	13.13±0.29
	C18:0	14.05±0.88	13.70±1.36	13.54±0.53	13.57±1.26
	∑SFA	29.21±1.33	28.97±1.93	28.88±1.29	28.86±1.73
MUFA	C16:1n7	2.81±0.47	3.11±0.36	3.22±0.11	3.20±0.34
	C18:1	17.37±0.61	17.44±0.86	17.21±0.66	17.06±1.06
	∑MUFA	20.18±1.08	20.55±1.22	20.43±0.77	20.26±1.40
(n-3) PUFA	C18:3n3	1.57±0.85	1.75±0.21	1.53±0.63	1.35±0.94
	C20:4n3	1.73±0.84	1.88±0.42	2.03±0.28	1.95±0.30
	C20:5n3	5.39±0.82	5.80±0.66	5.81±0.35	5.79±0.45
	C22:5n3	4.69±0.32	4.74±0.55	4.99±0.47	5.16±0.29
	C22:6n3	9.70±0.45	9.55±0.29	9.85±0.60	10.00±0.79
	∑(n-3)	23.08±3.28	23.72±2.13	24.21±2.33	24.25±2.77
(n-6) PUFA	C18:2n6	13.15±1.54	12.26±0.40	12.08±0.30	12.07±0.30
	C20:4n6	10.81±0.57	10.67±0.64	10.52±0.30	10.93±0.53
	C22:4n6	1.10±0.14	1.75±0.13	1.81±0.17	1.59±0.72
	∑(n-6)	25.06±2.25	24.68±1.17	24.41±0.77	24.59±1.55
	∑PUFA	48.14±5.53	48.40±3.30	48.62±3.10	48.84±4.32

Data are expressed as weight percentage of total fatty acids and presented as mean ± standard deviation. n = 7 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA was used to test for an effect of the treatments.

Ex Met, Excess Methionine; Bet, Betaine; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids

3.6 Effect of Diet on Hepatic Phospholipid Fatty Acid Composition

The phospholipid fatty acid composition of the liver in parenterally fed piglets in the control, betaine, excess methionine and betaine+excess methionine groups, expressed as both concentrations and relative percentages, is shown in tables 3.2 and 3.3 respectively. No significant differences were observed in the hepatic phospholipid SFA, MUFA, n-6 or n-3 PUFA concentrations (Table 3.2) or composition (Table 3.3).

Table 3.2: Effect of diet on hepatic phospholipid fatty acid concentration

		Control	Betaine	Ex met	Bet+Ex met
	Fatty acid		**(µmol/mg)**		
SFA	C14:0	0.08±0.04	0.06±0.03	0.07±0.05	0.05±0.03
	C16:0	1.58±0.24	1.69±0.83	1.61±0.58	1.40±0.84
	C18:0	2.18±0.32	1.80±0.77	1.97±0.67	2.03±1.40
	∑SFA	3.84±0.60	3.55±1.63	3.65±1.3	3.48±2.27
MUFA	C18:1	1.37±0.42	1.31±0.52	1.22±0.56	0.92±0.36
	∑MUFA	1.37±0.42	1.31±0.52	1.22±0.56	0.92±0.36
(n-3) PUFA	C20:4n3	0.21±0.12	0.18±0.13	0.18±0.15	0.12±0.10
	C20:5n3	0.09±0.03	0.07±0.03	0.09±0.04	0.07±0.02
	C22:5n3	0.22±0.09	0.18±0.08	0.19±0.10	0.13±0.06
	C22:6n3	0.22±0.07	0.16±0.05	0.21±0.08	0.15±0.03
	∑(n-3)	0.74±0.31	0.59±0.29	0.67±0.37	0.47±0.21
(n-6) PUFA	C18:2n6	0.79±0.20	0.61±0.26	0.75±0.26	0.60±0.19
	C20:4n6	0.41±0.16	0.33±0.14	0.38±0.16	0.29±0.05
	∑(n-6)	1.20±0.36	0.94±0.40	1.13±0.42	0.89±0.24
	∑PUFA	1.94±0.67	1.53±0.69	1.8±0.79	1.36±0.45

Data are expressed as mean ± standard deviation. n = 7 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA was used to test for an effect of the treatments.

Ex Met, Excess Methionine; Bet, Betaine; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids

Table 3.3: Effect of diet on hepatic phospholipid fatty acid percent composition

		Control	Betaine	Ex met	Bet+Ex met
	Fatty acid		**%**		
SFA	C14:0	3.35±0.67	3.36±0.74	3.42±0.68	3.18±0.59
	C16:0	16.65±1.28	18.10±2.02	17.60±1.43	17.44±1.49
	C18:0	19.92±1.86	18.91±2.08	19.71±2.34	21.17±2.21
	∑SFA	39.92±3.81	40.37±4.84	40.73±4.45	41.79±4.29
MUFA	C18:1	19.56±1.04	20.93±2.22	19.16±1.23	18.83±1.29
	∑MUFA	19.56±1.04	20.93±2.22	19.16±1.23	18.83±1.29
(n-3) PUFA	C20:4n3	5.63±1.27	5.49±1.73	4.97±1.72	4.67±0.90
	C20:5n3	3.64±0.39	3.65±0.91	3.92±0.71	3.87±0.43
	C22:5n3	5.85±0.92	5.74±0.94	5.67±0.95	5.29±0.59
	C22:6n3	5.92±0.82	5.52±1.14	5.99±1.08	5.85±1.44
	∑(n-3)	21.04±3.40	20.40±4.73	20.55±4.46	19.86±3.36
(n-6) PUFA	C18:2n6	11.42±0.88	10.55±1.70	11.53±0.66	11.54±1.15
	C20:4n6	8.07±1.20	7.74±1.66	8.12±1.46	8.15±1.66
	∑(n-6)	19.49±2.08	18.29±3.25	19.65±2.12	19.69±2.81
	∑PUFA	40.53±5.48	38.69±7.98	40.20±6.58	39.55±6.17

Data are expressed as weight percentage of total extracted phospholipid fatty acids and presented as mean ± standard deviation. n = 7 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA was used to test for an effect of the treatments.

Ex Met, Excess Methionine; Bet, Betaine; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid

3.7 Effect of Diet on Brain Total Fatty Acids

The fatty acid composition of total lipids in the brain of piglets receiving the control, betaine, excess methionine and betaine+excess methionine diets are shown in table 3.4. No differences were observed among the treatment groups in individual and total SFA, MUFA and PUFA.

Table 3.4: Effect of diet on brain total fatty acids

	Fatty acid	Control	Betaine (% w/w)	Ex met	Bet+Ex met
SFA	C14:0	2.44±0.24	2.38±0.16	2.38±0.16	2.47±0.18
	C16:0	16.90±1.17	15.95±0.66	16.15±0.41	16.37±0.88
	C18:0	16.69±0.90	16.49±0.92	16.57±0.67	16.22±1.04
	∑SFA	36.03±2.31	34.82±1.74	35.10±1.24	35.06±2.10
MUFA	C16:1n7	3.83±0.42	3.62±0.42	3.58±0.25	3.62±0.26
	C18:1	23.25±1.63	21.45±1.43	22.06±1.44	22.77±1.63
	∑MUFA	27.08±2.05	25.07±1.85	25.64±1.69	26.39±1.89
(n-3) PUFA	C20:5n3	2.86±0.79	2.37±0.65	2.59±0.39	2.45±0.57
	C22:5n3	1.78±1.00	2.14±0.92	2.18±0.08	2.29±0.99
	C22:6n3	10.51±0.80	10.52±0.45	10.41±0.51	10.53±0.77
	∑(n-3)	15.15±2.59	15.03±2.02	15.18±0.98	15.27±2.33
(n-6) PUFA	C18:2n6	3.33±0.76	6.75±3.43	5.43±3.01	3.81±1.96
	C20:4n6	11.49±0.85	11.35±0.44	11.54±0.42	12.01±0.83
	C22:4n6	6.93±0.48	6.98±0.54	7.11±0.23	7.46±0.38
	∑(n-6)	21.75±2.09	25.08±4.41	24.08±3.66	23.28±3.17
	∑PUFA	36.90±4.68	40.11±6.43	39.26±4.64	38.55±5.50

Data are expressed as weight percentage of total fatty acids and presented as mean ± standard deviation. n = 5 for Control, n = 8 Betaine, Ex Met and Bet+Ex Met. One-way ANOVA was used to test for an effect of the treatments.

