Long-term programming effects of feeding total parenteral nutrition to Yucatan miniature pigs during the neonatal period.

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Abstract

Early programming of adult diseases was initially described by Barker and colleagues and refers to long-term outcomes of nutritional or environmental insults in early life. Many epidemiological studies show a relationship between early nutrition and the risk of chronic diseases such as cardiovascular disease (CVD), type 2 diabetes, and metabolic syndrome. Changes in early nutrition during a critical period of life program the individual's health in adulthood, likely mediated via epigenetics. A key epigenetic mechanism is DNA methylation, and the availability of methyl nutrients during critical periods of early life is likely to alter the epigenome, thereby programming gene expression and risk for chronic diseases in adulthood. Metabolic syndrome is defined by the World Health Organization as a chronic pathogenic condition characterized by abdominal obesity, insulin resistance, hypertension, and dyslipidemia and has a high prevalence rate worldwide. Total parenteral nutrition (TPN) is a non-normal nutrition regimen given to infants who cannot tolerate oral feeding, commonly prescribed for preterm and intrauterine growth restricted (IUGR) infants as an essential part of medical management. Although TPN is a lifesaving feeding method, it exerts significant metabolic stress, and if this stress occurs during the epigenetic 'window' of programming, the metabolism can be programmed and increase the risk of adult diseases. Thus, modulation of methyl nutrients in TPN to prevent epigenetic programming and reduce the risk of adult diseases has great potential to improve the quality of life in infants who need TPN in early life as a life-saving measure. Although there is a wealth of knowledge on developmental programming, the metabolic consequences of being IUGR, and the acute effects of TPN feeding, the long-term effects of feeding TPN to infants and IUGR neonates are unknown. We hypothesized that the metabolic effects of feeding TPN in early life would persist into adulthood, increasing the risk of developing metabolic syndrome and that supplementing betaine and creatine, which are novel ingredients in TPN, would prevent this development. Twenty-four

female piglets (7 d old) were randomized to sow-fed (SowFed), TPN control (TPN-control), TPN with betaine and creatine (TPN-B+C); eight intrauterine growth restricted (TPN-IUGR) piglets fed TPN formed a fourth group. After 2 weeks of treatment, all pigs were fed a grower diet for 8 mo. At 9 mo, an arterial blood pressure telemeter and central venous catheters were implanted to conduct metabolic tests. TPN-IUGR pigs grew faster, and body measurements were not different among groups at 9 mo of age. Our findings indicate that feeding TPN in the neonatal period led to dyslipidemia in adulthood, as indicated by higher postprandial triglyceride (TG) levels in TPN-control pigs ($P<0.05$) compared to SF. Adding betaine and creatine to early TPN (TPN-BC) (to enhance methyl group availability) lowered mean arterial pressure ($P<0.01$) and systolic arterial pressure ($P<0.01$) in adulthood, compared to the TPN-control, thus reducing the risk of hypertension. IUGR piglets were particularly sensitive to TPN feeding effects as TPN- IUGR led to development of obesity and dyslipidemia in adulthood as revealed by a higher backfat thickness ($P<0.05$), elevated fasting non-HDL cholesterol ($P<0.01$), higher fasting non-esterified fatty acids ($P<0.001$), higher liver TG ($P<0.05$) and slower postprandial TG clearance ($P<0.05$) compared to TPN-control. Collectively, these data suggest feeding TPN in early life increases the risk for the development of biomarkers of metabolic syndrome in adulthood, especially in IUGR neonates, and supplementing betaine and creatine to TPN might reduce this risk.

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"The noblest pleasure is the JOY of understanding"- Leonardo da Vinci.

I am glad I chose to pursue a Ph.D.

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AA - arachidonic acid

ACC - acetyl-CoA carboxylase

ACE - angiotensin-converting enzyme

AGT - angiotensinogen

ALT - aspartate aminotransferase

ARA - arachidonic acid

AT1R - angiotensin type 1 receptor

AT2R - angiotensin type 11 receptor

BHMT - betaine-homocysteine methyltransferase

CETP - cholesterol ester transfer protein

CGL - cystathionine gamma-lyase

CHD - coronary heart disease

CM - chylomicrons

CpG - cytosine-guanine dinucleotides

CVD - cardiovascular disease

DHA - docosahexaenoic acid

DMG - dimethylglycine

DNMT - DNA methyltransferases

DOHaD - developmental origins of health and disease

FASN - fatty acid synthase

FFA - free fatty acids

GAA - guanidinoacetic acid

GGT - gamma-glutamyl transferase

GR - glucocorticoid receptor

HDL - high-density lipoprotein

IDL - intermediate-density lipoprotein

IGF2- insulin growth like factor 2

IL-6 - interleukin 6

IUGR - intrauterine growth restriction

IVGTT - intravenous glucose tolerance test

LCAT - lecithin-cholesterol acyltransferase

LC-PUFA - long-chain polyunsaturated fatty acids

LDL - low-density lipoprotein

LDL-r - low-density lipoprotein receptor

LPL - lipoprotein lipase

MS - methionine synthase

MTTP - microsomal triglyceride transfer protein

NCD - noncommunicable diseases

PC - phosphatidylcholine

PE - phosphatidylethanolamine

PEMT - phosphatidylethanolamine methyl transferase

PNALD - parenteral nutrition-associated liver disease

PPAR - peroxisomal proliferator-activated receptor

PPL - postprandial lipemia

RAS - renin angiotensin system

SAH - s-adenosylhomocysteine

SAM - s-adenosylmethionine

SCD - stearoyl-CoA desaturase

SMA - superior mesenteric artery

SR-B1 - scavenger receptor class B type 1.

SREBP1c - sterol regulatory element-binding protein 1c

TG - triglyceride

THF - tetrahydrofolate

TNF - tumor necrosis factor

TPN - total parenteral nutrition

TRLs - TG-rich lipoproteins

VLBW - very low birth weight

VLDL - very low-density lipoprotein

WHO - World Health Organization

1 Chapter 1- Introduction

1.1 Developmental origins of health and disease (DOHaD)- Concept

The Developmental Origins of Health and Disease (DOHaD) hypothesis proposes that exposure to certain environmental stimuli, such as non-normal nutrition, infections, chemicals, metabolite, or hormonal perturbations during critical periods of development and growth, may have significant consequences for an individual's short- and long-term health (Hoffman et al., 2017; Mandy & Nyirenda, 2018). When a developing fetus is exposed to such an environmental stimulus, it acquires 'adaptations' to improve immediate viability, which can re-emerge if the individual is confronted with a similar environmental stimulus. These adaptations are known as 'predictive adaptive responses,' and they include down-regulation/alteration of endocrine, metabolic, and organ function (Mandy & Nyirenda, 2018). These processes occur through disruptions in gene expression, cell differentiation, and proliferation with the goal of slowing the fetus's growth rate to match the nutrient supply in the uterine environment. As a result, long-term irreversible changes in vital organ development, structure, and function may occur. However, if the extrauterine environment is the opposite of that in utero, these individuals are predisposed to a higher risk of diseases in adulthood, exacerbated by postnatal life (Mandy & Nyirenda, 2018).

1.1.1 Role of nutrition in early programming

Amongst various environmental insults in early life, nutrition holds a great significance. Nutrition is a critical part of health and development, and undernutrition and overnutrition directly affect an individual's mental and physical state. Variations in the quality or quantity of nutrients consumed during critical windows of development (i.e., during pregnancy and infancy during the first year of life) can permanently affect developing tissues (Jiménez-Chillarón et al., 2012). This is because pregnancy and infancy are characterized by rapid organ growth, development, and maturation. Early studies of nutritional programming suggest that growth over the first year of life is an indicator of risk for disease in adulthood and that the key drivers of programmed diseases are poor fetal growth followed by rapid catch-up growth in infancy and childhood (Eriksson et al., 1999, 2001; Forsén et al., 1999).

In infancy, breastfeeding or formula feeding is the principal mode by which a newborn receives nutrition (Karmaus et al., 2017). However, there are other non-normal nutrition regimens that are also used to support infants who are unable to ingest food via the gastrointestinal tract for medical reasons (Lucchinetti et al., 2021). Moreover, an optimal diet is essential for enhancing the short- and long-term health of infants, children, and mothers. A substantial amount of research has shown that variations in the quality or quantity of nutrients available to a developing fetus during the perinatal period have long-lasting impacts on health (Langley-Evans, 2015).

1.1.2 Critical window of programming

The effects of various nutrition modifications on a wide range of critical programming periods, including periconception, pregnancy, and infancy to childhood, have been extensively studied. The periods when the fetus is vulnerable to plasticity and is sensitive to environmental

stimuli are called a critical window of development (Hoffman et al., 2017; Mandy & Nyirenda, 2018). For example, periods of rapid cell proliferation in the fetus and rapid growth during the postnatal period are critical windows. The period from periconception to the end of the first year is the most critical window of human development during which metabolic programming occurs (Vickers, 2011). However, each part within this critical window is uniquely significant in programming. The timing and duration of dietary disturbances contribute to programmed variations in the physiology and metabolism of the offspring, leading to permanent health impacts (Randunu & Bertolo, 2020). These programmed variations occur via epigenetics, which will be discussed later in this thesis. Besides the timing and duration of dietary disturbances, the target tissue in which programming occurs is also important in identifying programmed diseases later in life. For example, kidneys are more susceptible to programming during the prenatal phase of nephrogenesis (Langley-Evans et al., 1999). In contrast, the critical brain development period extends well into childhood due to the continued postnatal formation of neural connections (Plagemann et al., 2000a). Therefore, dietary perturbations that occur in the prenatal and/or postnatal phases differently affect the organs and, therefore, the diseases programmed in adulthood. Thus, adequate and appropriate nutrition during this critical period of life significantly impacts growth and development in early life and the health of the individual throughout life. However, most studies on nutritional programming, especially in rodents, have examined nutritional exposure throughout the prenatal (gestation) or both prenatal and postnatal (lactation) periods, making it difficult to separate the impact of these nutrients during different key windows (Randunu & Bertolo, 2020).

1.1.3 Intrauterine growth restriction and DOHaD

Many studies on DOHaD have revealed the consequences of maternal malnutrition on adult disease development. Intrauterine growth restriction (IUGR) is a condition in which the fetus does not reach its genetically assigned maximum size (Peleg et al., 1998). Eight to ten percent of pregnancies have been found to have IUGR, and depending on the timing of the damage, IUGRs are categorized as symmetric or asymmetric (Canadian Institute for Health Information., 2009; Peleg et al., 1998). Weight, head circumference and length are less than 10 centiles in symmetrical IUGR, whereas only weight is less than 10 centiles and the rest is as per gestational age in asymmetrical IUGR (Sharma et al., 2016). Although genetic, placental, and fetal factors play a role in IUGR, maternal malnutrition has been identified as the leading cause (Sharma et al., 2016). IUGRs can have significant short- and long- term implications on health through programming effects, predisposing individuals to long-term diseases (MacKay et al., 2012; Myrie et al., 2011, 2017). It is hypothesized that hemodynamic and metabolic modifications are made in IUGR to compensate for the unfavourable uterine environment, leading to permanent alterations in the organ's function and structure (Priante et al., 2019). This is because if postnatal environmental conditions change, the IUGR's response might be inadequate, increasing the risk of long-term repercussions (Maršál, 2018). For example, IUGR lowers blood flow to the kidneys and gastrointestinal tract while protecting blood flow to vital organs such as the brain, heart, and adrenal glands (Maršál, 2018); some of these alterations have been shown to persist into adulthood. Therefore, IUGR neonates are susceptible to numerous detrimental short-term and long-term metabolic changes. IUGR is also associated with endothelial dysfunction, increased aortic intima-media thickness, higher insulin sensitivity at birth, rapid postnatal development, insulin resistance with decreased insulin levels, and abnormalities in fatty acid metabolism, all of which increase the

risk of cardiovascular disease (CVD) (Cosmi et al., 2009; Lee et al., 2010; Longo et al., 2013; Muhlhausler et al., 2009; Norman, 2008).

It appears that the existence of low-fat mass in relation to lean mass and delayed skeletal muscle development in IUGR newborns may produce short-term impairments, while altered programming throughout the critical window of early life may cause long-term perturbations (Padoan et al., 2004; Yates et al., 2012). Reduced glycogen stores, poor gluconeogenesis, limited fat stores, and decreased counter-regulatory hormone response result in hypoglycemia, inability to oxidize free fatty acids, failure to spare tissue glucose, and hyperinsulinemia in IUGR newborns (Longo et al., 2013). A greater wealth of research demonstrates that IUGR neonates are at a higher risk of developing metabolic syndrome, which eventually leads to the development of CVD (Chernausek, 2012a). Furthermore, research conducted by our lab has demonstrated that IUGR neonates experienced catch-up growth, resulting in increased adiposity and poor lipid metabolism in adulthood (Myrie et al., 2017). According to these studies, IUGR pigs fed a high-salt, high-sugar, high-saturated- and trans-fat diet had elevated biomarkers for obesity, metabolic syndrome, cardiovascular disease, hypertension, dyslipidemia, and diabetes (Barker et al., 1989; Hales et al., 1991; Myrie, MacKay, et al., 2012; Myrie, McKnight, et al., 2012). Furthermore, human IUGR newborns demonstrated higher cholesterol production at ages 8-12 years, rapid growth at ages 6-8 years, and elevated blood pressure at ages 6-8 years of age (Mortaz et al., 2001; Singhal et al., 2007). These chronic disorders in IUGR occur due to irreversible changes in anatomy, physiology, and metabolism, which were likely caused by early-life insults. Together, these findings demonstrate that IUGRs are predisposed to short- and long-term metabolic disorders and that nutritional insults during the critical time may have contributed to the plasticity leading to altered metabolism and the development of adult chronic diseases.

According to reports, prenatal programming for insulin resistance, epigenetic changes

(described in section 1.2), and postnatal catch-up growth are implicated in developing metabolic syndrome in IUGR (Longo, 2013; Berends et al., 2013; Zhu et al., 2019; Chernausek, 2012). For example, Chernausek et al. (2012) suggest a specific epigenome signature in IUGR; nonetheless, the link between programmed genes responsible for growth and metabolism in IUGR is poorly understood (Chernausek, 2012b). Although the complete picture is not yet clarified, Einstein et al. (2010) demonstrated an epigenetic pathway connecting IUGR with adult-onset type-2 diabetes (Einstein et al., 2010), and Morgan et al. (2010) demonstrated a connection between a single nucleotide polymorphism and birth weight in obesity and diabetes-related genes (Morgan et al., 2010). Together, these findings indicate that IUGR is associated with an increased risk of developing chronic diseases later in life and that epigenetics may play a significant role in programming.

1.1.4 Importance of the postnatal period as a critical window

Early programming effects are exemplified by maternal malnutrition during pregnancy, which results in low birthweight infants due to reduced fetal growth. Similar to maternal nutrition, early postnatal nutrition plays a significant role in the programming of diseases in adulthood, as organ maturation continues into the early postnatal period. The body's metabolic and functional changes are governed by the hypothalamus, adipose tissue, liver, kidneys, and gut, as well as its hormonal axis. Because significant brain maturation occurs in the postnatal period in humans and many animal models, early postnatal life is just as important for programming as the prenatal period (McKee & Reyes, 2018). Many studies have demonstrated the isolated effects of postnatal nutrition on programming. Numerous human studies have demonstrated that immediate postnatal nutrition is a major determinant of noncommunicable diseases (NCD) such as atherosclerosis,

insulin resistance, and hypertension, particularly in preterm newborns. Breast-fed newborns had fewer atherosclerosis risk factors than formula-fed infants and were less likely to develop obesity later in life (Rich-Edwards et al., 2004; Singhal et al., 2004a). Similarly, preterm neonates with extremely low birth weight (VLBW) who consumed more energy and breast milk during the postnatal period had better health as adults (Sammallahti et al., 2017). Fasting insulin levels in young adults are influenced by the postnatal diet in very low birth weight (VLBW) infants (Suikkanen et al., 2018). A recent study found that postnatal nutrition during the first 1 to 8 weeks impacted the blood pressure of children aged 6.5 years who were born extremely prematurely (Zamir et al., 2019). Together, these studies demonstrate the importance of postnatal nutrition to long-term health and, most critically, its role in defining neonatal disease and long-term health outcomes after preterm birth. Similar to human studies, animal studies have also highlighted the impact of postnatal diet on the risk of obesity in adulthood (Patel & Srinivasan, 2011; Vogt et al., 2014). For instance, a rodent study revealed that the long-term obesity of rat pups was determined by their altered nutrition during lactation (Desai et al., 2014). A study found that a mother's diet during breastfeeding had a higher impact on the metabolic phenotype of her offspring than her diet during pregnancy (Sun et al., 2012). All these studies demonstrate that the postnatal period is a critical phase of development and that nutrition during the postnatal period has a significant impact on the health of the individual in adulthood. Hence, modifying nutrition during the "critical window" of development has a significant impact on diseases later in life and is likely involved in their programming.

1.2 Mechanisms of DOHaD – Epigenetics

Although the precise mechanisms are unknown, some critical mechanisms have been proposed to explain the effects of DOHaD. Some of them are epigenetic mechanisms, cellular stresses, metabolic adaptations, alterations to the microbiome, and social determinants (Hoffman et al., 2017). Specific changes, like epigenetic changes, are activated during the perinatal insult, whereas others, such as endoplasmic reticulum stress, influence metabolic diseases postpartum (i.e., during catch-up growth). Among these possible causes of how early nutritional insults program adult chronic diseases, epigenetics has been identified as the leading mechanism (Lillycrop & Burdge, 2015; McGee et al., 2018; Vickers, 2014). Epigenetics involves heritable changes in gene expression mediated by extracellular mechanisms that act on DNA without changing its sequence (Bird, 2007). For example, cell differentiation and organogenesis are controlled by epigenetic factors through variable regulation of gene expression. Epigenetic processes immediately modify gene expression in a tissue- and gene-specific manner to adjust to developmental plasticity windows, explaining many of the long-term repercussions of these perinatal insults. These epigenetic alterations can affect gene expression postnatally, too, causing metabolic diseases. Global, tissue or site-directed epigenetic alterations may cause various metabolic abnormalities corresponding to the scope of perinatal insults (Hoffman et al., 2017). The main epigenetic mechanisms involved in developing and differentiating various cell types are microRNA (noncoding RNAs), covalent histone modification, and DNA methylation. These epigenetic processes provide marks on the genome, which are responsible for activating or silencing genes leading to a specific phenotype (Bird, 2007). Thus, they are ultimately responsible for determining phenotypic plasticity (Margueron & Reinberg, 2010). However, studies conducted in this thesis did not address epigenetic mechanisms.

The interaction of nutrients with epigenetics is termed "nutri-epigenomics," where nutrients and their effects on health are mediated by epigenetic modifications. One of the best examples of this concept is the development of a honeybee with identical genomes into a queen or a worker depending on the different diets they feed on royal jelly for a queen or a diet of pollen or nectar, for the worker (Chittka & Chittka, 2010). Most of the epigenetic changes that take place during gametogenesis seem to be erased after a certain point of development. It is crucial to understand how epigenetic marks become heritable, as many marks are not completely erased during very early development and gametogenesis (Fan & Zhang, 2009; Trasler, 2009). Some methylated sites survive and replicate during cell division, and the marked DNA is passed along with the histones, leading to persistent influence of these marks on gene expression throughout life (Guibert et al., 2012). Moreover, many epigenetic changes occur throughout development, pre- and postnatally, and are preserved in mitotic cell division, providing the mechanism by which any epigenetic perturbation during development can persist into adulthood unchanged. Therefore, epigenetic modifications due to early nutritional exposures that affect the offspring's phenotype are not only taking place via germline modification but also mitotically in somatic cells with long-term effects on gene expression (Skinner, 2011).

1.2.1 DNA methylation

Particularly relevant to nutri-epigenomics, DNA methylation of promoter regions of genes is a key focus of research. DNA methylation is an epigenetic mechanism where methyl groups are added to DNA; DNMTs (DNMT3A, DNMT3b, DNMT1) catalyze the hydroxymethylation of carbon five in cytosine of the cytosine guanine dinucleotide (CpG). This methylation modifies the function of a gene, affecting its expression by (usually) inhibiting transcription (Figure 1.1). Differential methylation of promoter regions of various genes seems to be responsible for

the plasticity associated with early programming. Nutrients affecting either S-adenosylmethionine (SAM), the universal methyl donor, or S-adenosylhomocysteine (SAH), an inhibitor of methyltransferases, have the potential to modify methylation, and hence expression, of DNA (Tammen et al., 2013). Thus, dietary availability of methyl nutrients (choline, betaine, folate, methionine) influences DNA methylation.

DNA Methylation

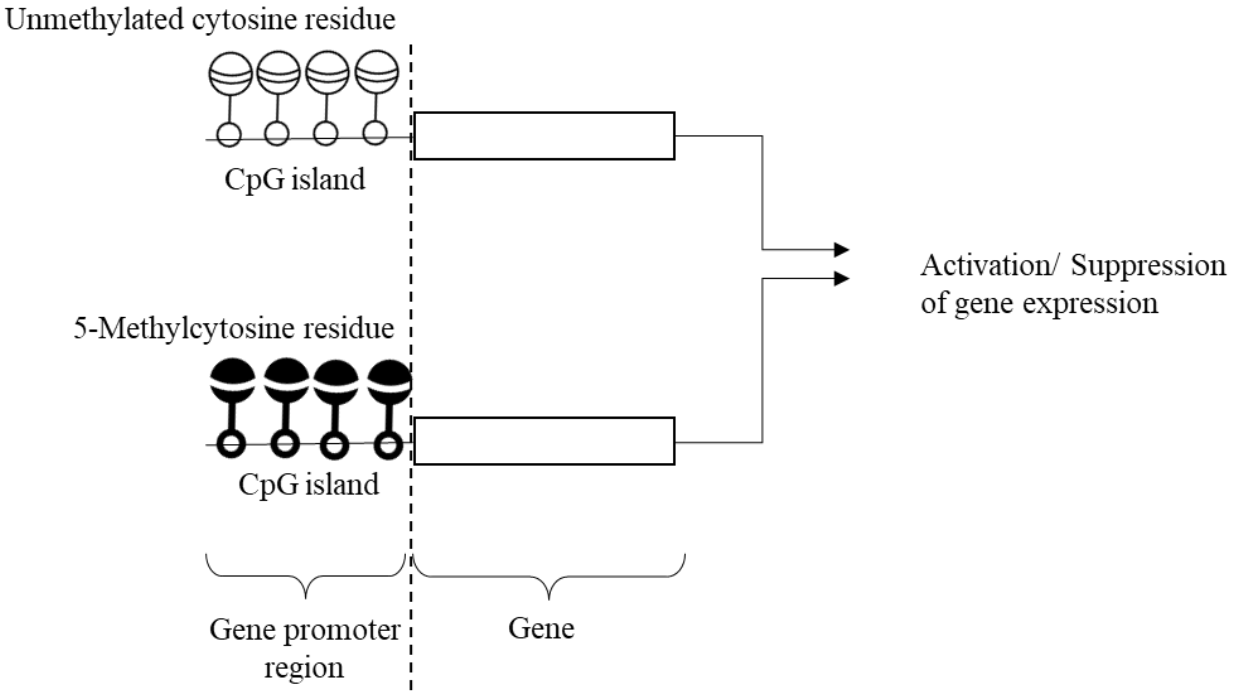

Figure 1:1 Methylation of cytosine residue in CpG island located in the gene promotor region.

Methylation or unmethylation in CpG islands in the gene promotor region determines whether a specific gene is activated or suppressed. CpG, cytosine guanidine dinucleotides.

1.2.2 The implication of methyl nutrients during the early critical window of epigenetics

The perinatal period, which includes both fetal and postnatal stages, acts as a critical window of development and a period in which epigenetic patterns are known to be modifiable. The epigenetic changes that occur within this critical window of development remain stable beyond the window and into adulthood (Perera & Herbstman, 2011). Thus, the epigenetic alterations caused by nutritional perturbations during this window are likely to persist into adulthood, potentially predisposing the individuals to an altered metabolism that can lead to NCD in later life (Glier et al., 2014). Most recent research has focused on the availability of methyl-related nutrients during this critical window of the postnatal period (discussed in sections 1.5 and 1.6), as these nutrients directly affect the availability of methyl groups used for methylation in epigenetics. Much research suggests maternal (i.e., prenatal) dietary methyl nutrients can affect the offspring's risk for NCD in later life via epigenetic alterations (McGee et al., 2018; Randunu & Bertolo, 2020). The early postnatal period is also part of the critical window, during which alterations in diet can also contribute to changes in epigenetics that may persist throughout a lifetime. However, whether dietary methyl nutrients during the early postnatal period can reprogram epigenetic alterations from prenatal perturbations is less understood. Indeed, postnatal epigenetic alterations due to changes in methyl nutrients in the infant diet and their persistence to later life is an emerging area of research in the field (McBreairty & Bertolo, 2016; Robinson & Bertolo, 2016). Moreover, defining the critical window of epigenetic susceptibility into postnatal life needs more clarification (Chmurzynska, 2010). Since affected traits predisposing the individual to NCD can be inherited via epigenetic changes during the perinatal period, longitudinal studies are required to understand whether early programming of metabolism genes by methyl nutrients is beneficial or detrimental to adult health. As a result, most mechanistic research must be conducted in animal models.

However, most animal studies supplement with a combination of methyl nutrients during the prenatal period, expecting to increase methyl metabolism and subsequent influence on epigenetics (Waterland, Dolinoy, et al., 2006). Thus, the effects on the epigenome result from multiple nutrients (Cordero et al., 2013), complicating the mechanistic explanation and limiting the nutritional relevance to humans since foods are not uniquely rich in methyl nutrients only. It is also important to note that the exposure time of methyl nutrient perturbation and the amount of methyl nutrients during the perinatal period will affect the phenotypic outcomes. Most studies expose their subjects to methyl nutrients throughout the periconception period, gestation, and lactation. Thus, it is challenging to identify the critical window of the perinatal period that is most susceptible to epigenetic alterations which predispose the individual to NCD in adult life (Randunu & Bertolo, 2020). Moreover, selecting animal models is critical, as different species are variably developed at birth, with some systems very immature and others mature. Timing of intervention is essential to identify not only the deleterious but also the beneficial epigenetic alterations that could not only predispose but also prevent the development of NCD in adult life.

1.3 Methyl and methionine metabolism

Methyl nutrients are dietary components necessary for methylation reactions that are essential for creating SAM, the primary methyl donor for DNA, RNA, protein, and phospholipids. Some methyl nutrients such as folate, riboflavin, vitamin B12, vitamin B6, choline, and betaine, as well as amino acids such as methionine, cysteine, glycine, and serine, are involved in the production of SAM. The pathways for the methyl/methionine cycle can be summarized as three cycles: transmethylation, in which methionine converts to homocysteine; transsulfuration, in which homocysteine converts to cysteine; and remethylation, in which homocysteine converts back into methionine. Methionine is an essential amino acid containing sulfur and is the principal

methyl donor for many transmethylation reactions that occur via the methionine cycle. Methionine is the primary dietary source of methyl groups, and newborns have a high metabolic requirement for it. Methionine is essential for the production of cysteine and taurine, is vital for protein synthesis, and plays a key role in delivering methyl groups for over 50 transmethylation processes, of which creatine, phosphatidylcholine (PC), and DNA methylation, are quantitatively the most important. The methionine cycle transfers the terminal methyl group of methionine to create these diverse methylated compounds and homocysteine. (i.e., transmethylation). Homocysteine can be irreversibly oxidized after the transfer of its sulfur atom for cysteine synthesis (i.e., transsulfuration), or it can be remethylated to methionine by using methyl groups derived either from betaine via choline or from methyltetrahydrofolate (THF) produced from the folate cycle. (i.e., remethylation) (Figure 1.2). Thus, dietary betaine, choline, and folate are vital in the remethylation pathways via betaine-homocysteine methyltransferase (BHMT) or vitamin B12-dependent methionine synthase (MS), and along with dietary methionine, these methyl nutrients determine the availability of methyl groups for transmethylation reactions. Among transmethylation events, methylation of DNA by DNA-methyltransferases (DNMT) using methyl groups from SAM, the universal methyl donor from methionine, is one of the most important mechanisms. Although the use of methyl groups for DNA methylation by DNMT consumes just 1% of the total dietary methionine in neonatal piglets (McBreairty et al., 2013, 2015; Robinson et al., 2016), this methylation route regulates the most important transmethylation reaction in terms of the long-term health of the offspring (Vickers, 2014; Williams & Schalinske, 2007). Early life methylation of CpG found in the promoter regions of DNA may not utilize quantitatively significant amounts of methyl groups, yet this suppression of gene expression continues throughout an individual's lifetime, making them susceptible to NCD.

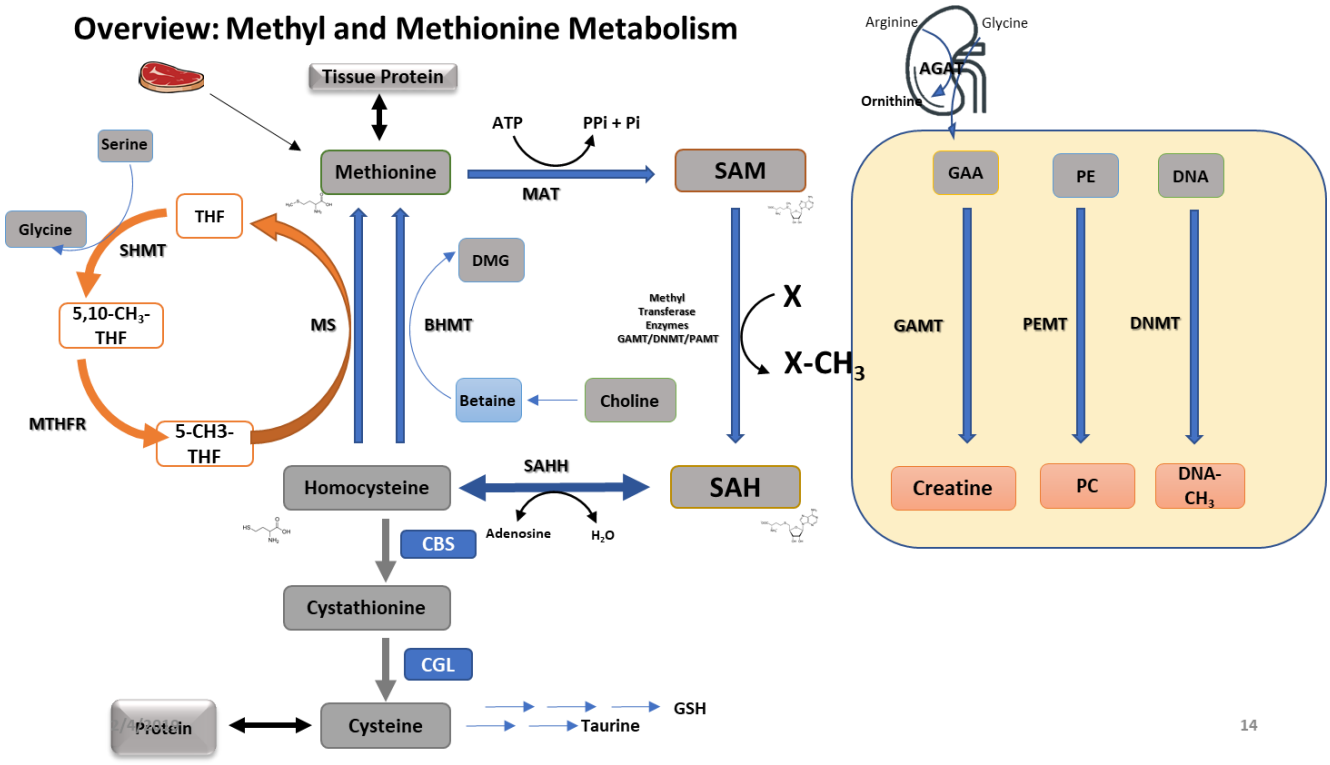

Figure 1:2 A summary of methyl and methionine metabolism.

Methionine is a ubiquitous amino acid acquired via tissue protein breakdown or dietary intake. Methionine is converted to SAM via irreversible adenosylation, which is used for transmethylation. Utilizing methyl groups from SAM, DNA, GAA, PE, glycine, and a number of other transmethylation precursors are transmethylated to methylated DNA, creatine, PC, sarcosine and other products, respectively. These transmethylation reactions are governed by the dietary availability of methionine, choline, betaine, and serine (via the folate cycle), which are the primary methyl donors. The common end product of all transmethylation reactions is SAH, which is then converted to homocysteine. Homocysteine is either irreversibly oxidized to cysteine via the transsulfuration pathway or remethylated to methionine via BHMT or MS. Enzymes involved in methyl metabolism are MAT, methionine adenosyltransferase; GAMT, guanidinoacetate methyltransferase; PEMT, phosphatidylethanolamine methyltransferase; DNMT, DNA methyltransferase; SAHH, S-adenosylhomocysteine hydrolase; CBS, cystathionine beta-synthase; CGL, cystathionine gamma-lyase; BHMT, betaine-homocysteine methyltransferase; MS, methionine synthase; SHMT, serine hydroxymethyltransferase; MTHFR, methylenetetrahydrofolate reductase. Metabolite abbreviations: SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; GAA, guanidinoacetic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DMG, dimethylglycine; THF, tetrahydrofolate; 5,10-CH3-THF, 5,10-methylenetetrahydrofolate; 5-CH3-THF, 5-methyltetrahydrofolate.

1.3.1 Significance of methyl and methionine metabolism in neonates

Methyl donors such as betaine (via choline) and 5-methyltetrahydrofolate offer methyl groups for BHMT and MS, respectively, to remethylate homocysteine to methionine. Since methionine has multiple activities, ranging from protein synthesis to donating methyl groups for transmethylation, the usual healthy individual's food intake must be sufficient to meet total methionine requirements (Robinson & Bertolo, 2016). During the early neonatal period, the demand for transmethylation products, such as creatine and methylated DNA (epigenetics) are high; therefore, the requirements for dietary methionine and methyl donors are high (McBreairty & Bertolo, 2016); moreover, neonates are exposed to a wide variety of dietary methionine and methyl donor levels. For example, most infants are exposed to wide variations in folate intake depending on maternal status, feeding practices and supplement use in Canada (Lamers, 2011). The prevalence of choline (or betaine) deficiency in humans is less understood because a food composition database was only available since 2003 (Zeisel et al., 2003). However, because choline is in very high demand during infancy, human milk contains large amounts of choline but negligible levels of betaine, whereas bovine- and soy-based formulas have little choline (Holmes-McNary et al., 1996). Thus, choline status in formula-fed neonates is low, and plasma choline is half that of breastfed infants (Ilcol et al., 2005). Importantly, as opposed to breast milk, the primary protein source for many infant formulas is casein (Martin et al., 2016), which is very high in methionine and low in cysteine. This will have implications on methyl metabolism. In addition, soy-based infant formulas represent 10% of the market and contain little choline and no creatine (Lauer et al., 1977), both affecting the methyl demands of the infant. Determining the absolute requirement for methionine by a neonate at a given time during early life is challenging. This is because demethylated methionine can be remethylated based on the availability of dietary methyl

donors such as folate and betaine, further complicating the absolute methionine requirement (Bertolo & McBreairty, 2013). Given the involvement of methionine and methyl nutrients to maintain a normal metabolism, the importance of methionine metabolism and its requirements throughout early development cannot be overstated.

Extensive important information about neonatal methionine metabolism has been uncovered through research utilizing piglets as a model. For example, these studies show that dietary cysteine can spare methionine requirement by up to 40% (Shoveller et al., 2003). They also determined that 50% of dietary methionine is used for transmethylation reactions in the early neonatal period and reduced to 30% around weaning (1 month) age (Bauchart-Thevret, Stoll, & Burrin, 2009b). The same research group showed that 45% of homocysteine is remethylated to methionine during the neonatal period, and it drops to 20% around weaning (Bauchart-Thevret, Stoll, & Burrin, 2009b). In addition, methionine and methyl metabolism is altered in IUGR neonatal pigs; IUGRs had 20-30% lower BHMT and CGL activity, indicating that IUGR neonates have a reduced capacity for remethylation and transsulfuration pathways (MacKay et al., 2012). All of these studies emphasize the increased need for methyl groups and the relevance of the methyl and methionine metabolic pathways during the neonatal period.

Importantly, among all transmethylation processes in piglets, creatine and phosphatidylcholine production requires the most methyl groups (McBreairty et al., 2013). The creatine accretion rate in suckling piglets is three times higher than creatine intake from sow milk, and a third of the dietary methionine is utilized for endogenous creatine synthesis (Brosnan et al., 2009). Similar to creatine synthesis, PC synthesis and DNA methylation are very active and have increased demand during the early neonatal period (McBreairty et al., 2013; McBreairty & Bertolo, 2016). As stated previously, despite the fact that just 1% of methyl groups are directed towards

DNA methylation (McBreairty et al., 2015; Robinson et al., 2016), it is arguably the most important pathway, as it influences the long-term health of offspring through epigenetic programming (Vickers, 2014; Williams & Schalinske, 2007). Recent research has demonstrated that if one methylation pathway is enhanced, it has an effect on the partitioning of limited methyl groups to other methylation reactions. In piglets, for instance, supplementation with GAA increased methyl incorporation to creatine synthesis, while decreasing PC and protein synthesis by 85% and 40%, respectively, with no change in DNA methylation (McBreairty et al., 2013). That investigation revealed that dietary composition influences methyl partitioning throughout transmethylation processes, and that methyl supply may be limited during the neonatal period, depending on demands for methylated products, and supply by the diet. This diet-methylation interaction plays an especially critical role in early life, when metabolic needs are high, and the neonate is susceptible to programming.

1.3.2 Importance of methyl donors and methyl recipients

Methionine and accessible methyl groups for transmethylation events, such as DNA methylation, are determined by the availability of dietary methyl donors and methyl group recipients (Niculescu & Zeisel, 2002). Thus, the availability of methyl donors and recipients during early life is crucial for growth requirements and epigenetics which are high in demand in a neonate. However, an optimum requirement of these vital nutrients throughout the critical programming window that results in beneficial health outcomes in adulthood has not yet been determined. Understanding the consequences of dietary methyl supply in neonates and the degree of transmethylation may assist in optimizing both infant and adult health, as the majority of newborns are exposed to dietary extremes of methyl-related nutrients during the critical period of early life.

Creatine, PC and DNA are the main transmethylation products, in other words, methyl recipients. Folate, choline, betaine, and methionine are the most important dietary methyl-related nutrients, as they play an important role in methyl metabolism as methyl donors. While these remethylation nutrients play critical roles during both pregnancy and infancy, the effects of maternal supplementation with these nutrients on programming are described in greater depth elsewhere (Randunu & Bertolo, 2020); thus, this literature review will focus on neonatal studies.

1.3.2.1 Creatine

Because of the requirement of a balance between methyl donors and recipients for optimal metabolism, some transmethylation products and remethylation nutrients are of higher importance among methyl nutrients. For instance, one of the most important transmethylation products is creatine, an energy buffer whose production is under great demand during early development (Brosnan et al., 2009). Creatine content in piglets increases by 70% throughout the neonatal period, but only 25% is supplied by sow milk (Brosnan et al., 2009). Consequently, there is a significant demand for methyl groups to create endogenous creatine in early life. As creatine levels in breast milk and infant formula vary greatly (Edison et al., 2013), infant nutritional intakes throughout the neonatal period are inconsistent and variable. As a result, when transmethylation reactions are at their peak and at high demand, the dietary supply of methyl nutrients can vary dramatically during early life. Importantly, one-third of a piglet's dietary methionine is utilized to synthesize creatine (Brosnan et al., 2007). Dietary methionine is required for synthesizing protein as well as transmethylation reactions (Asiriwardhana et al., 2022). Thus, low quantities of dietary creatine will drastically increase the need for methyl groups and methionine to synthesize creatine, which may reduce infant growth. Importantly, a recent study has shown that creatine supplementation to total parenteral nutrition (TPN) (discussed details in section 1.8) improved the accretion of creatine

and reduce the metabolic burden of creatine synthesis and reduced liver cholesterol levels in in rapidly growing neonatal piglets (Dinesh et al., 2018)

The evidence in favor of programming effects of early life creatine supplementation on metabolism in later life is limited. However, a review of creatine supplementation and age-related diseases revealed that creatine has the potential to modify age-related long-term diseases such as Parkinson's, Alzheimer's, Huntington's disease, and stroke (Gonzales et al., 2014). A study also demonstrated that 2% dietary creatine supplementation for one month can prevent neuronal damage in mice with stroke (Zhu et al., 2004). Although there are not many studies on the effects of creatine programming, these studies demonstrate the influence of creatine on programming. Although additional studies are required to confirm that creatine has an impact on programming, and the above data imply that creatine could be used as a prophylactic, dietary supplement in patients with risk of NCD.

As creatine synthesis consumes almost 40% of available methyl groups (Brosnan et al., 2011), it was hypothesized that creatine supplementation would decrease homocysteine production by saving methyl groups and reduce the risk of CVD (Clarke et al., 2020). Studies have also shown that creatine supplementation lowered homocysteine levels in rats, confirming the above hypothesis (Deminice et al., 2016; Stead et al., 2001). Moreover, creatine supplementation enhances vascular function, via the destruction of free radicals such as superoxide and peroxynitrite, which are related with CVD (Lawler et al., 2002; Rahimi, 2011; Sestili et al., 2006), and the management of inflammation (Kim et al., 2015; Nomura et al., 2003). These effects are all associated with a reduction in the risk of CVD. Therefore, it is clear that creatine supplementation has a potential to promote vascular health.

1.3.2.2 Phosphatidylcholine

PC is required for bile and lipoprotein production (Alvaro et al., 1986; Cole et al., 2011); hence it influences the neonate's lipid metabolism and is thus just as important as creatine synthesis to a neonate. PC is derived from two pathways: the CDP-choline pathway and the PEMT pathway, where labile methyl groups are required to form PEMT-derived PC (Robinson & Bertolo, 2016). PEMT-derived PC is particularly relevant since it is richer in arachidonic acid (ARA) and docosahexaenoic acid (DHA) than CDP choline-derived PC (DeLong et al., 1999). These long-chain fatty acids are essential in neonates and are absolutely critical in the developmental stages of the infant brain (Watkins et al., 2003). The methyl demand of the PEMT pathway is considered to be quantitatively greater than creatine and potentially all other transmethylation reactions because PEMT requires 3 moles of SAM to form 1 mole of PC (McBreairty et al., 2013; Stead et al., 2006). The importance of this pathway was demonstrated by Robinson et al. (2016), who found that dietary methyl deficiency led to a 60% increase in methyl incorporation into PC and a concomitant decrease by 30% methyl incorporation into creatine, demonstrating that limited methyl groups are prioritized for PC synthesis in the liver.

1.3.2.3 Choline and folate

Choline is significantly important in early development as choline status is positively associated with cognitive development, and breast milk levels reflect maternal choline status (Davenport et al., 2015; Zeisel, 2004, 2006). Since choline is a major methyl donor, dietary choline levels may affect the programming mechanisms of adult diseases. Most of the animal studies on choline and programming were done by modulating the maternal diet with various choline levels (Craciunescu et al., 2003; Mehedint et al., 2010; Niculescu M.D, Craciunescu C.N., 2006). These studies demonstrated that neurogenesis and angiogenesis are inherited via epigenetic mechanisms.

Moreover, memory recognition in relation to maternal choline levels was speculated to be inherited via epigenetic mechanisms (Bozon et al., 2003; Davison et al., 2009; Jones, 2001; Kovacheva et al., 2007; Mellott et al., 2007). Collectively these studies demonstrate the impact of methyl group availability in early life on persistent traits into adulthood, likely via epigenetic mechanisms. On the other hand, folate has been extensively studied as a methyl donor, as it determines the flux of methyl groups towards the synthesis of methionine and, thereby methylation of DNA (Crider et al., 2012). Thus, the deficiency of folate in the maternal diet has been shown to lead to decreased methionine synthesis, homocysteine accumulation (Forges et al., 2007) and an individual's predisposition to altered epigenetics and programming. Animal studies have shown that gestational folate and methyl donor deficiency led to increased plasma homocysteine and liver steatosis in dams, and in the offspring, it led to diminished body size (Bison et al., 2016; Pellanda et al., 2012), myocardial hypertrophy, defects in mitochondrial metabolism, and increased triglyceride levels (Garcia et al., 2011). Collectively, dietary intake of these methyl nutrients during early life plays a significant role in changing the epigenome and metabolism in infants.

1.3.2.4 Betaine

Betaine, which can be synthesized from choline and acts as an osmolyte and methyl donor, is another important nutrient for remethylation (Arumugam et al., 2021). An infant's primary source of betaine is choline, and intake via breast milk (Fischer et al., 2010). Dietary intakes of betaine are not well described, and estimates suggest that it is highly variable in both neonates and adults. In neonates, betaine intakes are low during suckling as breast milk contains very little betaine and largely depends on maternal betaine intake. Infant formulas were reported to be within the breast milk range, which was then largely dependent on maternal betaine intakes (Robinson & Bertolo, 2016). Thus, the intake of betaine during the neonatal period is variable. According to rat

studies, 85% of labelled dietary choline is recovered as betaine, and only 15% of glycerophosphocholine is converted to betaine (Cheng et al., 1996). As most formulas contain choline as glycerophosphocholine (McBreairty & Bertolo, 2016; Robinson & Bertolo, 2016), it is likely that formula-fed infants do not get adequate betaine. Indeed, variability of choline levels leads to variable betaine levels in infants; however, inadequate dietary choline or betaine levels can lead to inadequate methionine and methyl metabolism.