Ex Met, Excess Methionine; Bet, Betaine; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids

3.8 Effect of Diet on Brain Phospholipid Fatty Acid Composition

The phospholipid fatty acid composition of the brain in piglets, expressed as µmol/mg of brain, and as relative percentage of total fatty acids is given in tables 3.5 and 3.6, respectively.

The phospholipid DHA concentration was significantly higher in all 3 treatment groups compared to the control group ($P<0.01$) (Figure 3.7). Interestingly, the piglets receiving betaine+excess methionine diet had significantly higher DHA in phospholipid than the rest of the treatments ($P<0.01$) (Figure 3.7). Comparing the relative percentage of DHA in the phospholipids in all the treatment groups, the piglets receiving the excess methionine with or without betaine showed significantly higher phospholipid-DHA than the control and betaine groups ($P<0.0001$) (Figure 3.8).

The phospholipid–EPA concentration was significantly lower in the excess methionine, betaine and betaine+excess methionine groups compared to control group ($P<0.05$) (Figure 3.9). Similarly, phospholipid-EPA in all three treatment groups were significantly lower than the control group when expressed as relative percentage of total fatty acids ($P<0.0001$) (Figure 3.10).

Among the (n-6) phospholipid PUFA composition, the phospholipid-arachidonic acid (PL-AA) concentrations for the betaine group were significantly higher than the control and excess methionine groups ($P<0.05$), with betaine+excess methionine intermediate (Figure 3.11); however, no significant differences were observed when data were expressed as relative percentages, although a similar pattern was observed (Figure 3.12).

Among the phospholipid SFAs, the palmitic acid concentrations in the brain phospholipids of betaine piglets were significantly higher than the control ($P<0.01$), excess methionine ($P<0.01$), and betaine+excess methionine groups ($P<0.05$) (Figure 3.13). Interestingly, no differences across the groups were observed for palmitic acid when expressed as relative percentages (Figure 3.14).

For the phospholipid-stearic acid, the betaine group had significantly higher stearic acid when compared to other groups ($P<0.05$) (Figure 3.15); however, when expressed as relative percentage the betaine group was significantly different from the betaine+excess methionine group only ($P<0.05$) (Figure 3.16).

The phospholipid MUFA composition in the brain of piglets after 8 d of treatment did not differ among the treatments for any fatty acid, expressed either way (Tables 3.5, 3.6).

Table 3.5: Effect of diet on brain phospholipid fatty acid concentration

		Control	Betaine	Ex met	Bet+Ex met
	Fatty acid		**(µmol/mg)**		
SFA	C14:0	0.05±0.02	0.07±0.01	0.06±0.01	0.07±0.02
	C16:0	**1.44±0.39[a]**	**2.19±0.30[b]**	**1.59±0.21[a]**	**1.82±0.40[a]**
	C18:0	**2.30±0.37[a]**	**3.61±0.47[b]**	**2.52±0.35[a]**	**2.92±0.69[a]**
	∑SFA	3.97±0.78	5.87±0.78	4.17±0.57	4.81±1.11
MUFA	C16:1n7	0.06±0.01	0.08±0.07	0.07±0.03	0.07±0.02
	C18:1	1.45±0.63	1.94±0.48	1.49±0.25	1.71±0.39
	∑MUFA	1.51±0.64	2.02±0.54	1.55±0.28	1.78±0.41
(n-3) PUFA	C20:4n3	0.05±0.01	0.06±0.01	0.05±0.01	0.07±0.02
	C20:5n3	**0.08±0.01[a]**	**0.05±0.01[b]**	**0.04±0.01[b]**	**0.05±0.03[b]**
	C22:6n3	**0.13±0.03[a]**	**0.24±0.08[b]**	**0.24±0.06[b]**	**0.34±0.03[c]**
	∑(n-3)	0.26±0.05	0.35±0.10	0.33±0.08	0.46±0.08
(n-6) PUFA	C18:2n6	0.06±0.01	0.06±0.02	0.04±0.02	0.07±0.04
	C20:4n6	**0.15±0.08[a]**	**0.39±0.15[b]**	**0.19±0.06[a]**	**0.31±0.19[ab]**
	∑(n-6)	0.21±0.09	0.45±0.17	0.23±0.08	0.38±0.23
	∑PUFA	0.47±0.14	0.80±0.27	0.56±0.16	0.84±0.31

Data are expressed as mean ± standard deviation. n = 5 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA followed by Student Newman Keul's post hoc analysis ($P<0.05$) were used to test for differences among the treatments. Different superscript letters in a row indicate statistical difference.

Ex Met, Excess Methionine; Bet, Betaine; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids

Table 3.6: Effect of diet on brain phospholipid fatty acid percent composition

		Control	Betaine	Ex met	Bet+Ex met
	Fatty acid		**(% w/w)**		
SFA	C14:0	3.34±0.28	3.17±0.12	3.34±0.15	3.28±0.43
	C16:0	18.32±1.44	19.24±1.41	18.63±0.53	18.05±0.72
	C18:0	**24.07±1.23^{ab}**	**25.62±1.32^{a}**	**24.22±1.44^{ab}**	**23.56±1.16^{b}**
	∑SFA	45.73±2.95	48.03±2.85	46.19±2.12	44.89±2.31
MUFA	C16:1n7	3.61±0.41	3.53±0.24	3.62±0.67	3.47±0.24
	C18:1	24.14±3.04	23.87±1.28	23.90±1.075	23.31±1.52
	∑MUFA	27.75±3.45	27.40±1.52	27.52±1.745	26.78±1.76
(n-3) PUFA	C20:4n3	3.32±0.39	2.94±0.40	3.21±0.33	3.26±0.49
	C20:5n3	**4.06±0.44^{a}**	**2.78±0.33^{b}**	**2.92±0.30^{b}**	**2.75±0.50^{b}**
	C22:6n3	**5.41±0.94^{a}**	**5.98±0.69^{a}**	**6.94±0.70^{b}**	**7.60±0.77^{b}**
	∑(n-3)	12.79±1.77	11.70±1.42	13.07±1.33	13.61±1.76
(n-6) PUFA	C18:2n6	3.65±0.50	2.93±0.60	2.63±0.71	3.24±1.03
	C20:4n6	5.69±1.58	7.43±1.39	6.06±0.96	6.96±1.73
	∑(n-6)	9.34±2.08	10.36±1.99	8.69±1.67	10.20±2.76
	∑PUFA	22.13±3.85	22.06±3.41	21.76±3.00	23.81±4.52

Data are expressed as weight percentage of total extracted phospholipid fatty acids and presented as mean ± standard deviation. n = 5 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA followed by Student Newman Keul's post hoc analysis ($P<0.05$) were used to test for differences among the treatments. Different superscript letters in a row indicate statistical difference.

Ex Met, Excess Methionine; Bet, Betaine; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids

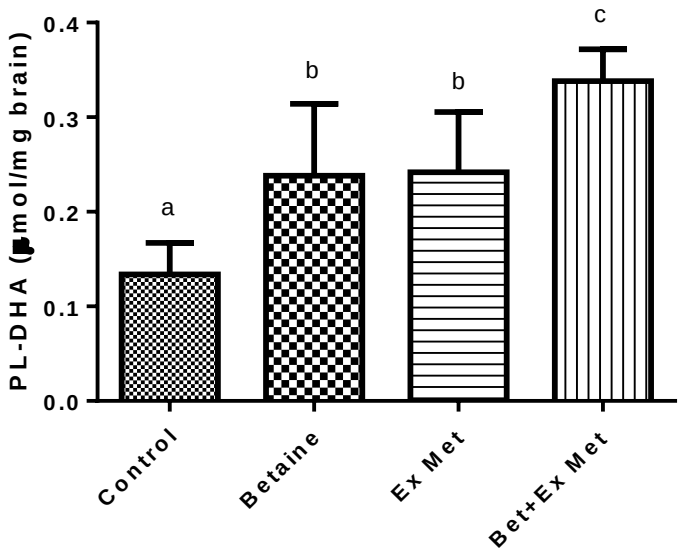

Figure 3.7: Effect of diet on phospholipid-DHA concentration in the brain: Values are mean ± standard deviations. n = 5 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA followed by Student Newman Keul's post hoc analysis ($P<0.05$) were used to test for differences among the treatments. Different letters indicate statistical differences.

Ex Met, Excess Methionine; Bet, Betaine; PL-DHA, Phospholipid-Docosahexaenoic acid

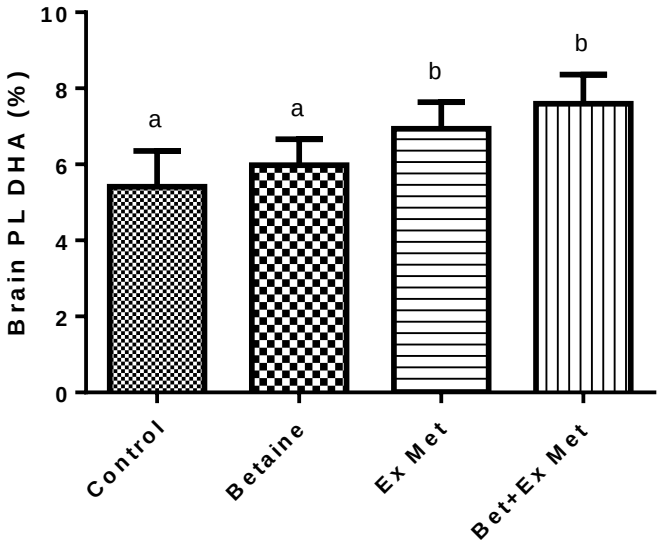

Figure 3.8: Effect of diet on phospholipid-DHA percent composition in the brain: Data are expressed as weight percentage of total extracted phospholipid fatty acids and presented as mean ± standard deviation. n = 5 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA followed by Student Newman Keul's post hoc analysis ($P<0.05$) were used to test for differences among the treatments. Different letters indicate statistical differences.

Ex Met, Excess Methionine; Bet, Betaine; PL-DHA, Phospholipid-Docosahexaenoic acid

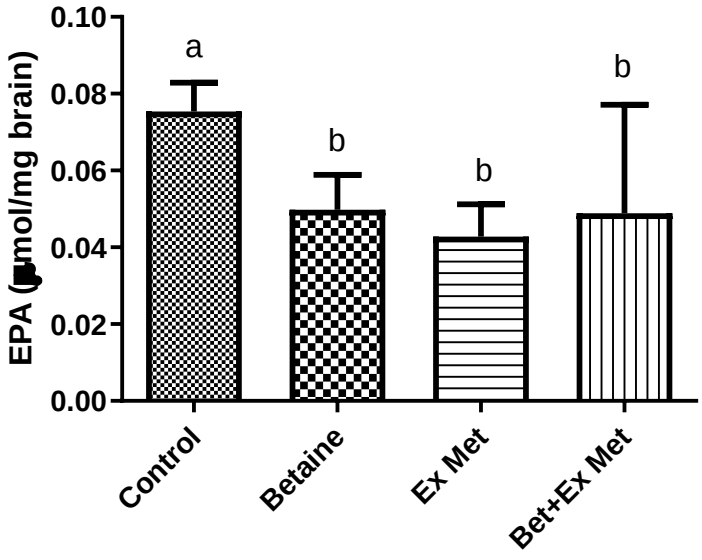

Figure 3.9: Effect of diet on phospholipid-EPA concentration in the brain: Values are mean ± standard deviation. n = 5 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA followed by Student Newman Keul's post hoc analysis ($P<0.05$) were used to test for differences among the treatments. Different letters indicate statistical differences.

Ex Met, Excess Methionine; Bet, Betaine; PL-EPA, Phospholipid-Eicosapentaenoic acid

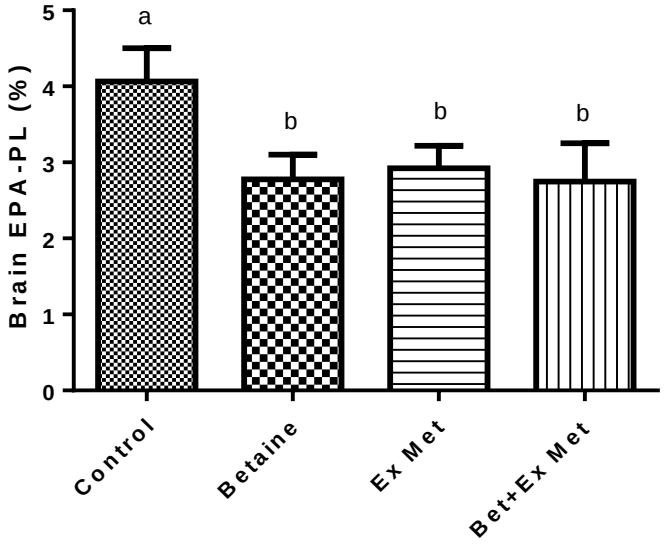

Figure 3.10: Effect of diet on phospholipid-EPA percent composition in the brain: Data are expressed as weight percentage of total extracted phospholipid fatty acids and presented as mean ± standard deviation for n = 5 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA followed by Student Newman Keul's post hoc analysis ($P<0.05$) were used to test for differences among the treatments. Different letters indicate statistical differences.

Ex Met, Excess Methionine; Bet, Betaine; PL-EPA, Phospholipid-Eicosapentaenoic acid

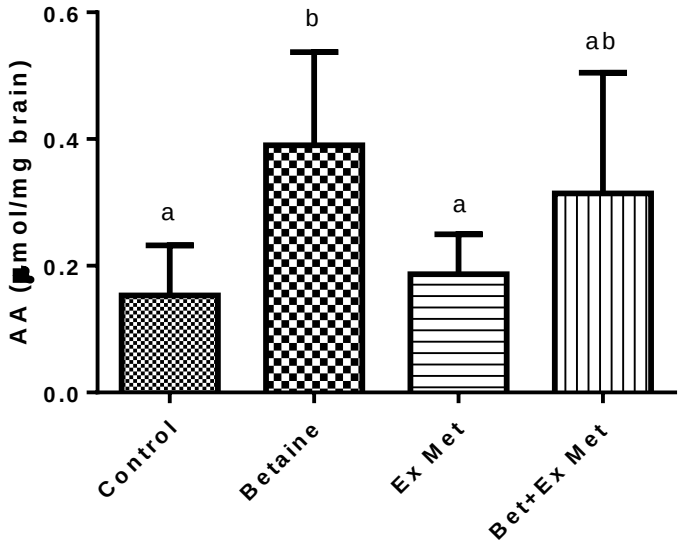

Figure 3.11: Effect of diet on phospholipid-AA concentration in the brain: Values are mean ± standard deviation. n = 5 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA followed by Student Newman Keul's post hoc analysis ($P<0.05$) were used to test for differences among the treatments. Different letters indicate statistical differences.

Ex Met, Excess Methionine; Bet, Betaine; PL-AA, Phospholipid-Arachidonic acid

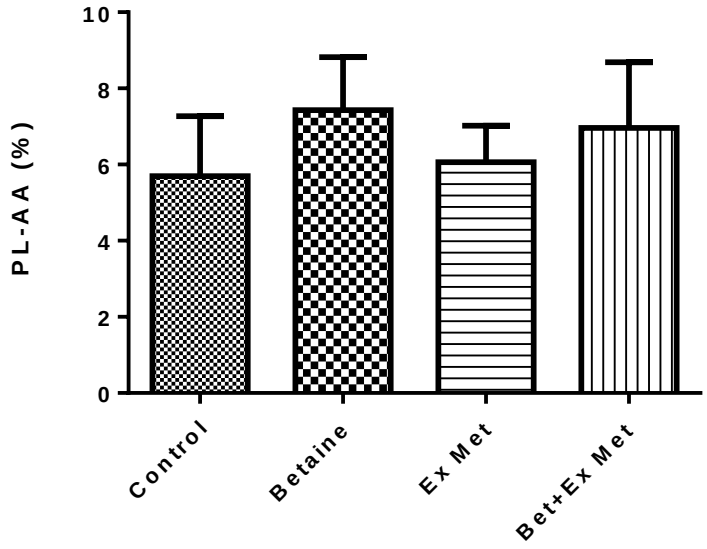

Figure 3.12: Effect of diet on phospholipid-AA percent composition in the brain: Data are expressed as weight percentage of total extracted phospholipid fatty acids and presented as mean ± standard deviation. n = 5 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA was used to test for an effect of the treatments.