Similar to choline, many maternal nutritional interventions in animals were performed to understand the effect of dietary betaine on programming. Studies have shown that maternal betaine supplementation leads to programmed regulation of fetal and postnatal growth intergenerationally via changing the epigenetics (Zhao et al., 2017). A sow study found that gestational betaine supplementation increased serum methionine and hippocampal mRNA and protein expressions of BHMT, glycine-N-methyltransferase, and DNMT1, as well as hypermethylation of certain genes such as insulin-like growth factor (IGF2) (Li et al., 2015). Another study in pigs showed that gestational betaine supplementation led to the enhanced glucocorticoid receptor (GR) expression (known to regulate via epigenetics) in hippocampus including alteration in micro-RNA expression (Sun et al., 2016). As GR is known to be an epigenetically regulated gene, it is likely that the effects may have been influenced by maternal betaine levels (Sun et al., 2016). Moreover, a swine study has shown that maternal betaine supplementation lowered plasma triglycerides in piglets, increased serum methionine and liver SAM/SAH ratio, and down-regulated hepatic expressions of the lipogenic genes such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD) and SREBP1c with DNA hypermethylation (Cai et al., 2014). Human studies showed that maternal folate and betaine supplementation were linked with offspring growth, metabolism, and appetite (Pauwels et al., 2017). Further, it was demonstrated that these

remethylation nutrient supplements were associated with lower leptin gene methylation in infants at birth and at six months of age (Pauwels et al., 2017). The longer-term persistence of these epigenetic changes is unknown but likely also depends on the postnatal nutrition of the infants. Collectively, it is clear that perinatal dietary betaine plays a role in lipid metabolism, and the effects are mediated through epigenetic mechanisms.

It is also important to note that recent studies in piglets showed that betaine is as effective as folate in resynthesizing methionine (McBreairty et al., 2016). Thus, both remethylation pathways, by BHMT and MS, have equal importance in resynthesizing methionine. Most of the above research has summarized the metabolic and epigenetic outcomes of maternal betaine on offspring in the neonatal stage; however, data on the effects of post-natal dietary betaine on the metabolic and epigenetic outcomes of the offspring and the impact on long-term health are scarce. Taken together, betaine exposure in early life can influence the methylation status of offspring in the perinatal period, and these effects can be permanent.

All of these studies investigated programming effects of methyl nutrients when they were given throughout conception, pregnancy, and lactation. However, there is no clear understanding of the effects of methyl nutrients on each specific developmental period. Understanding the separate effects of dietary methyl nutrients during different periods within the critical window is important in order to in modulate the methyl nutrients to offset programming effects.

1.4 Non communicable diseases - Metabolic syndrome and cardiovascular disease

Metabolic syndrome is defined as the concurrence of obesity, impaired glucose tolerance, dyslipidemia, and hypertension; it is a complex NCD where each component is an independent risk factor for CVD (Lee & Sanders, 2012). The combination of these risk factors increases the rate and severity of CVD. Statistics Canada reports that as many as 1 in 5 adults could have

metabolic syndrome, and the prevalence of this disease increases drastically with the age, predisposing individuals to a high risk of developing CVD (Statistics Canada. 2013. Metabolic Syndrome in Canadians, 2009 to 2011). Although advances have been made in understanding the etiology and implications of this multifaceted disorder, the underlying disease mechanisms have not yet been clearly identified. Since the development of NCD can be influenced by the environment, it is important to identify potential contributors and develop policies and precautions to reduce the incidence of this condition. In this context, early-life nutritional programming has a substantial influence on the development of NCDs later in life; therefore, it is important to investigate how the onset of NCDs in later life might be mitigated by modifying nutrition in early life.

1.4.1 Obesity

Obesity is a multifactorial condition, generally described as excess body fat with a complex pathogenesis that is associated with adverse health outcomes (Lin & Li, 2021). Adipose tissue is the defining organ of obesity; it is a diverse and highly plastic endocrine organ (Chait & den Hartigh, 2020). Although its development begins prenatally, adipose tissue is capable of unrestricted expansion into adulthood. Furthermore, adipose tissue is a very sexually dimorphic tissue, as are the related risks (Rodgers & Sferruzzi-Perri, 2021). In other words, accumulation and body distribution of adipose tissue are different between males and females. This may be because they are mediated through sex hormones and the sex chromosome complement. Hence, the related risks are different between males and females. It is apparent that environmental influences during prenatal and early postnatal development can influence the structure and function of adipose tissue, which has consequences for the development of obesity (Rodgers & Sferruzzi-Perri, 2021). Those who experience undernutrition or overnutrition throughout the critical period of development often

develop chronic adult disorders, such as obesity, which eventually lead to NCD such as metabolic syndrome and CVD.

1.4.1.1 Birth weight, postnatal growth and obesity

Epidemiological research has confirmed that both low and high birth weights increase the likelihood of childhood and adult obesity (Jornayvaz et al., 2016; Skilton et al., 2014). In particular, rapid postnatal growth has been linked to an increased risk of obesity, whereas slower postnatal growth reduces this risk. For example, newborns with IUGR or low birth weight have a greater risk of adult obesity and metabolic syndrome, especially those with rapid catch-up growth in the first few years (Gluckman et al., 2005; Simmons, 2008). Newborns with both low and high birth weights show signs of obesity development. In addition, the developmental programming impacts on infants born to mothers with obesity or gestational diabetes have recently been identified as a leading cause of the obesity epidemic (Ross & Desai, 2013).

1.4.1.2 Ectopic fat

Obese individuals have an increased quantity of visceral adipose tissue and greater ectopic fat accumulation (Neeland et al., 2019). Ectopic fat is characterized by the presence of extra adipose tissue in locations not usually associated with adipose tissue storage (Snel et al., 2012). Many cardiometabolic imaging investigations have revealed that both normal-weight and overweight individuals are at high risk of CVD if they have an excess of visceral adipose tissue because it is frequently accompanied by ectopic fat deposition, particularly in the liver, heart, and skeletal muscles (Neeland et al., 2019). The capacity for expansion of subcutaneous adipose tissue is one potential mechanism by which adipose tissue is deposited in ectopic depots (which surround organs and blood vessels) as opposed to non-ectopic depots (Neeland et al., 2019). Individuals

who are overweight or obese but have the potential to increase their subcutaneous adipose tissue mass may be at a lesser risk than expected. In circumstances of positive energy balance, surplus FFA are initially stored subcutaneously. However, once the capacity of subcutaneous adipose tissue is achieved, storage shifts to ectopic locations, such as the viscera, heart, skeletal muscles, and liver (Chait & den Hartigh, 2020). Hence, increased visceral adipose tissue and ectopic fat are connected with the risk of CVD in overweight and obese individuals (Piché et al., 2020).

1.4.1.3 Mechanisms of development of obesity

The exact mechanisms by which early life diet can cause obesity programming are not precisely understood. Changes in the structure and function of neural pathways in the brain, resulting in dysregulation of the pathways controlling energy balance, are among the underlying mechanisms that result in the programming of obesity (Martin-Gronert & Ozanne, 2013). For example, disruption of hypothalamic appetite pathway programming and adipogenic signals regulating lipogenesis may program obesity (Ross & Desai, 2013). In addition to modifications at the cerebral level, early feeding can have long-lasting negative impacts on peripheral physiological systems, such as fat storage and nutrient utilization, leading to even greater susceptibility to obesity. These mechanisms induce obesity in offspring by changing food sensors, epigenetic modifications, and modulating stem cell progenitors of appetite/satiety neurons and adipocytes (Martin-Gronert & Ozanne, 2013; Ross & Desai, 2013). Studies examining the epigenetic mechanisms have analyzed epigenetic alterations in adipose tissue and the adipogenic potential of mesenchymal stem cells from exposed offspring (Rodgers & Sferruzzi-Perri, 2021).

1.4.1.4 Programming obesity

Research demonstrates that maternal nutrition restrictions, particularly maternal low protein diets and maternal obesity/obesogenic diets, causes adiposity in offspring (Berends et al., 2013; Bispham et al., 2005; Budge et al., 2004; Guan et al., 2005; Long et al., 2012; Tchoukalova et al., 2014; Zhang et al., 2007). IUGR pups born as a result of maternal malnutrition consume much more food, resulting in rapid catch-up growth and adult obesity (Desai et al., 2005; Desai, Gayle, Babu, et al., 2007). This obese phenotype is the result of malfunction of the hunger/satiety pathway at several levels, as demonstrated by diminished satiety responses to leptin (Desai, Gayle, Han, et al., 2007) and enhanced responses to appetite stimulatory factors (i.e, ghrelin) (Jia et al., 2008). Furthermore, offspring may express a higher ratio of appetite/satiety genes in response to maternal malnutrition (Fukami et al., 2012; García et al., 2010; Plagemann et al., 2000b; Remmers et al., 2008). In IUGR offspring, there is evidence of adipocyte hypertrophy and enhanced de novo fatty acid synthesis (Desai et al., 2008; Yee et al., 2012). In addition, IUGR male progeny exhibited elevated adipogenic signalling cascades, shown by an increased expression of enzymes that promote adipocyte lipid synthesis and storage (Desai et al., 2008). While these changes occur early in life, prior to the emergence of obesity, they imply a regulated pathway of enhanced adipocyte differentiation and lipogenesis that likely contributes to the development of obesity and metabolic problems in IUGR offspring. Programmed adipocyte dysfunction then exacerbates low-grade inflammation, insulin resistance, and dyslipidemia, resulting in metabolic dysfunction (Salazar-Petres & Sferruzzi-Perri, 2022; Trivett et al., 2021).

Similar to IUGR, a maternal high fat diet also programs obesity, particularly via epigenetic modifications. For example, adult offspring of female mice fed a high fat diet (60% fat) showed hypermethylation at the promotor region of adiponectin and leptin receptor genes, as well as

decreased DNA methylation in the promotor region of leptin in visceral fat (Khalyfa et al., 2013). These findings indicate that epigenetic modifications may alter adiponectin and leptin expression in adipose tissue, potentially leading to metabolic syndrome (Lihn et al., 2005; Masuyama & Hiramatsu, 2012).

1.4.1.5 Methyl nutrients and obesity

Evidence shows that DNA methylation profiles are significantly associated with obesity or adiposity in infant and adult populations (Sharp et al., 2015; Rhee et al., 2017; Rzehak et al., 2017). For example, several CpG sites were differentially methylated in the cord blood of the offspring of obese mothers as opposed to offspring from normal-weight mothers (Sharp, et al., 2015). Among the other studied sections in the genome, certain gene body regions were hypomethylated in obese children, highlighting a unique cluster for obese children that was differentiated from the normal-weight children (Rhee, et al., 2017). Importantly, some of these methylated genes are implicated in differential body size and body composition in children (Rzehak et al., 2017). DNA methylation has largely been assayed from blood, given the challenges of more invasive sampling procedures to obtain target tissues such as adipose tissue in infants (Wu et al., 2020). Another human study observed a strong correlation between maternal blood leptin gene promoter DNA methylation and infant blood leptin gene methylation. Leptin is involved in adipose mass, lipogenesis and appetite regulation (Crujeiras et al., 2015; Ruchat et al., 2014; Vickers & Sloboda, 2012), all of which have direct implications on weight control and obesity risk. They further observed higher leptin gene promoter DNA methylation among small-for-gestational-age infants only (Lesseur et al., 2013). This higher leptin promotor DNA methylation could result in lower circulating leptin levels, then affect infant growth and development, given that small-for-gestational-age has been linked to an elevated risk of obesity during childhood. A study conducted

by Rzehak et al. (2017) on epigenome-wide association studies examined a potential association between epigenetic programming and childhood body composition and obesity. They found 13 genes in which DNA methylation variants were significantly associated with lipid metabolism and insulin activity, which are risk factors for overweight and obesity. Thus, it is clear that there is increasing evidence of a link between epigenetic modifications and obesity and that epigenetic changes have a possible impact on childhood obesity. On the other hand, there is evidence that the availability of dietary methyl donors is associated with obesity and related metabolism. For example, a recent study reported that overweight and obese human adolescents with higher dietary intakes of methyl-donor nutrients were less likely to be metabolically unhealthy (Poursalehi et al., 2022). Studies in human populations show associations of obesity with folate, vitamin B12 deficiencies, and higher homocysteine levels (Sánchez-Margalet et al., 2002; Karatela and Sainani, 2009). Differentiation of human adipocytes under low vitamin B12 conditions results in modification of miRNA expression of adipocyte differentiation and function, as well as modification to the expression of circulating miRNAs produced from adipocytes that target transcripts involved in adipogenesis (Adaikalakoteswari et al., 2017). DNA methylation is responsive to dietary methyl group availability (Anderson et al., 2012). Early life diets deficient in methyl donors might lead to epigenetic changes, which determine the degree of methylation of the above genes associated with obesity development. All the above findings indicate that the availability of dietary methyl nutrients can influence the risk of obesity programming and that the origins of obesity can be associated with one-carbon metabolism during development.

1.4.2 Dyslipidemia:

Dyslipidemia, defined as increased blood lipid and lipoprotein levels, is a key risk factor for CVD (Klop et al., 2013). Because the liver and adipose tissue are important organs involved in lipid and glucose metabolism, dyslipidemia results from impaired development and functioning of these organs, leading to obesity, insulin resistance, and metabolic syndrome (Armengaud et al., 2021). Dyslipidemia can result acute pancreatitis, hepatosplenomegaly, and symptomatic vascular disease, all of which can contribute to CVD (de Pretis et al., 2018). Obesity, or the combination of obesity and poorly controlled diabetes, may increase circulating free fatty acids (FFA), leading to increased hepatic very low-density lipoprotein (VLDL) synthesis. The triglyceride (TG)-rich VLDL then transfers TG and cholesterol to low density lipoprotein (LDL) and high-density lipoprotein (HDL), increasing the production of TG-rich, tiny, dense LDL and clearance of TG-rich HDL resulting in significant dyslipidemia (Feingold, 2020; Højland Ipsen et al., 2016; Lee et al., 2023). With prolonged lipemia, there is an increase in the exchange of core lipids between postprandial lipoproteins and LDL/HDL, resulting in small, dense LDL particles and decreased HDL cholesterol levels. This, eventually, will lead to atherosclerosis and CVD (Hyson et al., 2003).

1.4.2.1 Postprandial lipemia

Elevated TG levels in the blood during fasting are an independent risk factor for CVD (Gaziano et al., 1997). In Western countries, however, people spend the majority of their time in postprandial states, with fluctuating lipemia due to elevated levels of TG-rich lipoproteins (TRLs), such as chylomicrons (CM) and VLDL (Pirillo et al., 2014). Hence, changes in postprandial lipemia (PPL) are regarded as an independent CVD risk factor, much more significant than

changed fasting TG metabolism (Nakamura et al., 2016). PPL is a dynamic, non-steady-state situation defined by an increase in TG-rich lipoproteins after a meal (Figure 1.3). Several lines of evidence indicate that PPL increases the risk of atherogenesis. Clinical data demonstrate a link between postprandial lipoproteins, coronary artery disease progression, and carotid intimal thickness (Hyson et al., 2003). TG-rich lipoprotein remnants may have detrimental effects on endothelium and can enter the subendothelial region, according to mechanistic investigations. During PPL, hemostatic variables, such as clotting factors, platelet reactivity, and monocyte cytokine production, may be elevated. These findings suggest that assessment and therapy of atherosclerosis should incorporate PPL-related markers (Hyson et al., 2003). Postprandial lipid metabolism is influenced by several factors, including nutritional, physiological, pathological, and hereditary factors. Diet and genetic variables are among the most important moderators of PPL. Indeed, higher postprandial TG levels are related to coronary heart disease and obesity, suggesting that lifestyle adjustments and dietary modulations aimed at lowering postprandial TG levels could help reduce the prevalence of CVD (Pirillo et al., 2014).

Overview- Lipid and lipoprotein metabolism

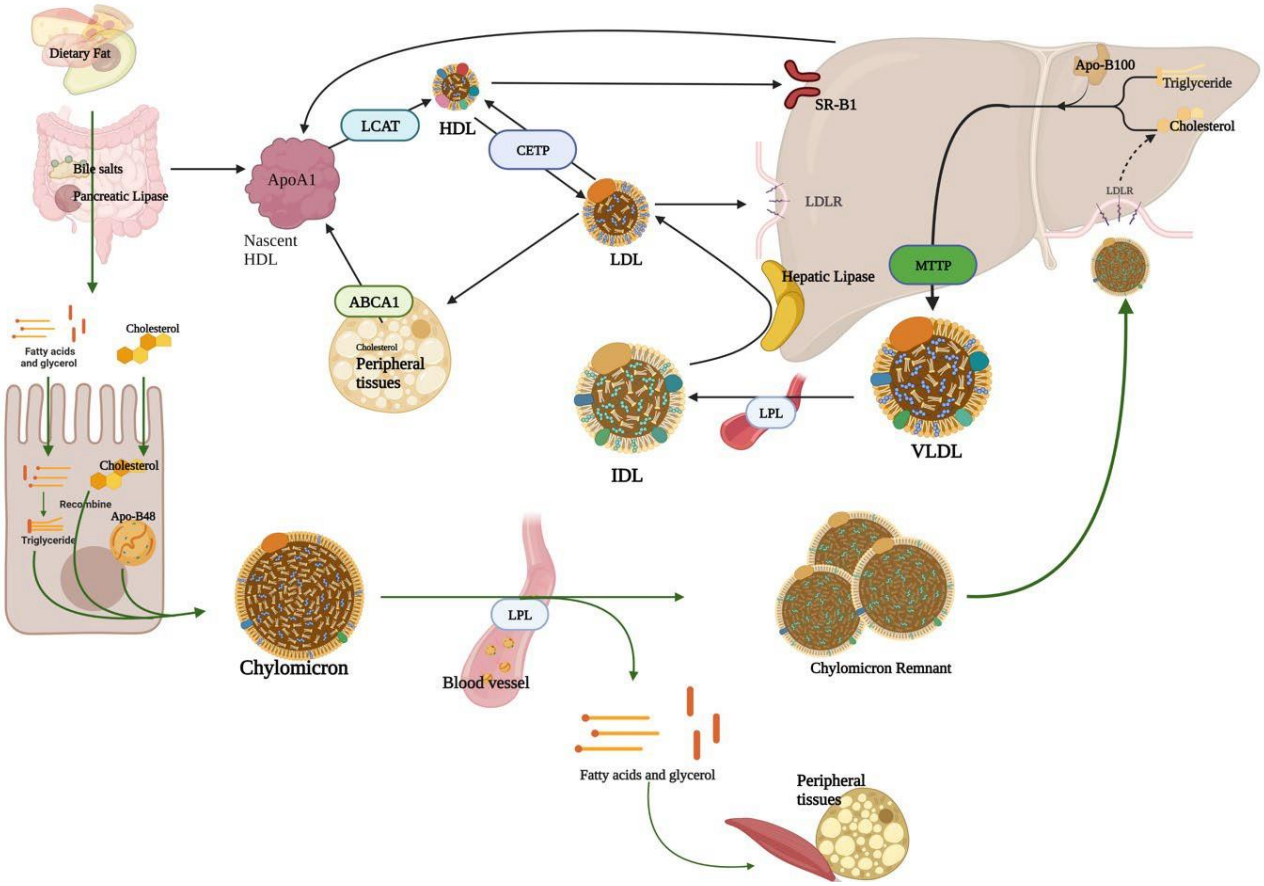

Figure 1:3 A summary of exogenous and endogenous lipid and lipoprotein metabolism.

Gastric and pancreatic lipases act on dietary TG and produce free fatty acids, monoglycerides and glycerol. These products then enter into the enterocytes and recombine to form TG. TG is packaged with cholesterol into large lipoprotein particles: CM. CM then leaves the enterocytes, eventually enters circulation, and delivers its TG to peripheral tissues via LPL. LPL is located in the capillary endothelium and hydrolyzes TG in the CM to free fatty acids and glycerol, enabling them to pass through the capillary wall to tissues, which are oxidized for energy or re-esterified for storage. Once TG is removed from the CM, the liver takes up the remaining CM remnant. This pathway is prominent during the postprandial stage and is the exogenous pathway of lipoprotein metabolism.

VLDL is synthesized by the liver, which contains TG and cholesterol and gets into circulation. The MTTP is essential for the VLDL packaging. The TG in VLDL is then extracted to peripheral tissues via LPL in the same way as CM. Once the TG is delivered to peripheral tissue, VLDL converts to IDL. Hepatic lipase acts on IDL and converts it into LDL. LDL has the highest cholesterol content and is the primary cholesterol carrier in circulation. High levels of LDL in the blood are associated with cholesterol plaque build-up and CVD. LDL distributes cholesterol to

peripheral tissues, and the liver takes the LDL remnant via the LDL-r receptor. This pathway is the endogenous pathway of lipoprotein metabolism.

The liver produces nascent HDL. HDL collects excess cholesterol from peripheral tissues and TRLs and returns it to the liver. HDL receives cholesterol from TRLs in exchange for TG via CETP. This is the reverse-cholesterol transport system.

LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; CM, chylomicron; LCAT, lecithin-cholesterol acyltransferase; CETP, cholesterol ester transfer protein; LPL, lipoprotein lipase; MTTP, microsomal triglyceride transfer protein; LDL-r, low-density lipoprotein receptor; SR-B, scavenger receptor class B type 1.

1.4.2.2 Programming dyslipidemia

Liver and adipose tissues are essential for proper lipid metabolism in mammals, and defective development and function of either of these organs results in dyslipidemia, obesity, insulin resistance and metabolic syndrome (Hoffman et al., 2017). Both human and animal studies have demonstrated that dyslipidemia has a developmental basis (Barker et al., 1993; Broholm et al., 2016; Sohi et al., 2011; Guan et al. 2004); thus, exposure to unfavourable prenatal environments can permanently modify physiological processing, resulting in the development of dyslipidemia in adulthood.

1.4.2.3 Programming dyslipidemia and IUGR

IUGR is a predisposing factor in the development of dyslipidemia. Reduced abdominal circumference at birth and elevated LDL-cholesterol later in life have been linked to liver size and function impairment due to dysmetabolism (Barker et al., 1993). Similarly, IUGR due to maternal malnutrition is frequently accompanied by reduced growth of the liver and kidneys (Neerhof, 1995; Valsamakis et al., 2006) predisposing individuals to dyslipidemia. In addition, altered adipose tissue functioning in IUGR offspring as a result of abnormalities in the expression of key genes involved in leptin secretion may also lead to the development of dyslipidemia (Broholm et al.,

2016). IUGR offspring that demonstrated nutrition-induced catch-up growth developed dyslipidemia and metabolic syndrome earlier than IUGR offspring who did not show accelerated growth (Eriksson, 2006; Finken et al., 2006; Martin et al., 2003; Yajnik, 2000). Severely damaged liver development, higher LDL/HDL ratio, and fast postnatal growth are likely attributable to altered hepatic cholesterol homeostasis in IUGR (Singhal et al., 2004). Collectively, these findings demonstrate that low birth weight, altered structure and function of liver and adipose tissue, and accelerated postnatal growth are closely associated with the development of dyslipidemia. In addition, a recent programming study demonstrated that dyslipidemia in IUGR persists into adulthood (Myrie et al., 2017). Additionally, dyslipidemia is associated with other CVD risk factors, such as hyperhomocysteinemia (Obeid & Herrmann, 2009). Studies have shown that lower plasma HDL cholesterol and hepatic fat accumulation are associated with hyperhomocysteinemia a key CVD risk factor (De Carvalho et al., 2013; Momin et al., 2017). IUGR have shown to have higher homocysteine levels in the liver as neonates (MacKay et al., 2012); however, whether this persists into adulthood need further investigation.

1.4.2.4 Dyslipidemia, nutritional programming, and epigenetics

Nutritional stressors in early life, such as maternal undernutrition, low-protein diets, and high-fat diets, cause epigenetic programming of lipid metabolism (Peral-Sanchez et al., 2022). For example, offspring with early exposure to hunger had low lipoprotein levels (Tobi et al., 2009, 2015). Recent research has established a link between epigenetic modification of genes involved in lipid metabolism and adipogenesis in offspring exposed to starvation during early gestation (Tobi et al., 2018). In a study by Jordan et al. (2023), C. elegans exposed to early life starvation developed developmental diseases, which resulted in altered lipid metabolism via widespread effects on adult gene expression (Jordan et al., 2022). This research further demonstrated that the

gene encoding a key lipogenic enzyme, fatty acid synthase (FASN), is one of several enzymes whose adult expression is increased as a result of early life food deprivation, affecting lipid levels. The livers of IUGR newborn mice subjected to a low-nutrition diet exhibited abnormal signalling and lipid metabolism and led to the development of non-alcoholic fatty liver disease in adulthood (Lee et al., 2016). Another piece of data revealed that when pregnant mice were fed a low-protein diet, the liver of the offspring exhibited higher expression of glucocorticoids and peroxisomal proliferator-activated receptor (PPAR) (Lillycrop et al., 2005). These gene-specific alterations may likely influence the development of hyperlipidemia later in life.

As discussed above, evidence shows that the programming effects of lipid metabolism are mediated through epigenetics. Lillycrop et al. studied epigenetic modifications of genes linked to lipid metabolism in weaning rats (Lillycrop et al., 2005). Maternal protein-restricted diet decreases DNA methylation levels of PPAR by 23% and glucocorticoid receptor (GR) by 21% in rat offspring after weaning. These epigenetic anomalies were detected on day 34 and day 80 following birth, indicating that perinatal starvation generates permanent epigenetic alterations in the genes involved in lipid metabolism. Collectively, epigenetics plays a crucial role in development of dyslipidemia.

1.4.2.5 Methyl nutrients and dyslipidemia

Lipid metabolism is connected to methyl metabolism via the PEMT pathway, which is essential for lipoprotein secretion by the liver. Reduced PC production decreases VLDL export, causing hepatic lipid accumulation (Młodzik-Czyżewska et al., 2021). PC is also involved in the transport of LC-PUFA to vascular endothelial cells and extrahepatic organs. Since metabolites from one-carbon metabolism participate in lipid biosynthesis, deficits in one-carbon nutrients may impact lipid metabolism, increasing the risk of fatty liver disease and metabolic syndrome

(Radziejewska et al., 2020; Tiihonen & Saarinen, 2016; van der Veen et al., 2017). Betaine is a lipoatrophic substance involved in the synthesis of L-carnitine, which is necessary for the transport of long-chain fatty acids and lipid metabolism (Obeid, 2013). Insufficient betaine may therefore disrupt normal lipid metabolism. In addition, a number of studies have demonstrated that obese individuals with abnormal lipid metabolism had lower levels of one-carbon nutrients than lean individuals, indicating that obesity may affect one-carbon nutrients (Chen et al., 2015; Da Silva et al., 2013; Elshorbagy, Kozich, et al., 2012; Gao et al., 2018; Mlodzik-Czyzewska et al., 2020). Collectively, these studies imply that dyslipidemia is strongly linked to early-life dietary programming and that the effects are most likely mediated via epigenetics, with methyl nutrients perhaps playing a substantial role in the onset of dyslipidemia.

1.4.2.6 Hypertension: Programming of hypertension

Hypertension is a highly prevalent disorder affecting one-fourth of the world's adult population, and is a major risk factor for CVD, cerebrovascular morbidities (i.e., myocardial infarction, stroke, atherosclerosis), and death (Mittal & Singh, 2010). It is a complex disease in which genetic and environmental factors play a substantial role, and it is mediated by abnormalities in several biological pathways. The prevalence of pediatric hypertension ranges from 1% to 2% and has consistently increased over the past decade in correlation with the prevalence of obesity (Jones et al., 2012). Studies have shown that nutritional manipulation in early life associated with programming of adult hypertension. For instance, severe and moderate protein restriction during pregnancy in rats led to hypertension and kidney structural abnormalities in a sex specific manner in offspring (Woods et al., 2001a, 2004, 2005). Many early-life stressors can significantly influence renal structure and function and renal compensatory mechanisms, (i.e., renal programming), resulting in hypertension and kidney disease in adulthood (Hsu & Tain, 2021). Vascular function, vasoactive systems, sympathetic nervous system activity, salt sensitivity, renin

angiotensin system (RAS) activity, nephron number, and epigenetics are some of the mechanisms by which early childhood nutrition impact the long-term risk of hypertension (Paauw et al., 2017).

1.4.2.7 Renin angiotensin system and programming hypertension

RAS plays a crucial role in the regulation of BP and interacts closely with other processes. RAS is comprised of classical and non-classical pathways. Angiotensin-converting enzyme (ACE), angiotensin II, angiotensin type 1 receptor (AT1R), and angiotensin type 11 receptor (AT2R) are involved in the majority of classical RAS pathways. Under pathological situations, activation of classical RAS pathway can induce vasoconstriction and inflammation, leading to hypertension and kidney damage (Forrester et al., 2018). In contrast, the ACE2-ANG-(1-7)-MAS receptor axis of the non-classical RAS pathway counteracts the deleterious effects of angiotensin II signalling. Notably, both RAS pathways are associated with fetal programming (Bogdarina et al., 2007; Chappell et al., 2013). Numerous nutritional intervention studies in animal models during pregnancy and lactation have demonstrated that adult hypertension is connected with changes in RAS mRNA expression. For example, male SD rats exposed to a high fructose diet during gestation and lactation had elevated blood pressure at 12 weeks of age, accompanied by decreased expression of AT2R mRNA (Hsu et al., 2016) and increased expression of renin (Tain et al., 2015). At 150 d of age, male Wistar rats that were born to dams fed a protein-restricted diet during lactation exhibited elevated blood pressure and glomerular filtration rate, as well as increased protein expression of AT1R and decreased protein expression of AT2R (Luzardo et al., 2011). Male SD rat offspring of dams fed a high-fat diet during gestation and lactation had elevated blood pressure at 16 weeks of age, along with increased angiotensinogen (AGT), ACE mRNA expression and AT1R protein expression (Hsu et al., 2019). Thus, RAS genes play a crucial role in programming adult hypertension. In addition to RAS, sympathetic nervous system activity is a

primary cause of hypertension and may contribute to the fetal programming of elevated CVD risk; however, the underlying processes remain unknown. It has been shown that stimulation of the renal sympathetic nerve mediates the development of hypertension after an early-life injury. Although the mechanism is unclear, modulation of sodium resorption along renal tubules may play a role in blood pressure regulation and hypertension development (Alexander et al., 2015).

1.4.2.8 Decreased nephrons in IUGR and programming hypertension

A lower number of nephrons in IUGR kidneys is another factor linking early childhood nutrition and hypertension risk in adulthood (Barker et al., 2006). Fetal growth restriction is associated with altered renal formation, decreased renal volume, and reduced number of nephrons (Hughson et al., 2003; Konje et al., 1997; Manalich et al., 2000; Silver et al., 2003; Spencer et al., 2001). For example, reduced fetal growth decreases the number of nephrons in animals, resulting in elevated blood pressure (Kwong et al., 2000; Woods et al., 2001a). A reduction in the number of nephrons results in glomerular hyperfiltration and a rise in glomerular pressure. Over time, this may cause glomerular hypertension, sclerosis, and premature nephron death (Barker et al., 2006). This process causes a self-sustaining negative loop of increased blood pressure and additional nephron loss, further contributing to hypertension (Eriksson et al., 2007). An estimated 10% of pregnancies worldwide are affected by uteroplacental insufficiency, which leads to IUGR as a result of hypoxia, altered hormone concentrations, and diminished nutrition (Jones et al., 2012). Nephrogenesis in humans is complete by 34-36 weeks of gestation; hence, premature infants will have undeveloped kidneys with decreased renal mass, diminished nephron and glomerular capacity, and higher protein excretion. In adulthood, the reduced number of nephrons, lowers the available filtration surface area, leading to hypertension (Brenner et al., 1988). Postnatal

development is also essential for kidney morphology since tubular length and glomerular size grow inversely with nephron number (Simeoni et al., 2011). Furthermore, kidney development is complete 3-4 d after birth in humans and pigs. Thus, IUGR may manifest as diminished nephrogenesis and RAS suppression in adulthood, resulting in hypertension (Woods et al., 2001b).

1.4.2.9 Endothelial dysfunction, IUGR and programming hypertension

Another mechanism that may explain the development of hypertension later in life is endothelial dysfunction in IUGR individuals (Goodfellow et al., 1998). Endothelial dysfunction may be affected by poor angiogenesis during fetal development, or by a decrease in the synthesis or function of nitric oxide (Goodfellow et al., 1998). According to research, elevated inflammatory cytokines and metabolic gene expression, such as increased expression and activity of ion transporters and sodium hydrogen ion exchanger 3, can contribute to hypertension in adults who were IUGR as infants (Bertram et al., 2001; Dagan et al., 2007).

1.4.2.10 Catch-up growth and programming hypertension

Post-natal growth is also associated with hypertension programming. For instance, rapid growth after two years of age was associated with a greater risk of hypertension among neonates who were light and thin than in those who stayed short and thin throughout childhood (Barker et al., 2005; Eriksson et al., 2007). Diastolic blood pressure is associated with rapid weight gain during the first month after birth, but systolic blood pressure is associated with growth during the toddler years (Ben-Shlomo et al., 2008), which indicates that high childhood growth rates are associated with hypertension later in life. Human studies have shown that immediate postnatal growth predicts diastolic blood pressure in term infants, and that birth weight and postnatal growth are associated with systolic blood pressure in young adults (Ben-Shlomo et al., 2008). Although

the underlying mechanisms are unknown, these studies indicate that prenatal and postnatal variables may influence blood pressure later in life. One possible explanation for the association between catch-up growth in IUGR and hypertension is that rapid growth in body size after delivery may exacerbate glomerular injury because a bigger body size results in an increased in excretory load (Eriksson et al., 2007). Thus, a mechanistic explanation of the relationship between early life growth rate and adult hypertension may be explained by low number of nephrons at birth in conjunction with low birth weight, followed by an increased excretory burden associated with rapid growth after birth.

1.4.2.11 Epigenetics and hypertension

Multiple genes and metabolic pathways contribute to the pathophysiology of hypertension. As prenatal insults can result in adult hypertension, it is believed that epigenetic pathways play crucial roles in the fetal programming of hypertension. Epigenomic research give novel insights into the underlying mechanisms of the developmental onset of hypertension. Specific epigenetic changes of genes expressed in the kidney have been linked to the development of hypertension, as revealed by a systematic investigation of epigenetic markers in the genome (Liang et al., 2013). Although epigenomic studies of hypertension are still in early stages, research involving parental and offspring diet and hypertension in rats has revealed that, dietary composition strongly influences salt-induced hypertension and renal disease (Liang et al., 2013). DNA methylation and mRNA expression patterns in renal medulla have also been implicated as a mechanism linking early nutrition to the development of hypertension characteristics.

Some research had demonstrated epigenetic alteration of specific genes associated with hypertension development. Binding glucocorticoids to mineralocorticoid receptors increase sodium reabsorption in the distal nephron, which may contribute to hypertension development. Increased 11-hydroxysteroid dehydrogenase type 2 expression in the distal nephron inactivates

glucocorticoids. The tissue-specific enrichment pattern of 11-hydroxysteroid dehydrogenase type 2 is inversely correlated with the degree of methylation of its promoter region, suggesting that 11-hydroxysteroid dehydrogenase type 2 promoter methylation may contribute to the homeostatic regulation of blood pressure (Alikhani-Koopaei et al., 2004). Renal 11-hydroxysteroid dehydrogenase type 2 was down-regulated from birth to postnatal day 21 in an IUGR rat model of hypertension, indicating DNA methylation changes.

Conversely, hypertension can result from the activation of systemic and/or local renin-angiotensin systems, as discussed previously. Lee et. al., (2012), reported that ACE1 was up-regulated in multiple organs, including the kidney, of spontaneously hypertensive rats compared to Wistar-Kyoto rats, (Lee et al., 2012) and that it was associated with increased binding of histone 3 acetylation (H3Ac) and, fourth lysine trimethylation (H3K4me3) and decreased binding of histone ninth lysine dimethylation (H3K9me2) (Lee et al., 2012). Prenatal changes in DNA methylation in rats lead to persistent changes in the expression of AGTR1 mRNA in the paraventricular nucleus, resulting in postnatal salt-induced hypertension (Kawakami-Mori et al., 2018). Moreover, studies have demonstrated a relationship between abnormal DNA methylation and developing hypertension. For example, increased AT1R expression is associated with progressive hypo-methylation of the AT1R promoter in spontaneously hypertensive rats at 20 weeks of age (Pei et al., 2015). All these findings demonstrate that hypertension has a developmental basis, and that epigenetics may be the underlying mechanism. Thus, the status of methyl nutrients in the early life is key in influencing the development of hypertension in adulthood.

1.4.3 Cardiovascular diseases

According to the World Health Organization (WHO), CVD was the leading cause of death

worldwide in 2019, accounting for 32% of all deaths worldwide. Notably, non-communicable diseases (CVD, metabolic syndrome) account for a third of all premature deaths among those under the age of 70. CVD is defined as a group of disorders of the heart and blood vessels that include coronary heart disease (CHD), cerebrovascular disease (stroke), peripheral artery disease, heart failure and other conditions where the main underlying cause of CVD is atherosclerosis. It has been predicted that by 2030, CVD will be responsible for 23.6 million deaths globally (WHO, 2017). However, by treating risk factors such dyslipidemia, hypertension, and obesity, it may be possible to prevent up to 80% of CVD (Buttar et al., 2005). Due to the nature of the CVD, it is critical to modulate early life diet to reduce the risk of development of CVD later in life.

1.4.3.1 Nutritional programming and cardiovascular disease

Among the causes of CVD, diet plays a crucial role in disease initiation, and numerous nutrigenomic and epigenomic studies have investigated the interactive effects of diet and genes on CVD. Although both genetic and dietary variables have a substantial role in the pathophysiology of CVD, dietary interventions are more effective in preventing CVD because they may be actively controlled. Changes in DNA methylation have been implicated as one of the fundamental processes of CVD, although the pathophysiology is only partially understood (Glier et al., 2014) Both human and animal studies have demonstrated an association between inadequate nutrient supply and IUGR during pregnancy and infancy with increased risk of CVD (Barker & Martyn, 1992; Jackson et al., 2011). The association between maternal malnutrition and increased risk of chronic diseases, is well illustrated by studies from the Dutch famine (1944-1945), where the Dutch famine birth cohort study found that the children of pregnant women exposed to famine were more susceptible to diabetes, obesity, cardiovascular disease, microalbuminuria and other health problems. In addition, rodent studies have demonstrated that postnatal overfeeding permanently increases body weight, adiposity, plasma insulin, leptin, and lipids, resulting in an

increase in heart disease (Josse et al., 2020). Even small periods of postnatal malnutrition result in permanent changes to the structure and function of the heart, hence raising the risk of CVD (Ferguson et al., 2019).

1.5 Programming effects of postnatal methyl nutrients and metabolic syndrome

It is clear that early nutritional insults play a significant role in the development of non-communicable diseases in adulthood via programming effects of epigenetics, especially DNA methylation. Epigenetic programming can lead to metabolic changes likely caused exposure to imbalanced dietary methyl nutrients during early life developmental windows. Studies have investigated the connection between dietary methyl groups and epigenetics, using maternal dietary interventions during the gestation period only, or during gestation and lactation have found that maternal methyl nutrient supplementation affects offspring metabolism (Randunu & Bertolo, 2020). For example, folate and vitamin B-related deficiencies are directly related to pathogenesis of several chronic diseases and are potentially modulated by DNA methylation (Sie et al., 2013). However, there has been limited research on the effects of postnatal dietary methyl nutrients on epigenetic programming of long-term adult diseases and if this can be mitigated by modifying postnatal methyl nutrients.

1.5.1 Postnatal methyl nutrients and insulin sensitivity

Several studies have investigated the effect of postnatal methyl nutrients on insulin sensitivity. For example, a postnatal methyl-deficient weaning diet (consisting of folic acid, vitamin B12, methionine and choline) permanently affected insulin-like growth factor-2 (IGF-2) expression by altering methylation patterns in mice (Waterland et al., 2006). The loss of IGF2

imprinting was evident by 60 d post-weaning, and effects persisted for a further 100 d even after mice were switched to a control diet. This suggests that the effects of postnatal methyl status can contribute to insulin sensitivity in adults.

The metabolic impacts of maternal diet during gestation on programming are already defined at birth. However, modulation of methyl nutrients in the postnatal diet can restore the maternal diet's epigenetic impact on insulin resistance. For example, a rat study showed that high maternal folic acid supplementation can increase insulin resistance in offspring, and supplementing matching folic acid levels in the postweaning diet can attenuate many of the effects of maternal diet (Huot et al., 2016). In another study, feeding rat pups a high folate diet postnatally reversed the obesogenic phenotype and impaired glucose response in offspring from dams fed a high folate diet. Furthermore, postnatal folate mediated these effects through methylation of the promoter of pro-opiomelanocortin (POMC), a hormone implicated in suppressing appetite (Cho, Sánchez-Hernández, & Reza-López, 2013). These results provide further evidence that postnatal methyl donors counteract insulin resistance programming during pregnancy and that these effects will likely persist into adulthood because of epigenetic programming. On the other hand, the availability of methyl nutrients, such as betaine, folate, and vitamin B12, during the postnatal period has been demonstrated to improve glucose metabolism in later life. For example, there is evidence that postnatal betaine improves glucose homeostasis in adulthood (Ribo et al., 2021). In a study using a IUGR rat model, enhanced insulin sensitivity was observed after 60 and 120 d in rats fed a postnatal diet rich in folate and vitamin B12 (Zhang et al., 2022). These findings suggests that methyl nutritional intervention in the early postnatal period can improve the immature metabolic system established during the fetal period and that lactation can be used as a window of opportunity for early nutritional intervention.

Similar to folate, postnatal betaine supplementation has demonstrated positive effects on

adult glucose metabolism. For example, a recent mouse study revealed that higher maternal betaine supplementation during lactation improved milk betaine concentration in dams and resulted in improved glucose homeostasis in adulthood of the offspring (Ribo et al., 2021). Taken together, these studies collectively suggest that methyl nutrients in the postnatal diet are capable of altering the epigenome and programming insulin sensitivity in adulthood.

1.5.2 Postnatal methyl nutrients and lipid metabolism

There are numerous reports on the effects of perinatal methyl nutrient supply on lipid metabolism (Blaise et al., 2007; Garcia et al., 2011; J. L. Guéant et al., 2013; Li et al., 2021; Pooya et al., 2012), however, very few studies have examined the effects of only postnatal methyl nutrient supply on lipid metabolism. In a rat model of IUGR, a postnatal diet supplement with high levels of folate and vitamin B12 improved hyperlipidemia significantly by day 60; however, effects diminished by day 120 (Zhang et al., 2022). While this does not completely repair lipid metabolism, it does indicate that methyl nutritional intervention in the early postnatal period can improve the immature metabolic system established during the fetal period and modulate lipid metabolism in adulthood. Postnatal methyl supplementation can rescue maternal programming effects on lipid metabolism. A rodent study demonstrated that postnatal folic acid supplementation reduced alcohol-induced hypercholesterolemia in pups and adults by reducing HMG-CoA reductase activity and increasing catabolism. These results indicate that alcohol intake provokes different lipid alterations in adults and in pups whose mothers were exposed to ethanol; however, folic acid supplementation alleviates these adverse effects by reducing HMG-CoA reductase activity (Ojeda et al., 2008). A study on rats demonstrated that postnatal methyl donor supplementation can reverse some of the detrimental effects induced by maternal separation, such as abnormal total and HDL cholesterol levels (Paternain et al., 2016). They also found that supplementing methyl donors increased total DNA methylation in the hippocampus of the

offspring. Collectively, these findings suggest that postnatal methyl nutrients can reverse the deleterious effects of maternal nutritional deficiencies on lipid metabolism and that some of these effects are likely to be mediated by DNA methylation and thus persist over time.

In animals, breast milk is the only source of nutrients and, therefore, the only dietary source of methyl nutrients for infants under normal conditions. Methyl donor supplementation during lactation in dams fed an obesogenic diet decreased the accumulation of fat in the liver of offspring as they aged (Cordero et al., 2014). Furthermore, methyl supplementation altered DNMT expression in offspring at 20 weeks of age, suggesting that epigenetics is involved in the accumulation of liver fat in adulthood. A recent study found that pigs with IUGR had abnormal circulating lipid parameters compared to normal birth-weight pigs. Furthermore, supplementing the diet of IUGR pigs with high levels of choline during the postnatal period improved liver lipid metabolism by decreasing the activity of NADPH-generating enzymes and altering the expression of genes related to lipid metabolism (Li et al., 2018). This suggests that postnatal methyl supplementation can positively impact lipid metabolism in neonates. These effects may persist into adulthood, as the mechanisms of lipid-lowering effects are associated with gene expression.

Lipid metabolism requires methyl nutrients because PC, which is required for lipoprotein secretion by the liver, is synthesized through the PEMT pathway (Guéant et al., 2014) (Figure 1.4). There is evidence that supplementing dietary transmethylation products such as PC in early life enhances lipid metabolism and is involved in negating the adverse effects of impaired dietary conditions. For example, a murine animal model fed a diet containing specific phospholipids during early life had improved liver oxidative capacity, mitochondrial fusion and lipid profiles, which persisted into later life under an obesogenic environment (Jelenik et al., 2022). This highlights the importance of dietary phospholipids in early life on metabolic health, including lipid metabolism

in later life and suggests that postnatal exposure to specific methyl nutrients impacts programming lipid metabolism of offspring, likely via epigenetic mechanisms.