Ex Met, Excess Methionine; Bet, Betaine; PL-AA, Phospholipid-Arachidonic acid

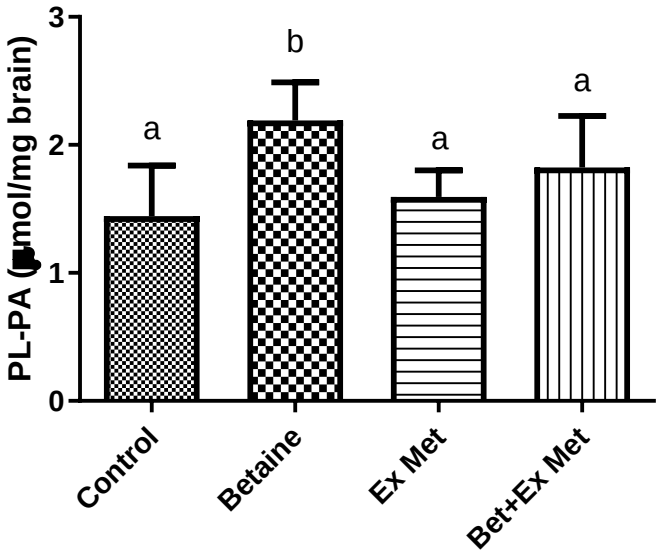

Figure 3.13: Effect of diet on phospholipid-palmitic acid concentration in the brain: Values are mean ± standard deviation. n = 5 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA followed by Student Newman Keul's post hoc analysis ($P<0.05$) were used to test for differences among the treatments. Different letters indicate statistical differences.

Ex Met, Excess Methionine; Bet, Betaine; PL-PA, Phospholipid-Palmitic acid

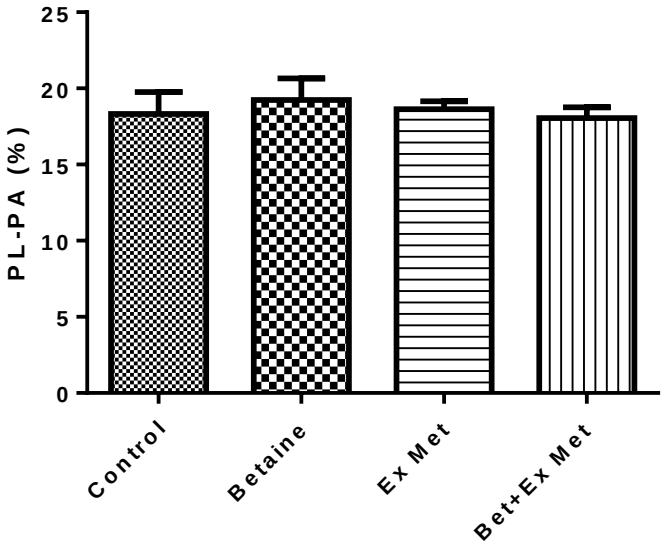

Figure 3.14: Effect of diet on phospholipid-palmitic acid percent composition in the brain: Data are expressed as weight percentage of total extracted phospholipid fatty acids and presented as mean ± standard deviation. n = 5 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA was used to test for an effect of the treatments.

Ex Met, Excess Methionine; Bet, Betaine; PL-PA, Phospholipid-Palmitic acid

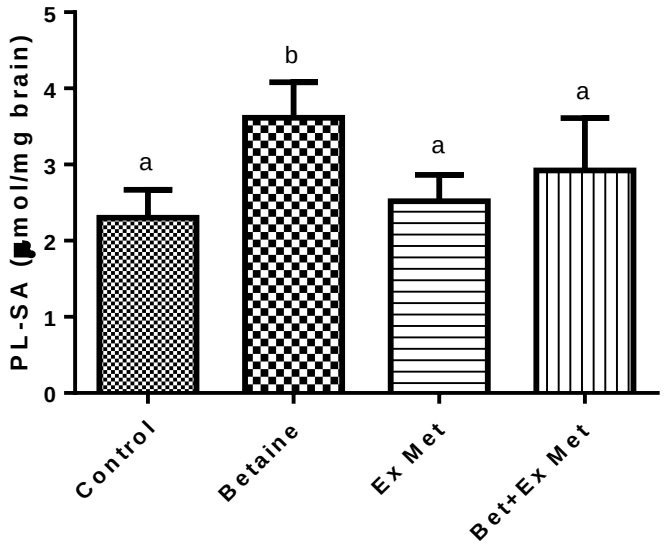

Figure 3.15: Effect of diet on phospholipid-stearic acid concentration in the brain: Values are mean ± standard deviation. n = 5 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA followed by Student Newman Keul's post hoc analysis ($P<0.05$) were used to test for differences among the treatments. Different letters indicate statistical differences.

Ex Met, Excess Methionine; Bet, Betaine; PL-SA, Phospholipid-Stearic acid

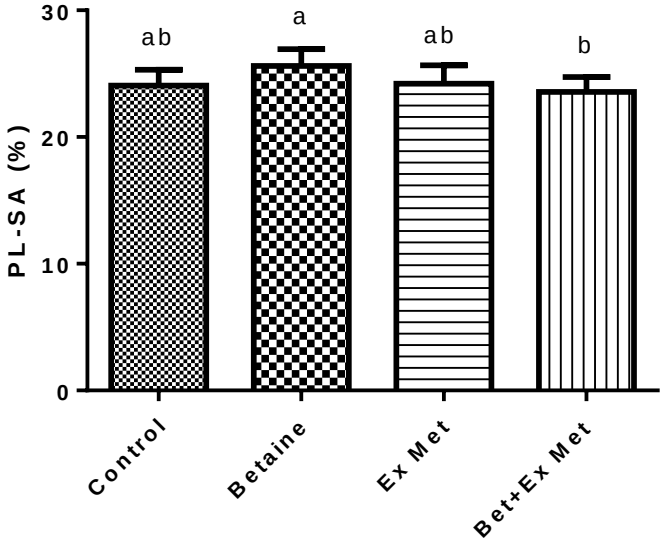

Figure 3.16: Effect of diet on phospholipid-stearic acid percent composition in the brain: Data are expressed as weight percentage of total extracted phospholipid fatty acids and presented as mean ± standard deviation. n = 5 for Control, n = 8 for Betaine, Ex Met and Bet+Ex Met. One-way ANOVA followed by Student Newman Keul's post hoc analysis ($P<0.05$) were used to test for differences among the treatments. Different letters indicate statistical differences.

Ex Met, Excess Methionine; Bet, Betaine; PL-SA, Phospholipid-Stearic acid

4. Discussion

Methionine is an indispensable amino acid used for protein synthesis as well as transsulfuration and transmethylation reactions (Robinson & Bertolo, 2016). However, the requirement for methionine depends on numerous factors such as mode of feeding and other nutrients in the diet. In the presence of excess cysteine, the pediatric methionine requirement during TPN has been shown to be 70% of the enteral requirement (Shoveller et al., 2003a). Methionine availability for protein as well as non-protein functions is affected by the presence of dietary methyl donors and consumers (Robinson & Bertolo, 2016). For example, substantial methionine is used to form PC via the PEMT pathway. The synthesis of PC from PE requires 3 mol of SAM to produce 1 mol of PC. Therefore, PC synthesis via the PEMT pathway relies highly on the methyl (and methionine) supply and has a higher methyl demand than the other transmethylation reactions. It is important to note that sufficient PC synthesis is required for hepatic VLDL secretion, and its impairment has been associated with fatty liver disease. Moreover, LCPUFA, especially DHA and AA, are critical to brain development in neonates and these fatty acids are preferentially incorporated into PEMT-derived PC (Pynn et al., 2011) and reach the peripheral tissues via VLDL. Thus, in order to enhance methionine and methyl availability, we sought to enhance methionine remethylation to enhance transmethylation. Betaine, a methyl donor, can remethylate homocysteine to form methionine and thus increase methionine availability, enhancing the flux towards transmethylation reactions. Betaine supplementation can also spare choline, which can be used for PC synthesis via the CDP-choline pathway. Both methionine and betaine have the potential to increase PC synthesis. However, dietary requirement for betaine is not yet established and it is unclear how betaine supplementation

affects the methionine cycle. We hypothesized that supplemental betaine could spare methionine and choline for PC synthesis, which in turn would enhance enrichment of PC with LCPUFA for distribution in circulation via VLDL. The main objectives of our study were to test the effect of betaine supplementation on alleviation of fatty liver and delivery of LCPUFA, particularly DHA and AA to the brain.

4.1 Fatty Liver and Lipid Parameters

NAFLD is a common and progressive liver disorder encompassing a broad spectrum of diseases ranging from simple fatty liver (steatosis) to cirrhosis and liver failure (Deminice et al., 2015; Dumitrascu & Neuman, 2018). PNALD is a sub-classification of NAFLD and its etiology is still an enigma (Kelly, 1998). In both cases, hepatic fat accumulation is one of the foremost markers of fatty liver development (Fon Tacer & Rozman, 2011) and the first hit of the two-hit model proposed by Day and James (Deminice et al., 2015; Fon Tacer & Rozman, 2011). Recently, impairment in lipid metabolism in fatty liver has been associated with lipoprotein abnormalities, particularly that of VLDL (Fon Tacer & Rozman, 2011). Specifically, insufficient PC synthesis leads to reduced VLDL secretion and consequent build-up of TG in the liver (Van Der Veen et al., 2012). Because of this recent hypothesis, we focused our investigation on overcoming limited PC synthesis in our piglet model of PNALD. Over the last few years, several studies have reported the hepatoprotective effect of betaine, in both alcoholic and non-alcoholic fatty liver disease (Abdelmalek et al., 2001; Kathirvel et al., 2010; Kawakami et al., 2012; Wang et al., 2014b). The beneficial effects of betaine are likely due to its methyl donating capacity, and subsequent increase in methionine and SAM facilitating further PC synthesis via PEMT (Deminice et al., 2015). Methionine supplementation can also enhance SAM and PC

synthesis; however, methionine supplementation can lead to hyperhomocysteinemia if homocysteine is not removed quickly enough. We compared betaine to methionine supplementation, where betaine is known to relieve hyperhomocysteinemia via remethylation of homocysteine to methionine.

Our data suggest that our piglets did not develop fatty liver. We measured total cholesterol and TAG in the plasma and liver of piglets with various treatments; there was no significant difference among the treatment groups. Additionally, no differences in total hepatic fat were found among the treatments. The hepatic and plasma lipid content were comparable to piglets fed a sow-fed diet, further suggesting that none of the TPN diets significantly altered lipid accumulation in the liver, which is similar to previously reported findings from our group (Dinesh et al., 2017). Hepatic lipid-induced complications associated with TPN tend to develop within two weeks of parenteral feeding and progress with the duration of TPN (Drongowski & Coran, 1989; Rayyan et al.; 2012; Guthrie et al., 2016; Vlaardingerbroek et al., 2014). However, our piglets were maintained on TPN for only 8 d, which could possibly be an insufficient time to observe overt effects of treatments on plasma and hepatic lipids. This is in line with a recent clinical study where short term TPN supplementation did not adversely affect the serum TAG in preterm infants (Rayyan et al., 2012). We had hypothesized that there would be detectable alterations in lipid metabolism in 8 days of TPN treatment, before overt fatty liver development. It should be noted that the lipid emulsion used in our study was SMOFlipid, which has been shown to reverse and prevent fatty liver, unlike the more traditional Intralipid that was associated with fatty liver (Rayyan et al., 2012; Vlaardingerbroek et al., 2014; Diamond et al., 2017). Thus, there has been a switch from Intralipid to SMOFlipid in neonates. We chose to

conduct our study using the more clinically relevant lipid emulsion SMOFlipid. We did not observe overt PNALD, likely because of short term exposure to TPN and/or because of the use of SMOFlipid.

The effect of betaine on VLDL secretion has been demonstrated in rodent model of fatty liver (Kharbanda et al., 2009). We had proposed that betaine supplementation would ameliorate fatty liver by increasing the synthesis of PC, which would then be readily available for the packaging of VLDL. The VLDL so formed is involved in the export of fat from the liver into the circulation. The increase in synthesis of PC by betaine supplementation has been suggested to occur due to enhanced remethylation of homocysteine to methionine, which would further make more SAM available for the donation of methyl groups to PE to form PC. Additionally, betaine supplementation has the potential to spare choline and make it available for PC synthesis by the CDP-choline pathway (Wang et al., 2013). The sparing effect of betaine on choline has been studied in chicks (Dilger et al., 2007) and supported the finding of increased choline concentration with betaine ingestion in mice (Wang et al., 2013). Increased choline levels have also been shown to mobilize the hepatic lipids via VLDL (Chandler & White, 2017). A recent study by Kharbanda et al. (2009) in mice fed an ethanol-containing diet demonstrated an increase in VLDL production rate by betaine supplementation (Kharbanda et al., 2009). These authors suggested that the enhanced VLDL production rate was a result of increased PC synthesis by the PEMT pathway caused by elevation of SAM levels through betaine ingestion. Several studies have reported that betaine supplementation increases hepatic SAM concentration, most likely by increased remethylation of homocysteine to methionine (Kawakami et al., 2012; Kharbanda, 2013; Kwon et al., 2009; Wang et al., 2010). Although

I did not measure VLDL secretion or SAM concentrations in my study, I would anticipate that under conditions of TPN induced fatty liver, betaine supplementation would increase VLDL secretion to prevent fatty liver. Alternatively, future studies should focus on measuring the concentrations of PC and choline in the plasma and liver, to provide further evidence in support of betaine's sparing effect.

Because there was no measurable accumulation of hepatic lipids, we failed to see any overt effect of parenteral betaine on the overall lipid profile in our piglets. TAG accumulation is the hallmark of NAFLD (Ducheix et al., 2017; Sookoian et al., 2016) and is considered the 'first hit' in the development of NAFLD (Kawakami et al., 2012). It has been reported that more than 90% of the plasma pool of TAG exists in the form of VLDL (Kharbanda et al., 2009). Thus, hepatic VLDL-TAG secretion can be reflected in the measurement of plasma TAG (Kharbanda et al., 2009). In my study, the plasma and hepatic TAG concentrations for the treatment groups did not differ from the control. We thus speculate that the VLDL secretion was normal and the dietary groups did not alter VLDL secretion. Although plasma total cholesterol is typically elevated in NAFLD (Malhi & Gores, 2008), our treatment groups did not differ from the control for hepatic or plasma total cholesterol concentrations, further suggesting that fatty liver likely did not develop. Additionally, no differences were observed among treatments for hepatic total fatty acids. Of particular interest were MUFAs (C18:1), which are storage fatty acids, and preferentially incorporated into TAG (Ibrahim et al., 2011). The latter was confirmed in a study where the absence of stearoyl-CoA desaturase-1 (SCD1), the enzyme which is involved in the formation of MUFA from SFA, led to reduced lipid accumulation (Li et al., 2009). The hepatic total MUFA did not differ among our treatment groups, further

suggesting that treatments did not affect TAG accumulation. This is not surprising because the source of lipid emulsion (SMOFlipid) used in our study was the same for all the treatments. SMOFlipid is a blend of pure soybean oil, medium chain triglycerides (MCT), olive oil and fish oil. Olive oil constitutes 25% of SMOFlipid and is a rich source of MUFAs. Previous studies have demonstrated the ability of MUFAs to inhibit TAG synthesis, as well as reduce hepatic apoB100 expression in mice with hepatic steatosis (Hussein et al., 2007; Lee et al., 2011). ApoB100, an important component of VLDL, is required for its assembly and secretion. Thus, MUFAs are involved in normal VLDL secretion and have been implicated in the prevention and treatment of NAFLD (Assy et al., 2009).