Lipid and lipoprotein metabolism and methyl and methionine metabolism.

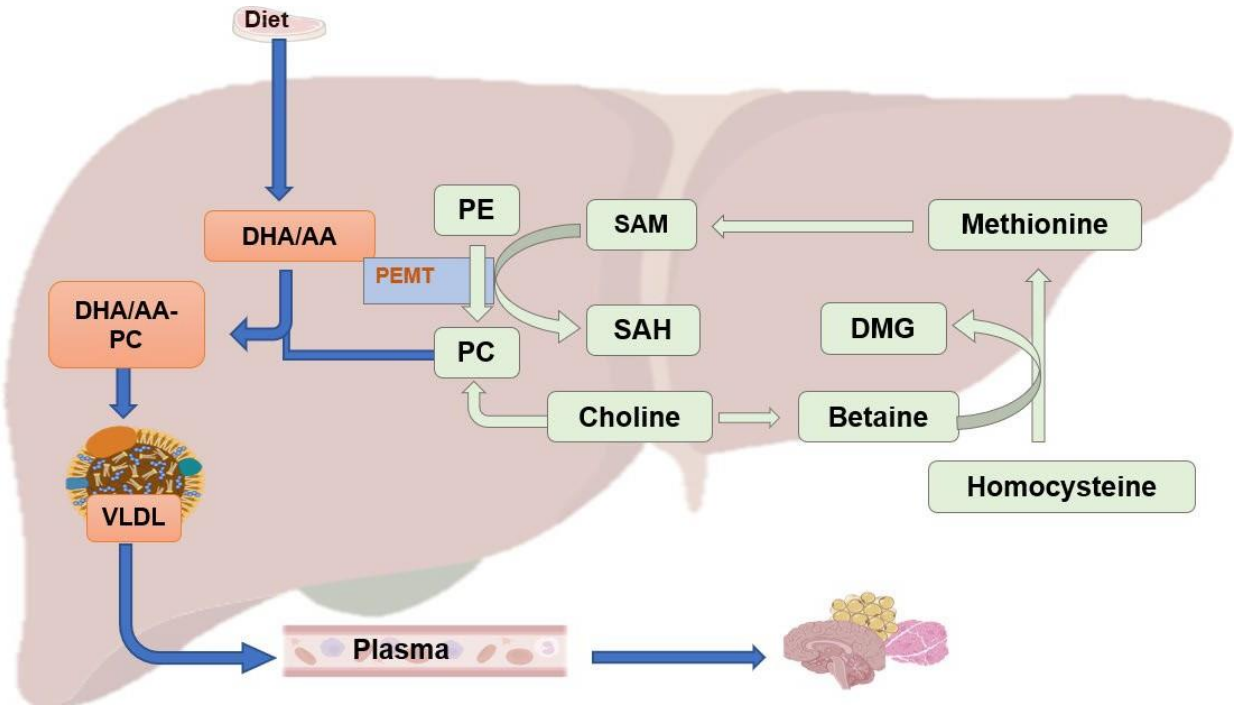

Figure 1:4 Schematic representation of connection between lipid and lipoprotein metabolism with methyl and methionine metabolism.

PC is one of the major transmethylated products in the methionine metabolism cycle and is synthesized via the PEMT pathway or the CDP-choline pathway from choline. Dietary DHA/AA gets incorporated into PC and assembled into VLDL in the liver. VLDL is then excreted to circulation, and fatty acids are distributed among peripheral tissues. PC, phosphatidylcholine; PE, phosphatidylethanolamine; DMG, dimethylglycine; PEMT, phosphatidylethanolamine N- methyltransferase; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; VLDL, very low-density lipoprotein; DHA, docosahexaenoic acid; AA, arachidonic acid.

1.5.3 Postnatal methyl nutrients and obesity

There are indications that obesity is also programmed by postnatal methyl nutrients, although research is limited. A study on rats revealed that dams administered a diet high in vitamins, especially folate, had offspring with metabolic syndrome, and this could be corrected by feeding the offspring a high folate diet postnatally. Furthermore, the effects of the postnatal diet were mediated by changes in the methylation of the pro-opiomelanocortin promotor, which is involved in appetite suppression (Cho, Sánchez-Hernández, Reza-López, et al., 2013). Another rat study showed that high maternal folic acid supplementation increased body weight and the expression of genes that govern food intake in a sex-dependent manner and that supplementing matching folic acid levels in the postweaning diet attenuated many of these effects (Huot et al., 2016). This highlights the ability of folate in the postnatal diet to rescue in-utero epigenetic programming by maternal diets.

Betaine supplementation in early life is also involved in programming obesity in adulthood. Ribo et al. (2021) demonstrated that increased betaine supplementation via maternal diet during lactation has been shown to reduce obesity in adult mouse offspring. This illustrates the long-term effects of postnatal dietary betaine supplementation on the development of adiposity in later life (Ribo et al., 2021). This study also revealed an association between low milk betaine content and high infant growth during the first mo of life (Ribo et al., 2021). Accelerated growth during infancy and childhood has been linked to adult obesity (Andersen et al., 2012; Aris et al., 2018; Stettler et al., 2005; Taveras et al., 2009), indicating there may also be a link between low milk betaine content and an increased risk of childhood obesity (Ribo et al., 2021).

There is further evidence demonstrating the ability of postnatal methyl nutrients to reduce antioxidant enzymes and lipid peroxidation associated with obesity. For example, a study on rats demonstrated that postnatal folic acid can significantly attenuate homocysteine induced

glutathione peroxidase activity and lipid peroxidation (Koohpeyma et al., 2019). Furthermore, folic acid was shown to significantly reduce total plasma total homocysteine levels. Together, these studies demonstrate that methyl nutrients in the postnatal diet play a role in the risk of developing obesity in adulthood.

1.5.4 Postnatal methyl nutrients and hypertension

The link between postnatal methyl nutrients per se and adult hypertension has not been investigated; however, both prenatal and postnatal methyl nutrients have been shown to be associated with adult hypertension. A study on rats found that a high maternal methyl donor diet during pregnancy and lactation led to the development of diet-induced hypertension and that maternal melatonin therapy can counteract these effects. A high methyl donor diet caused long-term alterations of ~800 renal transcripts in offspring; in particular, nutrient sensing signals and sodium transporters were implicated in methyl donor diet-induced programmed hypertension in offspring (Tian et al., 2018). Bai et al. (2012) found that maternal choline supplementation during periods of low protein exposure can normalize increased systolic blood pressure of offspring in later life (Bai et al., 2012). Collectively, these results demonstrate that postnatal methyl nutrients program obesity and dyslipidemia in adulthood, possibly through epigenetics, and that perinatal methyl nutrients are linked to the development of hypertension. Therefore, it is likely that methyl nutrients in early life, particularly during the postnatal period, play a crucial role in the adult development of metabolic syndrome in offspring.

1.6 Programming effects of postnatal methyl nutrients on cardiovascular disease

Although research is limited, gene methylation is implicated in the pathogenies of atherosclerosis. For example, tissue samples from bypass patients showed hypermethylated

atheroprotective genes in the aorta, internal mammary arteries, and veins (Lister et al., 2009). Global DNA hypomethylation is evident in the early stages of atherosclerosis with vascular aortic lesions in mice and modification of DNA methylation was found in each tissue type before the vascular damage occurred (Lin et al., 2016; Lund et al., 2004). Obesity and diabetes have been shown to be inversely related to the methylation status of leukocyte DNA (Kim et al., 2010), indicating that DNA methylation is an important element in the pathogenesis of CVD. Collectively, these studies implicate that a properly functioning cardiovascular system is controlled by epigenetics. DNA methylation and postnatal methyl nutrient status can play important roles in the programming of CVD in later life. However, it is worth noting that global DNA methylation indicates total methylation status across the entire genome, most of which is not modulated for gene expression control, while gene-specific DNA methylation status provides an analysis of the methylation status of a specific gene that is involved in the metabolic pathway expressed by the phenotype (Dahl & Guldberg, 2003).

1.7 Postnatal methyl nutrients and DNA methylation

Methyl nutrients can modulate the degree of DNA methylation in specific genes, playing a role in the programming of adult chronic diseases associated with methylation-altered genes. For example, a study on rats showed that a moderate dietary deficiency of folate during the postnatal period from early childhood to late childhood altered genomic DNA methylation in the liver, which persisted into adulthood following a return to a control diet at puberty (Kotsopoulos et al., 2008). Another study showed that site-specific CpG DNA methylation of selected genes in the liver of rat offspring was significantly increased by both maternal and postweaning folic acid supplementation; however, the effects were more consistent with post-weaning folic acid supplementation (Sie et al., 2013). These studies indicate that epigenetic alterations induced by

postnatal methyl nutrients may be long-lasting. Therefore, it is possible that methyl nutrients in the postnatal diet can influence adult diseases.

1.8 Total parenteral nutrition overview and significance for neonates

TPN is the intravenous provision of adequate daily nutrition. It is used to deliver nutrition to individuals, including neonates, who cannot ingest, digest, and absorb their daily nutritional requirements due to inadequate or contraindicated enteral feeding. Amongst the postnatal diets recommended for neonates, TPN is a non-normal nutritional regimen in which methyl nutrients are not optimized.

TPN was initiated in the late 1930s and has been used as a treatment since 1950; however, widespread clinical adoption did not begin until 1960 (Vinnars & Wilmore, 2003). Today, 8-9% of newborns are born preterm with a birth weight of less than 2.5 kg (Prolla et al., 2022), accounting for approximately 15 million live births yearly, and the incidence of IUGR among infants is 8-9% (Priante et al., 2019). In Canada, these infants account for 35-50% of hospitalizations in neonatal critical care units, costing the health care system 10 to 20 times more than a healthy infant, and account for 75% of perinatal deaths (Canadian Institute for Health Information., 2009). Most preterm and IUGR infants cannot feed and swallow and have underdeveloped digestive systems. Furthermore, they have higher nutrient requirements than term-born infants due to rapid growth rates, making them more susceptible to nutritional deficiencies. This also predisposes them to a broad spectrum of clinical disorders, including trauma, sepsis, shock, chronic intestinal failure, malignancies, inflammatory bowel disease, gastrointestinal obstruction, and short bowel syndrome. TPN is an essential lifesaving feeding strategy in treating newborns with conditions such as IUGR or prematurity and critically ill infants who require nutritional supplementation. TPN serves an important role in the medical management of critically

ill, preterm and IUGR newborns by bypassing the digestive tract to provide daily nutritional requirements. Furthermore, TPN is administered to 90% of newborns <1500 g and 80% of infants who fail to establish enteral feeds by day 3-5 (Bolisetty et al., 2020). Although the survival rates of low-birth-weight infants remain high, optimal nutritional requirements and health impacts are poorly understood (Brunton et al., 2000; Prolla et al., 2022). TPN is a non-normal dietary regimen, and it places infants under substantial metabolic stress. If this stress occurs during the "epigenetic window" of programming early in life, it can permanently reprogram the metabolism and increase the risk of adult disorders. A comprehensive understanding of the metabolic processes caused by TPN support is critical to designing nutritional management strategies that improve both short-term and long-term postnatal outcomes.

1.8.1 TPN limitations- Acute drawbacks

The lack of exposure to gut hormones, such as gastric inhibitory peptides and glucagon-like peptides, due to bypassing the important natural metabolic gatekeepers, such as the gut and liver, is primarily responsible for altered metabolism after TPN feeding (Drucker, 2007). Moreover, TPN-induced gut barrier dysfunction may result in gut-derived endotoxemia. This causes hepatic inflammation, which then causes metabolic dysfunction, resulting in altered hepatic lipid metabolism, steatosis, and insulin resistance (Kansagra et al., 2003). TPN is also associated with gut atrophy, gut epithelial leakage, dysbiosis, liver dysfunction, liver steatosis, parenteral nutrition-associated liver disease (PNALD), and significant metabolic derangements as acute adverse effects.

Gut atrophy during TPN feeding develops due to bypassing first-pass splanchnic metabolism and not utilizing the digestive and absorption processes of the gut. However, studies have shown that gut atrophy induced by TPN resolves once enteral feeding is initiated (Buchman

et al., 1995; Kamei et al., 2005). The cause of liver dysfunction is multifaceted; deficits of taurine and choline and lack of enteral nutrition, glucose, and lipids have all been linked to PNALD (Żalikowska-Gardocka & Przybyłkowski, 2020; Jain & Teckman, 2014). Although liver impairment in newborns might be benign and temporary (Lloyd & Gabe 2007), long-term TPN use can lead to end-stage liver disease. Concern exists about the extent to which the occurrence of such an incident during critical periods of development affects the long-term programming process. It is concerning that the stress that steatosis exerts on the mitochondria and several cell-signalling pathways in hepatocytes may modify the epigenome of the cell, which could be programmed for later life.

Lipid intolerance is a common complication in infants who are fed TPN, and many neonates who receive TPN show hyperlipidemia and hypercholesterolemia. For example, neonates receiving TPN during the first week of life have significantly higher plasma cholesterol levels than infants who did not receive TPN (Nghiem-Rao et al., 2016). The infants with low birth weight for gestational age or IUGR had the highest cholesterol levels, indicating that preterm infants and newborns with low birth weight are more susceptible to TPN-induced hypercholesterolemia (Nghiem-Rao et al., 2016). Intestinal sterol pumps such as ABCG5 and ABCG8 regulate cholesterol absorption and act as a barrier mechanism, preventing excess absorption of lipids. Administration of intravenous lipids bypasses this barrier mechanism, so lipids are directly infused into the circulation and may contribute to elevated cholesterol levels in TPN-fed patients.

Hypertriglyceridemia is a metabolic consequence of TPN use and is relatively common in this form of therapy (Antonio & Florit-Sureda, 2016; Llop et al., 2003; Mirtallo et al., 2010). The lipid profile of neonatal piglets administered TPN for 14 d revealed decreased HDL-cholesterol and increased TG concentration (Jain et al., 2012). The liver TG levels were also higher in the

TPN group compared to the enteral feeding group, with oil-red-O staining revealing significant microvascular lipid droplets (Jain et al., 2012). The etiology of hypertriglyceridemia in patients receiving TPN is linked to variations in plasma lipid clearance due to excessive supply or to decreases in lipoprotein lipase (LPL) activity (Llop et al., 2003; Miles et al., 2001). Several factors are known to enhance the risk of hypertriglyceridemia with TPN use. Among them are renal failure, sepsis, pancreatitis, hyperglycemia, diabetes, obesity, multiple organ failure, and the administration of specific medicines such as corticosteroids (McQueen et al., 2008). Infants who require TPN, such as those with IUGR, prematurity, or sickness, may be predisposed to develop hypertriglyceridemia during the TPN support period if they have the above conditions. In addition, various fat emulsions have distinct plasma TG clearances (Antonio & Florit-Sureda, 2016). In patients with moderate hypertriglyceridemia receiving TPN, substituting single-source fat emulsion (olive oil-based fat emulsion) with multiple-source oil fat emulsion decreased triglyceridemia (Antonio & Florit-Sureda, 2016). The observed alterations in dyslipidemia and associated gene regulation can be long-lasting. The precise mechanisms of these events are presumably related to the duration and the time of TPN exposure during the early critical period; however, these mechanisms are not fully understood. Thus, long-term research is required to determine if hypertriglyceridemia caused by TPN support during the early programming phase persists throughout later life, predisposing individuals to chronic disorders as adults.

Numerous animal and human research studies indicate that early postnatal TPN feeding increases infant body fat mass and systemic inflammation. For instance, Stoll et al. (2010) showed that feeding newborn pigs chronic TPN for 17 d altered their body composition, including increased fat deposition. Another study showed that 21 d of TPN feeding increased the proportion of body fat in young rats (Popp et al., 1982). In addition, TPN feeding for 10 d increased fat mass

and body weight in hospitalized children without changing fat-free mass (Schmid et al., 2006). Early TPN feeding increases inflammatory mediators such as IL-6 and TNF-α, indicating that TPN feeding can cause a systemic response and affect body composition (Stoll et al., 2010). Similarly, it has been demonstrated that IL-6 levels in premature newborns drop as TPN exposure time decreases (Cole et al., 2010). A human investigation aiming to examine the effects of TPN on the body composition of preterm newborns revealed that gestational age and duration of TPN are significant predictors of body fat percentage (Meyers et al., 2013). Although there are numerous reports on the acute effects of TPN feeding in the neonatal period on altered body composition (e.g., increased body fat content), the question arises whether these metabolic perturbances modify the epigenome during key programming windows. Moreover, if metabolic changes due to increased fat mass in the neonatal period influence the epigenome, the persistence of these effects into adulthood needs further clarification. Recent investigations have shown that the early support of TPN in neonates and children induces distinct DNA methylation patterns (Jacobs et al., 2021). Early TPN feeding in conjunction with critical illness can cause changes in DNA methylation within 3 d, and the majority of the modifications appear to be sustained with extended TPN feeding (Verlinden et al., 2020). However, it is unknown if these epigenetic alterations caused by TPN persist into adulthood, and there is very little long-term research examining the impact of early TPN on adult body composition. Given that the prevalence of obesity has tripled over the past three decades and 39 to 49% of the world's population is overweight or obese, it is crucial to investigate the causes of adult obesity to develop preventive and treatment strategies (Marousez et al., 2019).

1.8.2 Long-term effects of TPN during the postnatal period on chronic diseases.

Research has revealed the acute and complex problems associated with TPN feeding in neonates (Lucchinetti et al., 2021; Stoll et al., 2010, 2012). Among the acute adverse effects caused by TPN, gut atrophy, gut epithelial leakage, dysbiosis, liver dysfunction, liver steatosis, PNALD, and metabolic changes are the most prominent and significantly investigated issues (Ukleja & Romano, 2007). Studies have shown that feeding TPN alters sulphur amino acid metabolism in neonatal piglets (Shoveller, Brunton, Pencharz et al., 2003; Shoveller, Brunton, House et al., 2003; Shoveller et al., 2004). Alerted sulphur amino acid metabolism causes alterations in the methyl supply. Changes in methyl supply can permanently affect gene expression (McMillen et al., 2005), mainly if it occurs during the critical development period. Alerted gene expressions may persist into adulthood, causing permanent changes in metabolism. However, studies on the long-term effects of feeding TPN to newborns are scarce.

1.8.3 TPN support and dyslipidemia in adulthood

1.8.3.1 TPN, dyslipidemia, and CVD

Neonatal TPN support causes acute alterations in lipid profile, leading to hyperlipidemia (hypercholesterolemia and hypertriglyceridemia). These alterations to the lipid profile may have long-term impacts on the cardiovascular system. Studies indicate that cholesterol levels in later childhood and adulthood have links to early life cholesterol levels (Juhola et al., 2011; Sporik et al., 1991; Webber et al., 1991). Furthermore, studies indicate that early life cholesterol levels contribute to CVD risk later in life (Freedman et al., 1993; Napoli et al., 1997a, 1999; Papacleovoulou et al., 2013). This suggests that changes in cholesterol levels (dyslipidemia) in early life may programme diseases in later life, potentially through epigenetics, as these changes

occur during the critical programming windows. Lipid intolerance is a common complication in infants using TPN, and many neonates who receive TPN show acute hyperlipidemia and hypercholesterolemia (Nghiem-Rao et al., 2016). A nested case-control study of preterm infants who received TPN found significantly higher plasma cholesterol levels during the first 9 week of life (Lewandowski et al., 2011). Interestingly, they found that this elevation in plasma cholesterol induced by intravenous lipids during infancy was associated with an increased risk of metabolic syndrome, including hypertension and coronary heart disease, in adulthood. The study found a significant association between elevated plasma cholesterol levels and adult aortic and myocardial dysfunction. This study demonstrates the impact of circulating cholesterol during critical developmental periods on the cardiovascular system of adults. However, more research is needed to understand how changes in newborn lipid profiles result in long-term changes in the pathophysiology of the cardiovascular system.

Hyperlipidemia is a key driver of atherogenesis, and TPN-induced hyperlipidemia could lead to CVD. Lipid-rich fatty streaks are first visible in the aortic wall during fetal life and peak in prevalence during the first year of life (Faggiotto et al., 1984; Napoli et al., 1997b; R. Ross, 1993; Stary, 1990). Having a hypercholesterolemic mother with abnormal lipid profiles or exposure to hyperlipidemia after birth can predispose an infant to develop fatty streaks in the aorta, which can persist into adolescence. Long-term exposure to lipids causes aortic stiffness, which is the most plausible intermediate cardiovascular phenotype (Wilkinson & Cockcroft, 2007). The presence of increased cholesterol in the abdominal aorta during the critical period of development has long-term impacts. For instance, aortic development typically occurs during the third trimester, where elastin and collagen begin to deposit and contribute to arterial stiffness; however in premature neonates, elastin and collagen deposits during the postnatal period, influencing the early development of the aorta (Wells & Walter, 2010). Studies in cell culture and rabbits have shown

that cholesterol exposure reduces elastin-collagen cross-linking and negatively affects actin and myosin in aortic smooth muscle cells involved in aortic contractility (Massaeli et al., 1999; Rodríguez et al., 2002). Aortic stiffness is associated with elevated blood pressure (Mitchell et al., 2004); therefore, changes in aortic and cardiac function due to early exposure to cholesterol may result in hypertension that can amplify in the long term.

1.8.3.2 TPN, dyslipidemia and IUGR

Prematurity or intrauterine growth restriction as a neonate, as well as the need for TPN support, increases the risk of developing dyslipidemia in later life. In a 17-year retrospective investigation of pediatric patients with hypertriglyceridemia, early TPN feeding was identified as one of the primary causes of dyslipidemia (Richardson et al., 2018). This has been linked to intravenous lipid dose, the prevalence of hyperglycemia, and renal failure, all of which are frequently observed in premature or IUGR newborns. Besides, several patients acquired hypertriglyceridemia as a result of early TPN feeding alone, according to the study. Feeding TPN to premature or IUGR neonates who are predisposed to hyperlipidemia will result in pathophysiological events leading to a metabolically perturbed phenotype. TPN feeding is frequently associated with alterations in lipolysis and lipogenesis in patients with hyperlipidemia, leading to increased fat deposition in the liver via decreased triglyceride oxidation and increased liver lipogenesis (Golucci et al., 2018). Oxidative stress and the generation of cytokines contribute to the advancement of liver steatosis (Golucci et al., 2018). Hyperlipidemia and cholestasis are frequently found in neonates fed TPN (Madnawat et al., 2020), and VLDL synthesis may contribute to this hyperlipidemia, as well as fat accumulation in the liver. Furthermore, impaired bile flow during cholestasis can also increase plasma lipid levels (Nowak, 2020). LPL hydrolyzes lipid particles to release fatty acids, which are used for energy or stored in adipose tissue. Nutritional stress due to TPN feeding decreases LPL activity, resulting in dyslipidemia (Raman et

al., 2017). Taken together, exposure to dyslipidemia due to TPN feeding and as a consequence of being IUGR during a period that is sensitive to the window of programming increases the likelihood of permanently programming long-term metabolism via altering the epigenetics.

1.8.3.3 TPN, dyslipidemia and oxidative stress

Dyslipidemia resulting from TPN support is not only due to parenteral lipid content but can also be due to light exposure of TPN. A prospective randomized study found that photoprotection of TPN improves the progression of minimal enteral nutrition, which is the early introduction of small volumes of enteral feeding for the purpose of stimulating growth and development of the immature gastrointestinal tract of the preterm infant (Khashu et al., 2006). Because light exposure to TPN creates peroxides, which induce vasoconstriction, it is expected to alter not only feeding tolerance, but also gene signals involved in vasoconstriction, as well as long-term health implications. There is evidence that early-life oxidative stress caused by TPN feeding reprograms metabolism through epigenetics, causing changes that can persist into adulthood. For example, research in guinea pigs, demonstrated that administering TPN early in life causes sustained hypomethylation of DNA after inhibiting DNMT activity (Yara et al., 2013). Another study in guinea pigs found that the early life redox potential value has a significant effect on DNA methylation metabolism, resulting in an increase in DNA methylation as a function of increased oxidative stress (Mungala Lengo et al., 2020). These findings confirm that early-life oxidative stress caused by TPN can change the metabolism epigenetically. This research highlights the significance of improving the quality of parenteral nutrition solutions administered early in life, particularly to newborn infants.

1.8.4 TPN support and hypertension in adulthood

Although reports on TPN feeding and long-term blood pressure are scarce, a study in premature neonates found that receiving TPN during the first 14 d of life resulted in significantly higher diastolic blood pressure in adolescence compared to enterally fed infants (Ludwig-Auser et al., 2013). Previous explanations of how early-life dyslipidemia affects fatty steaks in the aorta and results in arterial stiffness may partially explain how TPN feeding programs blood pressure in adulthood; however, detailed molecular mechanisms are poorly understood.

1.8.5 TPN support and hyperglycemia in adulthood

1.8.5.1 TPN, β-cell proliferation and hyperglycemia

Hyperglycemia is another common condition that has been associated with the feeding of TPN to neonates. Insulin resistance, impaired pancreatic beta cell secretion, presence of naive glucose transport systems, and immaturity of the number of insulin dependent tissues are some reasons that cause hyperglycemia in TPN-fed neonates (Raney et al., 2008). Studies have also shown that regardless of parenteral glucose infusion, continuous liver glucose production could also contribute to hyperglycemia in premature infants (Chacko et al., 2011; Chacko & Sunehag, 2010). Stoll et al. (2010) showed that feeding chronic TPN for 17 d led to insulin resistance in the livers of neonatal pigs (Stoll et al., 2010). This dominant metabolic response was evident at both whole body and cellular levels. The study further highlighted that phosphorylation of the insulin receptor and insulin receptor substrates was significantly reduced in livers and skeletal muscles of TPN-fed piglets, which could be due to the presence of increased proinflammatory cytokines (Stoll et al., 2010). Furthermore, suppressed β-cell proliferation in the pancreas secondary to insulin resistance in TPN-fed piglets indicated that there could be long-term consequences on metabolic function due to TPN feeding. This is because β-cell proliferation in the pancreas occurs during the

critical periods of development in newborns, and if this mechanism is affected by feeding TPN during the 'critical window', it is likely to be programmed for the rest of life. Another study by the same research group showed that TPN feeding modality (continuous and intermittent) significantly affects metabolic outcomes in neonatal piglets and feeding continuous TPN as an elemental diet reduced insulin sensitivity. Furthermore, the authors suggest that reduced insulin sensitivity is likely due to defects in peripheral muscle tissue insulin receptor signaling (Stoll et al., 2012).

1.8.5.2 Timing of TPN, IUGR and hyperglycemia

Some human reports also show evidence that early parenteral feeding affects long-term neurodevelopment. Although IUGR neonates have a survival rate of 90%, higher nutritional intake via TPN is recommended to reduce the prevalence of extrauterine growth restriction in infants. This high parenteral nutrition intake in infancy may increase the risk of hyperglycemia in neonates. Human studies have found evidence that this parenteral nutrition-induced hyperglycemia influenced preterm infant survival and long-term neurodevelopment (Boscarino et al., 2021). A randomized controlled trial in critically ill infants showed that withholding TPN during the first week in the pediatric intensive care unit instead of initiating early TPN improved neurocognitive development at 2 years of age (Jacobs et al., 2019). In a 4-year follow-up study of the same critically ill infants, those who had received late administration of TPN had better physical, neurocognitive, and emotional/behavioural development than those who received TPN immediately, with altered DNA methylation as a potential biological mediator (Jacobs et al., 2020). Although these findings support the idea of delaying TPN use during critical illness in infants and children, certain medical conditions may require early TPN as the only survival measure. Furthermore, the aforementioned findings underscore the urgent need for research to modify the composition of TPN administered during the "critical window" to ensure that the metabolic effects of early TPN feeding are not detrimental in the long term.

1.8.6 TPN and methyl supply in TPN products

Indeed, the composition of TPN is critical to its positive outcomes and potential short- and long-term issues. Importantly, as discussed above, methyl nutrients in TPN have a tremendous effect on short- and long-term metabolic perturbations from early TPN feeding. However, neonates today are exposed to varied dietary methyl nutrient composition during their early life (Robinson et al., 2016), and the composition of the methyl nutrients in TPN is inconsistent.

Reports on methyl nutrients and their composition in TPN are rare. The addition of cysteine to neonatal TPN has been associated with an improved nitrogen balance (Soghier & Brion, 2006), which may support infant growth. Since cysteine may help to reduce the pH of TPN, it will allow increased calcium and phosphorus solubility with a lower risk of mineral precipitation, promoting adequate bone mineralization and growth (Greene et al., 1988). Furthermore, the addition of cysteine to TPN can spare methionine and methyl groups by reducing flux through the transsulfuration pathway, and homocysteine could be directed toward the remethylation pathway to produce methionine if folate and betaine are sufficient (Bertolo, 2021).

Similar to cysteine, adding the methyl donor betaine to TPN can also spare methyl groups, as betaine enhances remethylation and increases the resynthesis and availability of methionine. As methionine is the primary donor of methyl groups, the availability of methyl groups for various transmethylation pathways, especially DNA methylation, will be increased, ensuring adequate methyl precursors for appropriate epigenetic profiles (Randunu & Bertolo, 2020). Importantly, when proper DNA methylation occurs during the epigenetic window of programming, long-term effects on chronic diseases could be controlled simply by adding methyl nutrients like cysteine, betaine and creatine into TPN.

Supplementing TPN with methyl consumers, such as creatine, can reduce the demand for methionine by not requiring methionine for creatine synthesis. A study showed that feeding piglets

creatine supplemented TPN for 14 d downregulated creatine synthesis and led to higher creatine levels in plasma, muscle, liver, kidney and pancreas and reduced liver cholesterol (Dinesh et al., 2014). Given that one-third of methionine is devoted to synthesizing creatine, with only one-fourth of creatine being provided from milk and the rest of creatine needing to be synthesized in the body, the above finding suggests that supplementing creatine in TPN can increase/spare available methionine. If creatine is supplied through diet, available methionine can be spared and redirected to other methylation reactions, such as DNA methylation, which plays a critical role in epigenetically based programming. All of these findings emphasize the significance of the composition of TPN, particularly the presence of sufficient methyl groups for DNA methylation.

As mentioned previously, the methyl nutrients in current TPN solutions are highly variable. The primary commercial TPN products with methyl nutrients used to prepare TPN solutions for pediatric use are amino acid solutions, multivitamin solutions, and lipid emulsions. Breast milk is considered the ideal source of infant nutrition, and methionine and methyl-related nutrients in breast milk are listed elsewhere (Robinson & Bertolo, 2016). MVI (Multi12/K1 Pediatric, Baxter Corporation, Mississauga, ON, Canada) is the most frequently used multivitamin solution in TPN for pediatric patients. It includes folic acid and vitamin B12, but not betaine. Choline is obtained in the diet as free choline, glycerophosphocholine, phosphocholine, or phosphatidylcholine (Robinson & Bertolo, 2016) and is added to TPN lipid emulsions as purified egg phospholipids. Phospholipids in the most used commercial lipid emulsions (SMOF lipid and Intralipid) differ, with SMOF lipid containing 1.2 g of phospholipids and Intralipid containing 12 g of phospholipids per 100 mL (Fresenius Kabi, Uppsala, Sweden). Consequently, the amount of choline in various TPN solutions may vary considerably. Additionally, the different amino acid solutions used for TPN, as listed in Table 1.1, contain highly variable amounts of methyl-related nutrients (methionine, cysteine, glycine, serine). Overall, it is evident that infants receiving TPN during the

neonatal period are likely to be exposed to very different levels of methyl nutrients, which can predispose infants to develop chronic diseases as adults.

Table 1:1 Amino acid composition of TPN products used today (% of total amino acids by weight).

*Amino acids involved in methionine metabolism

Amino acid	Primene (Baxter)	Trophamine (McGaw)	Procalamine (B. Braun Medical Inc.)	Aminosyn (ICU Medical Canada Inc.)	HepatAmine (B. Braun Medical Inc.)	Prosol (Baxter)	Premasol (Baxter)	Aminoven (Fresenius Kabi)	Amino-plasmal (B. Braun Medical Inc.)	Travasol (Baxter)	Troph-Amine (B. Braun of Canada, Ltd.)	Vamiolact (Fresenius Kabi)	Vamin-14 (Fresenius Kabi)	Vamin-18 (Fresenius Kabi)
Isoleucine	6.7	8.2	7.2	6.0	11.3	5.4	8.2	5.0	3.9	6.0	8.2	4.7	4.9	4.9
Leucine	9.9	14	9.2	9.0	13.8	5.4	14.0	7.4	7.6	7.3	14.0	10.7	6.9	6.9
Valine	7.6	7.8	6.8	4.5	10.5	7.2	7.8	6.2	4.8	5.8	7.8	5.5	6.5	6.4
Lysine	10.9	8.2	7.5	9.5	7.6	6.75	8.2	6.6	5.3	5.8	8.2	8.6	8.0	7.9
Methionine*	**2.4**	**3.4**	**5.5**	**1.5**	**1.3**	**3.8**	**3.3**	**4.3**	**3.8**	**4.0**	**3.4**	**2.0**	**4.9**	**4.9**
Cysteine*	**1.9**	**0.1**	_	_	_	_	_	_	**0.2**	_	**0.2**	**1.5**	**0.5**	**0.5**
Methionine +Cysteine*	**4.3**	**3.5**	**5.5**	**1.5**	**1.3**	**3.8**	**3.3**	**4.3**	**4.1**	**4.0**	**3.6**	**3.5**	**5.4**	**5.4**
Phenylalanine	4.2	4.8	5.8	2.7	1.3	5	4.8	5.1	3.8	5.6	4.8	4.1	6.9	6.9
Tyrosine	0.9	2.3	_	2.4	_	0.25	2.3	0.4	0.3	0.4	2.4	0.8	0.2	0.2
Threonine	3.7	4.2	4.1	3.6	5.6	4.9	4.2	4.4	3.6	4.2	4.2	5.5	4.9	4.9
Tryptophan	2	2	1.6	1.8	0.8	1.6	2.0	2.0	1.4	1.8	2.0	2.1	1.6	1.7
Histidine	3.8	4.8	2.9	2.7	3.0	5.9	4.8	3.0	3.5	4.8	4.8	3.2	6.0	6.0
Arginine	8.4	12.2	9.9	9.2	7.5	9.8	12.2	12.0	10.7	11.5	12.0	6.3	9.9	9.9
Glycine*	**4**	**3.6**	**14.4**	**4.5**	**11.3**	**10.3**	**3.7**	**11.0**	**12.8**	**10.3**	**3.6**	**3.2**	**6.9**	**6.9**
Alanine	7.9	5.4	7.2	8.9	9.7	13.8	5.3	14.0	14.9	20.7	5.4	9.6	14.1	14.1
Aspartate	6	3.2	_	_	_	_	_	_	_	_	_	_	_	_
Glutamate	9.9	5	_	_	_	_	_	_	_	_	_	_	_	_
Proline	3	6.8	11.6	6.5	10.0	6.7	6.8	11.2	4.9	6.8	6.8	8.6	6.0	7.0
Serine*	**4**	**3.8**	**6.2**	**4.8**	**6.3**	**5.1**	**3.8**	**6.5**	**2.0**	**5.0**	**3.8**	**5.8**	**4.0**	**4.0**
Taurine	0.6	0.2	_	_	_	_	0.3	1.0	_	_	0.2	0.5	_	_
Ornithine	2.2	_	_	_	_	_	_	_	_	_	_	_	_	_
L-Aspartic acid	_	_	_	_	_	_	3.2	_	5.3	_	3.2	6.3	2.9	3.0
L-Glutamic acid	_	_	_	_	_	_	5.0	_	10.8	_	5.0	10.9	4.9	4.9

1.8.7 Methyl metabolism in IUGR and TPN

Consistent with epidemiological data (Hoffman et al., 2017), previous studies using IUGR pigs to examine postnatal nutrition and the risk of adult diseases have demonstrated compensatory growth and biomarkers for hypertension, dyslipidemia, and obesity (McKnight et al., 2012; Myrie et al., 2011, 2017; Myrie, MacKay, et al., 2012). In addition, studies have demonstrated that piglets with IUGR have alterations in their methionine metabolism, with a 20-30% decrease in the activity of homocysteine removal and transmethylation enzymes (MacKay et al., 2012). These findings demonstrate that methyl metabolism is likely implicated in the onset of adult diseases in IUGR neonates.

With regard to TPN, not only are the methyl components of TPN variable, but the route of diet delivery itself has an effect on methyl metabolism. The gut is primarily responsible for methionine metabolism (Bauchart-Thevret, Stoll, & Burrin, 2009a; Bauchart-Thevret, Stoll, Chacko, et al., 2009; Riedijk et al., 2007; Shoveller et al., 2005). In fact, 25% of the entire body's transmethylation and transsulfuration occurs in the intestines (Riedijk et al., 2007), making cysteine essential for TPN (Miller et al., 1995; Shoveller, Brunton, House, et al., 2003). Therefore, when the intestine is bypassed or atrophied, as in TPN feeding (Bertolo, Chen, et al., 1999), the entire body's methionine metabolism is reduced. When piglets are fed TPN with (Shoveller, Brunton, House, et al., 2003) or without (Shoveller, Brunton, Pencharz, et al., 2003) cysteine, their plasma methionine (Bertolo et al., 2000) and total body methionine requirements are 30-40% lower. The diminished capacity of methionine metabolism observed in IUGR (MacKay et al., 2012) is comparable to that observed in neonates receiving TPN (Miller et al., 1995; Shoveller et al., 2004). It is plausible that feeding IUGR with TPN will have a greater detrimental impact on

methyl metabolism than feeding TPN alone. Because a significant proportion of IUGR neonates are fed TPN at some stage in early life, they are at increased risk for developing adult diseases.

1.9 Summary and knowledge gaps

The postnatal period is a critical window for the programming of adult diseases, and exposure to environmental changes during this period plays an important role in epigenetic programming. TPN is especially significant in this regard because it is a lifesaving feeding strategy for many infants and is administered throughout critical periods of growth and development. Studies from neonatal piglet models indicate that early TPN feeding led to the development of an acutely disordered metabolic phenotype, indicated by metabolic markers similar to those of metabolic syndrome, type 2 diabetes, and obesity. These metabolic changes may be attributable to the mode of nutrition support and the TPN's composition. IUGR and preterm newborns are predisposed to chronic diseases as adults, and they are more likely to receive TPN in the immediate postnatal period, further increasing their risk of chronic adult diseases. Research on the acute metabolic consequences of early TPN feeding has shown that length and duration of neonatal TPN exposure may determine the short-term consequences of TPN in early life, and that specific molecular mechanisms can predict these effects. However, there is limited research exploring the long-term metabolic effects of postnatal methyl nutrients, as current studies have only examined the effects until weaning or adolescence. Because epigenetic changes can modify the expression of numerous genes involved in metabolism, it may be a potential way of programming long-term chronic diseases. Thus, more long-term studies are needed to understand the molecular mechanisms behind these effects. Methyl nutrients can alter epigenetics during the critical windows, therefore, adding methyl nutrients to TPN could modify the risk of adult chronic diseases. Betaine and creatine (section 1.3.2) are methyl nutrients that are currently not included

in commercial TPN products (Table 1.1); however, no research has been conducted on the long-term metabolic effects of feeding betaine- and creatine-supplemented TPN during the neonatal period. Current research is essential to understand the long-term adverse metabolic effects of early TPN feeding of both normal weight and IUGR infants. Ideally, this will lead to an epigenome-targeted postnatal diet/TPN designed to avoid the programming of adult diseases.

2 Chapter 2 - Experimental approach

2.1 Justification of the animal model

The Yucatan miniature pig model was established to investigate the development origin of adult disease, including methyl metabolism as a potential mechanism (McBreairty et al., 2013; McKnight et al., 2012; Myrie et al., 2017; Myrie, MacKay, et al., 2012). For example, research has shown that domestic piglets were sensitive to the presence of dietary cysteine (Shoveller, et al., 2003), and IUGR (i.e., runt) Yucatan miniature piglets exhibit perturbed sulfur amino acid metabolism (MacKay et al., 2012). Furthermore, the digestive physiology and metabolism of amino acids in pigs are very similar to those in humans, especially during development. Mini pigs provide an excellent animal model to study metabolic syndrome (Koopmans & Schuurman, 2015), as newborn pigs are the closest non-primate equivalent to human infants (Miller & Ullrey, 1987). Therefore, they are a valuable model for studying the acute and chronic effects of parenteral and enteral nutrition on whole-body metabolism and specific tissues (Puiman & Stoll, 2008). Studies have already used the Yucatan miniature piglet model to study TPN feeding (Dinesh et al., 2018; Dodge et al., 2012). The piglet has been used extensively for amino acid nutritional research, and its metabolism is sensitive to acute dietary perturbations, such as changes in dietary amino acid content of the meal (Brunton et al., 2007). Most importantly, compared to rodent models, the piglet's bigger size, tolerance to multiple catheterizations for extensive blood sampling, methyl and methionine metabolism techniques, steady state isotope kinetics and ability to consume experimental diets provide considerable advantages of using this model in the current study (Bertolo, Pencharz, et al., 1999). Moreover, this miniature piglet is smaller at birth than commercial strains and grows more slowly. Therefore, the maintenance methionine requirements

are proportionately higher than in commercial strains, which is advantageous experimental design. Due to their extensive use in agriculture, swine have well-defined nutrient requirements, which facilitates the design of elemental diets (National Research Council, 2012). Furthermore, the pig's dietary behaviour and nutrition, especially amino acid requirements, are very similar to humans. Hence, a wealth of information from studies with neonatal pigs has been applied directly to humans (Puiman & Stoll, 2008). Therefore, we decided that Yucatan miniature piglets would be the best animal model to investigate long-term effects of neonatal TPN feeding.

2.2 The Rationale of the Thesis/ Concept

There is a significant global prevalence of CVD and metabolic syndrome, a chronic disease characterized by abdominal obesity, insulin resistance, hypertension, and dyslipidemia. Both lifestyle factors and the developmental origin of disease are involved in the onset of chronic diseases in adulthood. Altered nutrition in critical development periods plays a significant role in the development of these chronic diseases. TPN is a lifesaving feeding method during the neonatal period; however, it is associated with changes in metabolism due to altered mode of feeding and bypassing the main metabolic gatekeepers such as the intestine and liver, which can increase the risk of chronic diseases later in life. Such metabolic changes can be permanent and may be caused by epigenetic changes due to imbalances of dietary methyl nutrients.

As discussed above, epigenetic programming occurs by changing the methylation patterns of the promotor regions of modifiable genes. These methylation patterns can be altered by dietary methyl supply (Burdge et al., 2009; Kotsopoulos et al., 2008; Waterland, Lin, et al., 2006). Dietary methyl groups are provided by methionine, which can be remethylated by betaine- and folate-dependent methylneogenesis. However, other transmethylation reactions compete with DNA methylation for these methyl groups, such as creatine and phosphatidylcholine synthesis. In

addition, TPN-induced intestinal atrophy reduces capacity of the methionine cycle, (Shoveller et al., 2005) causing even lower methyl availability for DNA methylation. Supplementing betaine and creatine, which are new to commercial TPN, is likely to increase the methyl availability through increased methylation and sparing methyl groups. Betaine in particular can increase the supply of methyl groups through the upregulation of the remethylation pathway without having to add more methionine to TPN. The amount of methionine that can be added to TPN is limited due to the toxicity of methionine and the accumulation of homocysteine. Supplementing creatine, on the other hand, would reduce consumption of methyl groups employed on creatine synthesis pathway (GAMT pathway) and spare methyl groups. As creatine synthesis consumes 1/3 of the methyl groups in the methyl and methionine cycle, supplementing with creatine would spare a considerable amount of methyl groups. The addition of both betaine and creatine to TPN will therefore ensure increased availability of methyl groups for other transmethylation reactions, including DNA methylation. Adequate availability of methyl groups for appropriate DNA methylation to occur during the critical window of development where epigenetic signatures are established would aid in reducing the programing of chronic diseases in adult life. Therefore, we hypothesized that the metabolic effects of feeding TPN in early life will persist into adulthood, along with increased risk of chronic diseases, and that supplementation with betaine and creatine will prevent these adverse metabolic effects.

Consistent with the developmental origins of adult disease, IUGR neonates are at higher risk of developing metabolic diseases later in life. A higher proportion of IUGR or premature neonates require TPN as a life-saving measure in early life. When TPN is fed, methionine metabolism is significantly affected as a considerable amount of methionine metabolism takes place in the intestine and liver which is bypassed due to feeding TPN. Studies have also shown a

reduction in sulfur amino acid metabolism due to TPN feeding, especially in IUGR (MacKay et al., 2012). Therefore, we hypothesized that IUGR neonates fed TPN during the early critical period will exacerbate the metabolic effects in adulthood. We utilized female pigs for the studies conducted in this thesis as females are more prone to develop metabolic syndrome (McKnight et al., 2012).

2.3 Diet

The control TPN diet we selected has been validated for use in piglets and the nutrient profile was modified for use in piglets (Dodge et al., 2012). An additional modification we made for this study was to replace the lipid source, substituting SMOF lipid for Intralipid, as SMOF lipid is now used routinely in clinics, to minimize PNALD.

Betaine and creatine were added to the control TPN diet to form a TPN B+C diet to maximize the methyl groups in the diet. As the principal source of methyl groups, methionine is essential for numerous transmethylation reactions; however, methionine is not typically increased in the diet due to concerns about homocysteine toxicity, a risk factor for CVD (Garlick, 2006). Alternatively, betaine supplementation promotes methionine re-synthesis and decreases homocysteine levels (McRae, 2013). Thus, adding betaine would increase the methyl group availability in TPN without increasing the homocysteine concentrations. Recent research has also demonstrated that most methyl groups are employed in synthesizing creatine and that enhancing one methylation pathway affects allocating the limited methyl groups to other methylation pathways (McBreairty et al., 2013). Therefore, if we supplement the diet with creatine, the available methyl groups will be spared for other methylation reactions, including DNA methylation. Thus, adding both dietary betaine and creatine can increase methyl group availability, with betaine increasing the remethylation of homocysteine to methionine, and creatine sparing methyl groups for other key

transmethylation reactions. Importantly, betaine and creatine are not used in commercial TPN products.

Studies have also shown that lower birth weight is linked with poorer health outcomes later in life (Huxley et al., 2007; Oken & Gillman, 2003). TPN feeding has become an essential part of the medical management of preterm and IUGR infants, such that ~90% of infants <1500 g and ~80% of infants who fail to establish enteral feeds by day 3-5 are prescribed TPN (Bolisetty et al., 2020). Moreover, in our previous research, we found that IUGR alone can program risk for chronic diseases in adult pigs (Myrie et al., 2012; 2017). Thus, IUGR piglets were fed with the control TPN diet to see if the combination of IUGR and TPN is particularly susceptible to programming effects.

Finally, sow milk fed piglets were selected as the clinical control reference group, representing normal neonatal nutrition and piglet development.

2.4 Objectives

2.4.1 General Objectives

The primary focus of this dissertation has three components. First, to determine whether TPN support throughout the newborn period causes permanent metabolic consequences in adulthood. Second, to determine whether TPN support supplemented with betaine and creatine during the neonatal period can reduce TPN-induced adverse metabolic effects in adulthood. Third, to investigate whether being born IUGR exacerbates TPN-induced deleterious metabolic effects later in life. The primary metabolic outcomes investigated in adulthood were dyslipidemia, hypertension and obesity. Furthermore, we investigated some mechanisms behind changes in these outcomes.

2.4.2 Specific Objectives

1. To determine if TPN feeding during the neonatal period permanently altered the lipid metabolism of pigs in adulthood; to evaluate whether supplementing betaine and creatine to neonatal TPN improves the lipid metabolism in adult pigs, compared to pigs fed a control TPN as neonates; to assess whether TPN-fed IUGR neonates show exacerbated effects of dyslipidemia in adulthood compared to pigs fed a control TPN as neonates - **Chapter 3**
2. To determine if TPN feeding during the neonatal period has long-term effects on blood pressure in adulthood; to evaluate whether supplementing betaine and creatine to TPN improves the programming effects of TPN on blood pressure; to assess whether IUGR will exacerbate the TPN-induced programming risk of hypertension- **Chapter 4**
3. To determine if previously observed acute effects of TPN feeding in the early neonatal period on adiposity and dyslipidemia persisted into adulthood; to evaluate whether feeding betaine- and creatine-supplemented TPN as neonates reduces the risk for obesity and dyslipidemia in adulthood; to assess whether IUGR neonates exacerbate TPN-induced obesity outcomes in adulthood- **Chapter 5**

2.5 Hypotheses

2.5.1 General hypothesis:

Feeding TPN during the neonatal period increases biomarkers of CVD in adulthood.

2.5.2 Specific hypotheses:

1. TPN feeding during the neonatal period permanently alters the lipid metabolism of pigs in adulthood, predisposing them higher risk of CVD. Supplementing betaine and creatine to neonatal TPN will improve lipid metabolism in adult pigs, compared to pigs fed control TPN as neonates. TPN-fed IUGR neonates will show exacerbated effects of dyslipidemia in adulthood compared to the control TPN-fed pigs as neonates- **Chapter 3**

2. TPN feeding during the neonatal period has long-term effects on blood pressure in adulthood. Supplementing betaine and creatine to TPN will improve the programming effects of TPN on blood pressure. TPN-fed IUGR will exacerbate the programming risk of hypertension- **Chapter 4**

3. Previously observed acute effects of TPN feeding in the early neonatal period on adiposity and dyslipidemia will persist into adulthood. Supplementing betaine and creatine to early TPN reduces the risk of obesity and dyslipidemia in adulthood. TPN-fed IUGR neonates will exacerbate the obesity outcomes in adulthood- **Chapter 5**

3 Chapter 3 - Feeding parenteral nutrition in the neonatal period programs dyslipidemia in adulthood in Yucatan miniature pigs.

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Authorship: R.F.B. and J.A.B. designed the study, and R.F.B. acquired the funding. L.H. conducted the initial animal work. K.A. assisted in conducting the animal experiments. E.W.R. provided technical support in analyzing samples. R.S.R. conducted the experiments; the analytical work, generated the results and prepared the initial draft of the manuscript. R.S.R and R.F.B. interpreted the results and wrote the manuscript. All authors read and agreed to the manuscript.

3.1 Abstract

Background: Early nutritional challenges can lead to permanent metabolic changes, increasing risk for developing chronic diseases later in life. Total parenteral nutrition (TPN) is a life-saving non-normal nutrition regimen, used especially in intrauterine growth-restricted (IUGR) neonates. Early TPN feeding alters metabolism, but whether these alterations are permanent is unclear. Programmed metabolism is likely caused by epigenetic changes due to imbalances of methyl nutrients.

Objectives: We sought to determine whether feeding TPN in early life would increase the risk of developing dyslipidemia in adulthood and whether supplementing the methyl nutrients betaine and creatine to TPN would prevent this development. We also sought to determine whether IUGR exacerbates the effects of neonatal TPN on lipid metabolism in adulthood.

Methods: Female piglets (n=32, 7 d old) were used in four treatments: 24 normal weight piglets were randomized to sow-fed (SowFed), standard TPN (TPN-control), and TPN with betaine and creatine (TPN-B+C); 8 IUGR piglets were fed control TPN (TPN-IUGR) as a fourth group. After 2 weeks of treatment, all pigs were then fed a standard solid diet. At 8 mo old, central venous catheters were implanted to conduct postprandial fat tolerance tests.

Results: Feeding TPN in the neonatal period led to dyslipidemia in adulthood, as indicated by higher postprandial triglyceride (TG) levels in TPN-control ($P<0.05$), compared to SowFed. IUGR piglets were particularly sensitive to neonatal TPN feeding, as TPN-IUGR piglets developed obesity and dyslipidemia in adulthood, as indicated by greater backfat thickness ($P<0.05$), higher liver TG ($P<0.05$), slower postprandial TG clearance ($P<0.05$), and elevated fasting plasma non-high density lipoprotein (HDL)-cholesterol ($P<0.01$), and non-esterified fatty acids (NEFA) ($P<0.001$), compared to TPN-control.

Conclusions: Feeding TPN in early life increases the risk of developing dyslipidemia in adulthood, especially in IUGR neonates; however, methyl nutrient supplementation to TPN did not prevent TPN-induced changes in lipid metabolism.

3.2 Introduction

Dyslipidemia is an important metabolic predictor of many chronic diseases, such as cardiovascular diseases (CVD), obesity, insulin resistance, and fatty liver (Bovolini et al., 2021). Recent evidence has shown that nutritional or environmental insults during early life (pre and post-natal) can program metabolism via epigenetics and increase the risk for these chronic diseases in later life (Hoffman et al., 2017). TPN is a severe nutritional intervention often given during the neonatal period to increase survival rates of neonates when they are born premature, IUGR, or when the enteral feeding is not compatible due to pathological conditions (Calkins et al., 2014; Vinnars & Wilmore, 2003). Feeding TPN to neonates leads to gut atrophy and altered metabolism (Bertolo et al., 1999), resulting in insulin resistance, fatty liver, and acute oxidative damage to organs (Lavoie et al., 1997; Lucchinetti et al., 2021a; Stoll et al., 2010, 2012). These metabolic consequences are associated with dyslipidemia, leading to CVD and metabolic syndrome (Lim et al., 2021; Rector et al., 2008). Importantly, TPN administered in early life, during an epigenetic programming window, can exert profound metabolic stress on the neonate during a particularly sensitive stage of development. However, it is unknown whether this TPN-induced metabolic dysfunction persists beyond adolescence into adulthood.

Studies have also shown that lower birth weight is linked with poorer health outcomes later in life (Huxley et al., 2007; Oken & Gillman, 2003). We have recently demonstrated in pigs that IUGR neonates develop a higher risk for chronic diseases in adulthood (McKnight et al., 2012; Myrie et al., 2017). Rapid catch-up growth during postnatal and early childhood is important in predicting future health problems such as dyslipidemia, which can predispose an individual to

develop metabolic syndrome and CVD later in life (Kerkhof et al., 2012; Singhal, 2017). TPN feeding has become an essential part of the medical management of preterm and IUGR infants, such that ~90% of infants <1500 g and ~80% of infants who fail to establish enteral feeds by day 3-5 are prescribed TPN (Bolisetty et al., 2020). Therefore, IUGR infants are particularly vulnerable to TPN-induced metabolic changes that can become lifelong.

Studies in animal models of early programming of adult disease suggest that dysregulation of methyl metabolism may explain programming that leads to adult chronic diseases by permanently altering epigenetic patterns of key genes (Randunu & Bertolo, 2020; Waterland & Jirtle, 2004). Indeed, methylation patterns can be altered by perinatal dietary methyl supply (Cai et al., 2014; Waterland et al., 2006), and some of these epigenetic changes are reversible with changes in dietary methyl supply (Burdge et al., 2009). However, it is unknown if changes in methyl supply and demand in early life can permanently alter epigenetics. Methionine is the primary source of methyl groups, and most transmethylation reactions occur in the liver, although the gut is also responsible for a significant proportion of methionine metabolism (Elango, 2020). Moreover, IUGR has also been shown to reduce hepatic methionine metabolism capacity (MacKay et al., 2012). Importantly, both the gut and liver often become dysfunctional during prolonged TPN feeding, which will reduce methionine and methyl metabolic capacity and potentially limit methyl availability for epigenetic processes (Bauchart-Thevret et al., 2009; Shoveller et al., 2004).

In total, the various transmethylation reactions consume a significant proportion of methionine flux; in particular, synthesis of creatine can consume ~35% of dietary methionine in neonatal piglets (Brosnan et al., 2009), and dietary provision of creatine can spare methionine for other reactions (Bertolo & McBreairty, 2013). We have recently shown that if one methylation pathway is enhanced (e.g., creatine synthesis), it diminishes the partitioning of the limited methyl groups among the remaining methylation reactions, especially phosphatidylcholine (PC) synthesis,

which can lead to fatty liver development (McBreairty et al., 2013). Indeed, creatine supplementation has been shown to prevent fatty liver (Deminice et al., 2015). Similarly, supplementing betaine, a methyl donor that can replenish methionine availability, was also shown to prevent fatty liver and reduce plasma homocysteine levels via increased remethylation (Deminice et al., 2015). Hence, betaine and creatine (B+C) can increase methyl group availability, although their role in epigenetic programming is unclear. Neither of these nutrients are currently provided in commercial TPN products.

The objectives of the current study were to investigate: 1) whether the metabolic effects of TPN feeding in the neonatal period persist into adulthood; 2) if the addition of betaine and creatine to TPN prevents the deleterious effects of TPN on lipid metabolism in adulthood; and 3) whether IUGR will exacerbate the effects of neonatal TPN on lipid metabolism in adulthood. We used the pig as our animal model as it is the most relevant species for modeling human lipid metabolism and has been established as a model for TPN and for investigation of early programming effects (Myrie et al., 2012, 2017).

3.3 Materials and methods

3.3.1 Animals and surgical catheter implantation

Animal care and handling procedures were approved by the Memorial University of Newfoundland Animal Care Committee, in accordance with Canadian Council on Animal Care guidelines. Suckling female Yucatan miniature piglets (n=32; 7 d old) were obtained from the Memorial University of Newfoundland breeding colony and used in four treatments: normal birthweight piglets fed standard TPN (TPN-control); normal birthweight piglets fed TPN supplemented with betaine and creatine (TPN-B+C); normal birthweight suckling piglets (SowFed); and IUGR piglets fed standard TPN (TPN-IUGR). Three normal birthweight piglets from each litter were randomized to the first three groups. IUGR was defined as ~65% of the birth weight of the largest littermate (Hegarty & Allen, 1978). At ~7 d old, all piglets underwent surgical procedures to implant two central venous catheters (Dodge et al., 2012a) (study day 0). TPN-fed piglets were housed for 14 d in individual metabolic cages fitted with a swivel and tether system (Lomir Biomedical, Notre-Dame-de-l'Île-Perrot, QC, Canada) that allowed for free movement while facilitating continuous intravenous diet infusion into a central vein catheter. Room temperature was maintained for piglets at approximately 27 ^{0}C and supplemental heat was provided via heat lamps; ambient lights provided 12-h light-dark cycles. SowFed piglets were returned to the sow after surgical recovery and allowed to suckle until study day 14 (all piglets resumed suckling within 2-3 hours of being returned to the sow). After 14 d of treatment, piglets were anesthetized as above, and catheters were removed. After recovery, all piglets were acclimated to a standard grower diet, which was fed until the end of the study. Groups of four pigs were housed together for 8 mo and fed 2% of their cumulative body weights as a group, adjusted every 2 weeks after body weights were measured. Water was available 24 h ad libitum and a 12-h day-night cycle was maintained (lights on 0800-2000 h).

At 8 mo of age, two venous catheters (inner diameter, 1.0 mm; outer diameter, 1.8 mm; Tygon Medical Tubing, Saint Gobain Performance Plastic Corp., Akron, Ohio, USA) were implanted in the femoral vein (Myrie et al., 2011) for blood sampling during the oral fat tolerance test (OFTT). Briefly, anesthesia was induced with a dexmedetomidine, azepromazine and alfaxalone mixture, and maintained with 1.5% isoflurane. Two blood sampling catheters were then inserted into the left femoral vein, tunneled under the skin, and exteriorized between the shoulder blades. After the surgery, 300 µg intravenous buprenorphine hydrochloride (Temgesic; Schering-Plough Ltd., Hertfordshire, UK) and intravenous trimethoprim 0.07 $mL \cdot kg^{-1}$, trimethoprim (40 $mg \cdot mL^{-1}$) and sulfadoxine (20 $mg \cdot mL^{-1}$) (Borgal; Intervet Ltd., Whitby, ON, Canada) were given for 3 d postoperatively and each pig was housed individually for the remainder of the study, including 8-10 d for post-surgical recovery (Myrie et al., 2017).

3.3.2 Diets

The TPN diets (Tables 3.1) were prepared according to Dodge et al. (Dodge et al., 2012b) under aseptic conditions. All vitamins and macro minerals were supplied at >100% of the estimated requirements for neonatal piglets (NRC, 2012). Just prior to infusion, multivitamins (Multi12/K1 Pediatric, Baxter Corporation, Mississauga, ON, Canada), iron dextran (Bimeda-MTC Animal Health, Cambridge, ON, Canada), trace elements (Sigma-Aldrich Canada, Oakville, ON, Canada), and SMOF lipid emulsion (Fresenius Kabi, Uppsala, Sweden) were added to each diet bag. For the TPN-B+C group, TPN was supplemented with betaine (235 $mg \cdot kg\ body\ weight^{-1} \cdot d^{-1}$) and creatine (118 $mg \cdot kg\ body\ weight^{-1} \cdot d^{-1}$). Betaine was supplied at a molar equivalent of the piglet methionine requirement (Shoveller et al., 2003), and creatine was supplemented to match the piglet creatine accretion rate (Brosnan et al., 2009). Diet bags were weighed routinely to record the amount of diet infuse

Table 3:1 Amino acid profile of TPN diets

	TPN control diet ($g \cdot L^{-1}$)	**TPN betaine + creatine diet ($g \cdot L^{-1}$)**
Alanine	5.89	5.89
Arginine	3.65	3.65
Aspartic Acid	3.32	3.32
Cysteine	0.76	0.76
Glutamic Acid	5.72	5.72
Glycine	1.47	1.47
Histidine	1.69	1.69
Isoleucine	2.51	2.51
Leucine	5.67	5.67
Lysine hydrochloride	5.58	5.58
Methionine	1.04	1.04
Phenylalanine	3	3
Proline	4.52	4.52
Serine	3.11	3.11
Taurine	0.27	0.27
Threonine	2.23	2.23
Tryptophan	1.14	1.14
Tyrosine	0.44	0.44
Valine	2.89	2.89
Betaine hydrochloride	0	1.29
Creatine monohydrate	0	0.57

After the dietary treatment period of 14 d (i.e., at 21 d of age), each piglet was acclimated with milk replacer (Grober Nutrition Inc., Cambridge, ON, Canada) for 2-3 d and weaned onto a standard pelleted grower pig diet (Table 3.2; Eastern Farmers Co-op, St. John's, NL, Canada). This grower diet delivered 67% of energy as carbohydrate, 12% as fat, and 21% as protein.

Table 3:2.Composition of the grower diet (12.1 MJ digestible energy·kg^{-1} and 154 g protein·kg^{-1}).

Energy (% total energy)	
Complex carbohydrate	67
Fat	12
Protein	21
Ingredients (g·kg^{-1} dry matter)	
Wheat shorts	400.5
Canola	49.0
Meat meal	19.0
Limestone	13.0
Corn gluten feed	40.0
Ground barley	297.0
Oats	175.0
Vitamin mix	0.8
Mineral mix	1.0
Sodium Chloride	4.7

3.3.3 Body measurements

Body weight was measured every day during the experimental dietary period (i.e., 7 d to 21 d of age), and body weight was measured every 2 weeks throughout the grower phase of the study (i.e., from 1 to 9 mo). Feed conversion ratio (FCR) was calculated by dividing the predicted feed intake of each pig (2% of their body weight) by the body weight they gained in each phase (1-4 mo /4-6 mo /6-8 mo). Because sexual maturity occurs in Yucatan miniature pigs around 4 mo, we divided the growth data by development 1-4 mo (pre-sexual maturity), 4-6 mo (peri-sexual maturity and 6-8 mo (post-sexual maturity) (Table 3) (McKnight et al., 2012). Fractional growth rate (FGR) was calculated by dividing body weight gain for a given period of time by the initial body weight of that time frame.

3.3.4 Oral fat tolerance test

After 8-10 d of surgery recovery at 8 mo old, adult pigs were fasted for 18 h prior to the

OFTT, and on the test day, a baseline blood sample (10 mL in ethylenediaminetetraacetic acid (EDTA) tubes (Becton, Dickinson and Company: Franklin Lakes, NJ, USA)) was collected via the venous catheter. Immediately after, pigs were presented with a high-fat meal bolus (1.5 g fat·kg body weight^{-1}), which was made with margarine (25%) (Central Dairies, St. John's, NL, Canada) and lard (75%) (Loblaw Inc., Toronto, ON, Canada). The fat bolus was blended with some ground grower pig diet (~5% of the total bolus) (Eastern Farmers Co-op, St. John's, NL, Canada) to enhance the absorption of fatty acids in the presence of some carbohydrates and protein (Kolovou et al., 2011) and to improve palatability. After the meal was presented to the pigs, they were allowed to eat for 1 h to consume the entire bolus, and water was provided ad libitum during the test. Starting from the bolus introduction, blood samples (10 mL), were collected hourly for the next 11 h. Half of each blood sample was immediately centrifuged at 4000 × g for 15 min at 4 ^{0}C to separate plasma, frozen at -80 ^{0}C and the other half was centrifuged at 15,500 × g for 20 min at 12 ^{0}C for separation of chylomicron (CM) and chylomicron-free (CMF) fractions (McAteer et al., 2003; Myrie et al., 2017). All plasma, CM and CMF fractions were analyzed for triglyceride (TG) concentrations using a commercially available enzymatic assay kit (Sekisui Diagnostics PEI Ltd., Charlottetown, PE, Canada). Total area under the curve (AUC) (calculated using the trapezoid method from baseline to final TG measurements for each plasma and CM fraction), peak TG concentration, peak TG concentration adjusted to the baseline TG, and time to peak TG were used to quantify the total plasma and CM TG responses during the OFTT (Myrie et al., 2017).

3.3.5 Necropsy

After the OFTT (~1 mo), the pigs were anesthetized with 105 mg·kg^{-1} sodium pentabarbitol (Euthanyl, Biomeda-MTC Cambridge, ON, Canada) and mechanically ventilated. Blood samples were collected into EDTA tubes by cardiac puncture and assayed for lipids, as described below. Organs were removed, weighed, and samples were flash-frozen using a freeze clamp and liquid

nitrogen and stored at -80 ^{0}C until further analyses. Subcutaneous fat thickness was measured with a ruler on the carcass at the midline of the back, immediately caudal to the last rib. Additionally, body measurements; crown to rump length, abdominal circumference, chest girth were measured in every pig at necropsy.

3.3.6 Plasma lipid analysis and cholesterol ester transfer protein (CETP) activity

Blood samples collected at necropsy and fasting monthly blood samples were centrifuged immediately at 4000 × g for 15 min to separate plasma, and fresh plasma was assayed for concentrations of plasma total cholesterol (TC), TG, high-density lipoprotein (HDL)-cholesterol, and free cholesterol using enzymatic assay kits (Sekisui Diagnostics PEI Ltd., Charlottetown, PE, Canada). Plasma non-HDL-cholesterol was calculated by subtracting values of HDL-cholesterol from TC. Plasma non-esterified fatty acid (NEFA) was assayed using enzymatic assay kit (Fujifilm Wako Diagnostics USA Corporation, CA, USA). Lipoprotein fractions were collected by sequential density ultracentrifugation of fresh necropsy plasma (Salter et al., 1998). Necropsy plasma was centrifuged at 15,500 × g for 20 min at 12 ^{0}C to separate CM and the infranatant was subjected to sequential density ultracentrifugation to collect lipoprotein fractions, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and HDL. TC and TG levels were measured in these fractions using enzymatic assay kits. Plasma CETP activity was measured using a CETP activity assay kit (ab196995 CETP activity assay kit II; Abcam Inc., Toronto, ON, Canada) following assay instructions.

3.3.7 Liver TG analysis and tissue LPL activity

Lipids were extracted from liver tissue (left median lobe) using the chloroform:methanol (2:1, v/v) extraction technique (Folch et al., 1957) and the lipid-containing phase was evaporated to yield dried lipids. Dried lipids were then dissolved using isopropanol and analyzed for TG and

TC by enzymatic assay kits. Quantitative measurement of LPL was measured in subcutaneous adipose tissue, gastrocnemius muscle, and cardiac tissue (left ventricle) using an LPL activity assay kit (Abcam Inc., Toronto, ON, Canada), following assay guidelines.

3.3.8 Gut parameters and histology

Different segments of gut (duodenum, proximal jejunum, mid jejunum, and ileum) were excised, emptied and collected during necropsy. First, the duodenum (from pyloric sphincter to the duodenojejunal flexure) was removed and emptied, and measured for length and weight, followed by the remaining small intestine. The first 30 cm of the remaining small intestine was considered as proximal jejunum, 30 cm from the midpoint of the small intestine was considered as mid jejunum, and the terminal 30 cm of the of the small intestine was considered as ileum. Sections (~1 cm) from each intestinal segment were placed in neutral-buffered 10% formalin (Sigma Chemical, St. Louis, MO, USA) for histological analysis. The data were expressed as small intestinal weight per length ($mg \cdot cm^{-1}$) for all four segments. Formalin fixed intestinal sections were processed and 10 measurements of villus height and crypt depths per sample were measured by the same individual in a blinded fashion, as described elsewhere (Dodge et al., 2012a).

3.3.9 Plasma and tissue metabolites

Concentrations of plasma betaine, choline and dimethylglycine (DMG) levels were quantified by high pressure liquid chromatography (HPLC) (Waters Alliance 2795, Waters Corporation, Milford, MA; Atlantis HILIC Silica 3 μm 2.1× 100 mm column) with a tandem mass spectrometer (MS) (Micromass Ultima Triple-Quad MS, Waters Corporation, Milford, MA), as previously described (Holm et al., 2003; Kirsch et al., 2010). Multiple-reaction monitoring mode was used to detect the compounds with following transitions; [$^2H_{11}$] betaine 129➔68, betaine 118➔59, [$^2H_9$ methyl] choline 113➔69, choline 104➔60, DMG 104➔58. Plasma concentrations

were calculated using calibration standards made using dialyzed plasma spiked with betaine, choline and DMG. [$^2H_9$-methyl] choline and [$^2H_{11}$] betaine were used as the internal standards (Holm et al., 2003). Final concentrations of betaine, choline and DMG were calculated using MassLynx Software (Waters Corporation, Milford, MA, USA).

Plasma total homocysteine, cysteine and glutathione concentrations were determined using reverse-phase HPLC and fluorescence detection of ammonium 7-fluoro 2-oxa-1,3-diazole-4-sulfonate thiol adducts (Vester et al., 1991) Liver homocysteine and cysteine concentrations were measured using the same method with modifications using 2-mercapto propionyl glycine as the internal standard (Thillayampalam, 2015). Plasma methionine concentrations were measured using reverse-phase HPLC after derivatization with phenylisothiocyanate (Bidlingmeyer et al., 1984).

After lipids were extracted from liver, PC, and phosphatidylethanolamine (PE) were isolated using thin layer chromatography as previously described (Thillayampalam, 2015). The quantification of liver PC and PE was then measured by measuring total phosphate using a modified Bartlett method (Bartlett, 1959) described elsewhere (Thillayampalam, 2015).

3.3.10 RNA extraction and real-time qPCR

Total RNA was extracted from liver samples using the Trizol method (Chomczynski & Sacchi, 2006). Genomic DNA contamination in RNA samples was eliminated using DNAse enzyme (cat. # M610A, Promega, USA). The concentration of the extracted RNA samples from the liver was determined using NanoDrop 2000 (Thermo Scientific, USA). Confirmation of the integrity of each RNA sample was then determined using 1.2% agarose gel. Complementary DNA (cDNA) was synthesized from the extracted RNA samples using reverse transcription (cat # A3500, Promega, USA). Real time qPCR primers for microsomal triglyceride transfer protein

(MTTP) (Chen et al., 2012), β-actin (Nygard et al., 2007) and glyceraldehyde 3-phosphate (GAPDH) (Park et al., 2015) (Integrated DNA Technologies, IA, USA) were verified using NCBI primer blast (www.ncbi.nlm.nih.gov/tools/primer-blast/). The forward and reverse sequence for each primer pairs were MTTP forward – GCCAGGTCTTCCAGAGCGAGTG; MTTP reverse- TGCCGTCCTGAGGTGCTGAATG; β-actin forward- CAC GCC ATC CTG CGT CTG GA; β-actin reverse- AGC ACC GTG TTG GCG TAG AG); GAPDH forward- ATCCTGGGCTACACTGAGGA; GAPDH reverse- TGTCGTACCAGGAAATGAGCT. GeneBank accession numbers for the primers were NM_214185 for MTTP, DQ845171 for β-actin and NM_001206359.1 for GAPDH. Primer efficiencies for MTTP, β- actin and GAPDH were 81.23, 104.50 and 84.70, respectively. The amplification of qPCR was initiated using SYBR Green Supermix (cat # 1708882, Bio-Rad, USA) and the samples were run using the Mastercycler ep realplex system (Eppendorf, ON, Canada). The delta Ct values were calculated for the MTTP and reference genes. Both β-actin and GAPDH were used as multiple reference genes to normalize the expression levels of MTTP gene, accounting for their primer efficiencies (Vandesompele et al., 2002). This method utilizes the geometric mean of the relative quantities for the reference genes to calculate gene expression. The formula is as below.

$$\frac{E_{GOI}\,\Delta^{Ct\,GOI}}{\text{Geomean}\,[E_{REF}\Delta^{Ct\,REF}]}$$

E = Amplification factor
GOI = Gene of interest
Geomean = Geometric mean
REF = Reference gene
ΔCt = Ct (Calibrator sample) – Ct (Test sample)

3.3.11 Statistical analyses

The experimental groups were compared using one-way ANOVA and the differences amongst groups were determined using Dunnett's post hoc test (GraphPad Prism 8.0; GraphPad

Software, San Diego, CA, USA), with TPN-control assigned as the control group. Our aim was to investigate the long-term effects of early TPN support; thus, we compared the TPN-control group to the Sowfed group, which served as the clinical control. The results are reported as mean ± SD. Pearson's correlation was used to compare the relationship between TG in VLDL and the plasma TG clearance rate. Differences were considered statistically significant if $P<0.05$.

3.4 Results

3.4.1 Growth

The growth data were partitioned into four developmental phases, with reference to sexual maturity, which in Yucatan miniature pigs occurs between 4 mo and 6 mo of age. The lower body weight of the TPN-IUGR piglets persisted throughout the TPN phase to 21 d old (Table 3.3). SowFed and TPN-B+C piglets grew not different from TPN-control piglets during the TPN feeding phase. By 1 mo old, body weights of TPN-IUGR piglets caught up to TPN-control and there was no difference in body weight compared to TPN-control group beyond 1 mo. The body weight growth rates were greater for TPN-IUGR pigs between 1-4 mo and 4-6 mo developmental stages compared to the TPN-control pigs, but there were no differences in the 6-8 mo phase among the groups. FGR, average FCR and body measurements at 9 mo were not different compared to TPN-control group (Table 3.3).

3.4.2 Oral fat tolerance test

After ingesting the bolus of fat, the AUC of plasma TG was lower in the SowFed group than for the TPN-control pigs (Fig. 3.1, Table 3.4). Further analysis showed that the SowFed group exhibited lower peak TG concentration and adjusted peak TG concentration (Table 3.4) compared to the TPN-control pigs. However, no differences were observed in the baseline TG concentrations among the groups (Table 3.4).

Table 3:3 Summary of body weight, growth rate, FGR, FCR and body measurements in TPN- control, TPN-B+C, TPN-IUGR and SowFed Yucatan miniature pigs.

Developmental period	Age	TPN-control	TPN-B+C	TPN-IUGR	SowFed
Body weight (kg)					
Birth	1 d	*0.98 ± 0.17*	1.02 ± 0.12	0.67 ± 0.10***	1.01 ± 0.15
Neonate -TPN start	7 d	*1.74 ± 0.18*	1.734 ± 0.24	1.25 ± 0.16**	1.80 ± 0.35
Neonate -TPN end	21 d	*3.10 ± 0.20*	3.15 ± 0.24	2.44 ± 0.21**	3.53 ± 0.88
Neonate - weaning	1 mo	*3.54 ± 0.88*	3.77 ± 0.61	3.57 ± 1.08	4.81 ± 1.87
Sexual maturity	4 mo	*14.99 ± 3.29*	15.83 ± 2.77	17.46 ± 2.54	18.06 ± 2.86
Post-sexual maturity	8 mo	*35.05 ± 7.62*	34.43 ± 5.49	41.74 ± 4.63	37.87 ± 7.49
Body weight growth rate ($g \cdot d^{-1}$)					
Neonate	7 d – 21 d	*97.1 ± 6.2*	101.1 ± 7.5	83.0 ± 8.5	117.9 ± 48.7
Pre-sexual maturity	1-4 mo	*130.1 ± 38.5*	135.6 ± 30.9	171.8 ± 25.1*	151.8 ± 31.9
Sexual maturity	4-6 mo	*143.7 ± 52.5*	148.5 ± 45.4	203.7 ± 28.7*	165.1 ± 57.1
Post-sexual maturity	6-8 mo	*190.2 ± 39.9*	169.0 ± 34.9	194.7 ± 51.0	164.0 ± 59.3
Fractional growth rate ($g \cdot kg$ body $weight^{-1} \cdot d^{-1}$)					
Neonate	7 d – 21 d	*40.1 ± 3.1*	41.6 ± 5.1	47.6 ± 4.3	45.4 ± 11.7
Pre-sexual maturity	1-4 mo	*13.9 ± 2.7*	13.7 ± 2.1	15.2 ± 3.3	13.0 ± 2.6
Sexual maturity	4-6 mo	*7.3 ± 1.6*	7.3 ± 1.7	8.5 ± 1.3	6.4 ± 2.6
Post-sexual maturity	6-8 mo	*6.1 ± 0.6*	5.3 ± 0.6	5.6 ± 1.2	5.0 ± 1.1
Average feed conversion ratio					
Pre-sexual maturity	1-4 mo	*1.6 ± 0.2*	1.6 ± 0.1	1.5 ± 0.3	1.7 ± 0.2
Sexual maturity	4-6 mo	*3.3 ± 1.0*	3.4 ± 1.1	2.6 ± 0.5	3.4 ± 1.1
Post-sexual maturity	6-8 mo	*3.8 ± 0.6*	4.5 ± 1.2	4.5 ± 1.3	5.4 ± 2.1
Body measurements					
Crown to rump length (cm)	9 mo	*112.1 ± 6.1*	111.8 ± 6.3	118.1 ± 3.2	116.3 ± 6.9
Abdominal circumference (cm)	9 mo	*84.7 ± 6.8*	82.8 ± 6.8	88.3 ± 4.1	84.4 ± 7.2
Chest girth (cm)	9 mo	*79.4 ± 4.3*	79.3 ± 3.7	82.9 ± 4.3	77.7 ± 4.0
Subcutaneous fat thickness (mm)	9 mo	*43.2 ± 5.5*	43.7 ± 3.8	50.0 ± 5.0*	45.1 ± 5.6

Values are means ± SD; n = 7-8.
TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled.
*P<0.05, **P<0.001, ***P<0.0001. 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

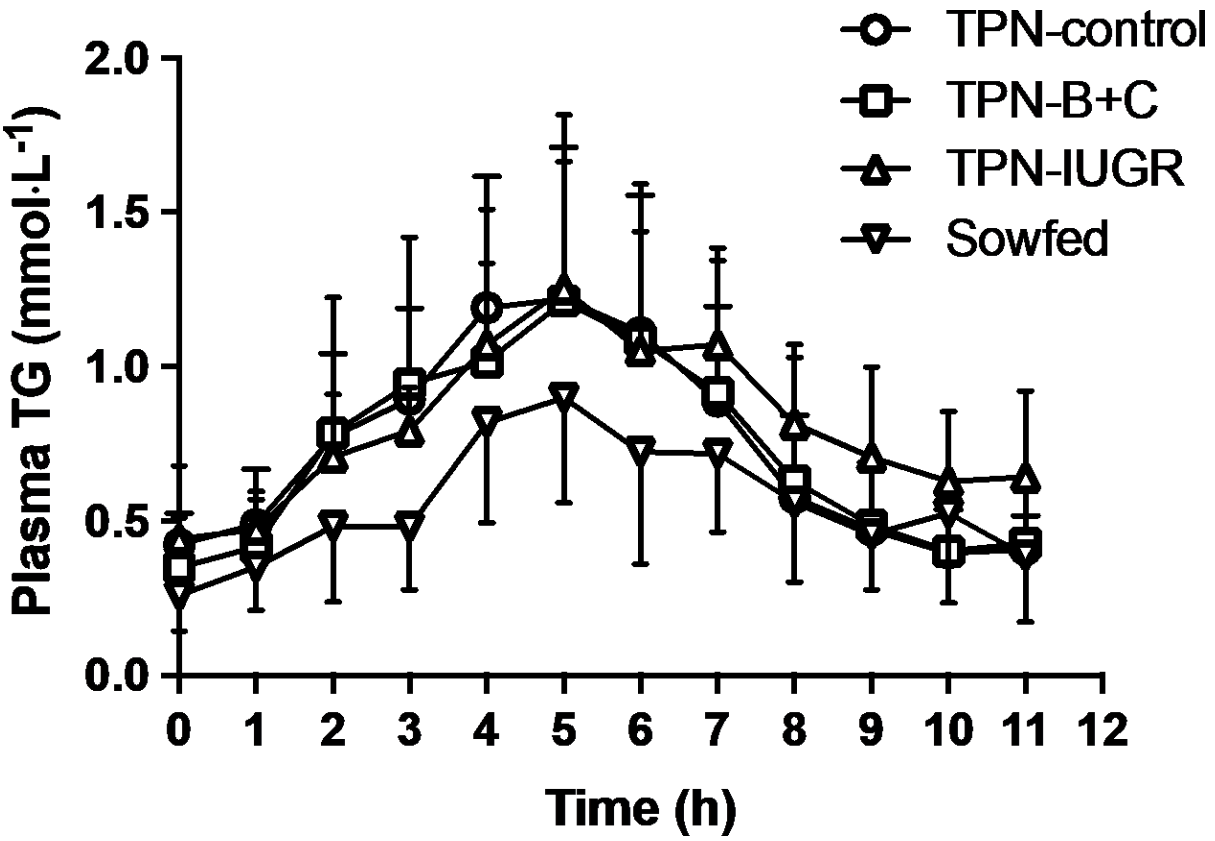

Figure 3:1. Plasma TG concentrations in TPN-control, TPN-B+C, TPN-IUGR and Sow-Fed Yucatan miniature pigs before and after administration of a high fat bolus.

Values are mean ± SD; n = 7-8. The fat bolus was given at time 0 h, and the pigs were allowed to consume the diet for 1 h. TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled.

Table 3:4 Summary of plasma TG in OFTT performed in TPN-control, TPN-B+C, TPN-IUGR and SowFed Yucatan miniature pigs at 9 mo.

	TPN-control	**TPN-B+C**	**TPN-IUGR**	**SowFed**
Baseline TG ($mmol \cdot L^{-1}$)	*0.42 ± 0.10*	0.35 ± 0.16	0.44 ± 0.24	0.26 ± 0.12
Total peak area ($mmol \cdot L^{-1} \cdot h^{-1}$)	*9.67 ± 1.80*	8.40 ± 3.09	9.45 ± 2.01	6.20 ± 2.07*
Peak TG ($mmol \cdot L^{-1}$)	*1.60 ± 0.32*	1.30 ± 0.39	1.39 ± 0.52	0.97 ± 0.34*
Adjusted peak TG ($mmol \cdot L^{-1}$) †	*1.14 ± 0.26*	1.03 ± 0.36	1.08 ± 0.36	0.67 ± 0.23*
Time to peak TG (h)	*5.1 ± 1.1*	4.7 ± 1.4	5.4 ± 1.1	5.2 ± 0.9
Rate of TG clearance (ln [TG])	*0.19 ± 0.05*	0.19 ± 0.06	0.11 ± 0.05*	0.13 ± 0.06

Values are means ± SD, n = 6-8.
TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled; †Adjusted for baseline TG within pig.
*P<0.05; 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

The rate of TG clearance was slower in the TPN-IUGR group than in the TPN-control group, suggesting delayed postprandial TG clearance in TPN fed IUGR pigs at adulthood. Similar to TG concentrations in plasma, the AUC of TG concentrations in CM were lower in SowFed pigs than in TPN-control pigs (Fig. 3.2, Table 3.5). Peak CM TG concentrations and adjusted peak CM TG concentrations were also lower in the SowFed group compared to TPN- control pigs, while the baseline CM TG levels show no difference among the groups. TG concentrations in VLDL fractions of fasted plasma were positively correlated with plasma clearance rate at OFTT (Fig. 3:3).

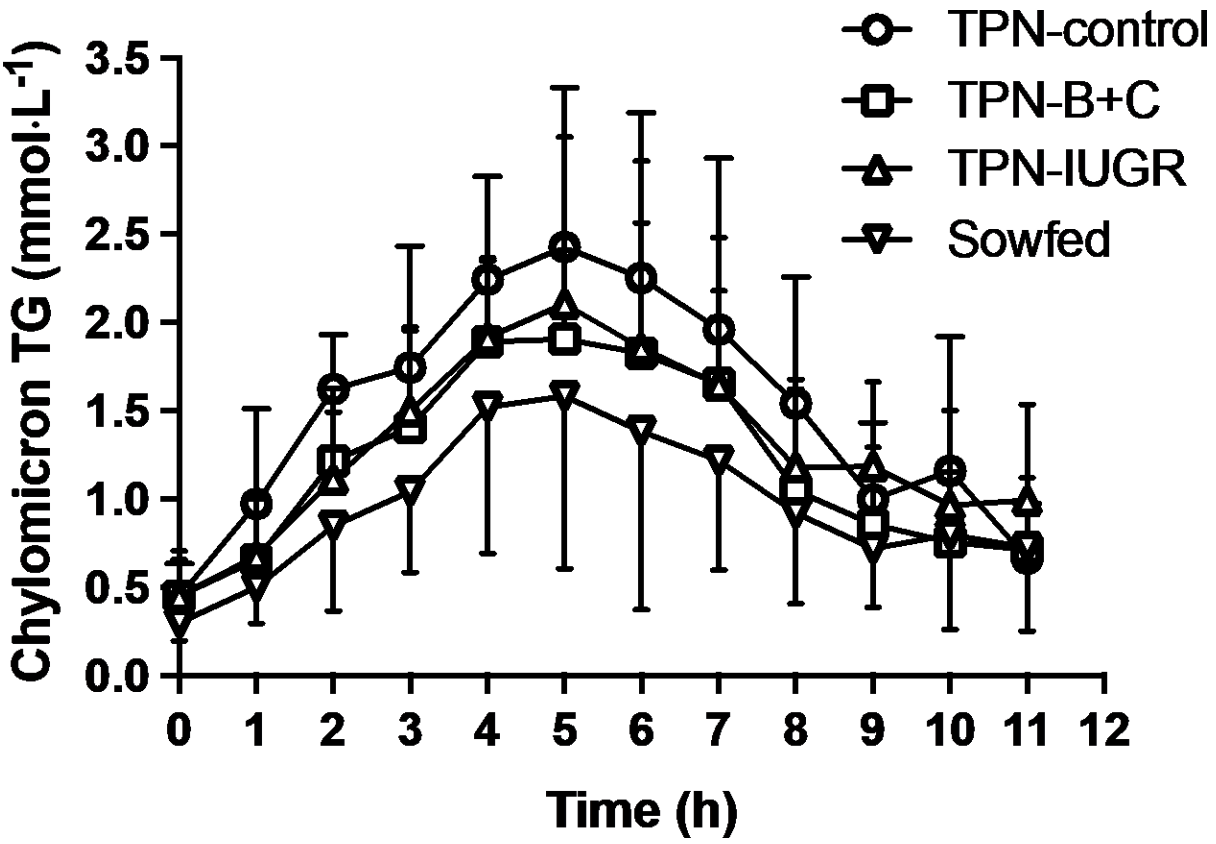

Figure 3:2 . Plasma CM TG concentrations in TPN-control, TPN-B+C, TPN-IUGR and SowFed Yucatan miniature pigs before and after administration of a high fat bolus.

Values are mean ± SD; n = 6-8. The fat bolus was given at time 0 h and the pigs were allowed to consume the diet for 1 h. TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled.

Table 3:5 Summary of CM TG in oral fat tolerance test in TPN-control, TPN-B+C, TPN-IUGR and SowFed Yucatan miniature pigs at 9 mo.

	TPN-control	**TPN-B+C**	**TPN-IUGR**	**SowFed**
Baseline TG ($mmol \cdot L^{-1}$)	*0.45 ± 0.19*	0.45 ± 0.26	0.45 ± 0.21	0.30 ± 0.10
Total peak area ($mmol \cdot L^{-1} \cdot h^{-1}$)	*17.49 ± 4.05*	13.89 ± 4.87	12.82 ± 4.34	10.49 ± 5.24*
Peak TG ($mmol \cdot L^{-1}$)	*2.96 ± 0.60*	2.38 ± 0.73	2.09 ± 0.85	1.84 ± 0.89*
Adjusted peak TG ($mmol \cdot L^{-1}$) †	*2.50 ± 0.64*	1.93 ± 0.69	1.64 ± 0.74	1.54 ± 0.82*
Time to peak TG (h)	*4.9 ± 0.9*	6.0 ± 1.3	4.9 ± 1.0	5.6 ± 1.1
Rate of TG clearance (ln [TG])	*0.22 ± 0.08*	0.21 ± 0.09	0.16 ± 0.07	0.20 ± 0.07

Values are means ± SD, n = 6-8.
TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled; †Adjusted for baseline TG within pig.
*P<0.05; 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

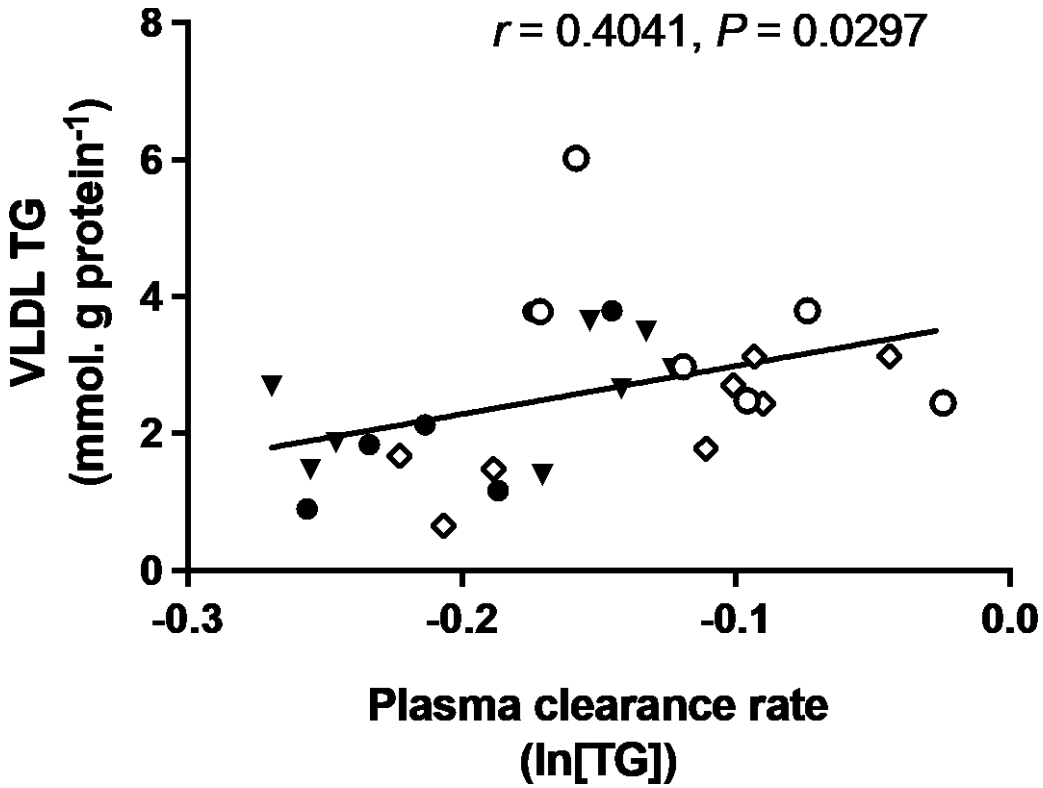

Figure 3:3 Correlation between TG concentration in VLDL fractions of fasted plasma at 9 mo (mmol·g protein^{-1}) and plasma TG clearance rate (ln [TG]) at OFTT.