Moreover, most studies demonstrating the hepatoprotective effect of betaine supplementation were conducted on animals that were fed very high fat diets (Kwon et al., 2009; Wang et al., 2014b). A high fat diet is a well-established method of inducing hepatic lipid accumulation and consequent liver damage in both humans and animal models (Wang et al., 2014b). A study by Joselit et al. (2018) showed that the mitigating effect of betaine supplementation on hepatic fat accumulation was only seen in mice fed a high fat diet and not in those on a normal fat diet. The authors further suggested that this might be due to some unique interaction between the high fat diet and betaine supplementation leading to increased consumption of betaine's methyl donating capacity or a lipotrope during metabolic syndrome. Our animal model, as well as the composition of the administered diet, is clinically more relevant to study the effects of TPN-induced fatty liver; however, short-term TPN administration failed to induce fatty liver in our model. Moreover, the effects of betaine on lipid metabolism are known to be variable (Li et al., 2017). In humans,

plasma betaine concentrations have been associated with improved fat profiles (Zhao et al., 2018). Additionally, dietary betaine supplementation has been demonstrated to decrease body fat and apoB levels (Lever et al., 2005). On the contrary, a higher dose of betaine >4 g/d, has been reported to increase TAG, total cholesterol and LDL cholesterol in human subjects (Olthof et al., 2005; Schwab et al., 2002). In animal models, betaine ingestion has been negatively associated with body fat, decreasing TAG, LDL- and HDL-cholesterol (He et al., 2015; Huang et al., 2006). Furthermore, it was suggested by Konstantinova et al. (2008) that the effect of betaine might differ depending on its source as well as dose, duration and species.

Although studies have demonstrated positive outcomes associated with betaine in rodent models with liver abnormalities (Kawakami et al., 2012; Kharbanda, 2013; Kwon et al., 2009), only one study has directly looked at its effect on the VLDL production rate (Kharbanda et al., 2009). In this study, betaine was shown to correct the diminished VLDL secretion in a rodent model of fatty liver, via increased PC synthesis through the PEMT pathway (Kharbanda et al., 2009). Other studies have observed increased serum lipid concentrations with betaine supplementation and proposed that betaine was involved with increased export of hepatic lipids via VLDL (Deminice et al., 2015; Kharbanda et al., 2009; Olthof et al., 2005) . Similarly, betaine has been shown to increase the availability of ApoB, which is required for normal VLDL secretion (Ji & Kaplowitz, 2003; Sparks et al., 2006). Moreover, betaine's effect on the normalization of microsomal triglyceride transfer protein (MTTP) in hepatic steatosis, a gene important for VLDL lipidation, suggests that betaine plays an important role in hepatic lipid metabolism (Wang et al., 2014b). Hence, further research investigating the role of betaine in lipid metabolism, specifically VLDL packaging

and secretion needs to be conducted in the future, perhaps using an extended period of TPN administration to induce fatty liver.

4.2 Liver Fatty Acids and Diet

We investigated the effect of diet on fatty acid composition of hepatic total lipid and phospholipid in neonatal piglets. The liver fatty acid composition is a reflection of the dietary fat and *de novo* synthesis. We initially measured the hepatic total lipid fatty acid composition to investigate if the treatments caused any measurable changes in specific fatty acids. As expected, the hepatic fatty acid composition of total lipids was not different among the treatments; this was not surprising given that all the treatment groups received the same lipid source throughout the study. Our next question was to investigate whether treatments induce any changes in hepatic PC fatty acid composition. PC is the major phospholipid class for which the synthesis by either pathway (i.e., CDP-choline or PEMT) occurs in the liver. Similar to the total lipid fatty acid composition, the hepatic total phospholipid fatty acid profile did not differ significantly with any of the treatments. This result is consistent with the fact that all pigs were fed the same dose of dietary lipids.

To focus the analyses on our phospholipid of interest, we further tried to analyze the fatty acid composition of PC. Hepatic PC was isolated from hepatic total lipids by TLC, as described previously, and the fatty acid methyl esters of the PC fractions were analyzed by GC-FID. However, our initial chromatograms showed a few, very minute peaks, which were not integratable. Depending upon the sample availability, we increased the amount of lipid spotted on the TLC plate to the maximum possible, and repeated the TLC procedure. This time, the GC-FID was able to detect a few fatty acids, namely myristic acid, palmitic acid and stearic acid, with the other major fatty acid peaks not detected. This was possibly

due to the sensitivity limits of our instrument. Also, the amount of lipid spotted might be insufficient to be detected by GC-FID. As a result, we were unsuccessful in obtaining hepatic PC fatty acid profile.

Several studies have shown the effects of betaine on hepatic fat in NAFLD. However, no study to date has investigated the effects of parenteral betaine or methionine supplementation on hepatic fatty acid composition. Our findings suggest that neither betaine nor methionine affects the hepatic fatty acid composition in parenterally fed neonatal piglets, when SMOFlipid is their source of fatty acids.

4.3 Brain Fatty Acid Composition and Diet

Ours is the first study investigating the effect of parenteral betaine and methionine on brain fatty acid composition. We followed a similar approach as the liver in analyzing brain fatty acids, where we first measured the fatty acid profile of total lipids extracted from the brain, followed by phospholipid fatty acid composition. There was no significant effect of treatments on brain total lipid fatty acid composition. The lipid-rich composition of the brain could be one of the reasons for not being able to detect changes in individual fatty acids in total lipid extracts. Interestingly, there were significant effects of the treatments on brain phospholipid fatty acid composition, possibly due to preferential incorporation of PUFAs in brain phospholipids .

4.3.1 Effect of Treatments on Brain DHA

The beneficial effects of DHA in brain development in infants are well established (Innis, 2008). It is known that the dietary supply of methyl groups greatly affects the synthesis of PC via the PEMT pathway (Robinson et al., 2018). Moreover, PEMT-derived PC is particularly rich in LCPUFA, especially DHA and AA, with DHA occupying the sn2

position (Pynn et al., 2011). Multiple studies have been conducted to demonstrate the ability of betaine to spare choline as well as to improve methionine availability, both of which can increase PC synthesis (Robinson & Bertolo, 2016). We hypothesized that supplemental betaine could resynthesize methionine and spare choline for PC synthesis, which in turn would enhance the overall PC biosynthesis and incorporation of DHA-enriched PC into VLDL, subsequently delivering more DHA to the brain. The piglets receiving the betaine and/or methionine supplemented diets had higher concentrations of DHA in brain phospholipids, supporting our hypothesis. The fact that the PL-DHA was not significantly different between the excess methionine and betaine+excess methionine groups agrees with our hypothesis that betaine's effect was likely due to enhanced remethylation of homocysteine to methionine and SAM. As discussed previously, betaine has the potential to influence the plasma homocysteine pool by altering the SAM:SAH ratio. Studies have reported increased concentrations of SAM with betaine intake and this can be explained by the increase in remethylation of homocysteine to methionine (Kawakami et al., 2012; Kharbanda et al., 2009; Kwon et al., 2009; Wang et al., 2010). The increased SAM availability enhances transmethylation flux and further increases the synthesis of PC from PE, which uses methyl groups from SAM. Measurements of hepatic SAM and methionine concentrations would confirm this hypothesis, which will be measured in the future. A recent study by Joselit et al. (2017) in rodent dams fed a high fat diet supplemented with betaine found an increased mRNA expression of FABP7 and FABP5 in the fetal brains. Both of these transporters have been associated with the transport of DHA (Joselit et al., 2017; Pan et al., 2015); thus it will be important to investigate the effects of treatments on the mRNA expression of FABP7/5.