TG concentration in VLDL is correlated positively with plasma TG clearance rate (r = 0.4041, P = 0.0297); n = 6-8. Each symbol represents an individual pig; TPN-control (●); TPN-B+C (▼); TPN-IUGR (○); SowFed (◊).

3.4.3 Fasting lipid concentrations at necropsy

Fasting plasma non-HDL cholesterol and plasma NEFA levels were higher in TPN-IUGR pigs than for TPN-control pigs at 9 mo (i.e., necropsy) (Table 3.6). TPN-fed IUGRs had higher liver TG levels than TPN-control pigs at 9 mo (Table 3.6). Lipoprotein subfraction analysis for lipid parameters at 9 mo showed a lower cholesterol level and higher TG level in VLDL subfraction in TPN-fed IUGR than for TPN-control pigs. A higher cholesterol level in LDL subfraction was also observed in the TPN-IUGR pigs compared to TPN-control pigs (Table 3.6).

3.4.4 Intestinal villi lengths and crypt depths

Because gut atrophy is a common outcome of neonatal TPN support, we measured intestinal villi length and crypt depth to determine whether intestinal atrophy persists into adulthood. Duodenum and proximal jejunum villus length and crypt depth did not differ among groups at 9 mo (i.e., necropsy) (Table 3.7). Unit weight of duodenum was higher in SowFed pigs compared to TPN-control pigs, but no differences were observed for other sections of intestine (Table 3.8).

3.4.5 Enzyme measurements

LPL activity in adipose, muscle and heart and plasma CETP activity were not different among the experimental groups at 9 mo (Table 3.9). LPL activity was measured in subcutaneous adipose tissue because during the postprandial stage, subcutaneous fat depots are the net importer of dietary fats (Fielding, 2011). The relative mRNA expression of liver MTTP was higher in TPN-IUGR pigs than that in the TPN-control group at 9 mo (Fig. 3.4).

Table 3:6 Liver, plasma fasting lipid profile, plasma fasting lipoprotein TG and TC profile in Yucatan miniature pigs at 9 mo in TPN-control, TPN-B+C, TPN-IUGR and SowFed groups.

	TPN-control	TPN-B+C	TPN-IUGR	SowFed
Cholesterol				
Liver, total (mmol· g protein^{-1})	*9.39 ± 2.53*	9.39 ± 2.32	11.16 ± 2.54	8.60 ± 4.07
Plasma, total (mmol· L^{-1})	*1.405 ± 0.268*	1.270 ± 0.216	1.617 ± 0.399	1.453 ± 0.117
Plasma, total HDL (mmol· L^{-1})	*0.974 ± 0.189*	0.958 ± 0.191	0.867 ± 0.240	0.938 ± 0.067
Plasma, total non-HDL (mmol· L^{-1})	*0.460 ± 0.190*	0.302 ± 0.095	0.750 ± 0.321*	0.530 ± 0.114
Plasma Free Cholesterol (mmol· L^{-1})	*0.608 ± 0.174*	0.484 ± 0.249	0.507 ± 0.104	0.536 ± 0.082
VLDL (mmol· g protein $^{-1}$)	*1.562 ± 0.656*	1.427 ± 0.491	0.883 ± 0.263*	1.421 ± 0.335
LDL (mmol· g protein $^{-1}$)	*2.284 ± 0.551*	2.390 ± 0.727	4.483 ± 2.062*	2.640 ± 0.649
HDL (mmol· g protein $^{-1}$)	*0.946 ± 0.364*	0.957 ± 0.489	1.438 ± 0.496	0.762 ± 0.461
Triglycerides				
Liver, total (mmol· g protein^{-1})	*3.77 ± 2.94*	4.13 ± 2.58	9.60 ± 5.73*	4.67 ± 4.37
Plasma, total (mmol· L^{-1})	*0.397 ± 0.110*	0.404 ± 0.113	0.422 ± 0.147	0.358 ± 0.106
VLDL (mmol· g protein $^{-1}$)	*2.125 ± 1.289*	2.522 ± 0.863	3.492 ± 1.208*	2.127 ± 0.873
LDL (mmol· g protein $^{-1}$)	*0.364 ± 0.222*	0.339 ± 0.148	0.254 ± 0.085	0.307 ± 0.209
HDL (mmol· g protein $^{-1}$)	*0.025 ± 0.015*	0.031 ± 0.018	0.020 ± 0.004	0.018 ± 0.013
Plasma NEFA (mmol. L^{-1})	*0.09 ± 0.04*	0.09 ± 0.04	0.15 ± 0.03*	0.08 ± 0.04
Liver				
Phosphatidylcholine (PC) (mmol· g protein $^{-1}$)	*0.66 ± 0.12*	0.61 ± 0.12	0.68 ± 0.12	0.68 ± 0.13
Phosphatidylethanolamine (PE) (mmol· g protein $^{-1}$)	*0.43 ± 0.06*	0.43 ± 0.07	0.47 ± 0.03	0.46 ± 0.06
PC/PE	*1.53 ± 0.22*	1.42 ± 0.16	1.57 ± 0.16	1.55 ± 0.35

Values are means ± SD, n = 6-8.
TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled.
*P<0.05; 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

Table 3:7 Summary of duodenal and proximal jejunum villus height and crypt depth in TPN- control, TPN-B+C, TPN-IUGR and SowFed Yucatan miniature pigs at 9mo

	TPN-control	TPN-B+C	TPN-IUGR	SowFed
Duodenum villus height (μm)	*421 ± 65*	353 ± 36	422 ± 72	383 ± 74
Duodenum crypt depth (μm)	*249 ± 26*	293 ± 84	294 ± 81	273 ± 88
Proximal jejunum villus height (μm)	*498 ± 58*	508 ± 60	502 ± 71	551 ± 92
Proximal jejunum crypt depth (μm)	*235 ± 36*	241 ± 48	292 ± 46	277 ± 75

Values are means ± SD, n = 6-8.
TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed with TPN-control diet; SowFed, suckled.
*P<0.05; 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

Table 3:8 Summary of intestinal total tissue weight in TPN-control, TPN-B+C, TPN-IUGR and SowFed Yucatan miniature pigs at 9 mo.

	TPN-control	TPN-B+C	TPN-IUGR	SowFed
Duodenum ($mg \cdot cm^{-1}$)	*651 ± 228*	792 ± 183	812 ± 519	957 ± 196*
Proximal jejunum ($mg \cdot cm^{-1}$)	*437 ± 72*	450 ± 105	462 ± 49	436 ± 67
Mid jejunum ($mg \cdot cm^{-1}$)	*400 ± 82*	377 ± 56	443 ± 74	468 ± 104
Ileum ($mg \cdot cm^{-1}$)	*470 ± 136*	547 ± 135	562 ± 146	468 ± 145

Values are means ± SD, n = 6-8.
TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled.
*P<0.05; 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

Table 3:9. LPL activity in adipose tissue, muscle and heart and plasma CETP activity in TPN-control, TPN-B+C, TPN-IUGR and SowFed Yucatan miniature pigs at 9 mo

	TPN-control	**TPN-B+C**	**TPN-IUGR**	**SowFed**
LPL Activity (mU·mg of protein $^{-1}$)				
Subcutaneous fat	*16.118 ± 6.474*	14.221 ± 6.418	14.553 ± 5.858	12.81 ± 5.3512
Muscle	*13.097 ± 3.255*	11.195 ± 1.737	13.772 ± 4.641	13.620 ± 1.876
Heart	*9.842 ± 3.038*	9.525 ± 1.797	10.617 ± 1.378	9.549 ± 2.644
CETP Activity (pmol·µL^{-1}·h^{-1})	*0.037 ± 0.008*	0.035 ± 0.009	0.036 ± 0.010	0.036 ± 0.005

Values are means ± SD, n = 6-8.
TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled.
*P<0.05; 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

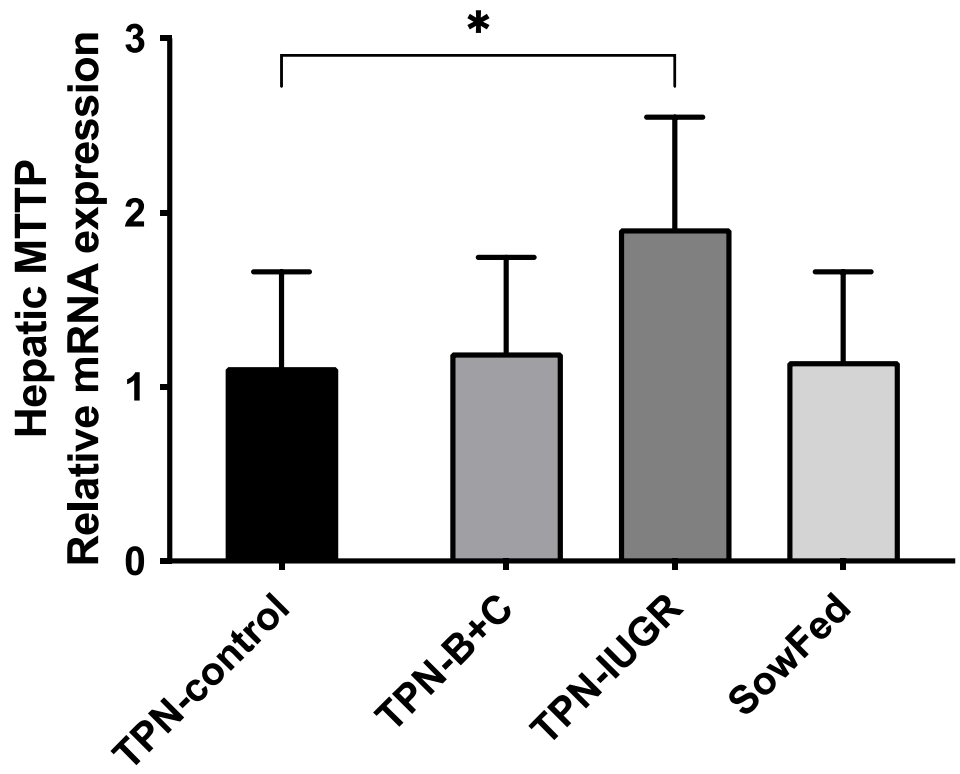

Figure 3:4 Effects of experimental diets in early life on relative mRNA expression of liver MTTP in TPN-control, TPN-B+C, TPN-IUGR and SowFed Yucatan miniature pigs at 9 mo.

Values are means ± SD; n = 7-8. Primer efficiencies for MTTP, β- actin and GAPDH were 81.23, 104.50 and 84.70, respectively. Geometric averaging of both β-actin and GAPDH was used to normalize the expression levels of the MTTP gene, and the data was corrected for the primer efficiency using Vandesompele et al., 2002 method. TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled. MTTP; microsomal triglyceride transfer protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *P<0.05; 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

3.4.6 Other biomarkers for cardiovascular disease

Cysteine and homocysteine levels were not different among the experimental groups at 9 mo in liver or plasma. Plasma DMG was higher in the TPN-IUGR pigs than the TPN-control pigs at 9 mo (Table 3.10). At the end of TPN feeding, plasma cysteine concentrations were not different; however, plasma homocysteine levels were higher in the TPN-IUGR group than in the TPN-control group (Table 3.10). DMG levels were higher in both TPN-B+C and sow-fed pigs compared to TPN-control pigs at 21 d (Table 3.10).

Table 3:10. Plasma and liver methyl-related metabolites in TPN-control, TPN-B+C, TPN- IUGR, and SowFed Yucatan miniature piglets.

	Age	**TPN-control**	**TPN-B+C**	**TPN-IUGR**	**SowFed**
Plasma					
Homocysteine (µmol· L^{-1})	21 d	*23.8 ± 3.4*	18.7 ± 4.8	46.2 ± 20.7**	25.6 ± 10.2
Cysteine (µmol· L^{-1})	21 d	*258.1 ± 42.2*	271.8 ± 50.4	239.3 ± 41.1	241.0 ± 23.2
Glutathione (µmol· L^{-1})	21 d	*12.3 ± 6.0*	8.0 ± 3.5	9.0 ± 3.5	8.6 ± 4.6
Dimethylglycine (µmol· L^{-1})	21 d	*5.3 ± 2.3*	18.6 ± 5.5***	4.4 ± 3.3	15.5 ± 3.3***
Betaine (µmol· L^{-1})	21 d	*64.2 ± 16.8*	358.4 ± 90.5***	35.2 ± 20.3	44.2 ± 11.4
Choline (µmol· L^{-1})	21 d	*8.7 ± 1.7*	11.8 ± 4.0	5.9 ± 2.2	8.0 ± 2.7
Homocysteine (µmol· L^{-1})	9 mo	*35.2 ± 5.1*	37.2 ± 7.1	34.6 ± 5.3	35.7 ± 4.9
Cysteine (µmol· L^{-1})	9 mo	*331.2 ± 60.9*	332.9 ± 42.3	335.9 ± 41.6	347.9 ± 31.3
Glutathione (µmol· L^{-1})	9 mo	*7.4 ± 0.8*	7.7 ± 1.7	7.1 ± 0.7	7.9 ± 1.3
Methionine (µmol· L^{-1})	9 mo	*8.7 ± 2.6*	7.7 ± *1.3*	10.0 ± *3.5*	9.4 ± *3.3*
Dimethylglycine (µmol· L^{-1})	9 mo	*1.40 ± 0.3*	1.5 ± 0.3	2.3 ± 0.8*	1.5 ± 0.4
Betaine (µmol· L^{-1})	9 mo	*105.8 ± 26.7*	117.9 ± 43.6	114.4 ± 32.9	129.4 ± 44.5
Choline (µmol· L^{-1})	9 mo	*6.4 ± 1.9*	5.8 ± 1.8	6.9 ± 1.8	5.5 ± 1.2
Liver					
Homocysteine (µmol· g^{-1})	9 mo	*0.097 ± 0.028*	0.088 ± 0.019	0.105 ± 0.043	0.083 ± 0.028
Cysteine (µmol· g^{-1})	9 mo	*0.096 ± 0.047*	0.071 ± 0.030	0.068 ± 0.024	0.075 ± 0.024

Values are means ± SD, n = 7-8.
TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed with TPN-control diet; SowFed, suckled.
*P<0.05, **P<0.001, ***P<0.0001, 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

3.5 Discussion

TPN is a lifesaving feeding method that has considerably increased infant survival rates since the early 1970s (Puntis, 2006; Vinnars & Wilmore, 2003). However, TPN feeding in the early neonatal period has been associated with changes in metabolism (Stoll et al., 2010, 2012). Studies have shown that TPN administration to neonates leads to acute dyslipidemia (Jain et al., 2012; Nghiem-Rao et al., 2016); however, the persistence of altered lipid metabolism into adulthood remains unknown. We hypothesized that metabolic alterations that occur due to early TPN feeding will persist into adulthood, highlighting the long-term effects of early TPN feeding. Supplementing TPN with betaine and creatine may negate this effect by enhancing the availability of methyl groups and prevent epigenetic changes that led to higher risk for dyslipidemia in adulthood. We expected to identify long-term effects of TPN feeding by comparing the TPN-control group to the Sowfed group (clinical control) at adulthood. Comparing TPN-control to TPN-B+C group will indicate whether feeding betaine- and creatine-supplemented TPN in the neonatal period will prevent TPN-induced metabolism in adulthood. And finally, comparing TPN-control to TPN-IUGR will answer whether IUGR exacerbates TPN-induced outcomes. Our study is the first to show that neonatal TPN feeding leads to dyslipidemia in adulthood. Importantly, these pigs were fed the same grower diet from 3 weeks of age to adulthood (9 mo) and dietary treatments only occurred for 2 weeks as neonates; thus, dyslipidemia observations in adulthood were entirely due to early TPN feeding. Moreover, as hypothesized, IUGR exacerbated TPN-induced dyslipidemia.

We demonstrated that feeding TPN to piglets early in life led to exaggerated postprandial triglyceridemia in adulthood, when compared to pigs that suckled as neonates. Postprandial dyslipidemia is strongly associated with endothelial dysfunction, and is thus a risk factor of atherosclerosis, CVD, and diabetes (Ansar et al., 2011). Therefore, our plasma and CM kinetics in

adulthood indicate that compared to feeding sow milk in the early neonatal age, TPN feeding during early life leads to a reduced ability to clear postprandial dietary lipids, which may increase the risk of developing atherosclerosis, CVD, and diabetes in adulthood.

Mechanistically, feeding TPN in the neonatal period did not seem to affect dietary fat digestion or absorption in adulthood, considering there were no difference in time to peak plasma TG or CM TG between the TPN-fed pigs and sow-fed pigs in adulthood. Studies have shown that because TPN bypasses the gut, this route of feeding suppresses blood flow to the intestine and leads to intestinal villus atrophy, especially in the duodenum and jejunum, where fat absorption takes place (Bertolo et al., 1999; Lucchinetti et al., 2021b; Niinikoski et al., 2004; Shaw, 2012). However, this TPN-induced villus and crypt atrophy in the neonatal period did not seem to persist into adulthood, as indicated by the lack of differences in duodenal or proximal jejunum villus height and crypt depth between the TPN-fed pigs and the sow-fed pigs. Taken together, because gut atrophy did not persist into adulthood, fat absorptive capacity was likely not altered and time to reach peak plasma TG after an oral fat bolus dose was not affected by early feeding treatment.

Compared to SowFed pigs, higher postprandial peak TG concentrations in TPN-control pigs suggest their inability to clear TG-rich lipoproteins from the circulation. The clearance of circulating CM in the postprandial state mainly depends on adipocyte and muscle LPL activities (Paglialunga & Cianflone, 2007; Watts & Playford, 1998). We measured adipose, muscle, and cardiac tissue LPL activity to evaluate the capacity of these tissues in the clearance of postprandial TG. However, LPL activity was not different in any measured tissue regardless of early diet. Among these tissues, adipose is the greatest net importer of dietary fat in the postprandial stage (Fielding, 2011). The absence of increased LPL specific activity in adipose tissue of the TPN-control group could explain the higher plasma TG concentrations postprandially in this group. However we could not measure total LPL activity in the blood, as we did not inject heparin at

necropsy, which would have compromised other parameters measured in the study.

It is unclear whether postprandial TG responses determine the fasting TG concentration or vice- versa. We observed higher postprandial TG in TPN-control pigs in adulthood, but no differences in the fasted plasma TG. This discrepancy suggests that the development of higher postprandial TG concentrations due to lack of clearance ability might be an earlier marker of dyslipidemia than fasting TG concentration. If the magnitude of the postprandial TG continues to remain high, the fasting TG concentrations may also eventually stabilize at higher levels. The addition of betaine and creatine to neonatal TPN did not affect growth performance nor lipid metabolism in later life. The ability to clear postprandial plasma TG and CM TG after consuming a fat bolus in TPN-B+C pigs did not differ from that of TPN-control pigs.

IUGR neonates make up approximately 10-15% (Suhag & Berghella, 2013) of all neonates worldwide, and they often receive TPN in early life. Previous studies have shown that IUGR neonates experience accelerated growth (i.e., catch-up growth) (McKnight et al., 2012; Myrie et al., 2017), greater adiposity, and impaired lipid metabolism as young adults (Myrie et al., 2017). Thus, comparing TPN-IUGR to TPN-control pigs will resolve whether being IUGR will exacerbate the deleterious effects of feeding TPN in early life on lipid metabolism in adulthood. We found that IUGR piglets had caught-up in body weight by one month, after TPN feeding. Catch-up growth was similar to our previous study when sow milk replacer formula was used in IUGR piglets (McKnight et al., 2012). The increased body growth rate of TPN-IUGR pigs compared to the TPN-control group during the 1-4 mo, and 4-6 mo is likely due to the development of obesity into adolescence, as seen previously in formula-fed IUGR pigs (McKnight et al., 2012).

Thus, our data confirm that IUGR pigs demonstrate catch-up growth around weaning age to the end of pre-sexual maturity age (4 mo), regardless of the mode of feeding. This catch-up growth was reduced in post sexual maturity, and body weights paralleled non-IUGR pigs in the postsexual maturity phase (6-8 mo). However, TPN-IUGR pigs were obese as adults, as indicated by increased backfat thickness and plasma NEFA concentrations compared to the TPN-control group.

The most interesting finding of the current study is that although early TPN feeding is a necessary life-saving measure in IUGR neonates, they are particularly sensitive to the long-term metabolic consequences of TPN, predisposing them to metabolic disorders such as dyslipidemia in adulthood. This conclusion was supported by the worsened fasting lipoprotein profiles and TG and TC content in the lipoprotein subfractions in TPN-fed IUGR pigs. Increased obesity in TPN-IUGR pigs, as indicated by more subcutaneous fat, may have led to higher fasting NEFA concentration in the circulation, which then enhanced the flux of FFA into the liver, causing higher TG in the liver compared to TPN-control pigs. Higher hepatic influx of FFA may have led to greater VLDL secretion, as indicated by higher MTTP gene expression, causing higher fasting non-HDL- cholesterol content and TG in VLDL subfractions. Although VLDL particles of TPN-IUGR pigs had greater TG content and reduced cholesterol concentrations compared to TPN-control pigs, plasma CETP activity, which facilitates transport of cholesterol esters and TG between lipoproteins, was not different between the groups.

The elevated levels of postprandial plasma TG in the TPN-IUGR pigs in adulthood is likely due to elevated VLDL content, as plasma TG clearance rate was significantly slower, but the CM TG clearance rate was not different. This conclusion is supported by the higher liver MTTP expression levels, as well as the positive correlation between plasma TG clearance rate and TG content in the VLDL subfraction. Other studies have also shown that the majority of the increment

of postprandial lipoprotein in the clearance phase is due to the presence of VLDL remnants, not CM remnants, based on the apo100/apo48 ratio and the particle size, which fluctuate over 6 h after a fat bolus intake (Nakajima et al., 2011). The slower VLDL clearance in TPN-IUGR pigs could be due to low LPL activity or low VLDL receptor in the extrahepatic tissues such as adipocytes, muscle, and brain, as the majority of the VLDL remnants are removed from the plasma by the VLDL receptor in peripheral tissues after removing TG from VLDL particles by LPL.

Previous studies showed that IUGR pigs have lower methionine cycle enzyme activity, including 30% lower betaine:homocysteine methyltransferase (BHMT) activity and 20% lower cystathionine gamma-lyase (CGL) activity (MacKay et al., 2012; McBreairty et al., 2015). These lower enzyme capacities limit disposal of homocysteine, which may explain the higher homocysteine concentrations in TPN-IUGR piglets in the TPN phase (21 d). However, adult pigs (9 mo) did not show hyperhomocysteinemia, suggesting that reduced BHMT and CGL activity in IUGR pigs may not persist into adulthood or homocysteinemia was maintained via increased renal excretion in adulthood.

When the TPN diet was supplemented with betaine (TPN-B+C), plasma concentration of DMG, the product of BHMT activity and a non-conventional biomarker for CVD (Svingen et al., 2015), were higher than TPN-control in the TPN-phase (21 d), but also were similar to the clinical reference group (SowFed). These data suggest that increased DMG concentration in the B+C supplemented group in the piglet stage is due to supplemented betaine to TPN and the resulting DMG concentrations represent normal levels. However, increased DMG concentration in the IUGR pigs at adulthood suggest that, although hyperhomocysteinemia did not persist into adulthood, TPN- IUGR pigs may still be at risk for developing CVD in adulthood.

Taken together, our data provide evidence that feeding TPN in the neonatal period permanently altered lipid metabolism of pigs, predisposing them to increased risk of CVD, and these deleterious effects persist into young adulthood. Supplementing betaine and creatine to neonatal TPN did not improve lipid metabolism in adult pigs, compared to pigs fed control TPN as neonates. Although it is common that IUGR neonates receive TPN as a life-saving measure early in life, this feeding regimen permanently programs poor lipid metabolism, predisposing them to develop obesity and dyslipidemia in adulthood. Understanding the effects of feeding TPN to IUGR neonates and finding novel nutrition interventions that reduce chronic disease risk has important implications in infant nutrition. The findings of this study will help establish phenotypic outcomes in adulthood as a result of feeding TPN in early life and will shed light on understanding the mechanisms of early programming. Thus, further novel nutritional interventions can be designed to offset the disease risk in adulthood.

4 Chapter 4 - Programming effects of early parenteral feeding on telemetric blood pressure measurements in adult Yucatan miniature pigs.

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Authorship: R.F.B. and J.A.B. designed the study, and R.F.B. acquired the funding. L.H. conducted the initial animal work. B.N.V. provided technical support in telemetry work. R.S.R. conducted the experiments; the analytical work, generated the results and prepared the initial draft of the manuscript. R.S.R and R.F.B. interpreted the results and wrote the manuscript. All authors read and agreed to the manuscript.

4.1 Abstract

Developmental programming is a key determinant of adult hypertension. Total parenteral nutrition (TPN) can exert nutritional stress during development and cause irreversible metabolic abnormalities. Nutritional programming of metabolism results from epigenetic modifications, likely caused by imbalances in dietary methyl nutrients. Betaine and creatine (B+C) can increase the availability of methyl groups and are novel to commercial TPN formulations. We hypothesized that receiving TPN during early life would increase the risk of hypertension in adulthood and that supplementing TPN with B+C would prevent this programming. We used 7-d-old female piglets (n = 24) who were randomly assigned to sow fed (SowFed), TPN control (TPN-control), and TPN with B+C (TPN-B+C). Eight intrauterine growth restricted (IUGR) piglets were fed TPN as a fourth group (TPN-IUGR). After 2 weeks of TPN, the pigs were fed a grower diet that was maintained until adulthood. At 8 mo, a telemeter was implanted to measure 24 h blood pressure (BP) before and after a high salt diet; expression of key renin angiotensin system (RAS) genes was measured using qPCR. BP parameters increased in response to a high salt challenge, but the neonatal diet did not affect the response. BP was not significantly different between TPN-control and SowFed adult pigs, indicating that feeding TPN in early life did not appear to program hypertension in adulthood. However, the addition of B+C to TPN reduced mean and systolic arterial pressure ($P<0.05$) in adulthood, compared to the TPN-control, which signifies a reduction in the risk of hypertension. However, the expression of key RAS genes was not altered in adult pigs. These data collectively suggest that TPN feeding in early life does not increase the risk of developing hypertension in adulthood but supplementing B+C with TPN may reduce the risk of hypertension.

4.2 Introduction

The most common treatable risk factor for cardiovascular disease is hypertension, which is one of the leading causes of premature deaths worldwide (WHO, 2021). One-third of the adult population has arterial hypertension, and the number of deaths attributed to hypertension has increased by 56.1% during the past decade (Valenzuela et al., 2021).Evidence suggests that developmental programming is one of the key determinants of adult hypertension (Nuyt et al., 2009).In particular, extremes in postnatal diet and catch-up growth after low birth weight are both associated with adult hypertension (Law et al., 2002; Singhal et al., 2007; Eriksson et al., 2007; Ben-Shlomo et al., 2008; Siewert-Delle et al., 1998) Animal models have been developed to study the mechanisms of this programming, including IUGR animal models (Lewis et al., 2002; Samuelsson et al., 2008; Ojeda et al., 2008; Myrie et al., 2011). Consistent with epidemiological data, our studies with IUGR Yucatan miniature pigs showed compensatory growth and early biomarkers of hypertension (McKnight et al., 2012; Myrie et al., 2011).

TPN is a lifesaving feeding method used to support IUGR and, premature neonates, as well as normal birth weight infants with gastrointestinal diseases (Chaudhari S, 2006). However, this extreme nutritional regimen can also be associated with permanent changes in metabolism if administered during a developmental window in early life. Although metabolic changes have been observed due to TPN feeding (Stoll et al., 2010, 2012), whether these changes are permanent has not been determined. Because these metabolic changes are likely caused by epigenetic alterations, particularly DNA methylation, the role of imbalances of dietary methyl nutrients has drawn particular attention (Randunu & Bertolo 2020). The gut and liver are the primary sites for metabolizing methyl nutrients (Bauchart-Thevret et al., 2009). Gut atrophy and bypassing the intestine and liver during TPN can disturb methyl metabolism. Indeed, evidence suggests that TPN feeding in neonatal piglets severely alters methionine metabolism, the primary methyl donor

in the body (Brunton et al., 2000). Such changes in methyl group supply can permanently affect gene expression, broadly affecting metabolism and leading to an increased risk of chronic diseases. We have shown that IUGR pigs have altered methyl metabolism capacity and developed early biomarkers of chronic disease (MacKay DS., et al., 2012; McBreairty LE., et al., 2013, Myrie SB., et al., 2011). However, it is unknown whether TPN feeding, relatively common in IUGR infants, exacerbates this programming.

Neonates have high demands for methyl nutrients (McBreairty & Bertolo, 2016), and TPN is given during this epigenetic window of programming, when requirements for methyl groups are critical. Reduction of dietary methyl nutrients during the neonatal period can limit methionine availability and affect whole-body and tissue-specific protein synthesis (Robinson et al., 2016), as well as the availability of methyl groups for normal epigenetic regulation of genes (Randunu & Bertolo, 2020). Recent studies in methyl nutrients have demonstrated that if one methylation pathway is enhanced (e.g., creatine synthesis), it impacts the partitioning of methionine and methyl groups among the remaining pathways (e.g., protein synthesis, phosphatidylcholine synthesis, DNA methylation) (McBreairty et al., 2013). For example, if creatine is provided in the diet, methionine is diverted to other methylation pathways. Moreover, methyl donors, such as betaine, can increase methionine availability by enhancing remethylation of homocysteine to methionine. Both betaine and creatine are novel to commercial TPN formulations and can increase the availability of the methyl groups. Although epigenetics of hypertension is an emerging area of research, studies have shown that several key genes in the RAS that control blood pressure (i.e., angiotensinogen (AGT), angiotensinogen receptor type 1 (AGTR1), and glucocorticoid receptor (GR)) are regulated by DNA methylation (Rivière et al., 2011; F. Wang et al., 2014; Wyrwoll et

al., 2007). Therefore, it is plausible to permanently program the regulation of blood pressure by dietary methyl nutrients.

This study was designed to determine whether early feeding of TPN during the neonatal period has long-term effects on blood pressure in adulthood and to determine if the supplementation of betaine and creatine to TPN will improve the programming effects of TPN on blood pressure. We also wanted to determine whether TPN fed IUGR exacerbates the programming risk of hypertension. Our Yucatan miniature pig model is ideal for studying the programming effects of TPN on blood pressure; these pigs are an established model used to study the developmental origins of various adult diseases, including hypertension, and for TPN feeding (Brunton et al., 2012; Myrie et al., 2011). This model has also been used to investigate methyl metabolism and regulation by dietary methyl supply (McBreairty et al., 2013).

4.3 Materials and Methods

4.3.1 Experimental design and animals

All animal procedures were conducted in accordance with the Canadian Council on Animal Care Guidelines and were approved by the Institutional Animal Care Committee at Memorial University of Newfoundland. After allowing for colostrum consumption in the first few d after birth, suckling female Yucatan miniature piglets from the Memorial University of Newfoundland swineherd (n = 7-8 per group, 7 d old) were divided into four treatment groups: twenty-four normal birth weight pigs were randomized into three groups: TPN-control, TPN supplemented with betaine and creatine (TPN-B+C), and suckling (SowFed) group; a fourth group was formed of IUGR piglets fed TPN (TPN-IUGR) that were ~65% of the birth weight of the largest littermate of each litter (Hegarty & Allen, 1978).

4.3.2 Catheter implantation in the piglet stage and grow-out period

Catheters were implanted in the femoral and jugular veins of all 7 d old piglets, as previously described (Study Day 0) (Dodge et al., 2012). Immediately post-surgery, piglets from the TPN-control group, TPN-B+C group, and TPN-IUGR group were fitted with jackets secured to individual metabolic cages (1 m diameter) with a swivel and tether system (Lomir Biomedical, Notre-Dame-de-l'Île-Perrot, QC, Canada). This setup allowed continuous TPN feeding with free movement and facilitated visual and aural contact with other piglets; toys were provided for further enrichment. All piglets received daily intravenous antibiotics (20 mg of trimethoprim and 100 mg of sulfadoxine; Borgal, Intervet Canada Ltd, Kirkland, Canada) and analgesic (0.03 mg/kg of buprenorphine hydrochloride; Temgesic, Schering-Plough, Whitehouse Station, New Jersey) for 3 d post-surgery. Lighting was maintained on a 12 h light: dark cycle and room temperature was maintained at 28 ^{0}C supplemented with heat lamps. Piglets received continuous TPN diet infusion

into the jugular vein for 14 d. The diet infusion was initiated at 50% of the targeted intake after surgery and increased to 75% the next morning for 12 hours and then to 100% (12 $mL \cdot kg^{-1} \cdot h^{-1}$) by evening. The piglets in the SowFed group were immediately returned to the sow after surgery and allowed to suckle for 14 d; all piglets successfully suckled within 2 hours of return to the sow. After receiving experimental TPN diets or sow milk for 14 d, all the piglets were weaned onto a milk replacer (Grober Nutrition Inc., Cambridge, ON, Canada) for 2-3 d and acclimated to a pig grower diet (Eastern Farmers Co-op, St. John's, NL, Canada) for the remainder of the study.

Once on the grower diet, pigs were group-housed with four pigs per pen, one from each experimental group, and fed ~2% of their cumulative body weight during the grow-out period. Body weights were measured, and blood samples were collected via jugular puncture every 2 weeks during the 8 mo. At 9 mo necropsy was performed and was measured crown to rump length, abdominal circumference, and chest girth. Subcutaneous fat thickness, which is an indicator of obesity in pigs were measured on the carcass at the midline of the back, immediately caudal to the last rib. Feed weights were adjusted every two weeks for body weight. Water was available 24 h ad-libitum and 12 h day-night cycle was maintained (lights on 0800-2000h) during the grow-out period.

4.3.3 TPN and grow-out diet

Parenteral diets were prepared under aseptic conditions following the procedures described elsewhere (Dodge et al., 2012), with modification to the lipid source as described below. Briefly, the elemental diet was prepared with crystalline L-amino acids, dextrose, and major minerals (Tables 4.1 and 4.2) dissolved in water at 60 ^{0}C under a nitrogen blanket. Diets were sterilized by cold filtering through a 0.22 µm filter into sterile intravenous bags (Baxter, Mississauga, Canada). Betaine and creatine were supplemented at 1.29 $g \cdot L^{-1}$ and 0.57 $g \cdot L^{-1}$ for the TPN-B+C diet. As the diet infusion rate was maintained at 12 $mL \cdot kg^{-1} \cdot h^{-1}$, piglets received betaine and creatine at 235

Table 4:1 Amino acid profile of TPN diets

Amino acid profile	TPN control diet ($g \cdot L^{-1}$)	TPN betaine + creatine diet ($g \cdot L^{-1}$)
Alanine	5.89	5.89
Arginine	3.65	3.65
Aspartic Acid	3.32	3.32
Cysteine	0.76	0.76
Glutamic Acid	5.72	5.72
Glycine	1.47	1.47
Histidine	1.69	1.69
Isoleucine	2.51	2.51
Leucine	5.67	5.67
Lysine hydrochloride	5.58	5.58
Methionine	1.04	1.04
Phenylalanine	3	3
Proline	4.52	4.52
Serine	3.11	3.11
Taurine	0.27	0.27
Tryptophan	1.14	1.14
Tyrosine	0.44	0.44
Threonine	2.23	2.23
Valine	2.89	2.89
Betaine hydrochloride	0	1.29
Creatine monohydrate	0	0.57

Table 4:2 Composition of the grower diet (12.1 MJ digestible energy·kg^{-1} and 154g protein·kg^{-1})

Energy (% total energy)	
Complex Carbohydrate	67
Fat	12
Protein	21
Ingredients (g·kg^{-1} dry matter)	
Wheat shorts	400.5
Canola	49.0
Meat meal	19.0
Limestone	13.0
Corn gluten feed	40.0
Ground barley	297.0
Oats	175.0
Vitamin mix	0.8
Mineral mix	1.0
Sodium Chloride	4.7

mg·kg^{-1} bodyweight·day^{-1} and 118 mg·kg^{-1} bodyweight·day^{-1}, respectively. Betaine was delivered at a molar equivalent of the piglet's methionine requirement (0.3 g·kg^{-1}·day^{-1}) (McBreairty et al., 2016; Shoveller et al., 2003), and creatine delivery matched the piglet's creatine accretion rate (Brosnan et al., 2009). The sterile diets were protected from light and refrigerated until fed to the piglets. All vitamins and minerals were supplied at >100% of the requirements for neonatal piglets (NRC, 2012). Vitamins (Multi-12/K1 Pediatric, Baxter Corporation, Mississauga, ON, Canada), trace minerals (Sigma-Aldrich Canada. Oakville, ON, Canada), iron dextran (Fe, 3.0 mg·kg^{-1}; Bimeda-MTC Animal Health. Cambridge, ON, Canada), and SMOF lipids (Fresenius Kabi, Uppsala, Sweden) were added to the TPN solution prior to feeding TPN. Diets were continuously infused intravenously by a pressure-sensitive peristaltic pump, and diet bags were weighed routinely to record the amount of diet infused.

The standard pig grower diet that was used during the grow-out period was based on wheat,

barley, and canola and contained 67% carbohydrates, 12% fat, and 21% protein (Table: 3). This diet has been used in our previous grow-out studies, with the same pig strain (Myrie et al., 2011).

4.3.4 Radiotelemetry system and surgical procedure at adulthood

At 8 mo of age, all pigs underwent surgery to implant an arterial blood pressure telemeter (M-10, DSI, St. Paul, USA) with 1.2 mm catheter thickness, 45 cm catheter length, and two blood sampling catheters. Briefly, anesthesia was induced using a dexmedetomidine, acepromazine, and alfaxalone mixture and maintained with 1.5% isoflurane. As this investigation was part of a larger study, two blood sampling venous catheters were also inserted into the left femoral vein (inner diameter, 1 mm; outer diameter, 1.8 mm; Tygon Medical Tubing, Saint Gobain Performance Plastic Corp., Akron, Ohio, USA). Blood sampling catheters were tunneled under the skin and exteriorized between the shoulder blades. The telemeter catheter was inserted 10 cm into the left femoral artery and was secured to the muscle. The body of the telemeter transmitter was implanted subcutaneously in the flank area of the hind limb. Intravenous buprenorphine hydrochloride 0.02 $mg \cdot kg^{-1}$ (Temgesic; Schering-Plough Ltd., Hertfordshire, UK) was given immediately after the surgery and one-day post-operative. Intravenous trimethoprim 15 $mg \cdot kg^{-1}$ (Borgal; Intervet Ltd., Whitby, ON, Canada) was given for 3 d post-operative, and each pig was housed -individually for 8-10 d for post-surgical recovery (Myrie et al., 2017).

BP and locomotor activity data generated by the telemeters were communicated via the transceivers to the communication link controller. Four transceivers were set up on top of the pig pens and positioned to maximize data acquisition, as recommended by the manufacturer (Data Sciences International, St. Paul, MN, USA). Data acquired by the controller, and the atmospheric pressure generated by an ambient pressure monitor, were transferred to a computerized data acquisition system (Ponemah Software 6.33, Data Science International, St. Paul, MN, USA) via a network switch. The data acquisition system generated a calibrated signal output after correcting

for the atmospheric pressure, and data were then processed offline. Pressure calibrations were assessed for each telemeter before implantation and after removal to account for data for any deviations from the original factory calibration. BP was sampled for 10 s at 30 s intervals for 48 h, and systolic (SAP), diastolic (DAP), and mean arterial pressure (MAP), as well as heart rate (HR), pulse pressure (PP), and activity were collected, and stored for further offline analysis.

4.3.5 Hemodynamic measurement and salt challenge protocol

SAP, DAP, MAP, HR, and PP were analyzed over a continuous 24 h period at 8-10 d post-surgical recovery. First, pigs were on the control grower diet (0.5% NaCl), and baseline recordings were collected over 48 h. Then, pigs were fed a high salt diet (4.5% NaCl) for 7 d, after which BP measurements were collected continuously for another 48 h. Finally, pigs were placed back on the control grower diet for a minimum of 5 d until necropsy (Myrie et al., 2011). The locomotor activity data were originated from the activity signal reported by the telemetry equipment and were expressed in arbitrary units, with 300 corresponding to the inactivity.

Telemetry data were averaged over 24 h, over 12 h light period, and 12 h dark period. The high-precision telemetry system generates accurate data without interfering with pig movement, resulting in minimum stress levels. However, one limitation of large animal implantable telemetry systems is that the elevation of the catheter tip relative to the telemeter body changes when pigs are standing versus lying down, potentially generating small but significant pressure differences ($\leq$ 8 mmHg). Because pigs mostly lie down at night and mostly stand up and are active during the day, we did not statistically compare parameters from the 12 h light period to the 12 h dark period to eliminate potential misinterpretation of data due to this postural artifact.

4.3.6 Necropsy and tissue collection

Pigs were anesthetized >5 d after finishing the high salt diet challenge, using 105 $mg \cdot kg^{-1}$ sodium pentobarbital (Euthanyl, Biomeda-MTC, Cambridge, ON, Canada), and were ventilated.

Tissues (i.e., kidneys, liver, heart) were removed, weighed, and the heart was immediately dissected into left and right ventricles, and samples of all tissues were freeze-clamped and frozen with liquid nitrogen, and samples were stored at -80 ^{0}C.

4.3.7 RNA extraction and real-time qPCR

The frozen kidney was pulverized to obtain a homogenous sample. Total RNA was extracted from the kidney using Trizol (Chomczynski & Sacchi, 1987). DNAse enzyme (Cat. # M610A, Promega, USA) was added to RNA samples to control for genomic DNA contamination. RNA concentration was confirmed using a NanoDrop 2000 (Thermo Scientific, USA), and the integrity of each RNA sample was verified using 1.2% agarose gel. Complementary DNA (cDNA) was synthesized from the extracted RNA samples using reverse transcription (cat # A3500, Promega, USA). Previously published real-time qPCR primers were used for AGT (Kim et al., 2015), β-actin (Nygard et al., 2007), GAPDH (Park et al., 2015). GR and AGTR1 primers were attained using NCBI primer blast (www.ncbi.nlm.nih.gov/tools/primer-blast/). All the primers were also verified using NCBI primer blast and obtained from Integrated DNA Technologies (IA, USA). SYBR Green Super-mix (Cat # 1708882, Bio-Rad, USA) was used to initiate qPCR amplification, and samples were run on a Mastercycler ep realplex system (Eppendorf, ON, Canada). Delta Ct values were calculated for the genes of interest and reference genes. Both β-actin and GAPDH were used as multiple reference genes to normalize the gene expression levels of interested genes accounting for their primer efficiencies using the Vandesompele method (Vandesompele et al., 2002). This method utilizes the geometric mean of the relative quantities for the reference genes to calculate gene expression. The formula is as below.

$$\frac{E_{GOI}{}^{\Delta Ct\ GOI}}{\text{Geomean}\,[E_{REF}{}^{\Delta Ct\ REF}]}$$

E = Amplification factor, GOI = Gene of interest, Geomean = Geometric mean, REF = Reference gene
ΔCt = Ct (Calibrator sample) – Ct (Test sample)

Table 4:3 Primer pairs used for real time PCR

Gene	Gene symbol	Forward	Reverse	Primer efficiency	Gene bank accession no
Angiotensinogen	AGT	CTC TCC CAC GCT CAC TAG ACT TG	ATG CAT GAA CCT GTT GAT CTT CTC	87.68	XM 021073990.1
Angiotensinogen receptor type 1	AGTR1	GCA CCC AAA CCC CAT ACC AG	ACA CTA CGC CAA ATG CAC CT	97.14	XM 003132469.4
Glucocorticoid receptor	GR	AGA AGG GGG CGG CTG TTT AC	CCC ACC GCA GCC GAG ATA AA	99.81	NM 001008481.1
Beta actin (kidney)	β actin	CAC GCC ATC CTG CGT CTG GA	AGC ACC GTG TTG GCG TAG AG	99.23	DQ845171
Glyceraldehyde-3-phosphate dehydrogenase (kidney)	GAPDH	ATCCTGGGCTACACTGAGGA	TGTCGTACCAGGAAATGAGCT	87.23	NM 001206359.1

4.3.8 Statistical analyses and hemodynamic data analyses

Differences in hemodynamic values between the 4.5% and 0.5% sodium chloride diets were calculated by subtracting baseline BP values for each pig from BP values after the salt challenge from the same pig. Feed conversion ratio (FCR) was calculated by dividing the estimated feed intake (i.e., 2% of body weight) divided by body weight gained during a period of time. Fractional growth rate (FGR) was calculated by dividing body weight gain for a given period by the initial body weight of that period and expressed as ($g \cdot kg\ bodyweight^{-1} \cdot d^{-1}$).