One limitation of our study is that we would need to measure PC-DHA in circulating VLDL to prove that enhanced brain DHA was a result of higher PC-DHA from PEMT. As previously discussed, PEMT-derived PC that is rich in DHA gets incorporated into VLDL during VLDL assembly, and exits the liver via VLDL secretion. The secreted VLDL containing the DHA esterified to PC is carried to the brain, where it is further hydrolyzed by lipases to form lyso-PC DHA. Lyso-PC DHA thus formed can be preferentially transported across the BBB by mfsd2a and gets incorporated into brain phospholipids. Thus, future experiments will focus on measuring PC-DHA in VLDL via LC-MSMS. We speculate that the betaine+excess methionine treated group would show highest PC-DHA in the VLDL when compared to the other groups since both betaine and methionine would be contributing to the synthesis of PC via the PEMT pathway. In general, we expect a trend in VLDL PC-DHA that corresponds to our current brain phospholipid-DHA findings. Moreover, the radiolabeled methionine and stable choline isotopes used in our study will also help us trace the two pathways for PC synthesis in the future.

Alternatively, recent studies have suggested that the pool of unesterified DHA in the plasma could represent the primary source of DHA for transport across the BBB, in contrast to lyso-PC (Chen et al., 2015). Unfortunately, because of limited samples, we were unable to quantify the plasma free fatty acid composition. Future studies involving gene expression of transporters involved in DHA transport and further investigating the uptake of DHA by brain might also provide us with additional data clarifying the mechanism of brain DHA accumulation after supplementing with betaine and methionine.

4.3.2 Effect of Treatments on Brain EPA

EPA is the precursor of DHA in the (n-3) pathway of synthesis (Fig. 1.3). However, there has been much debate over the differences in metabolism between EPA and DHA upon brain uptake (Chen et al., 2013). For example, the levels of EPA in the brain are consistently low and it has been suggested that this is due to rapid β-oxidation, reduced assimilation from the plasma unesterified pool, or reduced recycling within brain phospholipids (Chen et al., 2013). In a study by Chen et al. (2009), it was demonstrated that EPA undergoes mitochondrial β-oxidation 2.5-fold more readily than DHA when perfused with either radiolabeled EPA or DHA in situ. Furthermore, traces of radiolabeled saturated and monounsaturated fatty acids were found in rat brains treated with ^{14}C-EPA, suggesting that EPA gets catabolized via β oxidation to form acetyl CoA that can be used for fatty acid synthesis (Cunnane et al., 2003; Kaduce et al., 2008). Additionally, radiolabeled DPA (0.048% of the initial dose) and DHA (0.064% of the initial dose) were also found in brain phospholipids, suggesting that ^{14}C-EPA was partly involved in their syntheses (Kaduce et al., 2008). In another study by Chen and co-workers (2011), infusion of ^{14}C-EPA in rat brain was found to contribute only 0.03% to the brain phospholipid pool. Moreover, the same group demonstrated that there was rapid loss of the esterified EPA via de-esterification (Chen et al., 2011). This was attributed to the short half-life of EPA (5 days) compared to DHA (33 days) (Chen et al., 2011; DeMar et al., 2004). The short half-life of EPA can be further explained by the inefficient recycling of EPA (21% per day) compared to DHA (75% per day) (Chen et al., 2013). Furthermore, the conversion of EPA

into bioactive lipid mediators, for example, resolvins, might also contribute to the differences in the concentrations of these two fatty acids in the brain.

Unlike EPA, which contributes little to the phospholipid pool in the brain, the major proportion of radioactivity was found to be intact in the phospholipid DHA in the rat brain when radiolabeled DHA was infused intracerebroventricularly (DeMar et al., 2004). Moreover, the loss of DHA in the brain is slow when compared to EPA, due to a longer half-life when compared to EPA (Calder, 2016; DeMar et al., 2004). Also, DHA is preferentially transported to the brain by some carriers, and it is still unknown if any such selective carrier exists for the uptake of EPA.

An interesting finding of our study was that the PL-EPA in the treatment groups was significantly lower than that in the control group, which is in contrast with PL-DHA. A possible explanation may be the fact that EPA esterification to brain phospholipids from the plasma unesterified pool is one-third of that for DHA (Chen et al., 2013) and its de-esterification is more rapid than DHA (Chen et al., 2011). However, the EPA in the brain total lipid was not different across the treatments suggesting that de-esterification of EPA might be the possible cause for such contrasting data. Another noteworthy point is that our data are for total phospholipids in the brain, which is mostly PC, whereas EPA is found to be preferentially incorporated into the phosphatidylinositol class of phospholipids (Chen et al., 2011); thus future studies should involve investigating the effects of treatment on the fatty acid composition of individual phospholipid classes.

It should be noted that our EPA values are slightly higher than those reported for rodents (Chen et al., 2013). Although this could be a species difference, it is also important to note that previous studies showing low levels of accumulated EPA in the brain were

conducted using labeled unesterified EPA, whose entry into the brain is via diffusion (Chen et al., 2015; Yalagala et al., 2018). However, as previously discussed for DHA, recent findings have suggested a potential role of mfsd2a in the transport of PUFAs into the brain (Nguyen et al., 2014). Mfsd2a is known to be the major carrier of PUFA in the form of lyso-PC. A more recent study has suggested a 100-fold increase in brain EPA levels in mice by lyso-PC EPA via mfsd2a (Yalagala et al., 2018). The source of EPA in our piglet diet was the fish oil from SMOFlipid. Fish oil supplementation has been shown to increase plasma lyso-PC EPA (Ottestad et al., 2012). Analogously, intravenous infusion of EPA in the form of fish oil in lipid emulsions has been demonstrated to enrich blood lipids, the majority of which is PC (Al-Taan et al., 2013). However, there are also reports showing that brain EPA levels are unaltered by fish oil or EPA enriched supplements (Yalagala et al., 2018). To summarize, there are conflicting data regarding the form of EPA uptake by the brain, which further affects the brain EPA level. Further research needs to be conducted to identify the sources and mechanism of EPA enrichment in the brain. Liver EPA levels remain unchanged across all the treatment groups in our study; however, we did observe dramatic changes in the brain EPA levels and until the mechanism of transport is clarified, we can only speculate on why this is higher in our model.

4.3.3 Effect of Treatments on Brain AA

The brain has its own unique fatty acid composition (Chen et al., 2011; 2013), with arachidonic acid representing 50% of the brain PUFA (Liu et al., 2015). Arachidonic acid is essential to brain growth and development and is an important regulator of synaptic function (Darios & Davletov, 2006; Liu et al., 2015; Nishizaki et al., 1999). Indeed, AA is involved in neuroinflammation through the formation of its eicosanoids

(Funk, 2001) and disturbed AA metabolism with higher levels of brain incorporation of AA have been associated with the progression of Alzheimer's disease (Thomas et al., 2016), although contradictory results exist (Amtul et al., 2012; Hosono et al., 2015). However, the role of higher brain AA concentration in neurological development is unknown.

Like DHA, AA tends to be incorporated into phospholipids (Chalil et al., 2018), particularly phosphatidylinositol (Lee et al., 2012). Moreover, as with DHA, the PEMT-derived PC, which is the chief carrier for exporting PUFA from the liver to peripheral tissues, is also enriched with AA (Pynn et al., 2011).

As hypothesized, the supplementation of betaine increased the concentration of AA in the brain phospholipids. These data suggest that supplementation with betaine enhanced PC synthesis, increasing AA availability for esterification with PUFA, and further integration into VLDL for secretion into the circulation. Interestingly, the betaine group showed significantly higher accretion of AA than the excess methionine group, supporting betaine's significant role as a methyl donor to enhance SAM production. Thus, there appears to be enhanced export of the PL-bound AA to the brain via increased PC synthesis and subsequent incorporation of PUFA-rich PC into VLDL.

4.3.4 Effect of Diet on SFA Composition of the Brain

The synthesis of SFAs in the developing brain is mainly by de novo synthesis (Edmond et al., 1998). Additionally, transport of SFA from the circulation to the brain is also possible (Bourre et al., 1979). During development, SFAs rapidly accumulate and form MUFAs, accompanied by an increased activity of stearoyl CoA desaturase (DeWille & Farmer, 1992). MUFAs are essential for phospholipid synthesis, which in turn is required

for the development of neuronal membrane (Lengi & Corl, 2015) as well as myelin sheath formation.