Hemodynamic parameters and activity were compared between experimental groups using a one-way ANOVA, with a Dunnett's post hoc test (Graph Pad Prism 8.0; GraphPad Software, San Diego, CA, USA), using TPN-control as the reference group. By comparing to TPN-control: the TPN-B+C group was designed to identify the effects of adding B+C in TPN; the TPN-IUGR group was used to determine whether IUGR exacerbates the effects of TPN; and the SowFed group was used as a clinical reference group to determine the effects of TPN feeding. The results are reported as means ± standard deviation; differences were considered statistically significant if $P<0.05$.

A modified version of the Microsoft Excel 2000 template (HdStats) was utilized to inspect and analyze 24 h, 12 h light, and 12 h dark data sets, which we have also used to analyze BP data previously (Myrie, MacKay, et al., 2012; Myrie, McKnight, et al., 2012).

A paired *t*-test was used to assess whether the changes in 12 h light period, 12 h dark period, and 24 h BP responses were significant due to the high salt diet within the group.

Pearson's correlations were used to determine the relationship between HR and body weight vs. activity.

4.4 Results

4.4.1 Growth and feed intake

TPN-IUGR piglets were defined as ~65% body weight of the largest littermate (Hegarty & Allen, 1978) and the group had significantly lower body weight before and after TPN feeding compared to TPN-control piglets (Table 4.4). However, after one month, TPN-IUGR pigs reached the same body weight as piglets in the other experimental groups. Body weights remained insignificant amongst the groups until adulthood and were not different amongst the groups at 4 and 8-mo time points. Based on the developmental phases and sexual maturity in Yucatan pigs, growth rates were divided into time segments (7-21 d, 1-4 mo, 4-6 mo, 6-8 mo: Table 4.4). TPN-IUGR pigs showed higher body weight growth rates than TPN-control pigs over 1-4 mo and 4-6 mo. However, these groups did not significantly change body weight growth rates over 6-8 mo. Similarly, body measurements collected at 9 mo, including crown to rump length, abdominal circumference, and chest girth, were not significantly different amongst the experimental groups. However, subcutaneous fat deposition was higher in TPN-IUGR pigs compared to TPN-control pigs at 9 mo (Table 4.4).

4.4.2 Hemodynamics

4.4.2.1 Circadian hemodynamics and locomotor activity in Yucatan miniature pigs

Yucatan miniature pigs showed circadian variation in HR and locomotor activity (Figure 4.1). The HR and locomotor activity of adult pigs was greater in the light period than the dark period, regardless of neonatal diet. However, HR and activity were not correlated ($r = 0.2977$, $P = 0.1168$). Because of potential posture-dependent artifacts, circadian variations in BP were not analyzed (see Discussion).

Table 4:4. Summary of body weight, growth rate, body measurements, and feed conversion ratio in TPN control (TPN-control), TPN with B+C (TPN-B+C), IUGR fed with TPN control (TPN-IUGR), and sow fed (SowFed) Yucatan miniature pigs during the experimental period.

	TPN-control	**TPN-B+C**	**TPN-IUGR**	**SowFed**
Body weight (kg)				
Birth	0.98 ± 0.17	1.02 ± 0.12	0.67 ± 0.10***	1.01 ± 0.15
TPN start (7 d)	1.74 ± 0.18	1.74 ± 0.24	1.25 ± 0.16**	1.80 ± 0.35
TPN end (21 d)	3.10 ± 0.20	3.15 ± 0.24	2.44 ± 0.21*	3.53 ± 0.88
1 mo	3.54 ± 0.88	3.77 ± 0.61	3.57 ± 1.09	4.81 ± 1.87
4 mo	14.99 ± 3.29	15.83 ± 2.77	17.46 ± 2.54	18.06 ± 2.86
8 mo	35.05 ± 7.62	34.43 ± 5.49	41.74 ± 4.63	37.87 ± 7.49
Body weight growth rate (g· d^{-1})				
7 d – 21 d	97.13 ± 6.17	101.10 ± 7.48	82.99 ± 8.53	117.92 ± 48.72
1-4 mo	130.11 ± 38.55	135.58 ±30.91	171.78 ± 25.10*	151.85 ± 31.94
4-6 mo	143.67 ± 52.47	148.46 ± 45.43	203.69 ± 28.70*	165.07 ± 57.09
6-8 mo	190.19 ± 39.87	168.96 ± 34.92	194.75 ± 51.02	163.96 ± 59.28
Fractional growth rate (g· kg body weight^{-1}·d^{-1})				
7d - 21d	40.06 ± 3.15	41.64 ± 5.12	47.65 ± 4.33	45.42 ± 11.66
1-4 mo	13.88 ± 2.72	13.75 ± 2.06	15.24 ± 3.32	12.97 ± 2.63
4-6 mo	7.31 ± 1.65	7.27 ± 1.69	8.50 ± 1.30	6.41 ± 2.56
6-8 mo	6.11 ± 0.56	5.27 ± 0.61	5.61 ± 1.25	4.98 ± 1.15
Body measurements at 9 mo				
Crown to rump length (cm)	112.1 ± 6.1	111.8 ± 6.3	118.1 ± 3.2	116.3 ± 6.9
Abdominal circumference (cm)	84.7 ± 6.8	82.8 ± 6.8	88.3 ± 4.1	84.4 ± 7.2
Chest girth (cm)	79.4 ± 4.3	79.3 ± 3.7	82.9 ± 4.3	77.7 ± 4.0
Subcutaneous fat thickness (mm)	43.2 ± 5.5	43.7 ± 3.8	50.0* ± 5.0	45.1* ± 5.6
Average feed conversion ratio				
1-4 mo	1.56 ± 0.20	1.56 ± 0.12	1.47 ± 0.26	1.74 ± 0.23
4-6 mo	3.30 ± 0.98	3.45 ± 1.14	2.59 ± 0.49	3.43 ± 1.15
6-8 mo	3.83 ± 0.64	4.51 ± 1.20	4.55 ± 1.32	5.45 ± 2.13

TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled.
*P<0.05, **P<0.001,***P<0.0001. 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

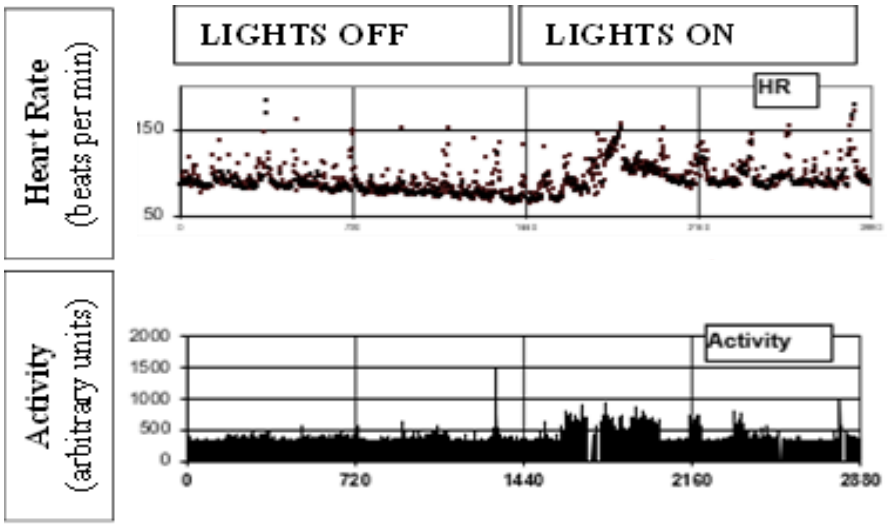


Figure 4:1 Representative 24 h recordings of heart rate (beats per min) and locomotor activity in a TPN-control Yucatan miniature pig (12 h light/dark cycle).

12 h light period heart rate and activity were not significantly different amongst the experimental groups when the pigs were fed a standard grower diet (0.5% NaCl) or after pigs were challenged with a high salt diet (4.5% NaCl; Table 4.5). The change in activity after the challenge with 4.5% NaCl diet was greater in TPN-IUGR pigs compared to TPN-control pigs. DAP and PP were not different amongst the dietary groups; however, SAP and MAP were significantly lower in TPN- B+C compared to TPN-control pigs (Table 4.5); other groups were not different from TPN-control.

12 h dark period BP parameters, heart rate, and activity were not significantly different amongst the experimental groups when the pigs were fed a standard grower diet with 0.5% NaCl or after pigs were challenged with a high salt diet containing 4.5% NaCl; the change in these parameters was also not different (Table: 4.6).

Table 4:5 12 h light period hemodynamic parameters for adult Yucatan miniature pigs fed four experimental diets in the neonatal period followed by a) standard grower diet for 8 mo, b) after a high salt diet challenge, and c) the difference of hemodynamic values between 4.5 and 0.5% NaCl diets (salt challenge and baseline diets)**.**

	TPN-control		TPN-B+C		TPN-IUGR		SowFed		
Variable	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	***P**
After 9 mo on standard diet (0.5% NaCl)									
DAP (mmHg)	86.6	6.2	81.8	3.7	89.2	3.8	86.1	4.2	0.035
SAP (mmHg)	134.8	6.4	125.3*	4.7	136.2	6.3	130.1	6.8	0.008
MAP (mmHg)	109.1	6.3	102.0*	3.0	111.1	4.3	106.9	5.4	0.010
PP (mmHg)	48.2	3.7	43.4	6.1	47.0	5.2	44.0	4.7	0.221
HR (beats·min^{-1})	96.1	6.3	95.5	6.4	102.1	11.8	100.2	7.9	0.379
Activity (Arbitrary Units)	440.9	41.1	431.0	35.2	436.4	43.6	423.3	25.8	0.833
After 7 d on high salt diet (4.5% NaCl)									
DAP (mmHg)	95.7	5.8	96.4	7.1	95.4	7.7	94.5	5.0	0.950
SAP (mmHg)	145.4	10.5	140.6	10.8	143.8	14.7	138.9	5.8	0.653
MAP (mmHg)	119.1	7.7	117.4	8.9	118.1	10.6	115.2	5.3	0.830
PP (mmHg)	49.7	5.6	44.2	5.7	48.5	9.1	44.4	2.7	0.240
HR (beats· min^{-1})	96.4	3.1	92.7	5.3	96.8	6.9	94.1	10.9	0.700
Activity (Arbitrary Units)	446.1	40.5	430.8	21.4	497.7	56.0	456.8	61.9	0.070
Change from 0.5 to 4.5 NaCl diets									
DAP (mmHg)	9.1†	7.8	14.6††	7.5	6.2†	7.2	8.4†	8.0	0.208
SAP (mmHg)	10.5†	8.6	15.3†	7.6	7.6†	11.5	8.7†	8.7	0.413
MAP (mmHg)	10.0†	8.2	15.4†	7.8	6.9†	9.3	8.4†	8.4	0.270
PP (mmHg)	1.4†	3.6	0.7†	3.4	1.4†	5.8	0.4†	2.9	0.353
HR (beats· min^{-1})	-1.8	11.2	-2.7	4.3	-5.4	6.7	-6.1	8.2	0.689
Activity (Arbitrary Units)	5.1	36.1	-0.2	27.7	61.4*	67.6	19.7	30.4	0.046

TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled; DAP, diastolic arterial pressure; SAP systolic arterial pressure; MAP, mean arterial pressure; PP, pulse pressure; HR, heart rate.

Significant differences among the groups were analyzed by one-way ANOVA followed by Dunnett's post hoc test comparing to TPN-control; *P<0.05.

Blood pressure change due to high salt diet was significantly different than zero. † P<0.05, †† P<0.001, assessed by paired t-test.

Table 4:6 12-h dark period hemodynamic parameters for adult Yucatan miniature pigs fed four experimental diets in the neonatal period followed by a) standard grower diet for 9 mo, b) after a high salt diet challenge, and c) the difference of hemodynamic values between 4.5 and 0.5% NaCl diets.

	TPN-control		TPN-B+C		TPN-IUGR		SowFed		
Variable	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	***P**
After 9 mo on standard diet (0.5% NaCl)									
DAP (mmHg)	*94.4*	*7.7*	89.9	6.1	92.9	6.6	91.7	7.2	0.657
SAP (mmHg)	*141.5*	*7.6*	133.6	9.9	141.8	8.5	136.7	10.2	0.275
MAP (mmHg)	*116.5*	*7.6*	110.1	7.1	115.6	7.0	112.9	8.7	0.403
PP (mmHg)	*47.0*	*3.4*	43.7	6.9	48.9	5.4	45.0	4.7	0.263
HR (beats· min^{-1})	*87.4*	*3.7*	81.0	6.3	91.1	10.5	87.3	11.7	0.205
Activity (Arbitrary Units)	*399.7*	*36.1*	371.4	52.0	375.8	28.0	381.2	50.1	0.616
After 7 d on high salt diet (4.5% NaCl)									
DAP (mmHg)	*97.4*	5.0	100.9	10.3	99.3	9.8	99.5	3.5	0.868
SAP (mmHg)	*147.7*	10.7	146.7	12.6	148.2	17.9	146.0	2.8	0.985
MAP (mmHg)	*120.6*	7.2	122.2	11.3	121.9	13.2	121.1	2.3	0.989
PP (mmHg)	*50.3*	6.3	45.8	5.6	48.8	9.9	46.5	4.0	0.095
HR (beats· min^{-1})	*82.5*	10.3	78.1	5.7	85.0	10.4	80.5	14.9	0.666
Activity (Arbitrary Units)	*410.0*	56.4	375.3	40.7	410.6	65.0	392.3	61.5	0.605
Change from 0.5 to 4.5 NaCl diets									
DAP (mmHg)	*3.3*	*2.9*	12.8†	8.0	6.1	7.3	8.1	7.3	0.098
SAP (mmHg)	*6.3*	*11.2*	13.1†	9.4	6.4	17.0	9.3†	9.5	0.644
MAP (mmHg)	*4.2*	*4.0*	13.7†	8.2	6.6	10.2	8.3†	7.8	0.156
PP (mmHg)	*3.3*	*4.6*	2.1	3.1	0.0	8.0	1.5	3.0	0.667
HR (beats· min^{-1})	*-4.9*	*10.7*	-2.9	6.4	-6.1	10.5	-6.9	9.9	0.867
Activity (Arbitrary Units)	*10.3*	*64.4*	3.9	40.1	34.8	57.5	11.1	36.4	0.683

TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled; DAP, diastolic arterial pressure; SAP systolic arterial pressure; MAP, mean arterial pressure; PP, pulse pressure; HR, heart rate.
Significant differences among the groups were analyzed by one-way ANOVA followed by Dunnett's post hoc test comparing to TPN-control; *P<0.05. Blood pressure change due to high salt diet was significantly different than zero. † P<0.05 assessed by paired t-test.

Only SAP and MAP values of TPN-B+C pigs on the standard diet in 24 h period showed a lower trend than TPN-control pigs (SAP, MAP, P = 0.0606, 0.0881 respectively), which is consistent with the 12 h light period data (Table 4.7). All other parameters were not different among groups.

Table 4:7. 24 h hemodynamic parameters of adult Yucatan miniature pigs fed four experimental diets in the neonatal period, followed by a) standard grower diet, b) after high salt diet challenge, and c) the difference of hemodynamic values between 4.5 and 0.5% NaCl diets. (Salt challenge and baseline diets)

	TPN-control		**TPN-B+C**		**TPN-IUGR**		**SowFed**		
Variable	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	***P**
After 9 mo on standard diet (0.5% NaCl)									
DAP (mmHg)	*90.5*	*6.4*	85.8	4.3	91.1	4.5	88.9	5.2	0.146
SAP (mmHg)	*138.1*	*6.5*	129.4	7.2	139.0	6.5	133.4	8.4	0.061
MAP (mmHg)	*112.8*	*6.4*	106.1	4.7	113.4	4.9	109.9	6.8	0.088
PP (mmHg)	*47.6*	*3.5*	43.6	6.3	47.9	5.2	44.5	4.5	0.251
HR (beats· min^{-1})	*93.1*	*2.2*	88.3	5.8	96.6	10.2	93.7	9.4	0.267
Activity (Arbitrary Units)	*419.0*	*36.8*	399.4	41.5	403.0	31.1	407.9	43.5	0.790
After 7 d on high salt diet (4.5% NaCl)									
DAP (mmHg)	*96.6*	*4.5*	98.7	8.6	97.4	8.2	97.0	3.0	0.938
SAP (mmHg)	*146.6*	*10.1*	143.7	11.6	146.0	15.8	144.1	10.8	0.583
MAP (mmHg)	*119.9*	*6.8*	119.8	10.0	120.0	11.4	118.2	2.5	0.970
PP (mmHg)	*50.0*	*5.8*	45.0	5.5	48.6	9.4	45.4	3.2	0.025
HR (beats· min^{-1})	*88.4*	*7.7*	85.4	5.2	90.9	8.4	83.2	5.9	0.186
Activity (Arbitrary Units)	*426.7*	*43.2*	401.3	25.1	451.0	59.7	421.3	54.9	0.282
Change from 0.5 to 4.5 NaCl diets									
DAP (mmHg)	*3.3*	*2.9*	12.8†	8.0	6.3†	7.3	8.1†	7.3	0.106
SAP (mmHg)	*5.6*	*6.5*	14.2†	8.1	11.0†	7.7	10.6†	12.4	0.433
MAP (mmHg)	*4.2*	*4.0*	13.7†	8.2	6.6	10.2	8.3†	7.8	0.200
PP (mmHg)	*2.4*	*4.0*	1.4	2.9	2.7	4.2	0.9	1.8	0.727
HR (beats· min^{-1})	*-3.4*	*9.6*	-2.9	4.6	-5.7	6.6	-6.5	8.7	0.757
Activity (Arbitrary units)	*7.6*	*41.7*	1.9	24.1	48.0	59.5	13.4	32.7	0.161

TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled; DAP, diastolic arterial pressure; SAP systolic arterial pressure; MAP, mean arterial

pressure; PP, pulse pressure; HR, heart rate.
Significant differences among the groups were analyzed by one-way ANOVA followed by Dunnett's post hoc test comparing to TPN-control; *P<0.05.
Blood pressure change due to high salt diet was significantly different than zero. † P<0.05 assessed by paired t-test.

Body weight at necropsy was negatively correlated to 24 h activity in adult pigs, suggesting that reduced activity led to higher body weight (Figure 4.2).

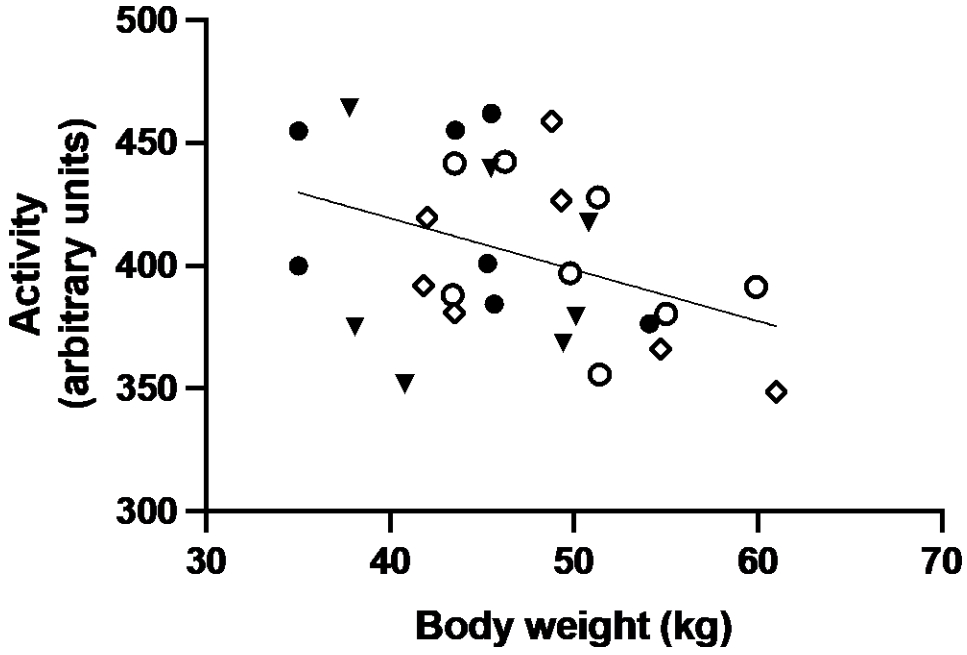


Figure 4:2 Effects of body weight (kg) on 24 h activity (arbitrary units) in adult Yucatan miniature pigs fed four experimental diets in the neonatal period.

r = -0.3859, P = 0.0387. Each symbol represents an individual pig. TPN-control (●); TPN-B+C (▼); TPN-IUGR (○), SowFed (◊).

The ratio of the left and right kidney to body weight and heart to body weight was significantly lower in TPN-IUGR pigs compared to TPN-control pigs (Table 4.8). There was no difference in other parameters.

Table 4:8 Renal and cardiac parameters in adult Yucatan miniature pigs fed one of four experimental diets a TPN-control, TPN-B+C, TPN-IUGR, and SowFed for 2 week during the neonatal period.

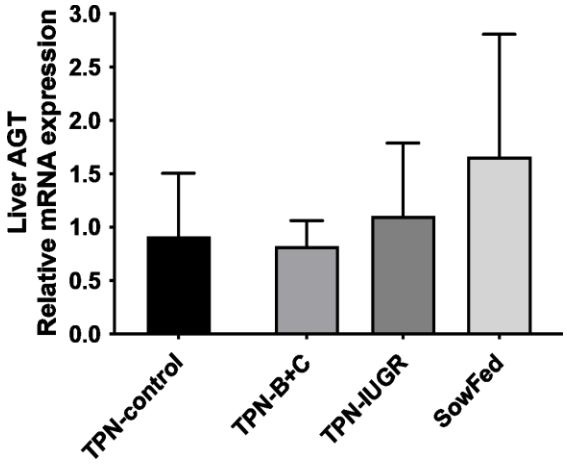
	TPN-control		**TPN-B+C**		**TPN-IUGR**		**SowFed**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Renal parameters								
Right kidney (g)	*72.396*	*14.077*	63.467	10.669	70.876	8.127	69.257	15.175
Left kidney (g)	*73.156*	*17.609*	68.801	15.778	68.499	4.689	67.046	13.691
Right kidney: body weight (%)	*1.693*	*0.277*	1.469	0.147	1.429*	0.209	1.485	0.056
Left kidney: body weight (%)	*1.699*	*0.281*	1.581	0.214	1.232*	0.525	1.445	0.102
Cardiac parameters								
Heart (g)	*174.604*	*20.634*	171.803	15.215	165.256	19.444	178.431	25.752
Heart/ body (%)	*4.097*	*0.453*	4.030	0.571	3.370*	0.438	3.847	0.341
Left ventricle (g)	*94.857*	*13.337*	95.555	7.323	91.783	4.995	97.709	16.366
Right ventricle (g)	*34.926*	*4.284*	33.879	4.643	32.867	2.809	33.960	4.904
Right/left ventricle ratio	*0.373*	*0.022*	0.355	0.049	0.361	0.033	0.339	0.028

TPN, total parenteral nutrition; TPN control diet fed (TPN-control), TPN with betaine and creatine (TPN-B+C); intrauterine growth restricted fed with TPN control (TPN-IUGR); Sowfed (SowFed). Significant differences among the groups were analyzed by one-way ANOVA followed by Dunnett's post hoc test comparing to TPN-control; *P<0.05.

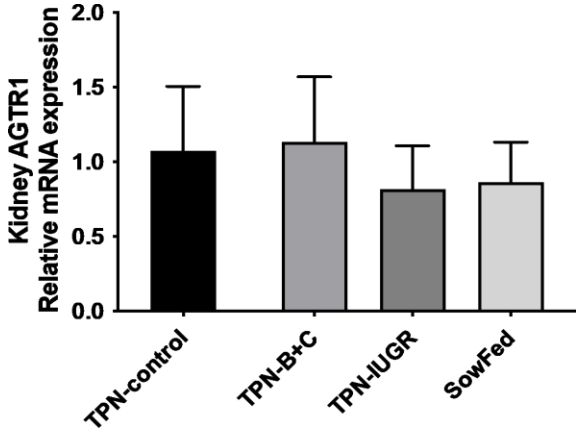
4.4.3 Relative mRNA expression

Several methylation-susceptible genes play a role in regulating blood pressure: AGT, AGTR1, and GR. There were no differences in relative mRNA expression of any of these three genes among the experimental groups in adulthood (Figure 4.3).

a)


Liver AGT
Relative mRNA expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
TPN-control
TPN-B+C
TPN-IUGR
SowFed

b)


Kidney AGTR1
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
TPN-control
TPN-B+C
TPN-IUGR
SowFed

c)

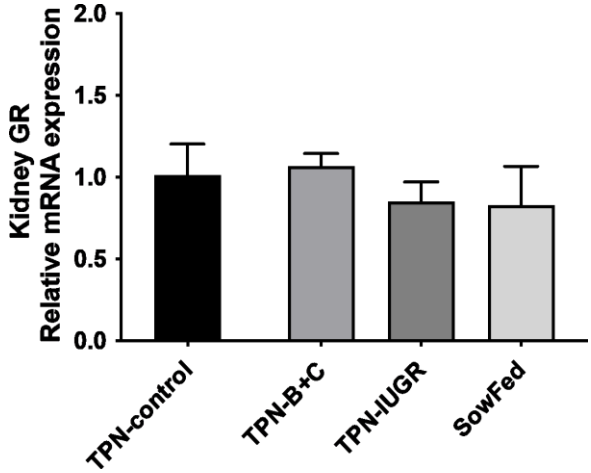

Kidney GR
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
TPN-control
TPN-B+C
TPN-IUGR
SowFed

Figure 4:3 Relative mRNA expression of a) liver AGT, b) kidney AGTR1, and c) kidney GR in adult Yucatan miniature pigs (at 8 mo) fed one of four experimental (TPN-control, TPN-B+C, TPN-IUGR, and SowFed) diet for 2 weeks during the neonatal period.

Values are means ± SD; analyzed using 1-way ANOVA with Dunnett's Post hoc test comparing to TPN-control. Primer efficiencies for liver β- actin and GAPDH were 104.50% and 84.70%, and kidney β- actin and GAPDH were 99.23%, 87.23%, respectively. Geometric averaging of both β-actin and GAPDH was used to normalize the expression levels of AGT, AGTR1 and GR genes, and the data was corrected for the primer efficiency using Vandesompele et al., 2002 method. TPN, Total parenteral nutrition; TPN-control, TPN control group; TPN-B+C, TPN supplemented with B+C; TPN-IUGR, IUGR piglets fed with TPN-control diet; SowFed, suckled; IUGR, intrauterine growth restricted; AGT, angiotensinogen; AGTR1, angiotensinogen receptor type 1; GR, glucocorticoid receptor.

4.5 Discussion

According to recent research, nutritional manipulation in early life is involved in the programming of hypertension in adulthood (Ojeda et al., 2008). TPN is a lifesaving feeding method for many neonates, but it often results in metabolic consequences (Kumar & Teckman, 2015). We are the first to examine the effects of early TPN feeding on BP in adulthood and whether changing the composition of TPN reduces the risk of developing hypertension in Yucatan miniature pigs. The current study found that BP parameters (SAP and MAP) were significantly lower when TPN was supplemented with B+C, compared to a control TPN (12 h light period). Since these pigs were fed TPN for 14 d in early life followed by a standard diet for 8 mo, the effects that were observed in adulthood are due to the programming effects of early TPN.

Ambulatory BP readings are advantageous over casual or less than 24 h blood pressure recording due to their ability to track circadian BP patterns. Nevertheless, 12 h day and night BP patterns are equally important in understanding the risk of hypertension (Wang et al., 2009). However, circadian variation in BP were not analyzed due to potential posture-dependent artifacts. This potential limitation is associated with BP measurements made in large animals by fluid-filled catheters (Myrie et al., 2011). Elevation of the catheter tip relative to the telemeter body changes when pigs are standing versus lying down, possibly generating small but significant pressure differences (≤ 8 mmHg). Although pigs are mostly lying down at night and mostly standing up and active during the day, exact posture throughout the recording period may not be consistent amongst all the pigs, and statistical comparison of parameters from the 12 h light period,12 h dark period and 24 h may result in misinterpretation of data. Because the variability of the data is low, we mostly focused on 12 h light data in interpreting the results. In our study, feeding TPN during the neonatal period did not program BP parameters in adulthood, as BP was not significantly

different between TPN-control and SowFed pigs. However, feeding TPN supplemented with B+C in early life reduced 12 h light period SAP and MAP during adulthood in TPN-B+C pigs.

Systolic hypertension suggests stiffening of the major arteries. Supplementing TPN with B+C during the neonatal period may reduce the stiffening of the artery walls with age, leading to a lower risk of hypertension in adulthood. Studies report that oral administration of betaine can acutely lower BP parameters via its anti-inflammatory effects (Wang et al., 2018; Yang et al., 2018). As B+C supplemented TPN is given during the early postnatal period, where the epigenetic window of programming takes place, lowering SAP and MAP in adulthood could be likely due to a change in epigenetic regulation of BP-related genes. A previous rat study showed that maternal choline supplementation during periods of low protein exposure during early life can normalize increased systolic blood pressure of offspring in later life (Bai et al., 2012). Another study showed that maternal methyl donor diet during pregnancy and lactation is associated with diet-induced hypertension and causes long-term alterations of ~800 renal transcripts in the offspring (Tain et al., 2018). Thus, the ability of B+C to increase the spare methyl group availability for epigenetic regulation may likely be involved in preventing the development of adult hypertension.

The molecular and biological mechanisms involved in the programming of hypertension are numerous and not completely understood (Liang, 2018). Epigenetic causes of hypertension are emerging research areas; however, evidence suggests that DNA methylation may regulate several genes relevant to BP regulation. AGT (Wang et al., 2014), AGTR1 (Rivière et al., 2011), and GR (Wyrwoll et al., 2007) are BP genes regulated by DNA methylation. However, there were no significant differences in expressions of these genes between TPN-control and TPN-B+C groups. It is possible, that other BP-relevant genes that are regulated via DNA methylation or other factors that govern BP, such as autonomic nervous system activity, may have caused the reduction

of SAP and MAP in the TPN-B+C group. Wyrwoll et al., 2007 investigated GR expression in the distal tubule of the nephron, whereas we used the homogenized kidney could be one reason we did not find significant differences amongst the groups. Further investigation of genes involved in BP regulation using RNA sequencing and methylation techniques could identify other potential targets to help explain the mechanisms behind SAP and MAP reduction in TPN-B+C pigs.

Salt sensitivity of BP refers to the BP responses to changes in dietary salt intake that produce meaningful BP increase or decrease and is a well-established phenomenon in hypertension (Choi et al., 2015). It has been suggested that abnormalities in the RAS, sympathetic nervous system, renal transmembrane sodium transport, nitric oxide system, and vascular endothelium are all involved in the pathogenesis of salt-sensitive hypertension (Choi et al., 2015). To determine if our treatments differentially affect salt-sensitive hypertension in adulthood, we challenged the pigs at 9 mo with an acute high salt diet for 7 d and measured BP parameters. We found that feeding TPN in the neonatal period did not significantly affect acute salt sensitivity in adulthood. All the pigs demonstrated higher BP parameters with acute salt challenge suggesting all the pigs were salt sensitive in adult life; feeding TPN during the neonatal period did not affect the degree of this salt sensitivity in adulthood. The observation of acute salt sensitivity is consistent with our previous findings in the same animal model fed a standard grower diet during the grow-out period and then fed a high salt version of the standard diet ad libitum (Myrie, McKnight, et al., 2012).

The body weight at birth in IUGR piglets was no longer different by one month of age, one week after TPN feeding ended. The increased body growth rate during the pre-sexual maturity stages of 1-4 mo and at 4-6 mo in TPN-IUGR pigs, which was also observed in McKnight et al., 2012, represents the continuation of the catch-up growth initiated in early life. Thus, our data confirm that IUGR pigs demonstrate catch-up growth from weaning age to the beginning of sexual

maturity (4 mo), whether they were fed with TPN as a neonate or fed sow milk replacer (Mcknight et al., 2012). Although body weight did not significantly differ amongst the dietary groups by 8 mo, TPN-IUGR were more obese, as indicated by increased subcutaneous fat thickness, than the TPN-control group. Thus, part of the rapid catch-up body weight growth in TPN-IUGR pigs is due to higher deposition of adipose tissue, rather than lean muscle. However, TPN-IUGR pigs did not show increased BP parameters despite being obese at 8 mo. Across all pigs, a significant negative correlation ($r = -0.386$) between body weight at necropsy (9 mo) and 24 h activity level demonstrates that higher body weight was associate with lower activity level, which may also increase the risk of CVD. Interestingly, the low ratio of kidney and heart weights to body weight in adult TPN-IUGR pigs suggests that reduced organ growth due to intrauterine growth restriction persisted into adulthood. Even though the kidneys and hearts of TPN-IUGR pigs were smaller than those of TPN-control pigs in adulthood, the functional contribution to maintaining BP was not different, as indicated by no difference in BP parameters. On the other hand, it is possible the smaller organ sizes were appropriate to the lean tissue growth of the TPN-IUGR pigs, as higher adipose tissue may have lowered this ratio. Supplementing betaine and creatine to TPN did not affect the growth rate of the pigs into adulthood.

In a previous study, we compared the BP of pigs with IUGR and normal birth weight pigs in adulthood and found a modest but significant increase in diastolic arterial pressure (DAP) (Myrie et al., 2011). However, in our current study, where IUGR pigs were fed TPN during the neonatal period, we did not observe any differences in BP parameters between the TPN-fed IUGR pigs and the TPN-control (normal weight) pigs. There are a few key differences between our study and the previous one conducted by Myrie et al. 2011. Firstly, the pigs in their study were ad libitum fed, whereas our pigs were subjected to feed restriction. Additionally, our study involved TPN

feeding for the IUGR and control pigs during the neonatal period, whereas the pigs in the study by Myrie et al. 2011 did not receive TPN. When comparing the BP parameters of the pigs in our study that were fed by sows and the pigs from Myrie et al. 2012, we found that they were quite similar, despite the difference in feeding regimens. Because the pigs in Myrie et al. (2012) were ad libitum fed, while our pigs were feed restricted suggests that feed restriction did not have an impact on BP parameters. Therefore, it is likely that the lack of differences in BP parameters between the TPN-IUGR pigs and the TPN-control pigs in our study can be attributed to the TPN feeding during the neonatal period.

Our study possesses significant strengths, including the use of a well-established pig model to study programming effects and parenteral feeding, as well as the application of high-precision telemetry measurements to assess BP in freely moving pigs. Based on our current data, we have observed that providing TPN to neonatal pigs does not have an impact on their blood pressure in adulthood. However, the addition of betaine and creatine to TPN during early life may potentially decrease BP parameters, thereby reducing the risk of hypertension in adulthood. Although AGT, AGTR1 and GR are methylation susceptible genes that involve in BP regulation, they are not involved in BP regulation in the current study. To gain a deeper understanding of the programming effect of betaine and creatine in TPN on adult BP, it would be beneficial to investigate other methyl-dependent genes involved in BP regulation. This could involve exploring the entire genome and examining the methylation status. Such investigations will aid in unraveling the underlying mechanisms behind this programming effect. Our findings cleared the avenues for further research into the molecular mechanisms implicated in the programming effects of early TPN feeding on hypertension in adulthood. By manipulating the composition of early TPN, it may be possible to alter the phenotypic outcomes of hypertension in adulthood. Ultimately, this

knowledge can inform the development of novel nutritional interventions aimed at mitigating the risk of hypertension later in life.

5 Chapter 5: Parenteral nutrition in early life exacerbated catch-up growth and obesity leading to ectopic fat deposition in IUGR Yucatan miniature pigs in adulthood.

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Authorship: R.F.B. and J.A.B. designed the study, and R.F.B. acquired the funding. L.H. conducted the initial animal work. R.S.R. conducted the experiments; the analytical work, generated the results and prepared the initial draft of the manuscript. R.S.R and R.F.B. interpreted the results and wrote the manuscript. All authors read and agreed to the manuscript.

5.1 Abstract

Total parenteral nutrition (TPN) is often used as a life-saving nutritional regimen in intrauterine growth restricted (IUGR) neonates. However, nutritional changes during the early critical period may permanently program the metabolism, possibly through epigenetic alterations, and methyl nutrient imbalances may be a cause. These epigenetic changes can lead to obesity and dyslipidemia later in life. Methyl group availability can be increased by betaine and creatine which are novel ingredients to commercial TPN. We hypothesized that TPN in early life would have long-term effects on obesity; moreover, IUGR will exacerbate these TPN-induced effects, and supplementing betaine and creatine will alleviate these effects. We used 7 d old female piglets (n = 24) that were randomized to suckled (SowFed), TPN control (TPN-control), and TPN with B+C (TPN-B+C). Eight IUGR piglets were selected as a fourth group and fed TPN (TPN-IUGR). After two weeks of TPN, all pigs were fed standard feed to adulthood (9 mo) when metabolic tests were conducted. Growth rates of TPN-IUGR were higher than TPN-control during 1-4 mo and 4-6 mo periods, indicating catch-up growth which led to greater adiposity, as revealed by a higher backfat thickness at 9 mo (P<0.05). Moreover, TPN-IUGR pigs had greater accumulation of ectopic triglyceride deposition in the liver (P<0.05) and skeletal muscle (P<0.05), possibly due to their higher fasting plasma non-esterified fatty acids (NEFA) (P<0.001). TPN-control was not different from SowFed or TPN-B+C pigs, suggesting that feeding TPN during the neonatal period did notresult in obesity later in life and methyl group supplementation to TPN had no effect. In conclusion, IUGR has a profound effect on developing obesity later in life, but TPN feeding per se does not lead to obesity in adulthood.

5.2 Introduction

Obesity is abnormal or excessive fat accumulation, which contributes to cardiovascular disease (CVD), dyslipidemia, liver steatosis, and metabolic syndrome (Agarwal et al., 2018). Although genetic susceptibility and adult lifestyles are involved in these trends, nutritional perturbations early in life could also increase the risk of developing obesity in later life (Agarwal et al., 2018). According to the developmental origins of health and disease (DOHaD) theory, environmental exposures during early life can permanently influence health and vulnerability to disease in later life (Hoffman et al., 2017). In this context, nutrition during the critical development window in early life plays an important role in programming diseases in adulthood. TPN provides essential nutrients intravenously when oral nutrition cannot be administered (Hamdan & Puckett, 2022) and is prescribed frequently in preterm newborns, IUGR neonates, and infants with certain medical problems. TPN support for neonates is associated with undernutrition, overnutrition, intestinal atrophy, and bacterial translocation due to a damaged, underused, malnourished gut (Lucchinetti et al., 2021), most likely predisposing them to altered metabolism, including the development of obesity. It is a non-normal nutrition regimen administered within the critical window of programming, which may program metabolism both acutely and permanently. Indeed, research has shown that feeding TPN to neonatal piglets leads to obesity 17 d later (Stoll et al., 2010); but to our knowledge, no study has examined the long-term consequences of feeding TPN to neonates and the possibility of developing obesity as an adult.

Previous studies have illustrated that IUGR leads to a thrifty phenotype, resulting in catch-up growth and increasing the risk of obesity in later life (Armengaud et al., 2021). Both TPN and IUGR independently alter metabolism in the neonatal period (Dessi et al., 2011; Golucci et al., 2018; Lianou et al., 2022; Liu et al., 2016; Nghiem-Rao et al., 2016) Since IUGR neonates are likely to receive TPN in their early life as a life-saving feeding method, we hypothesized that the

combination of TPN and IUGR will exacerbate changes in metabolism that lead to obesity in later life.

A growing body of evidence indicates that the risk of developing chronic diseases is influenced by genetics and epigenetic factors that impact gene expression, such as DNA methylation (Herrera et al., 2011; Mahmoud, 2022). Human and animal research demonstrates conclusively that early-life nutritional exposures can alter pre/post-natal development, leading to later obesity and obesity-related metabolic disorders, such as metabolic syndrome (Desai et al., 2015). For example, maternal diets low in methyl donors led to hypomethylation of the *Agouti* gene (Wolff et al., 1998), predisposing offspring to obesity (Pannia et al., 2016). Although there are many studies on the effects of perinatal methyl supply, including pregnancy and lactation, on metabolism (Randunu & Bertolo, 2020), reports on the impact of only post-natal methyl donors on long-term body weight and metabolism changes are scarce. Appropriate epigenetics require adequate methyl groups, and epigenetic changes might result from methyl nutrient imbalances (Bokor et al., 2022). As the principal source of methyl groups, methionine is essential for numerous transmethylation reactions; however, methionine is not typically increased in the diet due to concerns about homocysteine toxicity, a risk factor for CVD (Garlick, 2006). Alternatively, betaine supplementation promotes methionine synthesis and decreases homocysteine levels (McRae, 2013). Recent research has demonstrated that most labile methyl groups are employed in synthesizing creatine and that enhancing one methylation pathway affects allocating the limited methyl groups to other methylation pathways (McBreairty et al., 2013). Therefore, if we augment the diet with creatine, the available methyl groups for DNA methylation will be enhanced. Thus, dietary betaine and creatine can increase methyl groups' availability, with betaine increasing the remethylation of methionine and creatine, sparing methyl groups for other reactions. Notably,

betaine and creatine are currently not included in commercial TPN products. Therefore, we hypothesized that adding betaine and creatine to TPN would mitigate the negative consequences of feeding TPN during the neonatal period.

This investigation aimed to determine whether the previously observed acute effects of TPN feeding in the early neonatal period on adiposity and dyslipidemia will persist into adulthood. Moreover, we investigated whether supplementation of betaine and creatine to early TPN reduces the risk for obesity and dyslipidemia in adulthood. And finally, we were particularly interested in determining whether feeding TPN to IUGR neonates during the neonatal period will exacerbate obesity outcomes in adulthood.

5.3 Materials and Methods

5.3.1 Experimental design and animals

The Memorial University of Newfoundland Animal Care Committee approved the animal care and handling procedures in compliance with the Canadian Council on Animal Care guidelines. Thirty-two female Yucatan miniature piglets, aged 7 d (study day 0), were obtained from the breeding colony at the Memorial University of Newfoundland for the study. The piglets were categorized into four treatment groups: (1) TPN-control, consisting of normal birthweight piglets fed standard TPN; (2) TPN-B+C, consisting of normal birthweight piglets fed TPN supplemented with betaine and creatine; (3) SowFed, consisting of normal birthweight suckling piglets; and (4) TPN-IUGR, consisting of IUGR piglets fed standard TPN. Three normal birthweight piglets from each litter were randomly assigned to the first three groups. IUGR was determined as approximately 65% of the birth weight of the largest littermate (Hegarty & Allen, 1978). As previously described, all piglets underwent a surgical procedure to implant two venous catheters (Dodge et al., 2012). TPN-fed piglets were housed individually in metabolic cages equipped with a swivel and tether system (Lomir Biomedical, Notre-Dame-de-l'Île-Perrot, QC, Canada) for 14 d. This setup allowed the piglets to move freely while receiving continuous intravenous diet infusion through the central vein catheter. The temperature in the piglet room was maintained at 27 ^{0}C, and heat lamps were provided for additional heat. The lighting in the room followed a 12-hour light-dark cycle. The SowFed piglets were returned to the sow after they recovered from surgery and were allowed to suckle until study day 14; all piglets resumed suckling within 2-3 hours of being returned to the sow. After 14 d of treatment, the piglets underwent anesthesia and had their catheters removed. Following recovery, all piglets were weaned onto a milk replacer (Grober Nutrition Inc., Cambridge, ON, Canada) for 2-3 d, then transitioned to a standard grower diet which they were fed until the end of the study. Groups of four pigs were housed together for 8 mo

and were collectively fed 2% of their cumulative body weights, with adjustments made every 2 weeks based on body weight measurements. Water was available ad libitum for 24 hours, and a 12-hour day-night cycle was maintained with lights on from 0800 to 2000 hours. When the piglets reached 8 mo of age, they underwent a surgical procedure to implant two venous catheters (inner diameter: 1.0 mm; outer diameter: 1.8 mm; Tygon Medical Tubing, Saint Gobain Performance Plastic Corp., Akron, Ohio, USA) into the femoral vein (Myrie et al., 2011) to conduct various metabolic tests for a month. After surgery, each piglet was housed individually for the remainder of the study, including an additional 8-10 d for post-surgical recovery (Myrie et al., 2017).