There is some evidence demonstrating the positive effects of betaine in brain development. Betaine supplementation in homocysteinuric patients has been associated with normal brain structure and myelination in MRI scans (Engelbrecht et al., 1997; Jadavji, 2014). Also, in the elderly, betaine has been related to improved performance of construction, executive functioning and sensimotor speed (Jadavji, 2014). Interestingly, a study by Joselit et al. (2017) discovered that FABP5 mRNA expression was greatly increased in the fetal brain when high fat fed dams were supplemented with betaine. FABP5 is associated with the transport of SFA in the fetal brain (Joselit et al., 2017).

In our study, we observed that parenteral betaine led to more accretion of palmitic acid (C16:0) and stearic acid (C18:0) in brain phospholipids, compared to the control group. Rapid accretion of SFA in early life is critical to myelination and thus important during infant brain development (Edmond et al., 1998). There were no differences in the MUFA among our treatment groups. Interestingly, studies have shown that the PEMT-synthesized PC is predominantly enriched with C16:0 and C18:0 at the sn-1 position (Pynn et al., 2011). These findings support our hypothesis that betaine supplementation might have increased overall PC synthesis, which led to the accretion of SFA (C16:0 and 18:0) in the brain of piglets receiving parenteral betaine supplementation. Similar findings with betaine supplementation have been observed in the muscle tissue of pigs and was associated with improved meat quality (Hur et al., 2007; Hwang et al., 2010; Rojas-Cano et al., 2011). Ours is a novel finding highlighting the importance of future research to understand the mechanism behind betaine induced changes in brain fatty acids.

4.4 Conclusion and Future Directions

We hypothesized that betaine supplementation would both enhance methionine re-synthesis and spare choline for increased PC synthesis. The higher PC synthesis via PEMT would also result in more LCPUFA bound to PC which would be circulated via VLDL. We concluded that parenteral betaine improves accretion of DHA and AA in the brain. Interestingly, we also found the betaine supplementation increased the accretion of SFAs, particularly stearic acid and palmitic acid in the brain. Our data suggest that betaine may be a novel TPN ingredient for the neonate which may be used for brain development and to enhance methionine availability, which might be currently limiting in TPN solutions for optimal distribution of DHA and AA in the brain.

Interestingly, there brain phospholipid DHA and EPA responded oppositely to all treatments, which requires further investigation to understand the mechanisms. As discussed previously, this may be due to the different metabolism and brain uptake mechanisms that these fatty acids undergo. It would also be of interest to assess which pathway of PC synthesis contributed more to the accretion of specific fatty acids in the brain since betaine has the capacity to spare choline for the CDP-choline pathway and re-synthesize methionine for the PEMT pathway. Isotope tracer techniques using labeled methyl-methionine and choline will be used to delineate the roles of the two pathways in the future.

The mechanism by which betaine improves the accretion of fatty acids in the brain is still an enigma. Gene expression studies to measure the expression of transporters responsible for carrying specific fatty acids to the brain, and across the blood-brain barrier may provide further insights on the accretion of fatty acids in the brain. Overall, our

findings support that betaine should be included in parenteral nutrition in order to increase the accretion of LCPUFA for neonatal brain development.

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Appendices

Appendix I: Amino acid profile of experimental diets

	Control g/L	Betaine g/L	Ex Met g/L	Bet+Ex Met g/L
Alanine	5.89	5.89	5.41	5.41
Arginine	3.65	3.65	3.65	3.65
Aspartate	3.32	3.32	3.32	3.32
Cysteine	0.76	0.76	0.76	0.76
Glutamate	5.72	5.72	5.72	5.72
Glycine	1.47	1.47	1.47	1.47
Histidine	1.69	1.69	1.69	1.69
Isoleucine	2.51	2.51	2.51	2.51
Leucine	5.67	5.67	5.67	5.67
Lysine	5.58	5.58	5.58	5.58
Methionine	1.04	1.04	1.84	1.84
Phenylalanine	3	3	3	3
Proline	4.52	4.52	4.52	4.52
Serine	3.11	3.11	3.11	3.11
Taurine	0.27	0.27	0.27	0.27
Tryptophan	1.14	1.14	1.14	1.14
Tyrosine	0.44	0.44	0.44	0.44
Valine	2.89	2.89	2.89	2.89
Threonine	2.23	2.23	2.23	2.23
Betaine	0	1.13	0	1.13
Glycyl-tyrosine Dihydrate	1.631	1.631	1.631	1.631

Appendix II: D-glucose and major mineral concentrations in all diets

	g/L	g/kg/day
Dextrose	90.3	24.56
K2HPO4 Trihydrate	1.57	0.427
KH2PO4 Monobasic	1.085	0.295
K Acetate	1.47	0.3998
NaCl	2.17	0.5902
MgSO4	0.78	0.2122
ZnSO4	0.089	0.0242
Calcium Gluconate	6.41	1.744

Appendix III: Trace element concentrations in all diets

Element	Mineral Supplied	mg/kg/day	g/L of diet
Zinc	ZnSO4 7H2O	10.09	40.78
Copper	CuSO4 5H2O	0.86	3.12
Manganese	MnSO4 H2O	0.66	1.89
Chromium	CrCl3 6H2O	0.01	0.05
Selenium	SeO2	0.05	0.06
Iodine	NaI	0.02	0.02

Appendix IV: Multi-vitamin mix composition

Vitamin	Amount in 4 mL of vial 1	Dose (per kg/day)
Ascorbic acid (vitamin C) (mg)	80	17.41
Vitamin A (as palmitate) (IU)	2300	500.48
Vitamin D3 (as cholecalciferol) (IU)	400	87.04
Thiamine (vitamin B1) (as hydrochloride) (mg)	1.2	0.26
Riboflavin (vitamin B2) (as riboflavin-5-phosphate) (mg)	1.4	0.3
Pyridoxine HCl (vitamin B6) (mg)	1	0.22
Niacinamide (mg)	17	3.7
Dexpanthenol (as d-pantothenyl alcohol) (mg)	5	1.1
Vitamin E (dl-a-tocopheryl acetate) (IU)	7	1.52
Vitamin K1 (mg)	0.2	0.04
	Amount in 1 mL of vial 2	
Folic acid (µg)	140	4.35
Biotin (µg)	20	30.46
Vitamin B12 (cyanocobalamin) (µg)	1	0.22

Inactive ingredients in vial 1: 50mg polysorbate 80, sodium hydroxide and/or hydrochloric acid for pH adjustment.

Inactive ingredients in vial 2: 75mg mannitol, citric acid and/ or sodium citrate for pH adjustment.

Vial 2 was mixed with vial 1 prior to use, and 3mL was added to 750mL of diet to deliver the appropriate vitamin dose.

Appendix V: Fatty acid composition of SMOFlipid (Fresenius Kabi, Uppsala, Sweden)

Fatty acid	%
Oleic acid	23-35
Linoleic acid	14-25
Caprylic acid	13-24
Palmitic acid	7-12
Capric acid	5-15
Stearic acid	1.5-4
α-Linolenic acid	1.5-3.5
Eicosapentaenoic acid	1-3.5
Docosahexaenoic acid	1-3.5

Appendix VI: Amino acid profiles of experimental infusion diets

	Control g/L	Betaine g/L	Ex Met g/L	Bet. + Ex Met. g/L
Alanine	5.89	5.89	5.41	5.41
Arginine	3.65	3.65	3.65	3.65
Aspartate	3.32	3.32	3.32	3.32
Cysteine	0.76	0.76	0.76	0.76
Glutamate	5.72	5.72	5.72	5.72
Glycine	1.47	1.47	1.47	1.47
Histidine	1.69	1.69	1.69	1.69
Isoleucine	2.51	2.51	2.51	2.51
Leucine	5.67	5.67	5.67	5.67
Lysine	5.58	5.58	5.58	5.58
Methionine	0.91	0.91	1.71	1.71
Phenylalanine	3	3	3	3
Proline	4.52	4.52	4.52	4.52
Serine	3.11	3.11	3.11	3.11
Taurine	0.27	0.27	0.27	0.27
Tryptophan	1.14	1.14	1.14	1.14
Tyrosine	0.44	0.44	0.44	0.44
Valine	2.89	2.89	2.89	2.89
Threonine	2.23	2.23	2.23	2.23
Betaine	0	1.13	0	1.13
Glycyl-tyrosine Dihydrate	1.631	1.631	1.631	1.631