5.3.2 Diet (TPN and Grower)

The TPN diets described in Table 5.1 were prepared following aseptic protocols, as outlined by (Dodge et al., 2012), with changes in the lipid source. The vitamins and minerals in the diets were provided at levels exceeding 100% of the estimated requirements for neonatal piglets (NRC, 2012). Before infusion, each diet bag was supplemented with multivitamins (Multi12/K1 Pediatric, Baxter Corporation, Mississauga, ON, Canada), iron dextran (Bimeda-MTC Animal Health, Cambridge, ON, Canada), trace elements (Sigma-Aldrich Canada, Oakville, ON, Canada), and SMOF lipid emulsion (Fresenius Kabi, Uppsala, Sweden). In the TPN-B+C group, betaine (235mg·kg bodyweight^{-1}·d^{-1}) and creatine (118mg· kg bodyweight^{-1}·d^{-1}) were added as supplements. Betaine was provided in a molar equivalent to the piglet's methionine requirement (McBreairty et al., 2016), while creatine was supplemented to match the piglet's creatine accretion rate (Brosnan et al., 2009). The weight of the diet bags was regularly recorded to monitor the amount of diet infused. After the 14 d of dietary treatment, which concluded at 21 d of age, each piglet underwent a transition period where they were acclimated to a milk replacer (Grober Nutrition Inc., Cambridge, ON, Canada) for 2-3 d and then weaned onto a standard pelleted grower

Table 5:1. Amino acid profile of TPN diets

Amino acid profile	TPN control diet ($g \cdot L^{-1}$)	TPN Betaine + Creatine diet ($g \cdot L^{-1}$)
Alanine	5.89	5.89
Arginine	3.65	3.65
Aspartic Acid	3.32	3.32
Cysteine	0.76	0.76
Glutamic Acid	5.72	5.72
Glycine	1.47	1.47
Histidine	1.69	1.69
Isoleucine	2.51	2.51
Leucine	5.67	5.67
Lysine hydrochloride	5.58	5.58
Methionine	1.04	1.04
Phenylalanine	3	3
Proline	4.52	4.52
Serine	3.11	3.11
Taurine	0.27	0.27
Tryptophan	1.14	1.14
Tyrosine	0.44	0.44
Threonine	2.23	2.23
Valine	2.89	2.89
Betaine hydrochloride	0	1.29
Creatine monohydrate	0	0.57

pig diet (Table 5.2; Eastern Farmers Co-op, St. John's, NL, Canada). This grower diet is the same one used in our previous grow-out study (Myrie et al., 2011, 2017), providing 67% of energy from carbohydrates, 12% from fat, and 21% from protein.

Table 5:2. Composition of the grower diet (12.1 MJ digestible energy·kg^{-1} and 154 g protein·kg^{-1})

Energy (% total energy)	
Carbohydrate	67
Fat	12
Protein	21
Ingredients (g·kg^{-1} dry matter)	
Wheat shorts	400.5
Canola	49.0
Meat meal	19.0
Limestone	13.0
Corn gluten feed	40.0
Ground barley	297.0
Oats	175.0
Vitamin mix	0.8
Mineral mix	1.0
Sodium Chloride	4.7

5.3.3 Monthly blood samples and body measurements

Body weight was measured daily from 7 to 21 d of age and every two weeks during the grower phase of the study, which spanned from 1 to 9 mo. The feed conversion ratio (FCR) was calculated by dividing each pig's predicted feed intake (equivalent to 2% of their body weight) by the weight they gained during specific time intervals (1-4 mo, 4-6 mo, and 6-8 mo). The fractional growth rate (FGR) was determined by dividing the body weight gain during a given period by the initial body weight at the beginning of that time frame. Monthly, blood samples were collected from the jugular vein of the pigs after an overnight fasting period. The blood was collected using ethylenediaminetetraacetic acid (EDTA) tubes (Becton, Dickinson, and Company; Franklin Lakes, NJ, USA). The collected samples were immediately centrifuged at 4000 × g for 15 minutes at 4°C to separate the plasma. Lipid assays were conducted on fresh samples stored at 4°C, while the remaining plasma was stored at -80°C until further analysis (Myrie et al., 2017).

5.3.4 Necropsy

At 9 mo of age, the pigs were administered anesthesia using 105 $mg \cdot kg^{-1}$ of sodium pentobarbital (Euthanyl, Biomeda-MTC Cambridge, ON, Canada). They were mechanically ventilated during the collection of blood and organ samples. A cardiac puncture was performed to obtain blood samples, which were then placed in EDTA tubes. The blood samples were promptly centrifuged at 4000 × g for 15 minutes to separate the plasma. Using a freeze clamp, organs were removed, weighed, and rapidly frozen in liquid nitrogen. Both the organs and plasma samples were immediately stored at -80 ^{0}C until further analysis. The thickness of the back fat was measured in the carcass at the midline of the back, just caudal to the last rib, to assess the amount of subcutaneous fat. The crown-to-rump length, chest circumference (caudal to the stifle joint), and abdominal girth (measured at the naval) were recorded at necropsy.

5.3.5 Plasma lipid analysis

Fresh plasma was assayed for concentrations of plasma total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL)-cholesterol, and free cholesterol using enzymatic assay kits (Sekisui Diagnostics PEI Ltd., Charlottetown, PE, Canada). Plasma non-HDL-cholesterol was calculated by subtracting values of HDL-cholesterol from TC. Plasma NEFA was assayed using an enzymatic assay kit (Fujifilm Wako Diagnostics USA Corporation, CA, USA). Plasma from the necropsies was subjected to sequential density ultracentrifugation to isolate lipoprotein fractions (Salter et al., 1998). After centrifuging necropsy plasma at 15,500 × g for 20 minutes at 12 ^{0}C to remove chylomicrons, the supernatant was separated into very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and HDL fractions. Enzymatic assay kits were used to determine the concentrations of TC and TG in these subfractions.

5.3.6 Plasma adiponectin and leptin

Plasma leptin (Catalog # LS-F22387, LifeSpan BioSciences, Inc., Seattle, WA, USA) and adiponectin (Catalog # LS-F21779, LifeSpan BioSciences, Inc., Seattle, WA, USA) were assayed using ELISA kits following assay instructions.

5.3.7 Liver phosphatidylcholine (PC) and phosphatidylethanolamine (PE) concentration

After lipids were extracted from the liver using the Folch method (Folch et al., 1957), PC and PE were isolated using thin-layer chromatography as previously described (Thillayampalam, 2015). The liver PC and PE were then determined by measuring total phosphate using a modified Bartlett method (Bartlett, 1959), as described elsewhere (Thillayampalam, 2015).

5.3.8 Ectopic fat and cardiac muscle NEFA analysis

Lipids were extracted from known masses of the liver, skeletal muscle, and cardiac muscle samples using the chloroform: methanol (2:1, v/v) extraction technique (Folch et al., 1957). The lipid-containing phase was evaporated to obtain dried lipids. Then, they were dissolved in isopropanol and assayed for TG by enzymatic assay (Sekisui Diagnostic PEI Ltd., Charlottetown, PE, Canada), following assay instructions. Following assay guidelines, cardiac muscle NEFA was assayed using an enzymatic assay kit (Elabscience, TX, USA).

5.3.9 RNA extraction and real-time qPCR

Trizol was used to extract total RNA from liver tissues (Chomczynski & Sacchi, 1987). Genomic DNA contamination in RNA samples was removed using the DNAse enzyme (Promega, USA, Catalog # M610A). Using NanoDrop 2000, the concentration of extracted liver RNA samples was measured (Thermo Scientific, USA). The integrity of each RNA sample was verified using 1.2% agarose gel. Complementary DNA (cDNA) was produced from the extracted RNA samples using reverse transcription (catalog # A3500, Promega, USA). Real-time qPCR primers

(ACC-1: Acetyl coA carboxylase, FASN: Fatty acid synthase, SCD: Sterol co-A desaturase, DGAT-2: Diacylglycerol acyltransferase -2, SREBP-1C: Sterol regulatory element binding protein 1c, CPT1: Carnitine palmitoyl transferase I, CD-36: Fatty acid translocase, β-actin: beta-actin; GAPDH: Glyceraldehyde 3-phosphate) were taken from previously published articles (Table: 5.3). The primers were also verified employing NCBI primer blast (www.ncbi.nlm.nih.gov/tools/primerblast) and obtained from Integrated DNA Technologies (IA, USA). The forward and reverse sequences of each pair of primers are listed below (Table:5.3). SYBR Green Supermix (Catalog Number 1708882, Bio-Rad, USA) was used to begin qPCR amplification, and samples were processed on a Mastercycler ep realplex system. Then the deltaCt values for the genes of interest and the reference genes were calculated. β-actin and GAPDH were both used as multiple reference genes to normalize the gene expressions of interested genes, accounting for their primer efficiencies following the Vandesompele method (Vandesompele et al., 2002). This method utilizes the geometric mean of the relative quantities for the reference genes to calculate gene expression. The formula is as below.

$$\frac{E_{GOI}^{\Delta Ct\ GOI}}{\text{Geomean}\ [E_{REF}^{\Delta Ct\ REF}]}$$

E = Amplification factor
GOI = Gene of interest
Geomean = Geometric mean
REF = Reference gene
ΔCt = Ct (Calibrator sample) – Ct (Test sample)

Table 5:3. Primer pairs used for real-time PCR

Gene	Gene symbol	Forward	Reverse	Primer efficiency	Gene bank accession no	Reference
Acetyl co A carboxylase	ACC-1	ATA CCC GTG GGA GTA GTT GC	GCG GTT GAA GTC CTT GAT GG	89.46	NM_001114269.1	Xing *et al*., 2014
Diacylglycerol acyltransferase -2	DGAT-2	GCA GGT GAT CTT TGA GGA GG	GCT TGG AGT AGG GCA TGA G	92.08	NM_001160080.1	Cui, *et al*., 2011
Fatty acid synthase	FASN	ACA CCT TCG TGC TGG CCT AC	ATG TCG GTG AAC TGC TGC AC	88.79	NM_001099930	Madeira, *et al*., 2016
Sterol co- A desaturase	SCD	AGC CGA GAA GCT GGT GAT GT	GAA GAA AGG TGG CGA CGA AC	93.99	NM_213781	Madeira, *et al*., 2016
Sterol regulatory element binding protein 1c	SREBP-1C	AAG CGG ACG GCT CAC AA	GCA AGA CGG CGG ATT TAT T	95.81	NM_214157.1	Qiu *et al*., 2017
Carnitine palmitoyltransferase 1	CPT1	ACA AGC CAT AGT CTT AAC GAA A	GCC AGT CCA GGA TAA CAA A	103.05	NM_001129805	He, *et al*., 2011
Fatty acid translocase	CD36	GCA CAG AAA AAG TTG TCT CCA AAA AT	ATG TAC ACA GGT TTT CCT TCT TTG C	79.50	NM_001044622.1	Tonnac *et al*., 2016
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	ATCCTGGGCTACACTGAGGA	TGTCGTACCAGGAAATGAGCT	84.71	DQ845171	Park, *et al*., 2015
Beta actin	β actin	CAC GCC ATC CTG CGT CTG GA	AGC ACC GTG TTG CGC TAG AG	104.50	NM_001206359.1	Nygard *et al.,* 2007

5.3.10 Plasma amino acid analysis

Reverse-phase high-performance liquid chromatography (HPLC) was used to detect plasma amino acid concentration with the C18 column (Waters, Woburn, MA, USA), as a method described elsewhere, after plasma derivatization of plasma with PITC (phenylisothiocyanate) (Bidlingmeyer et al., 1984).

5.3.11 Measurement of lipid peroxidation and antioxidant capacity

The extent of lipid peroxidation in the liver and plasma was measured using TBRAS (Thiobarbituric Acid Reactive Substance) assay as described elsewhere (White JMB, 2022). Following assay instructions, liver antioxidant capacity was measured using SOD (Superoxide Dismutase) assay (ab65354 SOD activity assay kit; Abcam Inc., Toronto, ON, Canada).

5.3.12 Statistical analyses

The experimental groups were compared using one-way ANOVA, and the group differences were determined using Dunnett's post hoc test (GraphPad Prism 8.0; GraphPad Software, San Diego, CA, USA), with the TPN-control assigned as the control group. The results are presented as means ± SD. Pearson's correlation was used to compare the relationship between subcutaneous fat thickness and various parameters such as body growth measurements, plasma LDL and non-LDL-cholesterol at necropsy. If $P<0.05$, differences were considered statistically significant.

The experimental groups were compared for monthly lipid parameters using two-way ANOVA (GraphPad Prism 8.0; GraphPad Software, San Diego, CA, USA).

The FCR was calculated by dividing each pig's predicted feed intake (2% of body weight) by the body weight gain during each phase (1-4 mo /4-6 mo /6-8 mo). FGR was calculated by dividing the gain in body weight for a given period by the initial body weight of that period of time.

5.4 Results

5.4.1 Growth and feed intake

Since puberty in Yucatan miniature pigs occurs around 4 mo, we divided the growth parameters into the development phases as shown in Table 5.4, Figure 5.1, Figure 5.2. IUGR pigs were selected to have lower body weights than control pigs at birth, and the body weights were significantly lower than control pigs at the beginning of the experiment. The low body weight of the IUGR pigs remained significantly lower at the end of TPN feeding compared to the control pigs; however, the low body weights of IUGR caught up at 1 month (weaning) compared to the control group, which had normal body weights from birth. After one month, the body weights of all pigs in the pre-sexual maturity, sexual maturity, and post-sexual maturity phases did not differ between the groups until the end of the experimental period (9 mo). Body growth rates did not differ between groups during the TPN phase (7 d - 21 d); however, the growth rates of TPN-IUGR were higher during the pre-sexual maturity and sexual maturity phases (1-4 mo and 4-6 mo, respectively) compared to TPN-control pigs. At 9 mo, the experimental groups had no significant differences in body measurements.

Table 5:4 **Table 5:4** Growth parameters of Yucatan miniature pigs fed four experimental diets, TPN- control TPN-B+C, TPN-IUGR, and SowFed, during the neonatal period.

Age	TPN-control	TPN-B+C	TPN-IUGR	SowFed	Control:Sow	B+C:Sow	IUGR:Sow
Body weight (kg)							
Birth	*0.98 ± 0.17*	1.02 ± 0.12	0.67 ± 0.10***	1.01 ± 0.15	0.97	1.00	0.67
TPN start day (7d)	*1.74 ± 0.18*	1.74 ± 0.25	1.25 ± 0.16**	1.80 ± 0.35	0.97	0.97	0.70
TPN end day (21d)	*3.10 ± 0.20*	3.15 ± 0.24	2.44 ± 0.21*	3.53 ± 0.88	0.88	0.89	0.69
1 mo	*3.54 ± 0.88*	3.77 ± 0.61	3.57 ± 1.09	4.81 ± 1.87	0.74	0.78	0.74
4 mo	*14.99 ± 3.29*	15.83 ± 2.77	17.46 ± 2.54	18.06 ± 2.86	0.83	0.88	0.97
8 mo	*35.05 ± 7.62*	34.43 ± 5.49	41.74 ± 4.63	37.87 ± 7.49	0.93	0.91	1.13
Body weight growth rate (g· d^{-1})							
7d - 21d	*97.13 ± 6.17*	101.10 ± 7.48	82.99 ± 8.53	117.92 ± 48.72	0.82	0.86	0.71
1-4 mo	*130.11 ± 38.55*	135.58 ± 30.91	171.78 ± 25.10*	151.85 ± 31.94	0.86	0.89	1.13
4-6 mo	*143.67 ± 52.47*	148.46 ± 45.43	203.69 ± 28.70*	165.07 ± 57.09	0.87	0.90	1.23
6-8 mo	*190.19 ± 39.87*	168.96 ± 34.92	194.75 ± 51.02	163.96 ± 59.28	1.16	1.03	1.19
Fractional growth rate (g.kg body $weight^{-1}$·d^{-1})							
7d - 21d	*40.06 ± 3.15*	41.64 ± 5.12	47.65 ± 4.32	45.42 ± 11.66	0.88	0.92	1.05
1-4 mo	*13.88 ± 2.72*	13.75 ± 2.06	15.24 ± 3.32	12.97 ± 2.63	1.07	1.06	1.18
4-6 mo	*7.31 ± 1.65*	7.27 ± 1.69	8.50 ± 1.30	6.41 ± 2.56	1.14	1.13	1.33
6-8 mo	*6.11 ± 0.56*	5.27 ± 0.61	5.61 ± 1.25	4.98 ± 1.15	1.23	1.06	1.13
Body measurements at 9 mo							
Crown to rump length (cm)	*112.10 ± 6.11*	111.80 ± 6.27	118.10 ± 3.18	116.30 ± 6.86	0.96	0.96	1.02
Abdominal circumference (cm)	*84.69 ± 6.83*	82.79 ± 6.77	88.29 ± 4.07	84.43 ± 7.20	1.00	0.98	1.05
Chest girth (cm)	*79.44 ± 4.29*	79.31 ± 3.73	82.88 ± 4.30	77.69 ± 3.97	1.02	1.02	1.07
Subcutaneous fat thickness (mm)	*43.25 ± 5.52*	43.75 ± 3.81	50.00 ± 5.01*	44.33 ± 5.68	0.97	0.98	1.12
Average feed conversion ratio							
1-4 mo	*1.56 ± 0.20*	1.56 ± 0.12	1.47 ± 0.26	1.74 ± 0.23	0.89	0.89	0.84
4-6 mo	*3.30 ± 0.98*	3.45 ± 1.14	2.59 ± 0.49	3.43 ± 1.15	0.96	1.01	0.76
6-8 mo	*3.83 ± 0.64*	4.51 ± 1.20	4.55 ± 1.32	5.45 ± 2.13	0.70	0.83	0.84

Values are means ± SD; n = 7-8.
TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled.
*P<0.05, **P<0.001, ***P<0.0001. 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

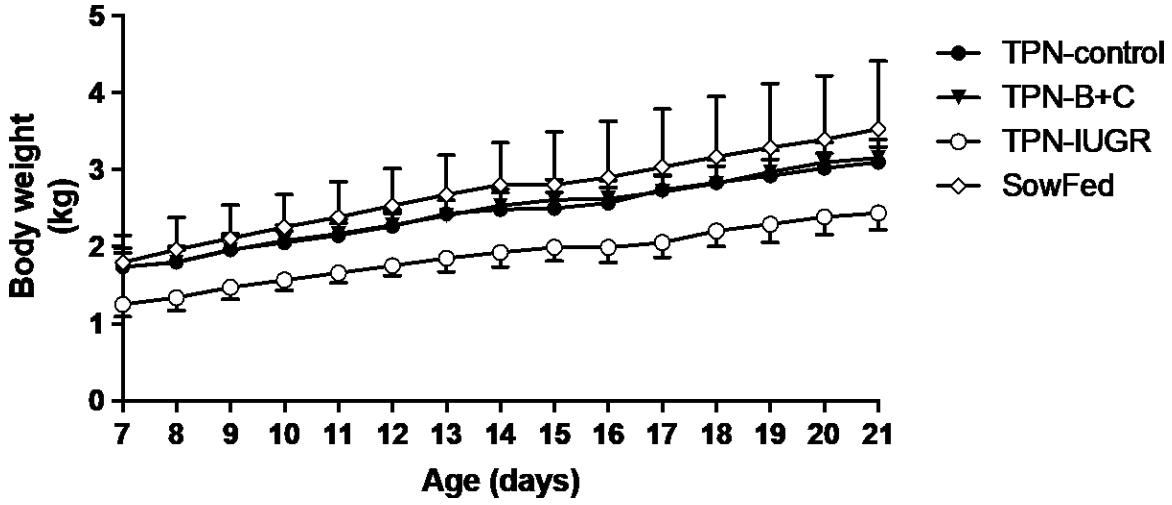


Figure 5:1 Body weights in TPN-control, TPN-B+C, TPN-IUGR, and SowFed Yucatan miniature pigs during the TPN feeding phase from 7 d to 21 d.

Each symbol represents the mean ± SD; n = 8. TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled.

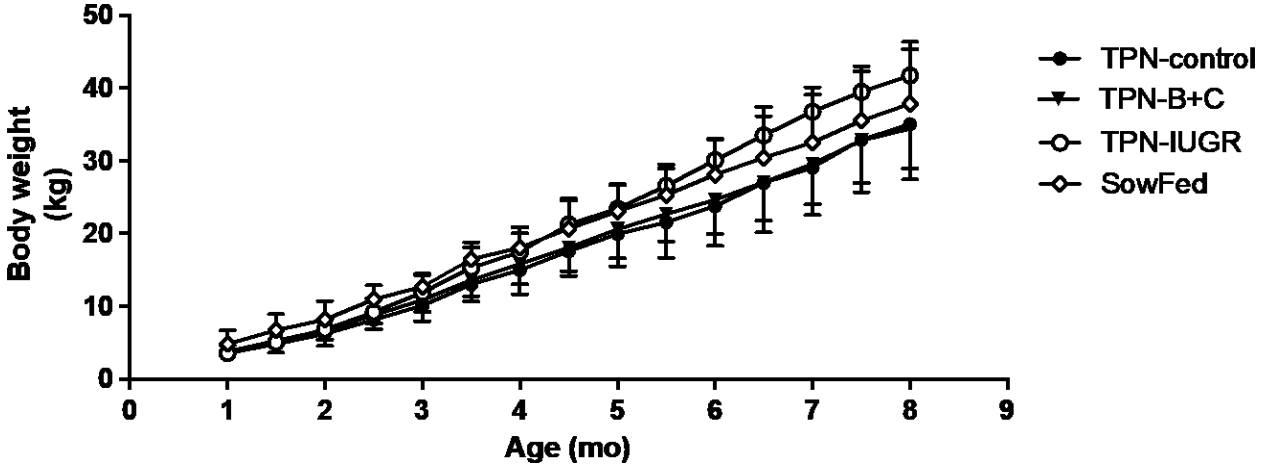


Figure 5:2. Body weights in TPN-control, TPN-B+C, TPN-IUGR, and SowFed Yucatan miniature pigs during the grow-out phase from 1 mo to 8 mo.

Each symbol represents the mean ± SD; n = 8. TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled.

5.4.2 Organ weights

Relative kidney, heart, and brain weights were significantly lower in the TPN-IUGR pigs compared to TPN-control pigs as young adults (Table 5.5). In contrast, TPN-IUGR pigs had an increased subcutaneous fat thickness compared to TPN-control pigs at 9 mo (Table 5.5).

Table 5:5. Relative organ weights and subcutaneous fat thickness at 9 mo in Yucatan miniature pigs fed four experimental diets during the neonatal period: TPN-control, TPN-B+C, TPN- IUGR, and SowFed.

Organ	TPN-control	TPN-B+C	TPN-IUGR	SowFed	*P* value
Liver	*17.80 ± 2.60*	17.19 ± 2.37	15.58 ± 1.47	16.83 ± 1.99	0.2355
Kidneys	*3.39 ± 0.54*	3.05 ± 0.34	2.63 ± 0.44*	2.88 ± 0.20	0.0078
Heart	*4.10 ± 0.46*	4.03 ± 0.57	3.37 ± 0.44*	3.85 ± 0.37	0.0268
Pancreas	*1.33 ± 0.44*	1.50 ± 0.39	1.29 ± 0.17	1.37 ± 0.38	0.6911
Stomach	*8.41 ± 1.12*	8.27 ± 0.75	7.11 ± 2.62	8.95 ± 2.01	0.3070
Brain	*1.93 ± 0.25*	1.92 ± 0.27	1.63 ± 0.17*	1.75 ± 0.20	0.0354
Subcutaneous fat (mm)	*43.25 ± 5.52*	43.75 ± 3.81	50.00 ± 5.01*	44.33 ± 5.68	0.0432

Values are means ± SD, n = 6-8.
TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled.
*$P<0.05$; 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

5.4.3 Tissue and plasma lipid profiles, lipid peroxidation and antioxidant capacity

Liver TG content is significantly higher in TPN-IUGR pigs than in TPN-control pigs at 9 mo (Table 5.6); however, liver cholesterol content did not differ amongst the groups. Plasma non-HDL cholesterol and NEFA contents were increased in TPN IUGR pigs compared to TPN-control pigs (Table 5.6); however, cardiac tissue NEFA concentration did not differ amongst the groups. VLDL-TG and LDL-cholesterol contents significantly increased whereas VLDL-cholesterol content was lower in TPN-IUGR pigs than in TPN-control pigs (Table 5.6). Interestingly, plasma TBARS was significantly higher in TPN-control pigs compared to SowFed control at 9 mo as young adult pigs (Table 5.6).

Table 5:6. Tissue and plasma lipid profiles, lipid peroxidation and antioxidant capacity of 9-month-old Yucatan miniature pigs fed a TPN-control, TPN-B+C, control TPN-IUGR and SowFed experimental diets during the neonatal period.

	TPN-control	**TPN-B+C**	**TPN-IUGR**	**SowFed**	***P***
Liver					
Total cholesterol (mmol· g protein^{-1})	*9.39 ± 2.53*	9.39 ± 2.32	11.16 ± 2.54	8.60 ± 4.07	0.3879
Total triglyceride (mmol· g protein^{-1})	*3.77 ± 2.94*	4.13 ± 2.58	9.60 ± 5.73*	4.67 ± 4.37	0.0360
PC (mmol· g protein^{-1})	*0.66 ± 0.12*	0.61 ± 0.12	0.68 ± 0.12	0.68 ± 0.13	0.6266
PE (mmol· g protein^{-1})	*0.43 ± 0.06*	0.43 ± 0.07	0.47 ± 0.03	0.46 ± 0.06	0.4135
PC/PE	*1.53 ± 0.22*	1.42 ± 0.16	1.57 ± 0.16	1.55 ± 0.35	0.6250
SOD activity (inhibition rate %)	*101.00* ± 3.36	101.55 ± 3.33	104.77 ± 3.05	102.07 ± 5.25	0.2674
TBARS (µmol· g protein^{-1})	*4.31* ± 0.83	5.06 ± 0.86	4.79 ± 1.10	4.77 ± 1.45	0.6473
Cardiac tissue					
NEFA (mmol· g protein^{-1})	*0.26 ± 0.04*	0.30 ± 0.05	0.25 ± 0.06	0.31 ± 0.07	0.1779
Plasma					
Total cholesterol (mmol· L^{-1})	*1.405 ± 0.268*	1.270 ± 0.216	1.617 ± 0.399	1.453 ± 0.117	0.1404
Total HDL cholesterol (mmol· L^{-1})	*0.974 ± 0.189*	0.958 ± 0.191	0.867 ± 0.240	0.938 ± 0.067	0.6652
Total non-HDL cholesterol (mmol· L^{-1})	*0.460 ± 0.190*	0.302 ± 0.095	0.750 ± 0.321*	0.529 ± 0.114	0.0056
Plasma free cholesterol (mmol· L^{-1})	*0.608 ± 0.174*	0.484 ± 0.249	0.507 ± 0.104	0.536 ± 0.082	0.5264
Plasma NEFA (mmol· L^{-1})	*0.09 ± 0.04*	0.09 ± 0.04	0.15 ± 0.03*	0.08 ± 0.04	0.0074
Total triglycerides (mmol· L^{-1})	*0.397 ± 0.110*	0.404 ± 0.113	0.422 ± 0.147	0.358 ± 0.106	0.7520
TBARS (µmol· L^{-1})	*6.52 ± 2.28*	4.89 ± 0.93	5.31 ± 0.52	4.27 ± 0.99*	0.0182
Plasma lipoprotein subfractions					
Cholesterol					
VLDL (mmol· g protein $^{-1}$)	*1.562 ± 0.656*	1.427 ± 0.491	0.883 ± 0.263*	1.421 ± 0.335	0.0489
LDL (mmol· g protein $^{-1}$)	*2.284 ± 0.551*	2.390 ± 0.727	4.483 ± 2.062*	2.640 ± 0.649	0.0023
HDL (mmol· g protein $^{-1}$)	*0.946 ± 0.364*	0.957 ± 0.489	1.438 ± 0.496	0.762 ± 0.461	0.0608
Triglycerides					
VLDL (mmol· g protein $^{-1}$)	*2.125 ± 1.289*	2.522 ± 0.863	3.492 ± 1.208*	2.127 ± 0.873	0.0538
LDL (mmol· g protein $^{-1}$)	*0.364 ± 0.222*	0.339 ± 0.148	0.254 ± 0.085	0.307 ± 0.209	0.6674
HDL (mmol· g protein $^{-1}$)	*0.025 ± 0.015*	0.031 ± 0.018	0.020 ± 0.004	0.018 ± 0.013	0.2351

Values are means ± SD, n = 6-8.
TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled; PC, Phosphatidylcholine; PE, phosphatidylethanolamine; SOD, super oxide dismutase; TBARS, Thiobarbituric acid reactive substance; NEFA, non-esterified fatty acid; HDL, high density lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein.
*P<0.05; 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

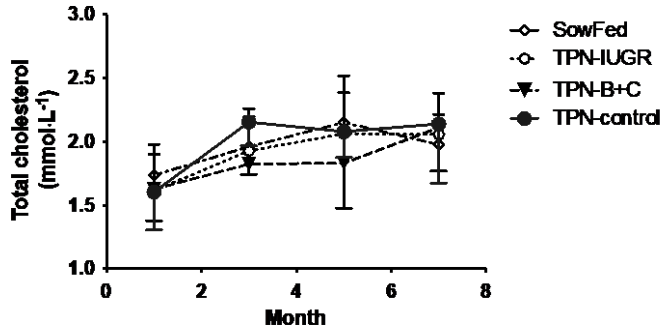
5.4.4 Monthly plasma lipid parameters

Monthly plasma lipid parameters did not significantly differ amongst the groups at 1,3,5,7 mo. However, regardless of the birth weight and feeding route in the neonatal period, TC, non-HDL cholesterol, LDL-cholesterol, and HDL-cholesterol increased with age in all groups (Figure 5.3).

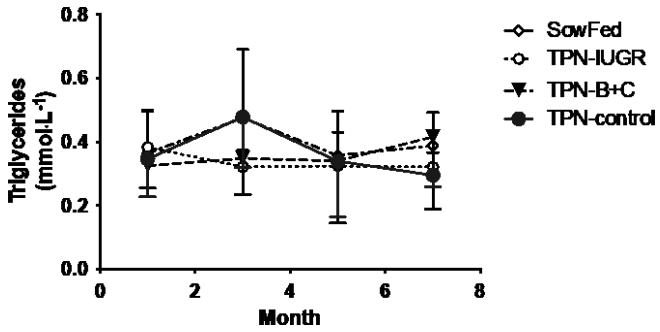
5.4.5 Ectopic fat content

Liver and skeletal muscle TG content was significantly higher in TPN-IUGR pigs compared to TPN-control pigs at 9 mo (Figure 5.4). Interestingly, TPN-B+C pigs showed a higher TG content compared to TPN-control pigs (Figure 5.4), while cardiac TG content of TPN-IUGR pigs were not different from that of TPN-control pigs. However, the absolute concentration of TG in the cardiac muscle is relatively low compared to the TG concentration in the liver and skeletal muscle.

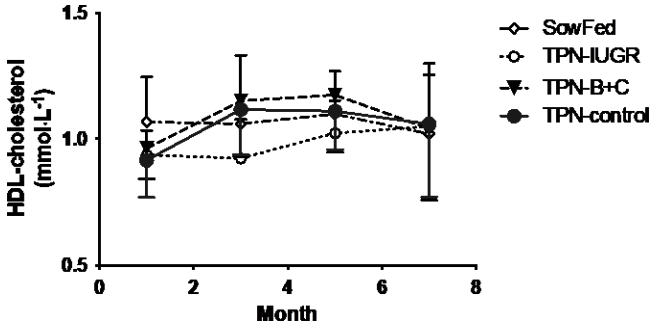
(a)


Total cholesterol (mmol·L⁻¹)
3.0
2.5
2.0
1.5
1.0
0
2
4
6
8
Month
SowFed
TPN-IUGR
TPN-B+C
TPN-control

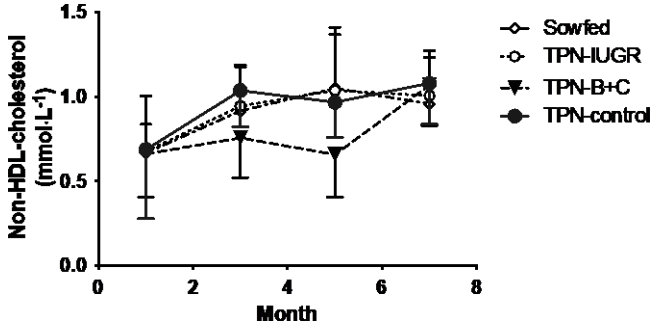
(b)


Triglycerides (mmol·L⁻¹)
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
Month
SowFed
TPN-IUGR
TPN-B+C
TPN-control

(c)


HDL-cholesterol (mmol·L⁻¹)
1.5
1.0
0.5
0
2
4
6
8
Month
SowFed
TPN-IUGR
TPN-B+C
TPN-control

(d)


Non-HDL-cholesterol (mmol·L⁻¹)
1.5
1.0
0.5
0.0
0
2
4
6
8
Month
Sowfed
TPN-IUGR
TPN-B+C
TPN-control

(e)

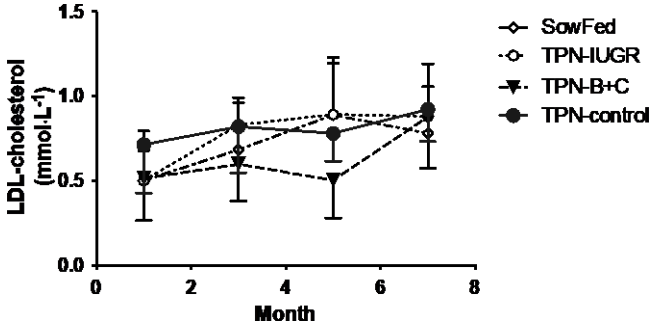
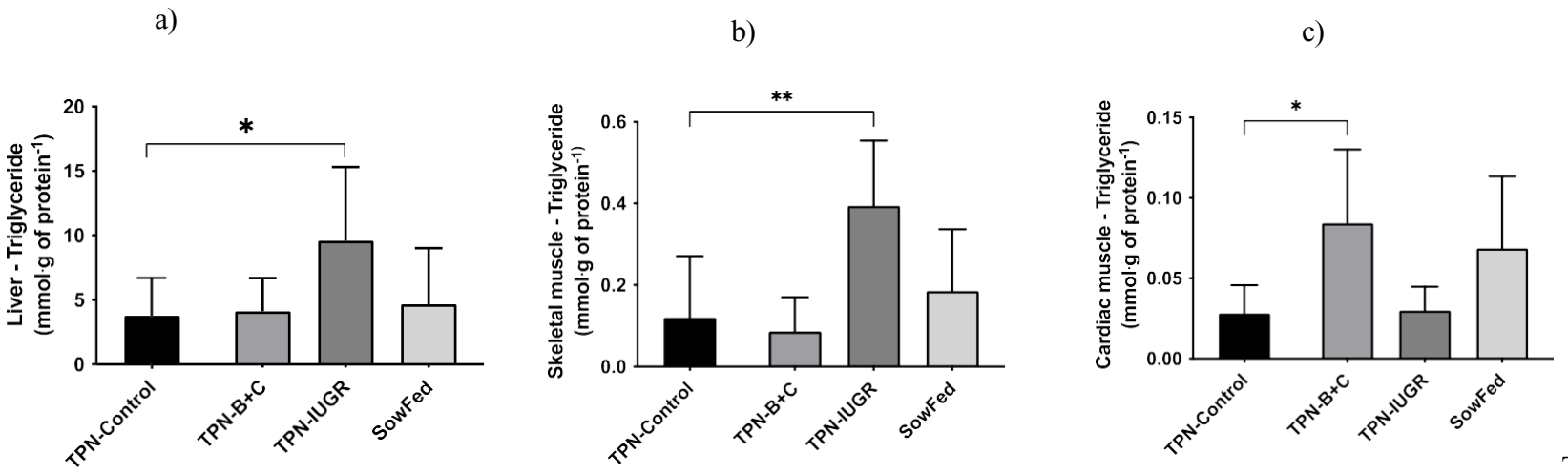

LDL-cholesterol (mmol·L⁻¹)
1.5
1.0
0.5
0.0
0
2
4
6
8
Month
SowFed
TPN-IUGR
TPN-B+C
TPN-control

Figure 5:3. Monthly fasting plasma total cholesterol (a), TG (b), HDL-cholesterol (c), non-HDL cholesterol (d) and LDL-cholesterol concentrations in Yucatan miniature pigs fed TPN-control, TPN-B+C, TPN-IUGR and SowFed during the neonatal period.

Values are mean ± SD; n = 7-8. TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled; TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein.



The

Figure 5:4. Ectopic fat (liver, skeletal muscle tissue, and cardiac muscle) a) Liver b) skeletal muscle tissue c) cardiac muscle TG content of adult Yucatan miniature pigs at 9 mo.

Values are means ± SD; n = 6-8. Data were analyzed using 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control. **P*<0.05, ***P*<0.001. TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed with TPN-control diet; SowFed, suckled.

5.4.6 qPCR analysis of lipogenic genes in the liver

The key genes involved in lipogenesis in the liver were not different amongst the experimental groups, except FASN. Sowfed pigs had higher expression of FASN mRNA in the liver compared to TPN-control pigs at 9 mo (Figure 5.5). CD-36 binds to long-chain FFA and facilitates their transport to liver cells (Tonnac et al., 2016) ; CPT-1a is active on the outer surface of mitochondria and serves as a regulatory site for fatty acid oxidation (He et al., 2011); ACC-1 and FASN genes are responsible for the rate- limiting enzymes of de novo fatty acid synthesis (Xing et al., 2014; Madeira et al., 2016); SCD is the gene responsible for the rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids (Madeira et al., 2016); DGAT-2 catalyzes the final reaction in the synthesis of triglycerides (Cui et al., 2011) and SREBP-1 involve in activation of lipogenic genes in the liver (Qiu et al., 2017).

5.4.7 Plasma adiponectin and leptin

Plasma concentrations of adiponectin at 9 mo were not significantly different amongst the experimental groups; however, adiponectin in TPN-IUGR pigs tended to be lower than TPN-control (P = 0.1469) (Figure 5.6). Plasma leptin concentrations were not significantly different between the groups.

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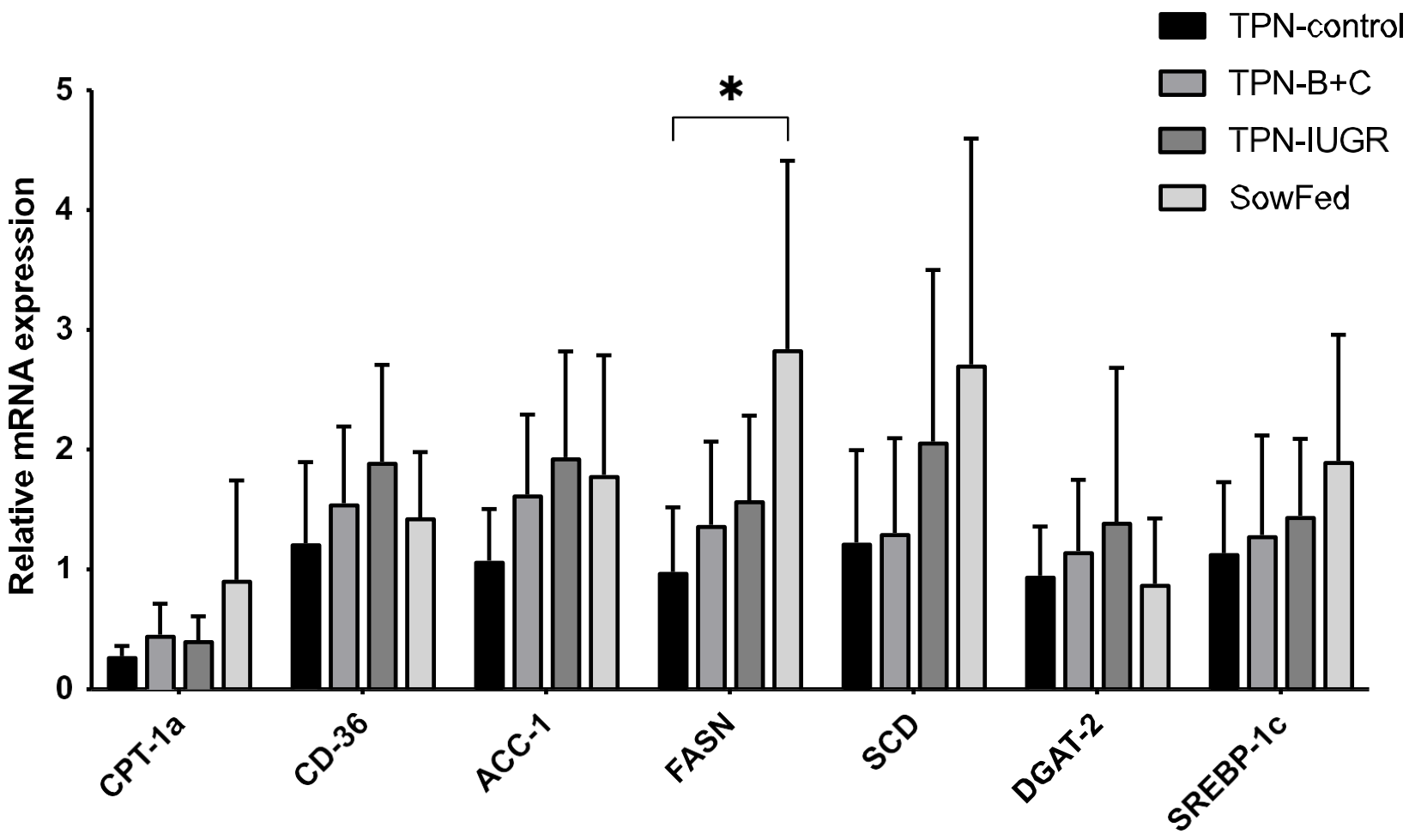

TPN-control
TPN-B+C
TPN-IUGR
SowFed
Relative mRNA expression
0
1
2
3
4
5
*
CPT-1a
CD-36
ACC-1
FASN
SCD
DGAT-2
SREBP-1c

Figure 5:5. Relative mRNA expression of lipogenic genes in the liver of adult Yucatan miniature pigs (9 mo) fed four experimental diets. Data are means ± SD, n = 6-8 pigs.

Data were analyzed using 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control. *P>0.05. Geometric averaging of both β-actin and GAPDH was used to normalize the expression levels of genes of interest, and the data was corrected for the primer efficiency using Vandesompele et al., 2002 method. TPN, Total parenteral nutrition; TPN-control, TPN control group; TPN-B+C, TPN supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed with TPN-control diet; SowFed, suckled; IUGR, intrauterine growth restricted; CPT1: Carnitine palmitoyl transferase I, CD-36: Fatty acid translocase, ACC-1: Acetyl co A carboxylase, FASN: Fatty acid synthase, SCD: Sterol co- A desaturase, DGAT-2: Diacylglycerol acyltransferase -2, SREBP-1C: Sterol regulatory element binding protein 1C; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

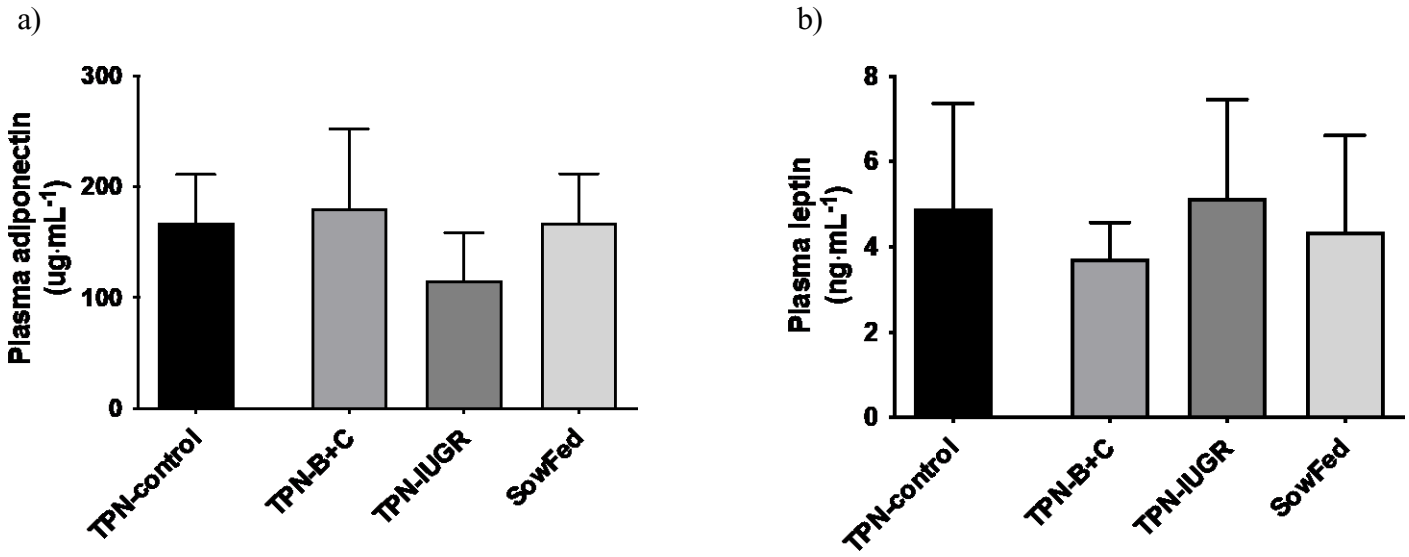


Figure 5:6. Plasma a) adiponectin (P = 0.0888) and b) leptin concentration of adult Yucatan miniature pigs (9 mo) fed four experimental diets; values are means ± SD, n = 7-8.

Data were analyzed using 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control. TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed with TPN-control diet; SowFed, suckled.

5.4.8 Plasma amino acid concentrations

Plasma isoleucine, leucine, and tryptophan have been shown to be higher during CVD (Zhang et al., 2021). Plasma amino acid profiles at 9 mo were not significantly different amongst the experimental groups; however, isoleucine, leucine, and tryptophan tended to be higher in TPN-IUGR pigs compared to TPN-control pigs.

Some amino acids (glycine, serine, tryptophan, alanine, tyrosine, proline, aspartate, and asparagine) are significantly higher in the SowFed compared to TPN-control piglets at 21 d of age (Table 5.8). These differences in plasma amino acid concentrations are related to differences in diet (TPN vs. sow milk) and feeding mode. However, adding B+C to TPN and being an IUGR did not affect plasma amino acid profiles during the TPN phase.

5.4.9 Correlation analyses

Correlation analyses between subcutaneous fat thickness with growth parameters at various stages of development (body weight, body weight gain, body measurements) and lipid parameters indicate that subcutaneous fat thickness is indicative of obesity and dyslipidemia (Figure 5.7; 5.8). Numerous indicators were correlated, although others did not show statistically significant differences.

Table 5:7 . Plasma amino acid concentrations (μmol·L^{-1}) of Yucatan miniature pigs at 9 mo fed four experimental diets: TPN-control, TPN-B+C, TPN-IUGR, and SowFed during the neonatal period.

	TPN-control	TPN-B+C	TPN-IUGR	SowFed	*P* Value
Amino acids related to methionine metabolism					
Glycine	*642.0 ± 143.6*	601.3 ± 183.9	521.6 ± 41.7	681.8 ± 144.7	0.218
Serine	*111.0 ± 23.0*	90.4 ± 22.6	96.8 ± 16.9	103.4 ± 20.2	0.303
Methionine	*8.7 ± 2.6*	7.7 ± 1.3	10.1 ± 3.5	9.4 ± 3.3	0.498
Taurine	*109.3 ± 31.1*	118.3 ± 82.6	128.7 ± 53.4	107.2 ± 33.9	0.847
Glutathione					
Indispensable amino acids					
Histidine	*41.5 ± 12.7*	41.3 ± 8.5	46.7 ± 18.5	43.5 ± 8.2	0.861
Isoleucine	*104.6 ± 15.0*	111.6 ± 15.6	119.3 ± 17.9	84.9 ± 40.2	0.083
Leucine	*184.9 ± 26.8*	195.7 ± 19.3	214.3 ± 42.1	176.1 ± 29.7	0.141
Lysine	*231.5 ± 55.5*	213.2 ± 47.5	221.7 ± 24.8	189.3 ± 34.7	0.312
Phenylalanine	*65.4 ± 8.2*	64.4 ± 6.4	57.4 ± 26.5	60.8 ± 6.3	0.867
Threonine	*118.6 ± 40.8*	96.2 ± 31.3	127.7 ± 54.7	83.6 ± 8.7	0.096
Tryptophan	*82.9 ± 31.1*	83.7 ± 43.3	93.4 ± 45.7	87.5 ± 23.6	0.084
Valine	*256.6 ± 41.4*	272.8 ± 39.2	310.8 ± 96.6	245.3 ± 51.1	0.256
Dispensable amino acids					
Alanine	*57.7 ± 18.4*	59.6 ± 10.9	55.6 ± 14.2	51.6 ± 10.0	0.393
Hydroxyproline	*36.4 ± 7.2*	32.5 ± 11.5	37.3 ± 12.8	37.2 ± 9.6	0.819
Tyrosine	*72.1 ± 9.4*	71.5 ± 12.7	75.2 ± 10.1	64.9 ± 12.0	0.394
Ornithine	*131.7 ± 35.8*	112.8 ± 40.9	100.0 ± 44.4	114.0 ± 33.4	0.476
Proline	*135.1 ± 38.3*	144.2 ± 43.7	128.8 ± 22.1	141.0 ± 34.2	0.878
Aspartate	*6.7 ± 2.2*	5.5 ± 1.4	7.5 ± 2.0	7.9 ± 3.0	0.325
Arginine	*57.7 ± 18.4*	59.6 ± 10.9	55.6 ± 14.2	51.6 ± 10.0	0.759
Glutamate	*13.1 ± 9.5*	13.2 ± 6.6	21.3 ± 10.1	12.1 ± 6.9	0.733
Glutamine	*207.3 ± 76.0*	200.1 ± 76.6	191.7 ± 24.9	295.5 ± 122.9	0.111
Asparagine	*28.3 ± 6.6*	21.7 ± 8.5	27.8 ± 4.4	28.9 ± 7.5	0.259
Citrulline	*24.0 ± 9.3*	27.3 ± 8.9	34.0 ± 15.4	29.0 ± 11.0	0.458

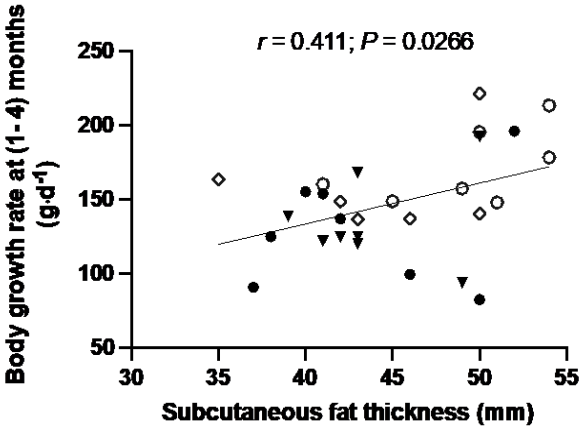
Values are means ± SD, n = 6-8.
TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled. 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

Table 5:8. Plasma amino acid concentrations (μmol·L^{-1}) of Yucatan miniature pigs at 21 d fed four experimental diets: TPN-control, TPN-B+C, TPN-IUGR, and SowFed during the neonatal period.

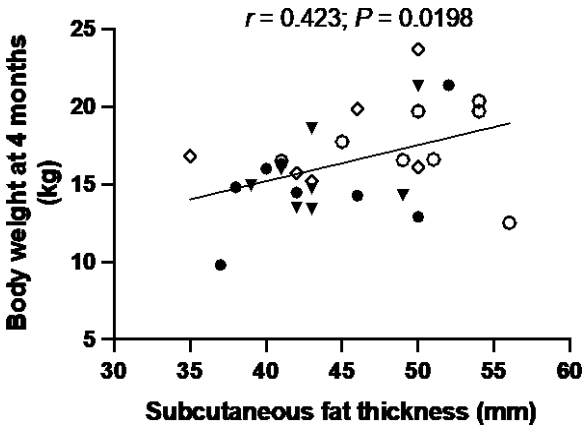
	TPN-control	TPN-B+C	TPN-IUGR	SowFed	P Value
Amino acids related to methionine metabolism					
Glycine	*209.3 ± 53.2*	190.4 ± 27.5	226.9 ± 62.8	332.0 ± 121.9*	0.0035
Serine	*621.2 ± 120.1*	574.6 ± 178.0	689.4 ± 152.6	336.3 ± 136.2*	0.0004
Methionine	*29.1 ± 13.1*	25.1 ± 9.1	28.8 ± 12.8	20.1 ± 11.8	0.4264
Taurine	*138.9 ± 35.3*	123.1 ± 28.6	129.6 ± 33.4	153.7 ± 53.9	0.4678
Indispensable amino acids					
Histidine	*89.9 ± 52.7*	119.3 ± 65.1	135.4 ± 86.2	93.0 ± 39.2	0.4299
Isoleucine	*197.8 ± 54.3*	218.3 ± 47.4	184.4 ± 55.0	177.1 ± 47.5	0.4203
Leucine	*351.8 ± 124.7*	387.0 ± 83.0	350.8 ± 133.9	253.7 ± 106.7	0.1693
Lysine	*240.5 ± 66.3*	305.0 ± 116.4	372.2 ± 190.0	222.3 ± 124.8	0.1366
Phenylalanine	*194.5 ± 54.9*	202.1 ± 35.	178.7 ± 39.6	253.2 ± 121.9	0.1797
Threonine	*152.1 ± 44.8*	163.8 ± 48.8	187.6 ± 64.1	160.5 ± 62.9	0.5754
Tryptophan	*288.1 ± 44.9*	263.7 ± 119.1	306.9 ± 63.4	139.7 ± 43.0*	0.0014
Valine	*421.5 ± 61.6*	491.3 ± 62.0	358.0 ± 102.3	431.9 ± 156.0	0.0686
Dispensable amino acids					
Alanine	*437.0 ± 33.0*	448.1 ± 120.8	453.9 ± 108.4	287.8 ± 85.4*	0.0062
Hydroxyproline	*119.2 ± 31.7*	122.6 ± 48.4	155.4 ± 44.3	139.9 ± 60.3	0.3416
Tyrosine	*82.1 ± 26.7*	132.4 ± 64.7	123.2 ± 74.8	177.2 ± 49.0*	0.0449
Ornithine	*137.1 ± 34.4*	118.3 ± 34.9	119.3 ± 42.7	150.9 ± 70.2	0.4797
Proline	*1023.0 ± 166.5*	1063.0 ± 93.43	942.7 ± 240.5	734.0 ± 287.3*	0.0357
Aspartate	*108.0 ± 50.0*	78.2 ± 26.3	78.0 ± 29.8	50.6 ± 13.8*	0.0435
Arginine	*157.0 ± 46.2*	184.3 ± 53.1	145.3 ± 71.4	106.5 ± 61.3	0.1122
Glutamate	*246.9 ± 97.0*	206.5 ± 65.5	219.7 ± 79.5	165.9 ± 53.2	0.2490
Glutamine	*54.4 ± 32.6*	75.7 ± 56.3	34.7 ± 34.0	54.1 ± 29.0	0.2321
Asparagine	*1405.0 ± 242.0*	1304.0 ± 489.1	1658.0 ± 361.8	829.6 ± 297.0*	0.0008
Citrulline	*111.3 ± 57.7*	103.2 ± 52.7	129.9 ± 69.9	162.7 ± 51.0	0.2410

Values are means ± SD, n = 7-8.
TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled.
*P<0.05; 1-way ANOVA with Dunnett's post hoc test comparing to TPN-control.

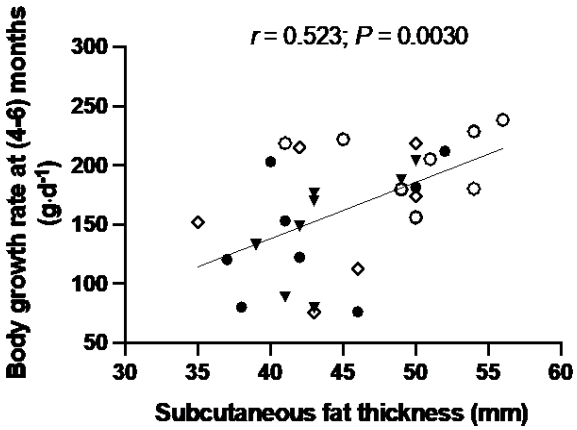
a)


r = 0.411; P = 0.0266
Body growth rate at (1 - 4) months (g·d-1)
Subcutaneous fat thickness (mm)
250
200
150
100
50
30
35
40
45
50
55

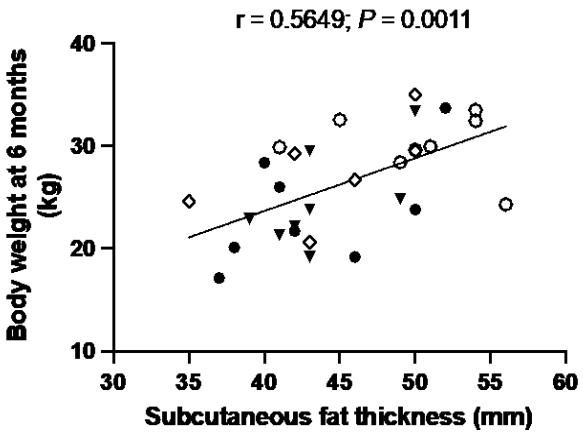
b)


r = 0.423; P = 0.0198
Body weight at 4 months (kg)
Subcutaneous fat thickness (mm)
25
20
15
10
5
30
35
40
45
50
55
60

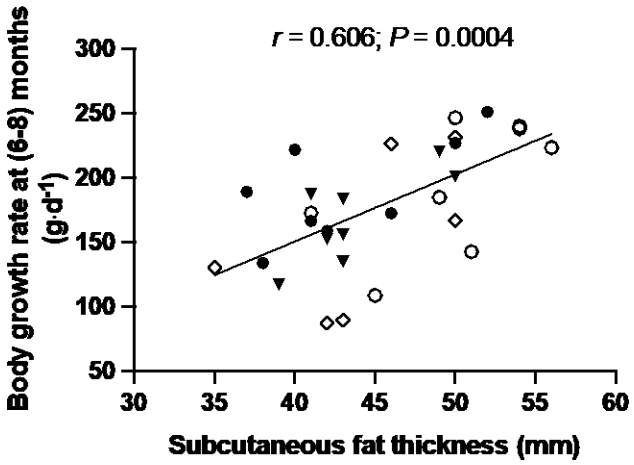
c)


r = 0.523; P = 0.0030
Body growth rate at (4-6) months (g·d-1)
Subcutaneous fat thickness (mm)
300
250
200
150
100
50
30
35
40
45
50
55
60

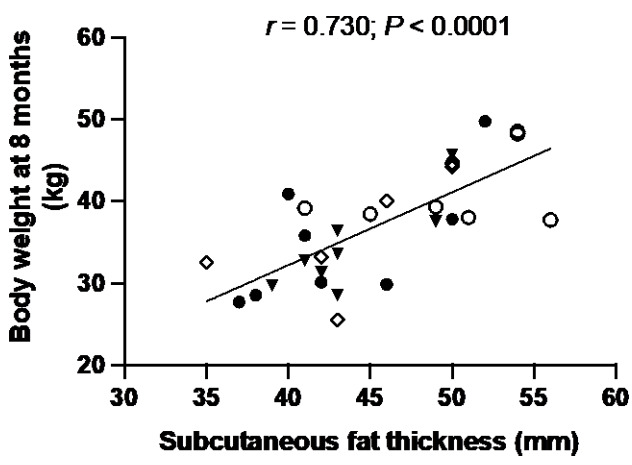
d)


r = 0.5649; P = 0.0011
Body weight at 6 months (kg)
Subcutaneous fat thickness (mm)
40
30
20
10
30
35
40
45
50
55
60

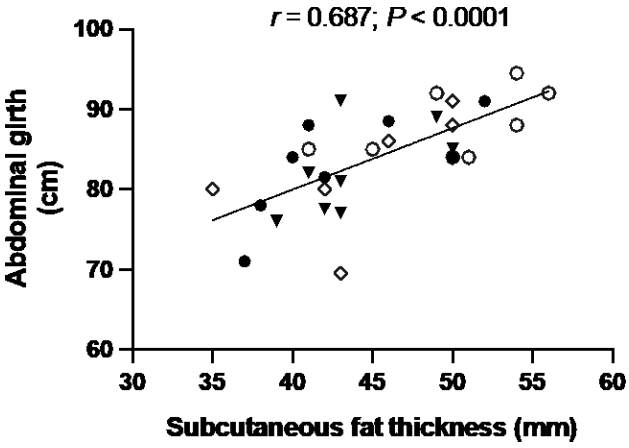
e)


r = 0.606; P = 0.0004
Body growth rate at (6-8) months (g·d-1)
Subcutaneous fat thickness (mm)
300
250
200
150
100
50
30
35
40
45
50
55
60

f)


r = 0.730; P < 0.0001
Body weight at 8 months (kg)
Subcutaneous fat thickness (mm)
60
50
40
30
20
30
35
40
45
50
55
60

g)


r = 0.687; P < 0.0001
Abdominal girth (cm)
Subcutaneous fat thickness (mm)
100
90
80
70
60
30
35
40
45
50
55
60

h)

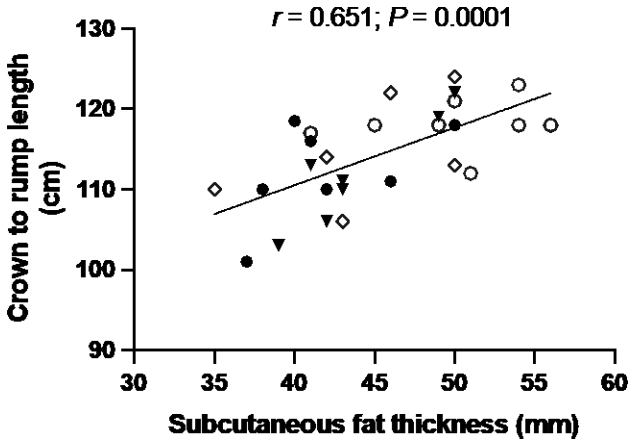
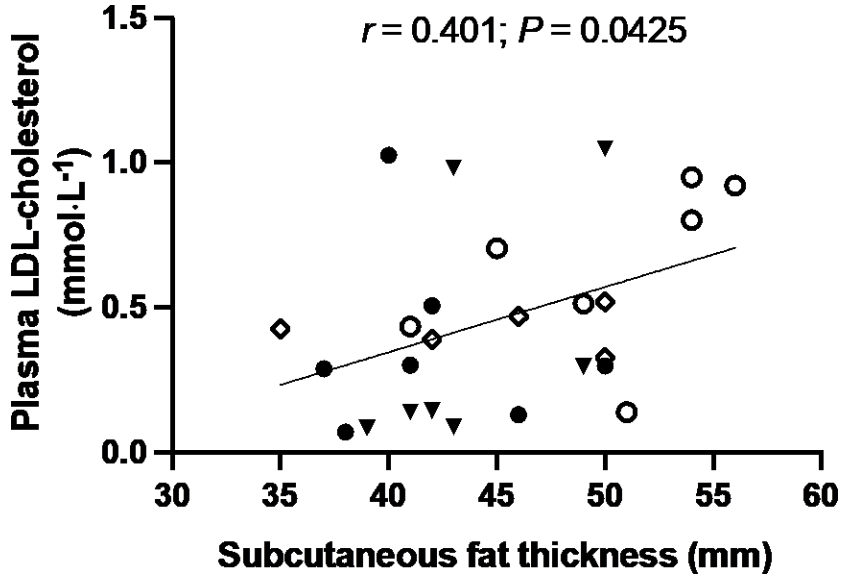

r = 0.651; P = 0.0001
Crown to rump length (cm)
Subcutaneous fat thickness (mm)
130
120
110
100
90
30
35
40
45
50
55
60

Figure 5:7. (A) Correlations between subcutaneous fat thickness and (A) body weight at 4 mo, (B) body growth rate at 1-4 mo, (C) body growth rate at 4-6 mo, (D) body weight at 6 mo, (E) body growth rate at 6-8 mo, (F) body weight at 8 mo, (G) abdominal girth at necropsy, (H) crown to rump length at necropsy in Yucatan miniature pigs fed four experimental diets in the neonatal period.

Data are given as mean ± SD; n = 7-8. Data were analyzed using Pearson correlation analysis. Each symbol represents an individual pig. TPN-control (●); TPN-B+C (▼); TPN-IUGR (○), SowFed (◊). TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled.

a)


r = 0.401; P = 0.0425
Plasma LDL-cholesterol (mmol·L-1)
1.5
1.0
0.5
0.0
30
35
40
45
50
55
60
Subcutaneous fat thickness (mm)

b)

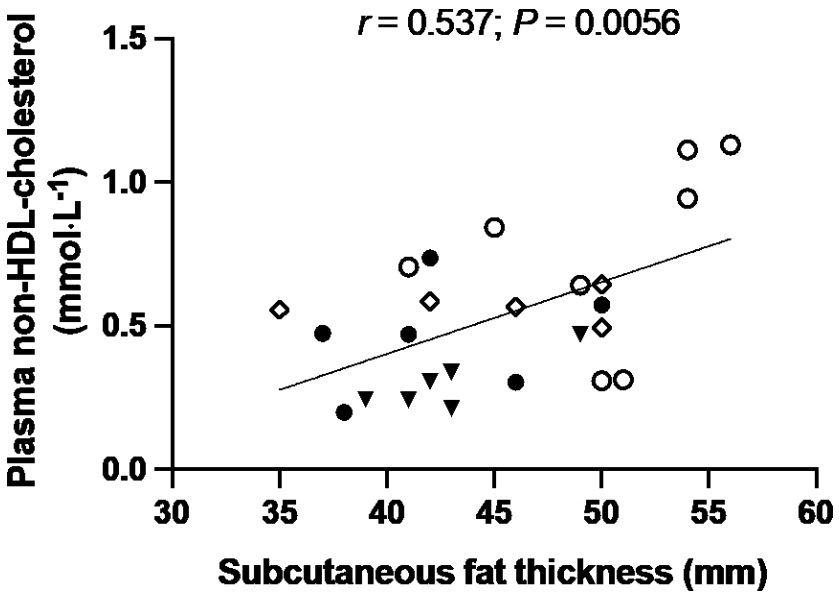

r = 0.537; P = 0.0056
Plasma non-HDL-cholesterol (mmol·L-1)
1.5
1.0
0.5
0.0
30
35
40
45
50
55
60
Subcutaneous fat thickness (mm)

Figure 5:8. Correlations between subcutaneous fat thickness and (A) plasma LDL-cholesterol at necropsy, (B) plasma non-LDL-cholesterol at necropsy in Yucatan miniature pigs fed four experimental diets in the neonatal period.

Data are given as mean ± SD; n = 7-8 pigs per group. Data were analyzed using Pearson correlation analysis. Each symbol represents an individual pig. TPN-control (●); TPN-B+C (▼); TPN-IUGR (○), SowFed (◊). TPN, Total parenteral nutrition; TPN-control, TPN control diet; TPN-B+C, TPN control diet supplemented with betaine and creatine; TPN-IUGR, IUGR piglets fed TPN-control diet; SowFed, suckled.

5.5 Discussion

TPN support is a non-normal life-saving nutritional regimen and has become an integral part of the medical management of preterm and IUGR neonates. Approximately 9% of births in Canada are preterm, and 8% to 10% are IUGR (Canadian Institute for Health Information., 2009). These infants constitute 35–50% of admissions to neonatal intensive care units, cost the health care system 10–20 times more than a healthy infant, and account for 75% of pre-natal deaths (Blackwell et al., 2005; Bolisetty et al., 2020). Studies demonstrated that although TPN is a lifesaving feeding method, it has short-term drawbacks, such as increased adiposity, hepatic steatosis, and inflammation (Kelly, 2010; Kubota et al., 2000; Stoll et al., 2010, 2012). When TPN is administered during the critical window of development in the neonatal period, it may have long-term adverse effects; however, nutritional requirements and interactions during the feeding regimen and their impact on long-term metabolic diseases are not well understood. In addition, research has demonstrated that neonates who are born IUGR are susceptible to developing catch- up growth, increased adiposity, and impaired lipid metabolism as young adults (McKnight et al., 2012; Myrie et al., 2017). Consequently, we hypothesized that metabolic alterations and increased adiposity resulting from early TPN feeding would persist into adulthood and that feeding TPN to IUGR neonates would exacerbate the effects of obesity and dyslipidemia in later life.

We demonstrated that TPN-fed piglets with a normal birth weight maintained a similar body weight as those fed sow milk throughout this early feeding phase. Although IUGR piglets grew in parallel to the TPN-control pigs while receiving TPN, they could not catch up in body weight at the end of the TPN feeding phase. This lack of catch-up growth during TPN feeding could be due to the restricted supply of TPN solution to their body weight (restricted feeding) during this phase. Therefore, IUGR piglets remained underweight at the end of the TPN feeding

phase (21 d of age). Following this, IUGR pigs appeared to experience a brief growth surge between 21 and 28 d of age when they were adapted to ad libitum grower diet. Because each pig adapts to the grower diet differently, they were not feed restricted, and it is possible that IUGR pigs had a higher feed intake, facilitating catch-up growth during this period; however, we did not monitor intakes throughout the acclimation period. Once the pigs were adapted to the grower diet, they were given a restricted diet for the 9 mo grow-out phase. In the current study, even if intakes are restricted, significant catch-up growth has been recorded in IUGR pigs between 1 to 4 mo and 4 to 6 mo, as demonstrated by considerably enhanced body growth rates. In another grow-out trial, IUGR pigs who were given ad libitum feed after weaning had a comparable catch-up growth spurt around the same age (McKnight et al., 2012). Both studies exhibited a resurgence of growth post weaning. Remarkably, the body weights of all pigs at the end of the current study were not different, although IUGR pigs had increased subcutaneous fat thickness. These data suggest that the overall catch-up 'growth' of TPN-fed IUGR pigs was primarily due to the accumulation of fat rather than lean mass. Significant positive correlations between body growth rates (Figure 7: A, B, C, D, E) and body weights to subcutaneous fat thickness further demonstrate that IUGR pigs developed obesity by the end of the study.

Based on the findings of the present investigation, it is evident that IUGR pigs developed obesity as young adults. In the event of obesity, excess circulating lipid will lead to increased FFA in the plasma. Continuous exposure of tissues to FFA will lead to accumulation of TG in tissues that do not normally store excess fat (ectopic fat deposition). We demonstrated that TPN IUGR pigs had higher ectopic fat accumulation in the liver, and skeletal muscles, further predisposing them to adverse metabolic effects. Because the liver serves as a metabolic nexus for various tissues such as skeletal muscle and adipose tissue (Rui, 2014), accumulation of TG in the liver could be a

key player in obesity and dyslipidemia (Alves-Bezerra & Cohen, 2017), biomarkers of which were observed in the TPN-IUGR pigs. To understand whether increased TG in the liver is due to increased lipogenesis and to investigate whether lipogenic genes have been programmed and differentially expressed in the adult IUGR pigs due to early TPN feeding, we measured the relative expression of lipogenic genes, including rate-limiting genes responsible for lipogenesis (ACC-1 and FASN) and TG synthesis (DGAT-2). However, we found that the relative lipogenic gene expressions were not significantly different in TPN-IUGR pigs compared to TPN-control pigs. Therefore, the TG accumulation in the liver of IUGR pigs could be due to delayed TG clearance via LPL (lipoprotein lipase). However, we could not measure total LPL activity in the circulation, as we did not inject heparin at the necropsy, which would have compromised other parameters measured in the study.

FASN catalyzes fatty acid synthesis, synthesizing a long-chain saturated fatty acid, palmitate, from acetyl-CoA and malonyl-CoA. Evidence shows TPN feeding influences the expression of genes involved in fat metabolism in the liver (Guthrie et al., 2016). Moreover, the gene for FASN has been demonstrated to be epigenetically controlled (Viscarra & Sul, 2020). Hence, the decreased relative expression of FASN in TPN-control pigs compared to SowFed pigs could be attributed to the effects of TPN feeding on FASN expression during the neonatal period, which persisted into adulthood. However, we did not measure expression of genes in neonates, so this expression change could also be due to other TPN- induced changes in lipid metabolism. Adult TPN-control pigs also had higher lipid peroxidation markers compared to Sowfed pigs. These data suggest that higher oxidative stress due to neonatal TPN feeding may have persisted into adulthood, likely via changes in epigenetics of genes related to antioxidant defence. The oxidative stress induced by TPN feeding during the first week of life is suspected to reprogram energy

metabolism in the liver (Teixeira et al., 2021). Generation of by-products of lipid peroxidation is an inherited drawback in using TPN, which can increase oxidative stress (Lavoie & Chessex, 2019) and reports have demonstrated novel associations between DNA methylation with oxidative stress (Hedman et al., 2016; Yara et al., 2013).

Obesity-related changes in adipocytes stimulate lipolysis and release fatty acids into the circulation (Guéant et al., 2014), as indicated by an increase in NEFA concentrations in the blood of 9- month-old obese TPN-IUGR pigs. Increased NEFA infiltration into the liver of TPN-IUGR pigs may also contribute to their elevated TG content. The increased hepatic influx of FFA may have resulted in increased VLDL production, as indicated by higher content of VLDL-TG and non-HDL cholesterol after fasting. Therefore, obesity was associated with the development of dyslipidemia in TPN-IUGR pigs. The positive correlation between subcutaneous fat thickness and plasma non- HDL cholesterol and LDL-cholesterol indicates that obesity and dyslipidemia are interconnected metabolic disorders. Adiponectin is a cardioprotective hormone that decreases as oxidative stress and inflammation increase, which is common in obese individuals with low-grade inflammation. We measured adiponectin in these pigs to determine the severity of the metabolic syndrome. Although adiponectin concentrations tended to be lower in TPN-IUGR pigs ($P < 0.088$), it is likely that IUGR pigs are not very far along in the development of metabolic syndrome. Given that these pigs are only young adults, this lack of overt metabolic syndrome is not surprising, and these markers are expected to worsen with age, as metabolic syndrome develops.

Myocardial TG within cardiomyocytes constitutes a critical fatty acid and energy reserve for the heart (Kienesberger et al., 2012). Metabolism of cardiac TG is highly dynamic (Lopaschuk et al., 2010) and it ensures a continuous fatty acid supply for mitochondrial oxidation, independent of short-term fluctuations in plasma fatty acid availability. Since intracellular TG hydrolysis

contributes significantly to the generation of ATP necessary for contractile function (O'Donnell et al., 2008; Saddik et al., 1991), TG reserves play a critical role in regulating cardiac function and reduced cardiac TG content is associated with cardiac dysfunction (Borradaile et al., 2005; Kienesberger et al., 2012). Therefore, it has been suggested that greater storage of TG in the cardiac muscle, to some extent, is considered to have a cardioprotective effect. Diverting infiltrating NEFA into TG in the cardiac muscle prevents the cardiac cells from lipo-toxicity effects of accumulated fatty acids (Liu et al., 2009). Thus, reduced cardiac TG reserves in the TPN-control pigs in the current study suggest that they may not have readily available energy stores for energy-demanding cardiac cells, whereas increased cardiac TG content in the TPN-B+C group compared to TPN-control group suggests a complementary effect on cardiac contractility and may likely have contributed to reduced hypertension parameters (Randunu et al., 2022). Interestingly, cellular NEFA concentration in cardiac cells was maintained at the same level, regardless of the treatment groups, preventing cardiac cells from being exposed to their lipo-toxicity effects.

TPN-IUGR pigs are anticipated to shift their amino acid profile towards the phenotype associated with obesity and CVD as their obesity develops with age. After 9 mo, there were no statistically significant differences between the fasting amino acid profiles of TPN-IUGR and TPN-control pigs; however, there were trends towards higher concentrations of isoleucine, leucine, threonine, and tryptophan in adult TPN-IUGR pigs. Higher concentrations of these amino acids have been associated with an increased risk of CVD. Recent research demonstrates that plasma amino acid profiles are altered in obese individuals, suggesting a correlation between fat accumulation and plasma amino acid profiles (Simonson et al., 2020; Takashina et al., 2016). Positive correlations of tryptophan, aspartate, alanine, leucine, isoleucine, and tyrosine at 9 mo with obesity-related parameters in the current study (TG content in skeletal muscle, 1–4-month growth rate, crown-to-

rump length, chest girth, and abdominal circumference) suggest that differences in amino acids will become more pronounced as TPN-IUGR pigs' obesity increases with age.

Overall, these findings imply that feeding TPN to neonates did not cause overt obesity in later life, and adding betaine and creatine to TPN also did not affect obesity in later life. However, IUGR does lead to obesity development, which exacerbates the effects of dyslipidemia in adulthood. Our findings will aid in comprehending the long-term programming effects of TPN feeding to IUGR neonates on obesity development, so that new nutritional interventions can be designed to reduce the programming risk of adulthood obesity.

6 Chapter 6: General discussion

6.1 Summary

Using Yucatan miniature pigs as an animal model, this project aims to investigate whether there are long-term metabolic implications of TPN feeding during the early neonatal phase. As TPN is administered during the epigenetic window of programming and the availability of methyl nutrients can alter epigenetics, we investigated whether supplementation with betaine and creatine, which increase and preserve adequate methyl groups for the proper epigenetic process, could reduce the deleterious metabolism effects in adulthood resulting from neonatal TPN feeding. IUGRs are susceptible to developing chronic disorders as adults. As 8-10% of births in Canada are IUGR neonates and are likely to receive TPN as a medical treatment or lifesaving feeding strategy, we explored whether giving TPN will exacerbate the adverse metabolic effects in adulthood.

In the current study, we assessed biomarkers of dyslipidemia, hypertension, and obesity among our four experimental groups. Because the pigs used in the current study were on the same grower diet from 3 weeks to 9 mo of age after feeding 2 weeks of TPN or sow milk during the neonatal period, the outcomes are solely due to experimental diets given in the neonatal period. The key observation in the current study is feeding TPN in the neonatal period resulted in dyslipidemia in adulthood. IUGR neonates are particularly sensitive to TPN feeding during the neonatal period and develop obesity as young adults. Similar to early programming studies, pigs from the current study showed increased weight gain throughout 1-4 mo and 4-6 mo developmental periods, representing catch-up growth. TPN support did not eliminate the catch- cup growth of IUGR pigs around 4 mo as neonates. Similar body weights, increased subcutaneous fat thickness as adults (9 mo), and catch-up growth in the IUGR pigs as adults further support the argument that

TPN-IUGR pigs developed obesity. The development of obesity may have led to increased NEFA in the circulation of TPN-IUGR pigs, which may have resulted in ectopic fat accumulation in the major organs, such as in the liver and muscles, after enhanced flux of FFA into the organs. Increased flux of FFA into the liver of TPN-IUGR pigs may have led to increased VLDL secretion, which was visible as increased TG in the VLDL fraction in TPN-IUGR pigs. The cholesterol distribution amongst lipoprotein subfractions of TPN-IUGR pigs (increased cholesterol in the LDL subfraction and non-HDL cholesterol) indicates susceptibility to atherosclerosis and CVD. After an oral fat tolerance test, a lower postprandial TG clearance rate in TPN-IUGR pigs suggests an increased TG resident time in the plasma. All these findings (Figure 6.1) collectively indicate that IUGR develops obesity and dyslipidemia in adulthood, changes that persisted long after feeding TPN as neonates. Supplementing TPN with betaine and creatine did not affect the development of obesity or dyslipidemia in later life; however, these supplements assisted in lowering blood pressure parameters in adults, which may be beneficial for preventing hypertension.

Schematic representation of the summary of outcomes related to TPN-IUGR

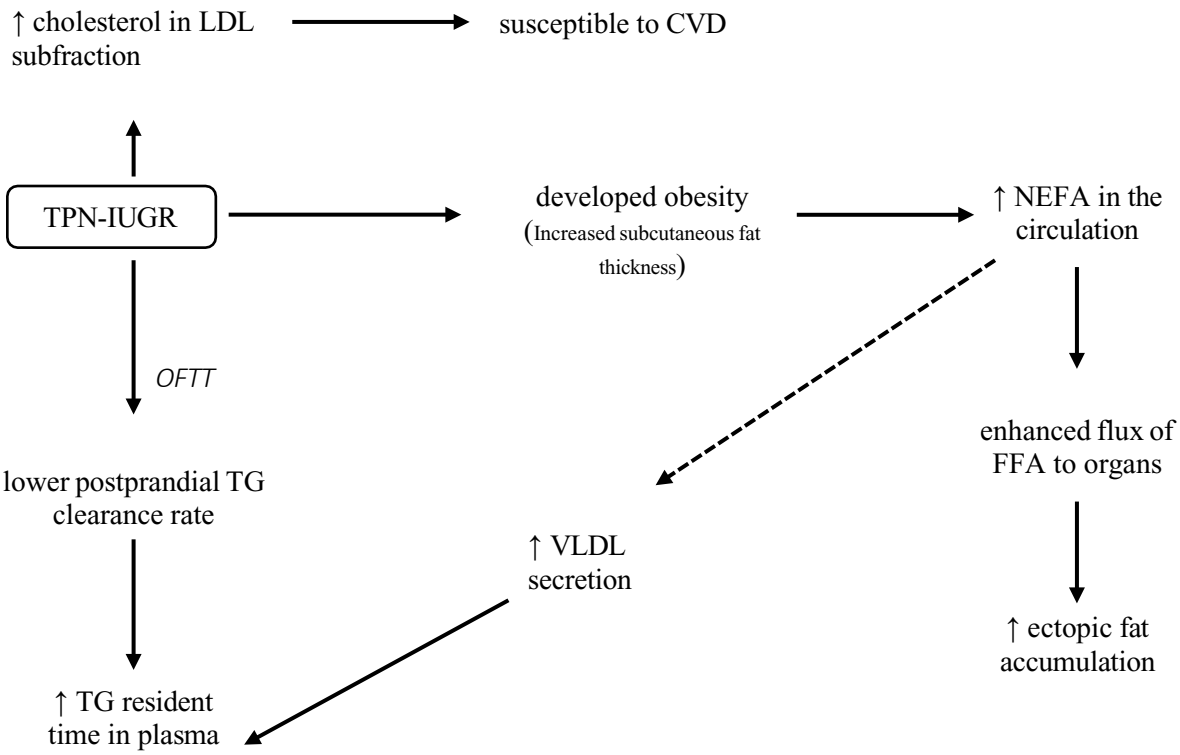


Figure 6:1 Schematic representation of the summary of outcomes related to TPN-IUGR.

LDL, low-density lipoprotein; CVD, cardiovascular disease; TPN-IUGR, total parenteral nutrition fed intrauterine growth restricted; OFTT, oral fat tolerance test; TG, triglyceride; VLDL, very low-density lipoprotein; NEFA, non-esterified fatty acids; FFA, free fatty acids.

6.2 Future directions

The current study established an adult female metabolic phenotype due to early-life TPN support, with some mechanistic explanations. Advancing this research, establishing all the potential regulatory pathways underlying the perturbations observed will aid in understanding the mechanisms in programming. As evidence on the long-term metabolic changes due to early TPN feeding is scarce, it is inefficient, time consuming and expensive to investigate specific pathways one at a time that could be affected by programing due to early TPN support. Thus, a better approach to identify most of the possible pathways that could explain the phenotypic changes is necessary to accomplish the aforementioned. For this, bulk-cell RNA-seq profiling could be used to discover genes differently expressed to explain phenotypes between experimental groups. Bulk RNA-seq measures the average gene expression levels across a population of cells and provides a snapshot of the overall gene expression profile for a given sample, allowing for the identification of differentially expressed genes and analysis of pathways. By employing this method, we could have a broader identification of what metabolic pathways had been affected by early TPN feeding. This approach will allow us to identify genes of interest that must be explored for methylation studies. Given that TPN support is administered within the epigenetic window of programming, it is likely that epigenetic modifications contributed to the observed metabolic alteration. Thus, characterization studies of epigenetic processes (methylation studies) can be conducted using methylome sequencing to comprehend the role of epigenetics in programming mechanisms. Here, a bisulfite conversion-based approach or next-generation sequence-based platforms can be utilized (Sant et al., 2012). This approach will assist in capturing complete mechanistic pathways affected by programming due to early TPN feeding.

Based on the observations of the preceding studies in the current thesis, some specific advances can be made to understand the mechanisms behind certain outcomes. The current study revealed that TPN support during early life programmed the lipid metabolism into adulthood, and TPN-fed IUGR pigs developed dyslipidemia and obesity in later life. Notably, the postprandial lipid metabolism in adulthood was affected when fed TPN in early life. In addition, TPN-IUGR showed higher plasma non-HDL cholesterol concentration in adulthood compared to TPN-control pigs suggesting cholesterol metabolism had also been programmed due to neonatal TPN feeding. Lipoprotein metabolism is a complex system in which abnormal concentrations of various lipoprotein particles can result from alterations in their production, conversion, and catabolism rates. Conventional approaches to measuring plasma lipoprotein concentrations offer limited insight into the dynamics of lipoprotein metabolism and fail to provide detailed mechanistic information (Borén et al., 2020). In contrast, the utilization of stable isotope-labeled tracers along with mathematical modeling offers a robust methodology for investigating lipid and lipoprotein kinetics in vivo. This approach enables us to delve into the underlying mechanisms of dyslipoproteinemia (Adiels et al., 2015), including the rates of lipoprotein secretion and fractional catabolism in the circulation. Hence, an additional application of kinetic studies to elucidate impairments in lipid metabolism amongst the treatment groups, such as kinetic studies on lipoprotein subtype metabolism (CM, CM remnant, VLDL, HDL, and LDL metabolism) and cholesterol metabolism, would provide more comprehensive evidence of the mechanistic insights. Stable isotopes commonly used as metabolic tracers include [$^{2}H_3$]-leucine for apolipoprotein turnover; [$^{2}H_5$]-glycerol (or ^{3}H-glycerol) and [^{13}C]-palmitate for VLDL–triglyceride metabolism; [1,2-^{13}C]-acetate and [^{13}C]-palmitate for fatty acid metabolism and hepatic de novo lipogenesis; $^{2}H_2O$ for de novo lipogenesis in the liver; [$^{13}C_5$]-cholesterol and [$^{2}H_6$]-cholesterol for intestinal

cholesterol absorption; and [^{13}C]-acetate for assessing HDL-mediated reverse cholesterol transport. We expect to observe differences in CM concentrations and possibly VLDL concentrations between TPN-control and SowFed groups because plasma TG concentrations were higher in the TPN-control group than SowFed during the postprandial stage. Moreover, VLDL apoB fractional catabolic rate and CM remnant clearance is impaired in metabolic syndrome, which can be assessed with a stable isotope breath test. We expect to observe a deleterious lipoprotein profile, including impaired CM remnant clearance in the TPN-IUGR group compared to the TPN-control group, as TPN-IUGR pigs are more sensitive to developing metabolic syndrome in later life. As our TPN-fed IUGR adult pigs showed some attributes of metabolic syndrome at 9 mo, a longer duration of the grow-out phase to examine, complete metabolic phenotype could be beneficial, followed by aforementioned kinetic studies to understand the mechanism of dyslipidemia.

The role of betaine in mammals is poorly understood, and there is very little information on betaine intake within the population (Ross et al., 2014). It is also unclear if the neonate has a dietary betaine requirement. In the current study, although supplementing betaine and creatine to TPN did not change the TPN effects on dyslipidemia and obesity programming, these supplements were involved in reducing blood pressure parameters in adult pigs, suggesting supplementing betaine and creatine may reduce the risk of metabolic syndrome in adulthood. Therefore, establishing in vivo choline and betaine kinetics would improve the understanding of how dietary methyl donors in the neonatal diet contribute to programming. To my knowledge, whole-body betaine and choline kinetics in neonates are unknown, although choline plays a critical role during fetal development, which may likely have long-term programming effects (Zeisel, 2006). Therefore, kinetic relationships of remethylation can be determined by selecting the appropriate

isotope of betaine and choline. Previous research shows that remethylation and transsulfuration are not governed strictly by the supply of labile methyl donors, and maximal remethylation capacity is likely predetermined by the transmethylation requirement (McBreairty et al., 2016). Thus, folate and choline are essential, but betaine may be a conditionally dispensable nutrient. Focused studies on betaine kinetics would assist in understanding how much betaine is synthesized by the neonate and if there is a dietary requirement for betaine. This understanding will assist in designing novel early nutritional interventions (e.g., TPN) that may reduce the metabolic burden in adulthood due to programming.

The prevalence of metabolic syndrome is higher in females (Beigh & Jain, 2012). Thus, only females were focused on disease biomarker outcomes in the current study, as females were predisposed to develop metabolic syndrome. Most of these metabolic differences are mediated in a tissue-specific manner and are attributed to differences in body fat distribution, glucose homeostasis, insulin signaling, ectopic fat accumulation, and lipid metabolism in response to hormonal or nutritional imbalance mediated through sex hormones and the sex chromosome complement. Thus, future research characterizing the metabolic phenotype in detail, including in both males and females, would allow us to compare the effects of sex on the programmed metabolic outcomes. Also, generating long-term programming data on both male and female of early TPN support would be interesting and important to establish as a reference, which can be utilized for further nutritional interventions designed to reduce deleterious long term metabolic effects.

Adding a TPN-supplemented B+C-fed IUGR group to the four experimental groups in the current study would aid in isolating specific conclusions. A TPN-B+C-IUGR group will help distinguish the effects of B+C-supplemented TPN on IUGR pigs. In addition, tissues were

collected and analyzed at 9 mo in the current study. However, if tissues are biopsied following TPN support, it would be possible to evaluate the state of metabolism immediately after TPN feeding and later in adulthood, allowing us to confirm the acute and long-term programming effects of TPN feeding.

6.3 Conclusions

Understanding the long-term effects of feeding TPN to neonates, including IUGR neonates, has important implications for infant nutrition. Overall, this study signifies that although TPN is an essential part of the medical management of IUGR infants, feeding TPN may have long-term consequences exacerbating the risk of chronic diseases in adulthood. Further, it demonstrated that the effects of feeding TPN in early life persisted into adulthood, especially dyslipidemia. The findings from this comprehensive study helped identify the long-term programming effects of early TPN feeding, provided some mechanistic insights, and paved the way for novel nutritional interventions designed to reduce the risk of adult chronic diseases.

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